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Reversed-phase high-performance liquid chromatography of phospholipids with fluorescence detection

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ABSTRACT

Reversed-phase high-performance liquid chromatographic (HPLC) behavior of fluorescent labeled phospholipids (PLs) was studied. Molecular species of phosphatidylethanolamine (PE) derivatized with fluorescein-, thiocarbamoyl-, pyrenesulfonyl-, and dimethylaminonaphthalenesulfonyl (dansyl)-fluorophores were separated on octadecylsilica with a mobile phase of acetonitrile–methanol–water in the presence of tetraalkylammonium phosphates (TAAPs). Under similar HPLC conditions, dansylated phosphatidylserines and PE plasmalogens (ether-linked PLs) were also resolved. Incorporation of the fluorescein moiety to the parent PE appeared to facilitate further resolution of its subcomponents. Subcomponents of dansylated PE derived from egg phosphatidylcholines were quantifiable. Effects of the type and concentration of TAAP on capacity factors of PL solutes were indicative of an ion-pair separation processes. HPLC with high-molecular-mass TAAPs favored the separation of the components that remained unresolved with mobile phases containing low-molecular-mass TAAPs. The HPLC–fluorescence detection method provided a useful approach to quantitative analyses of various PL structures. Compositions of PL subcomponents were determined directly from peak areas.

INTRODUCTION

Separations and quantitative measurements of phospholipids (PLs) in crude and degummed soybean oil are important analytical procedures in studying effects of oil storage on the polar lipid compositions. Normal-phase high-performance liquid chromatography (HPLC)–evaporative light scattering detection techniques (ELSD) have been used to monitor the changes in all soybean PL classes as well as in molecular species subclasses of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) [1–5]. For negatively charged PLs, molecular species have been separated by reversed-phase (RP) ion-pair HPLC with UV detection [6–9].

As demonstrated in a recent study [10], detection of neutral PLs (*e.g.* PE and PC) by normal-phase HPLC–ELSD was considerably less efficient and less sensitive than by RP-HPLC–UV with mobile phases containing tetraalkylammonium phosphate (TAAP) additives. However, limitations inherent with the RP-HPLC–UV detection systems are: (a) its inability to quantify molecular species simultaneously from the UV detector signals [11], (b) its requirement of fraction collection of individual molecular species, and (c) quantitative analysis of each fraction by phosphorimetry [12,13]. In other words, compositions of PL subcomponents can not be determined by direct computer integration of individual peak areas on chromatograms. An additional drawback of the RP-HPLC system with mobile phase electrolytes is the incompatibility of the mobile phases with an ELSD or a mass

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spectrometer for quantitation or characterization of molecular species.

In common analytical practice, derivatization of PLs with fluorescent materials should allow not only quantitative analyses of the products with specific fluorescence detection, but also lead to substantial enhancement in detection sensitivity. Few publications are available in the literature on the analysis of PL subclasses using fluorescence-labeled derivatives. In several known procedures [14–19], the PL structure is disrupted for the removal of the polar head group prior to HPLC analysis. Thus, fluorescent derivatives of diradylglycerols prepared from treatment of PLs with phospholipase C were the analyte fragments produced by molecular cleavage at the glycerol oxygen–phosphorus bond of PLs. Although a few other papers [20–22] have dealt with fluorescent derivatives of PLs, quantitative analyses of intact PL molecular species by HPLC–fluorescence detection have not been thoroughly investigated. This paper reports the RP-ion-pair HPLC behavior of fluorescence-labeled PLs. Examples of utilizing the ion-pair HPLC-fluorescence detection (FL) technique for the quantitative determination of molecular species compositions are presented.

EXPERIMENTAL

Chemicals and reagents

Dansylated egg PE was purchased from Molecular Probes (Eugene, OR, USA). Dansyl-, pyrenesulfonyl- and fluoresceinthiocarbamoyl-PE obtained by transphosphatidylation of egg PC were purchased from Avanti Polar Lipids (Pelham, AL, USA). Dansylated brain PE (mainly PE plasmalogen) and brain PS were also products of Avanti. Dansylated plant PE was prepared from plant PE (Avanti) by the procedure described in the following paragraph. Tetramethylammonium phosphate (TMAP) was prepared by treating tetramethylammonium hydroxide (Aldrich, Milwaukee, WI, USA) with phosphoric acid (Fisher, Fair Lawn, NJ, USA) until reaching pH 6.5. Tetrabutylammonium phosphate (TBAP), pentyltriethylammonium phosphate (PTAP), heptyltriethylammonium phosphate (HPTAP), octyltriethylammonium phosphate (OTAP), and dodecyltriethylammonium phosphate (DTAP) were purchased from Regis Chemicals (Morton Grove, IL, USA). HPLC solvents acetonitrile and methanol were obtained from J.T. Baker (Phillipsburg, NJ, USA). HPLC water was obtained by filtering distilled water through a Millipore (Bedford, MA, USA) Milli-Q water purifier.

General procedure for the preparation of dansylated phospholipids

A previously reported procedure [23] was modified as follows: PE (10 mg) dissolved in 0.5 ml chloroform in a reacti-vial was treated with dansyl chloride (4 mg) and triethylamine (50 μl) in 0.5 ml chloroform. The mixture was stirred at room temperature for two hours and then chromatographed onto a Varian (Sunnyvale, CA, USA) Bond Elut-Si column for purification of products. The column was extracted with methanol–chloroform (1:1). After evaporation, the residue was dissolved in 1 ml methanol (or chloroform) to exact volume for quantitative HPLC analysis. For qualitative analysis, the residual crude reaction mixture was streaked on a HP-TLC plate coated with silica gel-60 (E.M. Science, Gibbstown, NJ, USA) and developed in ethyl acetate. The product band was scraped off and extracted with methanol–chloroform (1:1) to give samples suitable for analyses.

High-performance liquid chromatography

A Spectra-Physics (San Jose, CA, USA) Model SP8800 liquid chromatograph coupled with an Applied Biosystems (Foster City, CA, USA) Model 980 programmable fluorescence detector was used in all HPLC experiments. PL analytes were detected at various wavelengths depending on the type of fluorophores used: dansyl-PL, excitation 338 nm, emission 470 nm; fluorescein-PL, excitation 489 nm, emission 550 nm; pyrene-PL, excitation 342 nm, emission 389 nm. Mobile phases were prepared by mixing desired amounts of tetraalkylammonium phosphates (TAAP) to acetonitrile–methanol–water. The ternary solvents were adjusted to suit different structural types of fluorescent PL. The mobile phase solutions were filtered, degassed,

and pumped through a RP column at a flow-rate of 1 ml/min. All samples were freshly prepared from frozen materials prior to analysis. Aliquots (10 μl) of the samples containing 1–2 mg/ml of PL analytes were injected onto the column via a Rheodyne (Cotati, CA, USA) Model 7125 injector fitted with 10-μl loop. Two different RP columns were used. These include (1) a Beckman Instruments (San Ramon, CA, USA) Ultrasphere ODS column, 150 × 4.6 mm I.D., 5 μm, and (2) a Waters (Milford, MA, USA) NovaPak C18 column, 300 × 3.9 mm I.D., 4 μm.

In HPLC experiments where the analyte components were well resolved, individual chromatographic peaks were isolated, and converted to fatty acid methyl esters by a published HCl–methanol method [24]. Fatty aldehyde liberation from PE plasmalogens were performed following a known procedure [25]. Both fatty aldehyde and fatty acid determinations were carried out on a Varian (Sugarland, TX, USA) Model 3400 gas chromatograph interfaced with a flame ionization detector. Fatty acid components were separated on a 30 m × 0.25 mm I.D. fused-silica capillary column coated with 0.2 μm SP 2330 (Supelco, Bellefonte, PA, USA), using helium as the carrier gas. The column temperature was programmed by holding initially at 200°C then increasing to 220°C at 10°C/min. Peak areas were automatically integrated by a built-in data processor.

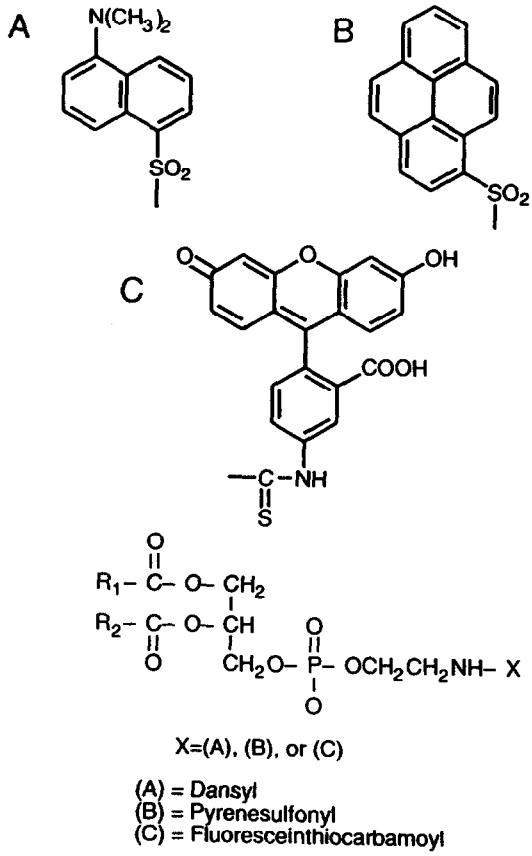

Fig. 1. Structures of investigated fluorescent derivatives of phosphatidyl ethanolamine (PE).

RESULTS AND DISCUSSION

In RP-HPLC with mobile phases containing TAAP quaternary ammonium salts, subcomponents of neutrally charged PLs (PC and PE) were presumably separated via an ion-suppression mechanism [10]. Derivatization of the amino group in PE with fluorescent materials (fluoresceinthiocarbamoyl, pyrenesulfonyl, and dansyl compounds) yielded the corresponding fluorescent labeled PE as shown in Fig. 1. Each of the derivatized polar lipids was consequently left with a negative charge in the molecule. As expected, the retention behavior of these fluorescent compounds was different from that of the parent PE, but was very similar to that of negatively charged PLs [phosphatidylinositol (PI), phosphatidic acid (PA), phosphatidylserine (PS), phosphatidylglycerol (PG) and diphosphatidylglycerol (DPG)] reported previously [6–9].

The purpose of introducing three fluorescent groups (dansyl, fluorescein, and pyrene) to the underivatized parent polar lipids was to see if these fluorescent substituents would contribute some degrees of differential selectivity for PL subcomponents. Although hydrophobicity of the parent PL was enhanced as a result of derivatization, HPLC without the use of the TAAP electrolytes in mobile phases normally led to little retention of PL solutes on octadecylsilica.

It was of interest to note that HPLC separations of subcomponents of PLs were dramatically influenced by the nature of fluorophores attached to the head groups. As illustrated in Fig. 2A and B, only three major egg PC species were

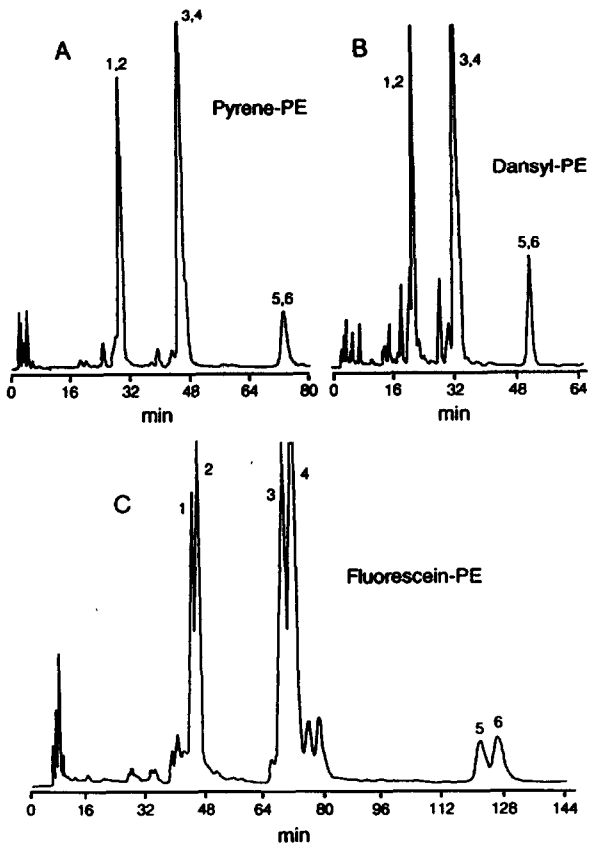

Fig. 2. RP-ion pair-HPLC separations of fluorescence-labeled PE derived from egg PC. Column, NovaPak C18; mobile phases, 5 m*M* DTAP in acetonitrile–methanol–water [(A) and (B) 70:28:2; (C) 70:22:8]. For peak identification, see footnote to Table I.

resolved on a NovaPak C18 phase, when the PE amino group was derivatized with either pyrene- or dansyl-fluorophores. However, upon conversion of the parent PE to the fluoresceinthiocarbamoyl derivative, each of the three components was further split into two subcomponents as shown in Fig. 2C. The chromatograms shown in Fig. 2 are obtained under optimized HPLC conditions where the mobile phase for Fig. 2A and B is different from that for Fig. 2C. When the HPLC experiments for all three derivatives of PE were carried out under the same conditions as for Fig. 2C (fluorescein-PE), separations of subcomponents of pyrene- and dansyl-PE (retention times were too long to be of any practical value) were not improved. Thus, the observed differences in component resolution among the three PE derivatives investigated were not simply due to the use of a different mobile phase containing a high percentage of water (Fig. 2C *vs.* Fig. 2A and B). This is the first example of substituent effects of the PL head group on the RP separation processes. The results of the present study appeared to contradict earlier findings that hydrophobic interactions of analyte solutes with a hydrocarbonaceous stationary phase were confined to the tail groups of perbenzoylated PI [6], which is also a negatively charged PL. In a PE molecule, the fluorescein substituent on the amino group is situated two carbons remote from the phosphorus center where the inositol group of PI is attached.

Inspection of the retention data in Table I and II revealed a decreasing order of retention characteristics of analyte components: k'(pyrene-PE) > k'(dansyl-PE) > k'(fluorescein-PE). This follows the reverse order of polarity of the fluorescent moieties: pyrenesulfonyl < dansyl < fluoresceinthiocarbamoyl irrespective of that of the total number of carbon atoms of the fluorescent ring systems.

Three sets of plots were selected to show linear relationships between ln k' of fluorescent-labeled PL and the total number (N) of carbon atoms in TAAP and are presented in Fig. 3. Solvophobic interactions in RP-ion pair-HPLC of the compounds of interest were found to discriminate different structure types (*i.e.* symmetrically substituted TAAP *vs.* unsymmetrically substituted TAAP) of TAAP employed. As noted in the figure, a deviant of each set of linear correlation lines is located at $N = 16$ where TBAP was used as the counter ion. This is due to the fact that TBAP is in a different structural series from the primary alkyltriethylammonium phosphate series investigated (Table III). The observations are parallel to earlier results [7–9] from HPLC studies of PA, PS, and DPG. However, the results of the present study are in contrast with the linear correlation data for PI and PG [6,9] that showed independence of ln k' values on TAAP structural types. It is not clear whether the hydroxy functionalities of PI and PG at the close proximity to the phosphorus center

TABLE I

HPLC SEPARATION OF SUBCOMPONENTS OF FLUORESCEIN-LABELED PE DERIVED FROM EGG PC (ON A BECKMAN ULTRASPHERE ODS, 150 × 4.6 mm I.D. COLUMN)

Mobile phase			Capacity factor (k') of component[b]								
Ratio[a]	TAAP	Concentration (mM)	1	2	α_{1-2}	3	4	α_{3-4}	5	6	α_{5-6}
70:28:2	TMAP	10.0	0.0	0.0	1.0	0.0	0.0	1.0	0.1	0.1	1.0
70:28:2	PTAP	5.0	0.1	0.1	1.0	0.1	0.1	1.0	0.2	0.2	1.0
70:28:2	HPTAP	10.0	0.3	0.3	1.0	0.5	0.5	1.0	1.1	1.1	1.0
70:22:8	PTAP	10.0	0.5	0.5	1.0	1.2	1.2	1.0	2.2	2.2	1.0
70:22:8	OTAP	10.0	3.8	3.8	1.0	6.7	6.7	1.0	11.7	11.7	1.0
		20.0	150	152	1.01	175	178	1.02	287	296	1.03
70:22:8	DTAP	2.5	0.2	0.2	1.0	0.5	0.5	1.0	1.1	1.1	1.0
		5.0	4.5	4.8	1.07	7.7	8.2	1.07	13.8	14.7	1.07
		7.5	17.2	18.0	1.05	28.5	30.2	1.06	54.8	63.5	1.16
		10.0	39.0	41.5	1.06	71.3	76.2	1.07	139	159	1.15

[a] Ratio acetonitrile–methanol–water.
[b] Component identification: (1) + (2) 16:0–18:2, (3) + (4) 16:0–18:1, and (5) + (6) 18:0–18:2 corresponding to fatty acid chains with R_1 and R_2 groups in PE derivatives (Fig. 1). Fatty acid designations: 16:0 = palmitic; 18:0 = stearic; 18:1 = oleic; 18:2 = linoleic. Exact structures within component pairs 1–2, 3–4, and 5–6 were not determined.

TABLE II

HPLC SEPARATIONS OF SUBCOMPONENTS OF DANSYLATED AND PYRENE-LABELED PE DERIVED FROM EGG PC (ON A BECKMAN ULTRASPHERE ODS, 150 × 4.6 mm I.D. COLUMN)

Mobile phase conditions[a]		Capacity factor (k') of component[b]					
TAAP	Concentration (mM)	1	2	3	4	5	6
Dansylated PE							
TMAP	10.0	2.2	2.2	3.8	3.8	6.5	6.5
HPTAP	5.0	1.2	1.2	2.0	2.0	2.7	2.7
	10.0	4.5	4.5	7.2	7.2	11.6	11.6
Pyrene-labeled PE							
TMAP	10.0	3.0	3.0	4.8	4.8	7.9	7.9
HPTAP	5.0	2.2	2.2	2.3	2.3	6.3	6.3
	10.0	5.3	5.3	8.2	8.2	13.2	13.2

[a] Mobile phase: TAAP in acetonitrile–methanol–water (70:28:2).
[b] All component pairs 1–2, 3–4, and 5–6 were not resolved ($\alpha = 1.0$). For component identification, see footnote to Table I.

have any bearing on the availability of the total area of tetraalkyl groups in TAAP for solvophobic interactions [26,27]. The linear plots are useful for the prediction of k' values of PL components analyzed under HPLC conditions where an unknown member of TAAP within the same structural series is present in a mobile phase.

As previously observed in HPLC work involving negatively charged PL, retention times or capacity factors (k') values of subcomponents of fluorescent-labeled PE derived from egg PC (Table I and II) were invariably longer in HPLC with mobile phases containing TAAP of larger size and higher concentration (positive concentration effects). Evidently, the RP separation

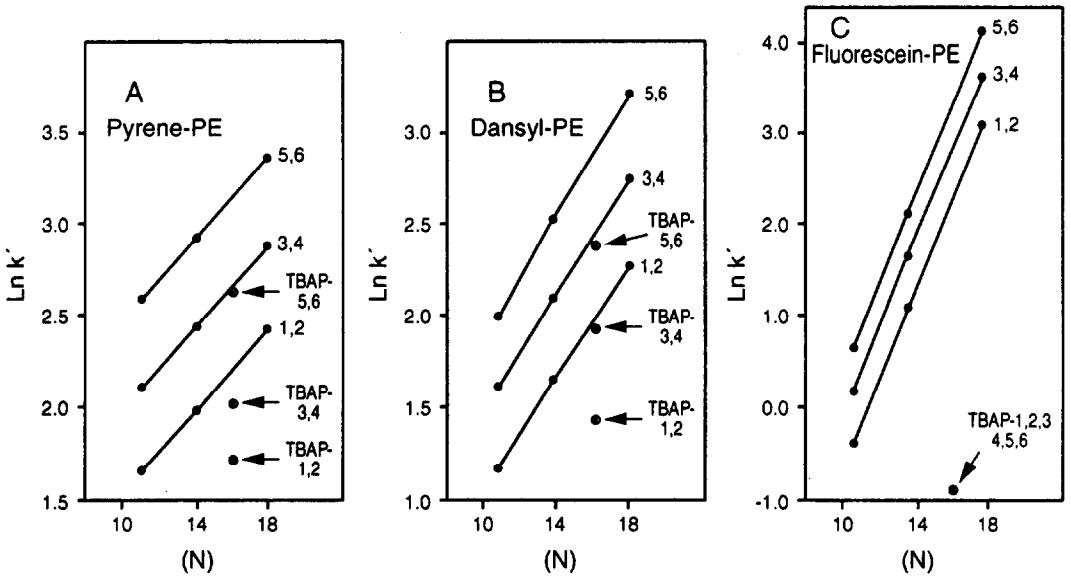

Fig. 3. Linear correlation between the total number (N) of carbon atoms in TAAP and ln k' of fluorescence-labeled PE derived from egg PC. HPLC conditions are same as in Fig. 2 except various TAAP were used. The deviant points for TBAP are shown for components 1, 2, 3, 4, and 5 of the three different derivatives studied.

TABLE III

EFFECTS OF TAAP STRUCTURES ON CAPACITY FACTORS, k', OF SUBCOMPONENTS OF FLUORESCENT DERIVATIVES OF PE DERIVED FROM EGG PC (ON A NOVAPAK C_{18}, 300 × 3.9 mm I.D. COLUMN)

TAAP[a]	Capacity factor (k') of component					
	1	2	3	4	5	6
Fluorescein-labeled PE [acetonitrile–methanol–water (70:22:8)]						
TBAP	0.4	0.4	0.4	0.4	0.4	0.4
PTAP	0.6	0.6	1.2	1.2	2.0	2.0
OTAP	3.1	3.1	5.4	5.4	9.0	9.0
DTAP[b]	21.8	22.6	36.0	37.2	61.5	65.3
Dansyl-labeled PE [acetonitrile–methanol–water (70:28:2)]						
TBAP	4.2	4.2	7.0	7.0	11.0	11.0
PTAP	3.2	3.2	5.0	5.0	8.0	8.0
OTAP	5.2	5.2	8.2	8.2	12.6	12.6
DTAP	9.8	9.8	15.6	15.6	25.0	25.0
Pyrene-labeled PE [acetonitrile–methanol–water (70:28:2)]						
TBAP	5.6	5.6	7.6	7.6	14.1	14.1
PTAP	5.2	5.2	8.2	8.2	13.4	13.4
OTAP	7.2	7.2	11.5	11.5	33.6	33.6
DTAP	11.5	11.5	18.0	18.0	29.3	29.3

[a] Concentration of TAAP is 5 mM.

[b] The α values of component pairs 1–2, 3–4, and 5–6 are 1.04, 1.03 and 1.06, respectively. All other component pairs 1–2, 3–4, and 5–6 shown in this table were not resolved ($\alpha = 1.0$). For component identification, see footnote to Table I.

processes were typical of an ion-pair retention mechanism. Interestingly, fluorescein-labeled PE complex was inadequately resolved into three major components, when a low member of TAAP in the series was present at a low concentration in the mobile phase of relatively low water content (*e.g.* 2% water shown in Table I). Examples of the poor resolution of the PE mixture can also be seen in the table where 2.50 m*M* of DTAP or 10 m*M* of PTAP (or OTAP) was used in mobile phases containing 8% of water. Separation factors (α) for the unresolved component pairs 1–2, 3–4, and 5–6 were 1.00. However, a total of six major components (three sets of doublets each having α values greater than 1.00) were obtained with mobile phases containing either 20 m*M* of OTAP or DTAP at concentrations greater than 5 m*M* in acetonitrile–methanol–water (70:22:8). For dansyl- and pyrene-labeled PE, resolution of subcomponents was apparently incomplete because only three major components (each unresolved pair has an α value of 1.00) are visible from the HPLC data in Table II.

Table IV shows the positive concentration effects of TAAP on the k' values of the subcompo-

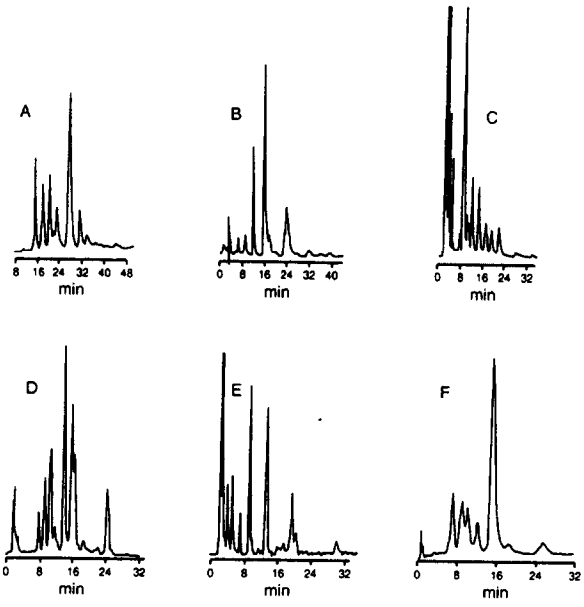

Fig. 4. Comparisons of HPLC–UV chromatograms of underivatized parent phospholipids (PLs) [(A) Egg-PE; (B) soybean-PE; (C) brain-PS] with HPLC–fluorescence chromatograms of the corresponding dansylated PLs (D, E, F). Columns, (A, B, C) NovaPak C18; (D, E, F) Beckman Ultrasphere ODS. Mobile phases, acetonitrile–methanol–water [(A, B, D, E) 70:28:2; (C, F) 70:22:8] containing (A, B) 25 m*M* TMAP; (C) 5 m*M* TMAP; (D, E, F) 10 m*M* HPTAP.

TABLE IV

HPLC SEPARATIONS OF SUBCOMPONENTS OF DANSYLATED EGG PE AND BRAIN PS (ON A BECKMAN ULTRASPHERE ODS COLUMN, 150 × 4.6 mm I.D. COLUMN)

Mobile phase conditions		Capacity factor (k') of component[c]					
TAAP	Concentration (m*M*)	1	2	3	4	5	6
Dansylated egg PE							
TMAP[a]	10.0	1.8	2.2	2.7	3.8	4.6	7.5
HPTAP[a]	5.0	1.2	1.3	1.7	2.5	3.1	5.0
	10.0	2.2	2.8	3.3	4.6	5.7	9.1
Dansylated brain PS							
HPTAP[b]	5.0	2.5	3.3	3.7	4.5	7.8	9.8
	7.5	9.0	11.5	13.6	16.3	20.2	25.7
	10.0	21.0	26.3	31.8	39.2	48.5	79.2

[a] Mobile phase TAAP in acetonitrile–methanol–water (70:28:2).
[b] Mobile phase: HPTAP in acetonitrile–methanol–water (70:22:8).
[c] Component identification: PE, (1) 18:2–18:2, (2) 16:0–20:4, (3) 16:0–18:2, (4) 18:0–20:4, (5) 16:0–18:1 + 18:0–18:2, (6) 18:0–18:1. PS, (1) 16:0–20:4, (2) 18:0–20:4, (3) 16:0–18:1, (4) 18:0–18:2, (5) 18:0–18:1, (6) 18:0–20:1. Fatty acid designations: 20:1 = eicosenoic, 20:4 = arachidonic; for others, see footnote to Table I.

nents of dansylated egg PE and brain PS. Negative concentration effects have been observed in HPLC of the underivatized parent PE or PC [10], in which the charge is internally neutralized. Conversion of the parent neutral PL to negatively charged fluorescent derivatives led to a corresponding change in a retention mechanism from ion-suppression to an ion-pair separation processes. Such a mechanistic change reflected opposite effects of the counter ion concentrations. Comparisons of HPLC–UV chromatograms of underivatized parent egg PE, soybean PE, and brain PS shown in respective Fig. 4A, B, and C with corresponding HPLC–FL chromatograms of their dansylated derivatives (Fig. 4D, E, and F) indicated that generally the component peak patterns are fairly simple and quite similar despite obvious differences in peak intensities. On the other hand, the HPLC–FL chromatogram of dansylated brain PE (Fig. 5B) is much more complex than the HPLC–UV chromatogram of the parent brain PE (Fig. 5A). In Fig. 5, the later-eluting peaks of both the parent and dansylated materials correspond to the subcomponents of PE plasmalogen as confirmed by the characterization of the major components (Table V). Interestingly, the retention behavior of PE plasmalogens exhibiting increased retention times relative to the corresponding diacyl PE is similar to that of alkenylacyl acetylglycerols relative to the diacyl analogues [28]. Most of the peaks attributable to the dansylated brain PE are significantly more intense than those of corresponding underivatized components. Apparently the UV absorptions of the ether lipids having large alkyl mem-

TABLE V

COMPOSITION DATA FOR COMPONENTS OF DANSYLATED PHOSPHOLIPIDS DERIVED FROM NATURAL SOURCES

A Beckman Ultrasphere ODS (150 × 4.6 mm I.D.) was used; mobile phases varied depending on the PL structural type. Values in parentheses are coefficients of variation. Major component identification: brain PE plasmalogen, (3) 18:1–22:6, (4) 18:1–20:4, (5) 18:0–22:6, (7) 18:0–20:1, (9) 18:1–18:1, (10) 16:0–18:1; egg PE, (3) 18:2–18:2, (4) 16:0–20:4, (5) 16:0–18:2, (7) 18:0–20:4, (8) 16:0–18:1, (9) 18:0–18:2, (16) 18:0–18:1; soybean PE, (1) 18:2–18:2, (3) 16:0–18:2, (6) 16:0–18:1, (10) 18:0–18:2; egg PC, (1) 16:0–20:4, (2) 16:0–18:3, (3) 18:1–18:2, (4) 16:0–18:2, (5) 16:0–18:1, (6) 18:0–18:2, (10) 18:0–18:1; for PS components, see footnote to Table IV. Fatty acid designation: 22:6 = docosahexaenoic; for others, see footnotes to Table I and IV.

Component	Composition (%)[a]				
	Brain PS	Brain PE	Egg PE	Soybean PE	Egg PC
1	11.9 (1.5)	2.9 (6.0)	0.1 (9.5)	29.3 (2.0)	3.0 (5.5)
2	12.8 (5.0)	3.8 (4.5)	0.3 (9.0)	1.8 (9.0)	4.2 (5.0)
3	12.3 (3.0)	8.7 (4.0)	4.2 (7.0)	37.7 (1.0)	26.3 (0.5)
4	9.6 (1.5)	10.6 (4.0)	7.6 (6.0)	1.1 (9.5)	7.5 (5.0)
5	47.8 (0.5)	13.4 (3.5)	14.3 (4.0)	1.8 (9.0)	41.3 (0.5)
6	1.7 (9.0)	4.8 (5.0)	4.2 (8.0)	17.6 (2.5)	6.2 (4.5)
7	3.9 (5.5)	8.2 (5.0)	22.5 (1.5)	6.0 (5.0)	0.8 (8.0)
8		6.5 (5.0)	24.5 (1.0)	0.1 (9.5)	0.1 (9.5)
9		10.8 (3.5)	6.1 (3.0)	0.1 (9.5)	0.3 (9.5)
10		11.1 (4.0)	0.6 (8.5)	4.2 (5.0)	10.4 (3.0)
11		4.3 (6.5)	2.5 (6.0)		
12		2.8 (8.0)	0.6 (7.5)		
13		3.1 (6.5)	0.3 (9.5)		
14		3.6 (7.0)	0.7 (9.0)		
15		1.2 (9.5)	1.0 (9.5)		
16		1.7 (9.5)	10.2 (3.0)		
17		2.7 (8.5)	0.2 (9.0)		
18			0.3 (9.0)		

[a] Mean values of triplicate determinations.

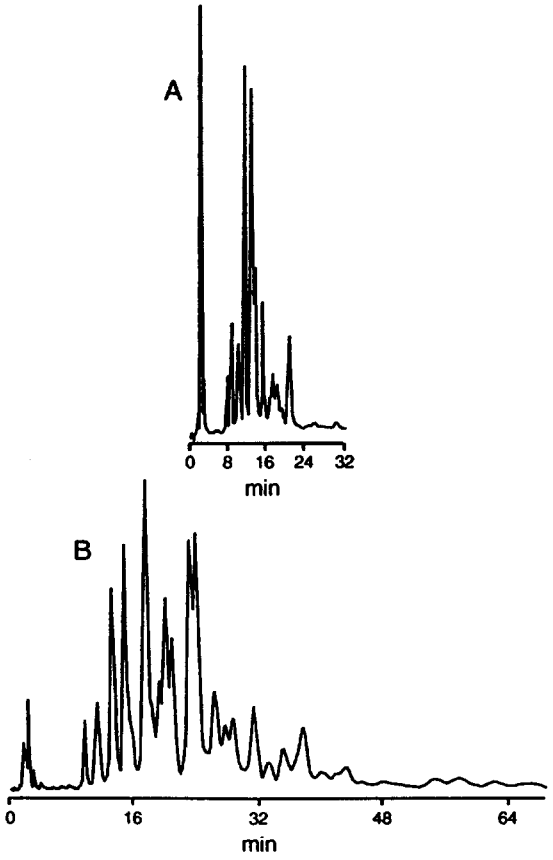

Fig. 5. Comparison of (A) HPLC–UV chromatogram of underivatized parent brain PE and (B) HPLC–fluorescence chromatogram of the dansylated brain PE. Columns, (A) NovaPak C18; (B) Beckman. Mobile phases, acetonitrile–methanol–water (70:28:2) containing (A) 25 m*M* TMAP; (B) 5 m*M* TMAP.

bers of the non-polar tail groups (Fig. 1) were relatively weak.

Compositions of subcomponents of fluorescent-labeled PL were determined directly by computer data processing of the FL detector signals as shown in Table V and Table VI. This is the first report on sensitive, reliable quantitative analyses of intact molecular species of negatively charged PLs. The analytical data for peak integration gave coefficients of variation ranging from 0.5 to 9.5%. On the contrary, direct computation of UV detector responses has proved futile because of variations in absorbance of different molecular species at a given UV wavelength. In HPLC with FL detection, peak areas

TABLE VI

COMPARISONS OF COMPOSITION DATA FOR SUBCOMPONENTS OF VARIOUS FLUORESCENT LABELED PE DERIVED FROM EGG PC

A Beckman Ultrasphere ODS column (150 × 4.6 mm I.D.) was used; mobile phases varied depending on the PL structural type. Values in parentheses are coefficients of variation. Major component identification: pyrene-PE, (1) 16:0–20:4, (2) 16:0–18:3, (3) 16:0–18:2, (6) 16:0–18:1, (9) 18:0–18:2; fluorescein-PE, (3) 16:0–20:4, (4) 16:0–18:2, (5) 16:0–18:2, (8) 16:0–18:1, (9) 16:0–18:1, (10) 18:0–18:2; dansyl-PE, (1) 16:0–20:4, (2) 16:0–18:3, (3) 16:0–18:2, (5) 16:0–18:1, (8) 18:0–18:2. For fatty acid designations, see footnotes to Table I and IV.

Component	Composition (%)[a]		
	Pyrene-PE	Fluorescein-PE	Dansyl-PE
1	2.5 (5.5)	0.8 (7.5)	3.3 (5.0)
2	2.5 (5.5)	0.9 (7.0)	4.5 (5.5)
3	27.7 (1.5)	3.1 (5.0)	26.8 (2.0)
4	1.1 (9.0)	15.3 (4.0)	6.2 (5.0)
5	2.2 (6.0)	21.9 (2.0)	48.2 (1.0)
6	50.5 (0.5)	0.7 (8.5)	0.6 (8.5)
7	4.8 (4.5)	0.3 (9.0)	0.4 (9.0)
8	0.6 (8.0)	20.0 (2.0)	10.1 (4.0)
9	7.9 (5.0)	23.1 (1.5)	
10		3.9 (6.0)	
11		4.4 (5.5)	
12		2.5 (7.5)	
13		3.1 (6.0)	

[a] Mean values of triplicate determinations.

of individual lipid subcomponents were linearly correlatable within the normal range 5 ng–50 μg of analytical samples assayed. The fluorophores of individual analytes were solely responsible for the linearity of peak integrals with the amounts injected as well as for the detection specificity and sensitivity. The minimum FL detection limit at a signal-to-noise ratio of 4/1 was 4 ng, which was substantially lower than that of UV detection (100 μg).

CONCLUSION

In conclusion, the FL detection techniques developed in this study facilitate a convenient means for the analysis of a variety of PL structures. In addition to PE, subcomponents (or molecular species) of PC may be quantified in

the forms of PA, PG, and PS via sequential transphosphatidylation and derivatization with a suitable fluorigenic compound. If a high degree of component resolution is required, fluorescein is the preferred derivatizing agent because of its unusual tendency to resolve structurally similar subcomponents. In view of the abundant occurrence of PC and PE in the nature, the RP-ion pair-HPLC–FL detection method may provide a useful alternative approach to the analysis of these compounds with high reproducibility, sensitivity, and specificity.

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