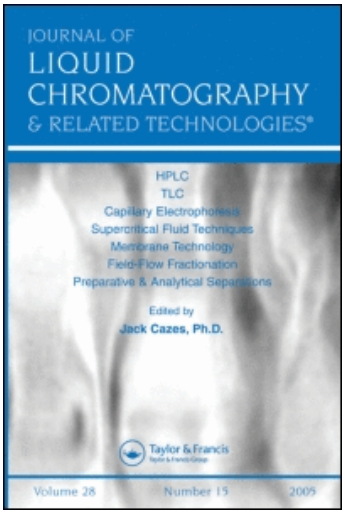

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Reversed-Phase Retention Behavior of Fluorescence Labeled Phospholipids in Ammonium Acetate Buffers

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REVERSED-PHASE RETENTION BEHAVIOR OF FLUORESCENCE LABELED PHOSPHOLIPIDS IN AMMONIUM ACETATE BUFFERS

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ABSTRACT

Subcomponents of fluorescent derivatives of phosphatidylethanolamine (PE) and phosphatidylserine (PS) were resolved by reversed-phase high-performance liquid chromatography (HPLC) with mobile phases containing acetonitrile, methanol, water, and ammonium acetate. The fluorescence labeled phospholipids (PL) include N-(rhodamine B sulfonyl)-PE, N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-PE, N-(5-fluoresceinthiocarbamoyl) (FL)-PE, N-(1-pyrenesulfonyl)-PE, and N-(5-dimethylaminonaphthalene-1-sulfonyl)-PE. Among the compounds studied, FL-PE exhibited the highest degree of selectivity for component resolution. The HPLC behavior of the five PE derivatives was examined under variable concentrations of ammonium acetate. Capacity factors of the PL subcomponents increased with increasing concentrations of the acetate buffers. Incorporation of triethylamine into the mobile phase alleviated peak

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broadening and improved detection sensitivity of polar PL (FL-PE and PS). Fatty acid structures of molecular species in FL-PE were studied by particle beam (PB)-LC-mass spectrometry (MS). Compatibility of the HPLC method with PB-LC-MS is demonstrated.

INTRODUCTION

In general, phospholipids (PL) can be enzymatically or chemically transphosphatidylated into phosphatidylethanolamine (PE). The presence of an amino group in a PE molecule facilitates conversion of the parent compound to various derivatives. Incorporation of a fluorigenic reagent into PE yields a fluorescent derivative which can be sensitively measured with a fluorescence detector.

Molecular species or subcomponents of PL have been separated by reversed-phase high-performance liquid chromatography (HPLC) (1-9). Structurally intact PL subclasses have been analyzed by reversed-phase ion-pair HPLC-UV detection (10-13). HPLC analyses of fluorescent derivatives of diradylglycerols obtained by enzymatic hydrolysis of PL have been documented (14-19). A few reports on HPLC assays of fluorescent derivatives of PL are available in the literature (20-22). In a recent study (23), a new reversed-phase HPLC-fluorescence detection technique has been developed for the direct quantitative determination of compositions of PL molecular species without isolation of components for phosphorus analysis.

Conventionally, molecular species of PL are characterized by fatty acid analyses of isolated individual PL subcomponents. Simultaneous separation and characterization of the lipid subcomponents requires hyphenated techniques such as LC-mass spectrometry (MS) or

LC-nuclear magnetic resonance spectrometry. In LC-MS methodology, mobile phases containing tetraalkyl ammonium phosphates (23) suffer from severe limitations because of their incompatibility with a LC-MS interface. Fortunately, mobile phases with ammonium acetate buffers are vaporizable and therefore amenable to LC-MS analyses. An HPLC study on the retention behavior of various fluorescent derivatives of PE in acetate buffers should pave the way to subsequent structural studies of PL molecular species by on-line LC-MS.

A number of publications have appeared in the literature dealing with off-line MS analyses of PL using various soft ionization techniques (24-30). Although there are two published reports concerning on-line LC-MS studies with moving belt- and thermospray interfaces (31,32), particle beam (PB)-LC-MS analyses of PL molecular species have not been investigated. In this paper, the results of structural verification of subcomponents of a sample of FL-PE by PB-LC-MS are presented.

Our continuous studies of the deterioration of soybean PL during storage and the composition of the polar lipids in modified oil required the development of analytical methods for the separation of PL subcomponents. Analysis of PL in soybean oil provides useful information on the oil stability and the impact of genetic modification on the distribution of plant constituents. In view of the analytical capability of the LC-MS technique for the characterization of individual PL molecular species, the HPLC behavior of several derivatives of PE was studied under mobile phase conditions simulating the LC-MS interface systems. The HPLC results along with the optimization data are reported in this paper.

EXPERIMENTAL

Materials:

Fluorescent derivatives of phosphatidylethanolamine (PE) [N-(rhodamine B sulfonyl) (NRD)-PE, N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) (NBD)-PE, N-(5-fluoresceinthiocarbamoyl) (FL)-PE, N-(1-pyrenesulfonyl) (PY)-PE, and N-(5-dimethylaminonaphthalene-1-sulfonyl) (DAN)-PE] were obtained from Avanti Polar Lipids, INC. (Pelham, AL). All these polar lipids were derived from egg phosphatidylcholine. Egg DAN-PE was obtained from Molecular Probes, Inc. (Eugene, OR). Plant DAN-PE was prepared from plant PE, dansyl chloride and triethylamine in chloroform solution as described previously (23). Brain dansylated phosphatidylserine (DAN-PS) was the product of Avanti. Commercial phospholipids (PL) were used as received without further purification. PL samples were stored in a freezer at -30°C whenever not in use. Dodecyltriethyl ammonium phosphate and triethylamine were obtained from Regis Chemicals (Morton Grove, IL). HPLC grade ammonium acetate and HPLC solvents acetonitrile and methanol were obtained from J. T. Baker, Inc. (Phillipsburg, NJ). Ultrapure HPLC grade water was obtained by filtering distilled water through a Milli Q water purifier system (Millipore Inc., Bedford, MA).

Methods:

High-performance liquid chromatography: A Model SP8700 liquid chromatograph (Spectra Physics, San Jose, CA) interfaced with a Model 980 Programmable Fluorescence detector (Applied Biosystems Inc., Foster City, CA) was

used for most of the HPLC separations unless otherwise specified. Wavelength parameters for various fluorophores were: DAN-PE (and DAN-PS), excitation 338 nm, emission 470 nm; FL-PE, excitation 489 nm, emission 550 nm; PY-PE, excitation 342 nm, emission 398; NRD-PE, excitation 563 nm, emission 585 nm; NBD-PE, excitation 460 nm. emission 534 nm. Mobile phases were acetonitrile-methanol-water containing 5-50 mM ammonium acetate and were prepared immediately before use. Aliquots of freshly prepared analytical samples (10-20 ul of 2 mg/ml solutions) were injected via a Model 7125 injector (Rheodyne Co., Cotati, CA) (25-ul loop) onto a reverse-phase HPLC column. The column packings were of NovaPak C18 prepacked in a stainless steel column (300 mm x 3.9 mm I.D., 4 um) as supplied by Waters Associates (Milford, MA). In all HPLC analyses, mobile phases were filtered, degassed, and pumped through the column at a flow rate of 1 ml/min. Capacity factors (k') were determined from the equation $k' = t/t_o - 1$, where t and t_o are the retention times of an analyte and an unretained solute, respectively.

Capillary gas chromatography: Molecular species of fluorescent PL were isolated by collecting individual HPLC peak components which were then treated with methanol and hydrochloric acid to yield fatty acid methyl esters using a published procedure (33). The fatty acid methyl esters were analyzed with a Varian (Palo Atto, CA) Model 3400 gas chromatograph equipped with a flame ionization detector. A fused silica capillary column (0.25 mm x 30 m) coated with 0.2 um SP 2330 (Supelco Inc., Bellefonte, PA) was used throughout the analyses. In a typical GC analysis, the column temperature was initially held at 200°C for 15 min and then increased from 200 to 220°C at a rate of 10°C/min.

Particle beam liquid chromatography-mass spectrometry: PB-LC-MS analyses were performed in the EI mode on a Vestec Model 201 LC-MS (Vestec Corp., Houston, TX), equipped with a Universal Interface. The chromatographic system used was a Kratos Spectroflow 400 Ternary Pumping System (Kratos Analytical, Ramsey, NJ) equipped with a 400 nm UV detector. A sample (100 ug) of FL-PE was injected onto an unused NovaPak C18 column (different from the one used in HPLC analyses) and eluted with a mobile phase of acetonitrile-methanol-water (70:20:10) containing 35 mM ammonium acetate at a flow rate of 1.2 ml/min. The Teknivent Vector/One Mass Spectrometry Data System (Teknivent Corp., St. Louis, MO) was used for acquiring and processing data.

RESULTS AND DISCUSSION

Fig. 1 shows structures of the five fluorescence labeled PE investigated. Of these derivatives, FL-PE (Fig. 1B) and NRD-PE (Fig. 1A) are of particular interest for comparative study of their HPLC behavior because of the similarity in ring structures. In the absence of mobile phase electrolyte, the polar lipids each having a negative charge at the phosphoryl moiety exhibited little retention on a reversed-phase column. However, HPLC with mobile phases containing ammonium acetate buffers led to variable degrees of analyte retention as well as component separations, depending on the nature of fluorophores attached to the amino group of PE. The observed retention of PL components on the hydrocarbonaceous stationary phase as a result of adding ammonium acetate to mobile phases is parallel to that found in HPLC with and without tetraalkyl ammonium phosphates in mobile phases (23).

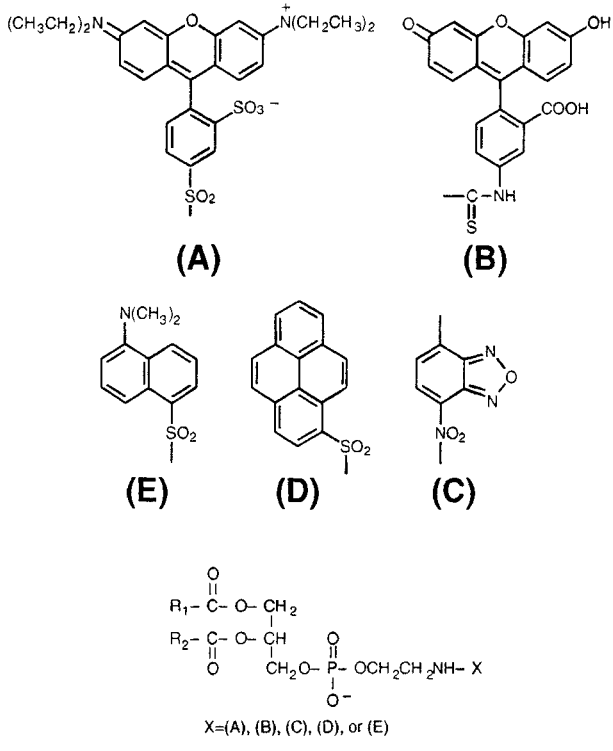

Fig. 1. Structures of fluorescent derivatives of phosphatidylethanolamine (PE) investigated: (A) NRD-PE, (B) FL-PE, (C) NBD-PE, (D) PY-PE, and (E) DAN-PE.

Fig. 2 shows separations of molecular species of different fluorescent derivatives of PE derived from egg PC. The three major components 1-3 in Fig. 2A-2E correspond to those listed in Tables I-III. It is noteworthy that each of the three major components in FL-PE were further split into a-b doublets (Fig. 2B and 2B'). Although the rhodamine fluorophore in NRD-PE possesses the same aryl tricyclic ring structure as the fluorescein counterpart in FL-PE (Fig. 1A vs 1B), only three major

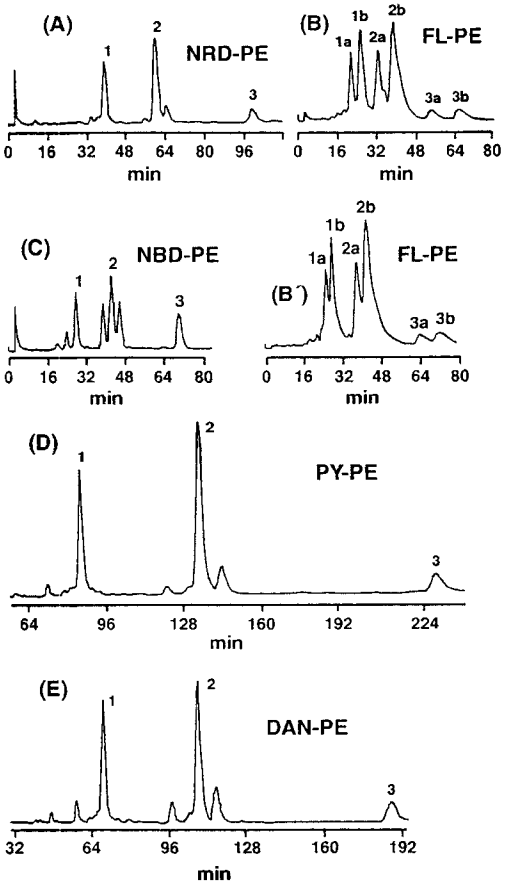

Fig. 2. HPLC separations of subcomponents of fluorescent derivatives of phosphatidylethanolamine derived from egg phosphatidylcholine. Mobile phase: acetonitrile-methanol-water (70:25:5) containing 50 mM ammonium acetate. Triethylamine (50 mM) was added to the mobile phase in (B'). Column: used NovaPak C18. Flow rate: 1 ml/min.

peaks are visible in the chromatogram of the former derivative (Fig. 2A vs 2B). The differences in the types of substituents on the ring system evidently brought about the differences in the chromatographic outcome. Nonetheless, the exact nature of the structural effect of the fluorescein moiety on component resolution is unclear. Incorporation of triethylamine into the mobile phase used in Fig. 2B led to a nearly four-fold increase in detection sensitivity at the slight expense of selectivity for component resolution as shown in Fig. 2B. This may be explained in terms of reduced adsorption of FL-PE analytes on the stationary phase used. The acidic carboxyl- and hydroxy groups in fluorescein appeared to cause adsorption of analyte solutes on the octadecylsilica (ODS) phase.

Retention of all resolved components of PE derivatives on ODS was quite sensitive to the variation in water content of mobile phases employed. As demonstrated in Table I, a small increase in the percentage of water in mobile phases resulted in a drastic increase in capacity factors (k') values. The retention data in Table I provide optimal ranges of mobile phase solvent compositions so that k' values of individual analyte components can be measured within reasonable limits of retention times during routine PL assays. The observed short retention times of all the compounds studied in HPLC without ammonium acetate (at zero concentration) suggested that the PL solutes were in the form of ionized species in the separation processes. Addition of variable amounts of ammonium acetate to mobile phases resulted in retention of the polar lipid on ODS, which is indicative of diminished ionic characteristics (or increased hydrophobicity) of the analyte species. Table II shows the general trend of concentration effects of

Table I. Effect of mobile phase solvent composition on k' values of various fluorescence labeled PE derived from egg PC

Subcomponent	k'*				
Mobile phase NH_4OCOCH_3 (25 mM)	PL compound				
	FL-PE	DAN-PE	PY-PE	NBD-PE	NRD-PE
Acetonitrile-methanol-water (70:25:5)					
1	8.0 (9.2)	23.0	26.6	12.2	19.4
2	12.2 (14.4)	36.0	42.3	18.5	30.2
3	20.8 (25.4)	58.4	70.0	31.0	50.4
Acetonitrile-methanol-water (70:28:2)					
1	4.6 (5.1)	11.0	13.3	6.3	8.1
2	7.0 (7.8)	17.4	20.4	9.0	12.0
3	10.8 (12.2)	27.4	32.6	13.2	19.8

* Values in parentheses are for the additional FL-PE peaks further resolved.

ammonium acetate on k' values of the major components of various PE derivatives. Higher k' values (longer retention times) were obtained with mobile phases containing higher concentrations of ammonium acetate. The mobile phase variables (solvent compositions and acetate concentrations) discussed above can be used for optimization of retention parameters to meet separation requirements for specific HPLC experiments.

Table III compares the HPLC results obtained with different mobile phase electrolytes, ammonium acetate and

Table II. Effect of ammonium acetate concentration on k′ values of various fluorescence labeled PE derived from egg PE

Subcomponent	k′**				
Mobile phase* NH_4OCOCH_3 concentration	PL compound				
	FL-PE	DAN-PE	PY-PE	NBD-PE	NRD-PE
50.0 mM					
1	6.0 (7.25)	13.6	16.4	7.2	9.7
2	9.1 (10.6)	22.0	25.2	10.6	14.4
3	14.2 (17.2)	34.6	40.2	16.5	23.4
12.5 mM					
1	3.4	7.2	9.0	5.1	6.5
2	5.0	11.2	13.8	7.7	10.0
3	7.4	17.4	21.2	11.8	16.2

* Mobile phase:acetonitrile-methanol-water (70:28:2) containing variable concentrations of ammonium acetate.

**Values in parentheses are for the additional FL-PE peaks further resolved.

dodecyltriethyl ammonium phosphate (DTAP). The presence of a hydrocarbonaceous electrolyte (DTAP) at a relatively low concentration (5 mM) in the mobile phase considerably enhanced hydrophobic interactions between PY-PE (and DAN-PE) solutes and the octadecylsilica phase. For three other derivatives (FL-PE, NBD-PE, and NRD-PE), much higher concentrations of electrolytes were required to show significant differences between the k′ values obtained with

Table III. Comparison of retention data for fluorescence labeled PE studied in two different mobile phase electrolyte systems

Subcomponent	k'**				
Mobile phase* electrolyte (5 mM)	PL compound				
	FL-PE	DAN-PE	PY-PE	NBD-PE	NRD-PE
Ammonium acetate					
1	3.6 (4.0)	10.4	12.6	6.4	10.2
2	5.6 (6.2)	17.0	20.5	10.4	16.2
3	9.4 (10.6)	28.2	35.4	16.8	27.8
Dodecyltriethyl ammonium phosphate					
1	3.2	27.4	39.8	9.4	13.0
2	5.4	45.0	64.2	14.8	19.8
3	8.6	78.1	111	25.6	33.3

* Mobile phase:acetonitrile-methanol-water (70:25:5) containing an electrolyte (indicated above) at pH 6.5.
**Values in parentheses are for the additional FL-PE peaks further resolved.

the two electrolyte systems. The ammonium acetate mobile phase appeared to have a greater tendency for resolving the a-b subcomponents than the tetraalkyl ammonium phosphate mobile phase. Examination of HPLC data compiled in Tables I-III revealed that variations in the magnitude of k' values among the PL derivatives reflect disparity in polarity of different PL structures. Accordingly, the retention order (k'-PY-PE > k'-DAN-PE > k'-NRD-PE >

k'-NBD-PE > k'-FL-PE) for the PE compounds in the series follows an increasing order of polarity of the polar lipids (PY-PE < DAN-PE < NRD-PE <NBD-PE < FL-PE).

Examples for analyses of dansylated PL derived from other natural sources are presented in Fig. 3. For separations of dansylated egg PE (Fig. 3A) and dansylated soybean PE (Fig. 3B), there was a striking similarity between the separation profiles obtained with mobile phases containing ammonium acetate and those with mobile phases containing tetraalkyl ammonium phosphate reported previously (23). On the other hand, HPLC of dansylated brain PS with ammonium acetate mobile phase produced peak broadening and tailing (Fig. 3C). The carboxyl group in PS seemed to cause severe adsorption of the PS components on the reversed-phase column. However, separations of components were substantially improved by the addition of triethylamine to the ammonium acetate mobile phase (Fig. 3C).

Capillary GC fatty acid analyses of hydrolysates of individual fractions 1a, 1b, 2a, 2b, 3a, and 3b isolated from HPLC of FL-PE (Fig. 2B) showed that these components were attributed to molecular species containing fatty acids 16:0-18:2 (1a-b), 16:0-18:1 (2a-b), and 18:0-18:1 (3a-b). When the same sample of FL-PE was analyzed under different HPLC conditions such as those used in the later LC-MS experiment with a new NovaPak column, different mobile phase conditions (see Experimental on LC-MS procedure), two additional minor components 2c and 2d were resolved (Fig. 4).

Initial attempts at using a thermospay-LC-MS technique for the analysis of FL-PE were unsuccessful. Subsequent

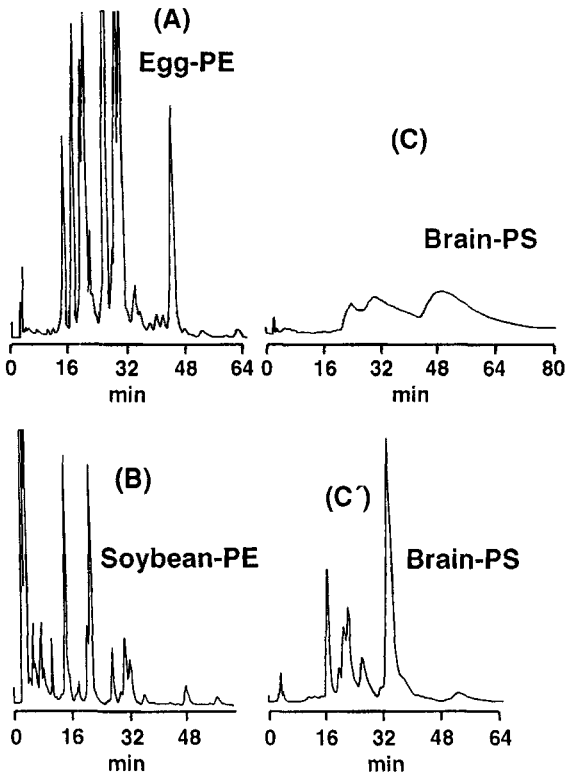

Fig. 3. HPLC separations of subcomponents of dansylated phospholipids derived from animal and plant sources. Mobile phases: acetonitrile-methanol-water (70:28:2) containing 25 mM (A, B) or 5 mM (C, C') ammonium acetate. Triethylamine (15 mM) was added to the mobile phase in (C'). Column: used NovaPak C18. Flow rate: 1 ml/min.

elaboration of a particle beam (PB)-LC-MS instrument system on the FL-PE sample met with some success. With the exception of components 3a and 3b whose intensities were too weak to be measurable, the total ion chromatogram showing the separation of components 1a, 1b, 2a, 2b, 2c,

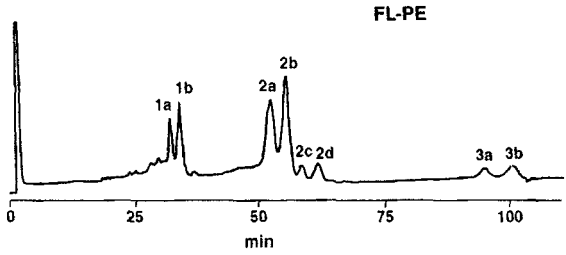

Fig. 4. HPLC separation of molecular species of fluorescein (FL) labeled phosphatidylethanolamine (PE) derived from egg phosphatidylcholine. Column: unused NovaPak C18. Mobile phase: acetonitrile-methanol- water (70:20:10) containing 35 mM ammonium acetate at a flow rate of 1.2 ml/min.

and 2d was very similar to the HPLC profile in Fig. 4. Since the PB-LC-MS system was operated in the EI mode, molecular ions of the polar lipid components were not detected. Each of the EI mass spectra of ion peaks exhibited major mass fragments of fatty acid chain moieties and those of the fluorescein fluorophore. The fragmentation patterns for the fatty acid region were particularly informative for the characterization of PL molecular species. The individual EI mass spectra of molecular species were closely superimposable with composite spectra of two standard fatty acids. The MS data (not shown here) indicated that the EI fragmentation patterns between a- and b-components (or between c- and d-component) were indistinguishable.

In summary, molecular species of fluorescence labeled PL can be analyzed by reversed-phase HPLC with ammonium acetate mobile phases. For enhancing detection of polar PL (FL-PE and DAN-PS), adsorption of analytes on a column can

be eliminated by adding triethylamine to the ammonium acetate mobile phase. The LC-MS technique used for the characterization of fatty acid structures has proven useful and may be applied to the analysis of other PL compounds.

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