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Reversed-phase high-performance liquid chromatography of molecular species of phospholipid derivatives[1]

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Abstract

Molecular species of phospholipid (PL) derivatives in two structural series phosphatidic acid (PA) and phosphatidylethanolamine (PE) were separated on octadecylsilica by reversed-phase high-performance liquid chromatography (HPLC)–UV detection. Using a mobile phase of acetonitrile–methanol–water–quaternary ammonium phosphate (QAP), the individual PLs derived from egg phosphatidylcholine were resolved into six major molecular species. The QAP electrolytes served as ion-pair counter-ions or ion-interactants depending on PL structures and on the QAP concentrations. Correlation of logarithmic capacity factors (k') of anionic PLs with the number of carbon atoms in QAP established linear relationships. The $\ln k'$ values of compounds in PA and PE series containing respective O–H and N–H functionalities were not linearly proportional to the number of carbon atoms in their corresponding O-alkyls and N-methyls. The k' values of negatively charged PLs increased with increasing QAP concentrations. Conversely, inverse relationships between the QAP concentrations and k' values of PE derivative were observed in HPLC with low QAP concentrations. PA derivatives were useful for trace analyses.

Keywords: Mobile phase composition; Phospholipids; Quaternary ammonium compounds; Phosphatidic acid; Phosphatidylethanolamine; Phosphatidylcholine

1. Introduction

Phospholipids (PLs) are important substances found in cell membranes of animals and plants. Molecular species of individual PL classes exist in nature as complex mixtures particularly in samples of animal origins. In general, the structures of molecular species are very similar differing only in the alkyl or alkenyl groups of fatty acyl chains at the C_1 and C_2 positions of the glycerol backbone (Fig. 1). It is recognized that variations in the distribution of PL molecular species alter chemical and physical properties of cell membranes. Our continuous interest in the PL analytical methodology provides impetus to further explore the separation potential of various PL subclass species.

Because of the complexity of closely related structures with ionic charges, mixtures of PL molecular species have not been readily resolved by conventional reversed-phase high-performance liquid chromatography (HPLC) with simple solvents. In the

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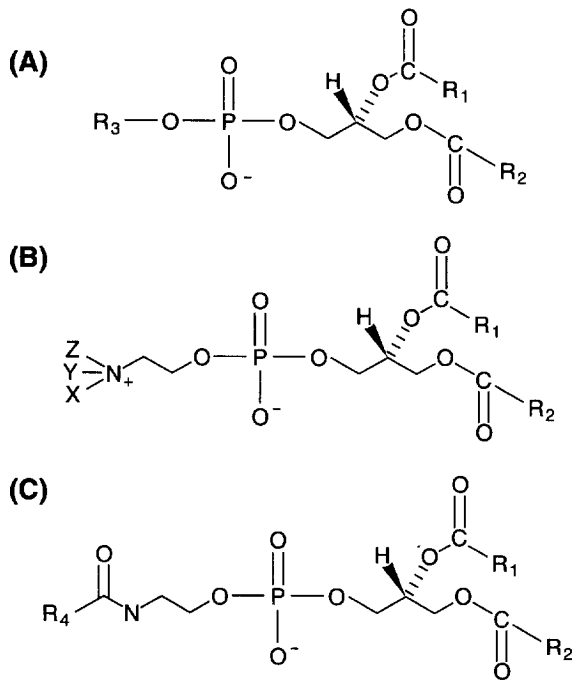

Fig. 1. Structures of investigated PL derivatives where R_1 and R_2 represent respective alkyl and alkenyl groups of fatty acid chains. (A) PA, R_3=H; Phosphatidylalkanol, R_3=alkyl(C_1–C_4). (B) PE, X=Y=Z=H; PEMM, X=CH_3, Y=Z=H; PEDM, X=CH_3, Y=CH_3, Z=H; PC, X=Y=Z=CH_3. (C) PECA, R_4=aminopentyl; PEDC, R_4=carboxydecanyl; PEDA, R_4=aminododecanyl.

absence of mobile phase electrolyte additives, negatively charged PL [e.g., phosphatidic acid (PA)] species elute through an alkylsilyl reversed-phase column with little retention [1], while neutrally charged PL molecular species [e.g., phosphatidylcholine (PC) and phosphatidylethanolamine (PE)] are partially adsorbed on the alkylsilica phase (e.g., an octadecylsilica (ODS) column) resulting in unusually long retention times and peak broadening [2]. The latter chromatographic behavior of the neutral PLs would lead to inaccurate quantification by reversed-phase HPLC.

We recently demonstrated that incorporation of electrolytes into mobile phases significantly improved the chromatographic behavior of molecular species of both negatively and neutrally charged PL species on ODS [1–7]. In these studies, reversed-phase ion-pair and ion-interaction HPLC techniques were utilized in conjunction with the use of quaternary ammonium phosphates (QAPs) in mobile phases for the separation of the PL molecular species.

Numerous other reversed-phase HPLC methods for the analysis of PLs have been reported in the literature [8–16]. Few studies have dealt with the HPLC separation of the polar lipids with structural modification at the nitrogen- and oxygen moieties of their head groups. In an earlier study, we observed a notable enhancement in the resolution of molecular species of PA-monomethyl ester in comparison to the underivatized parent PA [1]. Here we report the results of a systematic study on the reversed-phase HPLC separation of selected derivatives of PA and PE (PC is included as a PE derivative). Factors affecting their retention are delineated.

2. Experimental

2.1. Materials

PLs and their derivatives were obtained from Avanti polar lipids (Alabaster, AL, USA). Two structural types of PLs were used. Type-I structures include PA, phosphatidylmethanol (PAMM), phosphatidylethanol (PAME), phosphatidylpropanol (PAMP) and phosphatidylbutanol (PAMB). Type-II structures include PE, monomethyl-PE (PEMM), dimethyl-PE (PEDM), PC, N-caproylamine-PE (PECA), N-dodecanylamine-PE (PEDA) and N-dodecanyl-PE (PEDC). All phospholipid samples were stored in amber vials at −30°C in a freezer. Tetrabutyl ammonium phosphate (TBAP), pentyltriethyl ammonium phosphate (PTAP), heptyltriethyl ammonium phosphate (HTAP), octyltriethyl ammonium phosphate (OTAP) and dodecyltriethyl ammonium phosphate (DTAP) were obtained from Regis Chemicals (Morton Grove, IL, USA). Homologues of tetraalkyl (C_1–C_3) ammonium phosphates (TAAP) were freshly prepared from the corresponding tetraalkylammonium hydroxide (Aldrich, Milwaukee, WI, USA) as described previously [4]. Acetonitrile and methanol were HPLC-grade products of EM Science (Gibbstown, NJ, USA) and Fisher Chemicals (Fair Lawn, NJ, USA), respectively. Pure HPLC water was obtained by passing distilled water through a Milli-Q water purifier (Millipore, Bedford, MA, USA).

2.2. High-performance liquid chromatography

A Thermo Separation Products (Fremont, CA, USA) Model SP8700 liquid chromatograph was used in all HPLC experiments. The liquid chromatography (LC) solvent delivery system was coupled to a SpectroMonitor D multiple-wavelength UV detector (LDC Analytical, Riviera Beach, FL, USA). Column effluents were monitored at 208 nm. Mobile phases were freshly prepared by mixing solutions of acetonitrile in methanol with water that contained different concentrations of QAPs at pH 6.5. The HPLC eluents were degassed with helium sparge, filtered through a 0.02-μm filter, and pumped under isocratic elution at a flow-rate of 1 ml/min.

Aliquots (1–2 μl) of sample solutions (10 mg/ml) were injected onto a reversed-phase column via a Rheodyne (Cotati, CA, USA) Model 7125 injector fitted with a 10-μl loop.

Three different commercially prepacked ODS columns were used. These columns include (1) Ultrasphere ODS, 5 μm, 250×4.6 mm I.D. (Beckman Instruments, San Ramon, CA, USA), (2) NovaPak C_{18}, 4 μm, 300×3.9 mm I.D. (Waters Chromatography, Milford, MA, USA), and (3) Prodigy ODS-2, 5 μm, 250×4.6 mm I.D. (Phenomenex, Torrance, CA, USA).

Capacity factors ($k' = t/t_o - 1$) were computed from retention times of analytes (t) and uracil (t_o). The retention times were average values obtained from three replicate injections of individual samples. Separation factors ($\alpha = k'_{c+1}/k'_c$, where "c" denotes an analyte component) were measured for adjacent molecular species components.

The HPLC peaks were identified by peak matching using synthetic standards or by fatty acid analyses of collected peak components as described below.

2.3. Capillary gas chromatography (GC)

Molecular species of egg PC were collected and converted to fatty acid methyl esters by transesterification with methanolic hydrochloric acid following a published procedure [17]. The methyl esters were determined by capillary GC. A Varian Model 3400 gas chromatograph equipped with a flame ionization detector was used. The methyl ester samples were injected onto a fused-silica capillary column (30× 0.25 mm I.D.) coated with Supelco 0.2 μm SP2330. 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. Since all other investigated PA and PE compounds were derived from egg PC, their molecular species were not collected for fatty acid analyses and their peak components were presumably the same as those of the egg PC species characterized.

3. Results and discussion

Structures of the PL derivatives under consideration are depicted in Fig. 1. Basically, two structural series were chosen for reversed-phase HPLC evaluation. The PA and PE series contain ionic structures having either negative charges or neutral charges at the polar head groups. The compounds in the PA series consist of the parent PA and four homologues of monoalkyl esters (Fig. 1A). The compounds in the PE series are further divided into two sub-series which include (i) the parent PE, three N-methyl-PEs (Fig. 1B), and (ii) three N-acyl-PEs (Fig. 1C). Modifications of PL structures and ionic characteristics have been shown to have significant bearing on their chromatographic behavior [1,2,6].

A comparison of HPLC chromatograms in Fig. 2 indicated that the separation of molecular species of the monobutyl ester derivative, PAMB (Fig. 2B), was remarkably better than that of the underivatized parent PA (Fig. 2A). The latter PA complex remained virtually unresolved ($\alpha = 1.00$ for each adjacent pair of molecular species) under the identical HPLC conditions employed. The α values for the PA derivatives ranged from 1.05 to 1.38 (Table 1).

Similarly, when HPLC chromatograms (Fig. 3) of PE and its derivatives, N-trimethyl-PE (PC) and N-dodecanylamine-PE (PEDA), were compared, discernable improvement in the separation of the six major components (having identical fatty acid chains for the corresponding species) in the three PE compounds was observed. In these cases, the weak UV-absorbing species in both PC (Fig. 3B) and PEDA (Fig. 3C) were more distinctly separated from the strong UV-absorbing components than the underivatized PE species (Fig. 3A). The α values for

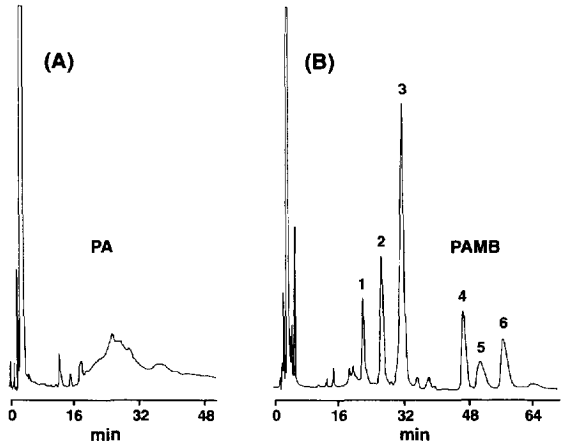

Fig. 2. Comparison of reversed-phase HPLC separations between PA (A) and its butanol ester derivative PAMB (B) on a NovaPak C_{18} column. Mobile phase: CH_3CN–CH_3OH–H_2O (70:22:8) containing 5 m*M* PTAP at pH 6.5. Component identification: (1) 16:0–22:6, (2) 16:0–20:4, (3) 16:0–18:2, (4) 16:0–18:1, (5) 18:0–20:4, (6) 18:0–18:2.

the investigated compounds in the PE series ranged from 1.06 to 1.42 (Table 2).

The retention data in Table 1 and Table 2 show that capacity factors (k') of molecular species of respective PA and PE derivatives increased with increasing molecular masses of QAPs. Thus, k' (PTAP)<k' (HTAP)<k' (OTAP)<k' (DTAP). Within the same structural series, the PL derivatives with longer carbon chain length or with more alkyl substituents were more strongly retained by an ODS phase. Under identical mobile phase conditions, a normal elution order for reversed-phase HPLC was observed for compounds in each of the three structural series investigated: (1) for PA-O-alkyl derivatives, k' (PAMM)<k' (PAME)<k' (PAMP)<k' (PAMB), (2) for PE-N-alkyl derivatives, k' (PE)<k' (PEMM)<k' (PEDM)<k' (PC), (3) for PE- aminoalkylcarbonyl derivatives, k' (PECA)<k' (PEDA). Of the PE species evaluated, PEDC showed least retention on ODS due to the presence of a polar anionic carboxylate functionality at its head group (Fig. 1).

Representative trends of QAP structural effects on PL retention characteristics of selected PL derivatives are illustrated in Fig. 4 and Fig. 5. Correlation of ln k' values of PL molecular species with the total number of carbon atoms (N) in QAPs produced two different sets of straight lines (Fig. 4). The magnitude of slope values were dependent on whether symmetrically or unsymmetrically substituted QAPs were used in the mobile phases. Generally, HPLC with the unsymmetrically substituted QAPs led to steeper (greater slope values) correlation lines than with the symmetrical QAP counter-ions. In other words, retention times of PL analytes in HPLC with the unsymmetrical QAPs were more responsive to variations in hydrophobicity of QAP homologues than those obtained with the symmetrical QAPs. The linear relationships observed in these cases are indicative of a reversed-phase HPLC retention mechanism in which the total areas of the QAP tetraalkyl groups were available for solvophobic interactions.

On the other hand, correlation of ln k' values of alkyl analogues of PLs with the number of carbon atoms (N) in their alkyl substituents yielded nonlinear curves as demonstrated in Fig. 5. Two separate sets of seemingly concave and convex curves were obtained for PA-O-alkyl esters (Fig. 5A) and PE-N-methyl derivatives (Fig. 5B), respectively. Under the same experimental conditions, the k' values of the monoalkyl esters (N=1–4) of PA were much higher than those of the parent PA (N=0), while a relatively small increase in k' values with increasing carbon chain length (N>0) were manifested by the homologues of PA esters (Fig. 5A). In contrast, a sharp decrease in k' values was noted when the retention characteristics of PE (N=0) and other N-substituted PE (N=1 or 2) were compared with those of PC (N=3) (Fig. 5B). Inspection of the plots in Fig. 5B revealed that the change in the k' values with N among the N-methyl-PE analogues containing polar N–H bonding was comparatively small. The permethylated derivative, PC (i.e., the N-trimethylated PE derivative), seemed to acquire the high degree of hydrophobicity (high k' values) by virtue of deprivation of N–H bonding from its molecule.

Effects of QAP concentrations on the retention characteristics of PL were found to depend not only on PL structures but also on levels of QAP concentrations (Table 3, Table 4, Table 5). Without exceptions, HPLC with more concentrated solutions of QAPs in mobile phases resulted in greater retention (higher k' values) of the PA-derived molecular species on ODS (Table 3). The normal relationships (defined as positive QAP concentration effects)

Table 1
Effects of the type of quaternary ammonium phosphates (QAPs) on capacity factors, k', of molecular species of monoalkyl esters of PA derived from egg PC[a]

QAP pH 6.5	Molecular species										
	1		2		3		4		5		6
	k'	α	k'	α	k'	α	k'	α	k'	α	k'
PTAP (10 m*M*)											
PAMM	3.75	1.22	4.58	1.16	5.33	1.36	7.25	1.11	8.08	1.05	8.50
PAME	4.08	1.21	4.92	1.18	5.83	1.36	7.92	1.10	8.67	1.08	9.25
PAMP	4.53	1.21	5.47	1.17	6.42	1.35	8.67	1.12	9.68	1.07	10.4
PAMB	5.01	1.20	5.99	1.18	7.07	1.36	9.59	1.12	10.7	1.07	11.4
HTAP (10 m*M*)											
PAMM	4.74	1.20	5.67	1.16	6.58	1.38	9.08	1.08	9.84	1.08	10.6
PAME	5.16	1.19	6.15	1.18	7.26	1.35	9.83	1.09	10.7	1.08	11.5
PAMP	5.59	1.21	6.75	1.17	7.92	1.38	10.9	1.08	11.8	1.07	12.6
PAMB	6.00	1.22	7.32	1.18	8.67	1.37	11.9	1.09	13.0	1.08	14.0
OTAP (10 m*M*)											
PAMM	5.26	1.21	6.36	1.19	7.54	1.35	10.2	1.10	11.2	1.08	12.1
PAME	5.64	1.19	6.69	1.20	8.00	1.34	10.7	1.10	11.8	1.09	12.9
PAMP	6.42	1.16	7.46	1.22	9.12	1.36	12.4	1.10	13.7	1.07	14.6
PAMB	6.96	1.23	8.58	1.20	10.3	1.33	13.7	1.11	15.2	1.07	16.3
DTAP (10 m*M*)											
PAMM	8.35	1.22	10.2	1.19	12.1	1.31	15.8	1.15	18.2	1.07	19.4
PAME	8.50	1.22	10.4	1.17	12.2	1.37	16.7	1.11	18.5	1.07	19.8
PAMP	10.3	1.21	12.5	1.18	14.8	1.36	20.2	1.10	22.3	1.08	24.0
PAMB	11.9	1.22	14.5	1.19	17.2	1.37	23.5	1.10	25.8	1.08	27.8

PAMM=phosphatidylmethanol, PAME=phosphatidylethanol, PAMP=phosphatidylpropanol and PAMB=phosphatidylbutanol. For identification of molecular species, see Fig. 2.
[a]Column: Beckman Ultrasphere ODS. Mobile phase solvents: acetonitrile–methanol–water (49:49:2).

between QAP concentrations and k' values were typical of reversed-phase ion-pair HPLC of anionic PLs [1,3–7]. The presence of high QAP concentrations appeared to enhance hydrophobic interactions between ODS and analyte solutes presumably via ion-pairing partitioning processes. Differences in k' values of PA-related species obtained with 50 m*M* and 10 m*M* of TMAP were substantially larger than those found in experiments with 10 m*M* and 5 m*M* of HTAP (Table 3).

On the contrary, the QAP concentration effects observed in HPLC of PE derivatives (Table 4) were more intriguing and seemed to be much less straightforward than those observed with the PA derivatives, as discussed above. The HPLC data in Table 4 indicate that all but PC exhibited positive concentration effects at relatively high TMAP concentrations between 10 m*M* and 50 m*M*. For PC, a small inverse concentration effect (defined as a negative QAP concentration effect) was observed in this concentration range. Thus, a 20% decrease in TMAP concentrations (50→10 m*M*) caused a slight increase in k' values of PC species. Likewise, at lower QAP electrolyte concentrations, retention characteristics of both PC and PECA were inversely related to the HTAP concentrations ranging from 5 m*M* to 10 m*M* (Table 4).

When sample analyses were performed using identical HPLC conditions except for the QAP concentrations, the k' values of the rest of compounds in the PE series (PE, PEMM, PEDM, PEDC, and PEDA) increased with increasing HTAP concentrations from 5.00 m*M* to 10.00 m*M* (Table 4). These latter findings were confirmed by the chro-

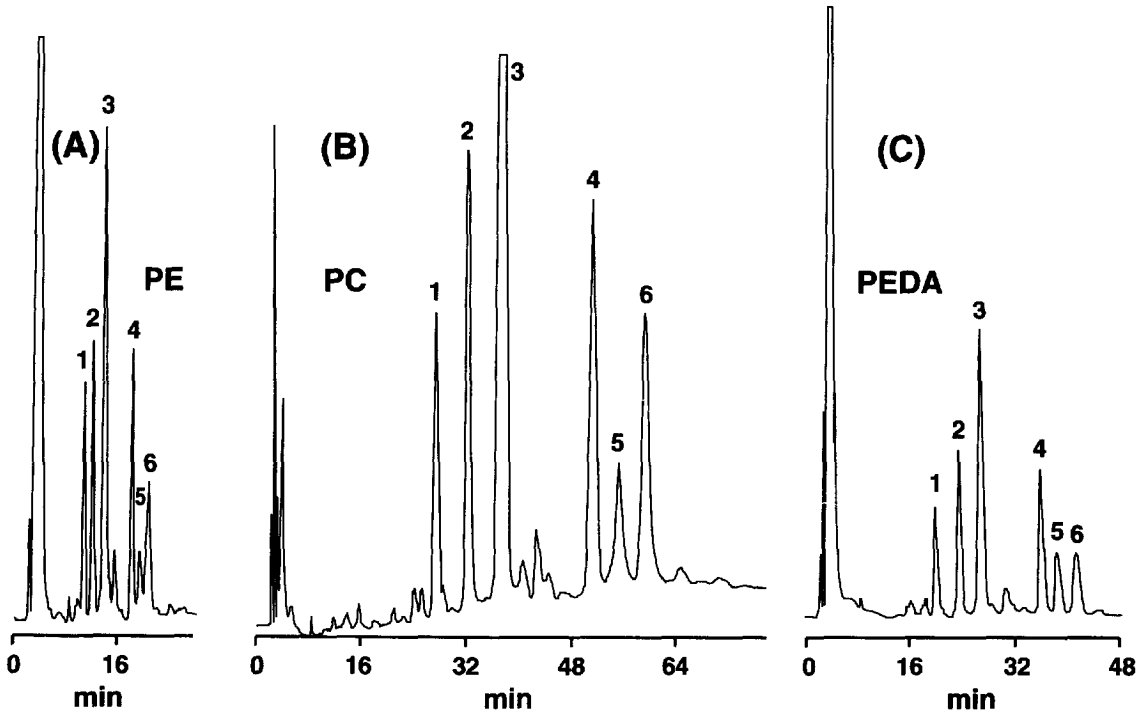

Fig. 3. Comparison of reversed-phase HPLC separations between PE (A) and two PE derivatives, PC (B) and PEDA (C), on a Beckman Ultrasphere ODS column. Mobile phase: CH_3CN–CH_3OH–H_2O (49:49:2) containing 10 m*M* TMAP at pH 6.5.

matographic data obtained using same mobile phase conditions (5.00–10.00 m*M* HTAP) but with a different ODS column as shown in Table 5.

It is noteworthy that HPLC of the PE derivatives with relatively low concentrations of QAPs yielded results which were different from those obtained at relatively high concentrations. Relevant data in Table 5 show inverse relationships between k' values of neutrally charged PE compounds (PE, PEMM, PEDM, PC, PECA and PEDA) and HTAP concentrations in the 0.32 m*M*–1.67 m*M* range. In agreement with published HPLC data for other nitrogen-containing ionic compounds [2,18,19], the k' values of all the neutrally charged PE compounds investigated invariably decreased with increasing HTAP concentrations at low levels. The observed negative concentration effects may be explained in terms of an ion-exchange or an ion-interaction retention rationale [20,21]. Further, interactions of QAP electrolytes with ODS might prevail during the HPLC processes leading partly to diminished analyte adsorption on the octadecylsilyl stationary phases. Because of the existence of an anionic carboxylate functionality in the molecule, PEDC is the only negatively charged compound studied in the PE series (Fig. 1). The expected positive concentration effects on k' values of the PEDC molecular species paralleled those of the negatively charged PA derivatives described earlier.

From the HPLC data in Tables 4 and 5, and the plots in Fig. 6 it can be summarized that the QAP concentration effects on the retention of PE derivatives on ODS were dramatically influenced not only by the QAP concentrations used, but also by the nature of N-substituents in mobile phases. Within the constraints of experimental variables, the inverse QAP concentration effects on analyte k' values were noticeable at concentrations up to 10 m*M* HTAP and 50 m*M* TMAP for PC, and up to 10 m*M* HTAP for PECA (Fig. 6). Although there was no change in the mode of QAP concentration effects on PC retention throughout the concentration range used in this study, the variation in the magnitude of the negative concentration effect was fairly small in comparison

Table 2
Effects of the type of QAPs on the capacity factors, k', of molecular species of PE derivatives derived from egg PC

QAP ph 6.5	Molecular species										
	1		2		3		4		5		6
	k'	α	k'	α	k'	α	k'	α	k'	α	k'
PTAP (10 m*M*)											
PE	3.50	1.14	4.00	1.21	4.84	1.38	6.67	1.09	7.26	1.08	7.82
PEMM	4.00	1.21	4.82	1.17	5.66	1.39	7.85	1.09	8.59	1.06	9.07
PEDM	4.31	1.20	5.16	1.17	6.04	1.35	8.16	1.10	9.00	1.07	9.66
PC	11.6	1.19	13.8	1.15	15.9	1.42	22.5	1.06	23.9	1.10	26.3
PECA	4.17	1.21	5.05	1.18	5.97	1.37	8.16	1.08	8.82	1.08	9.55
PEDC	1.00	1.25	1.25	1.20	1.50	1.39	2.09	1.08	2.25	1.11	2.50
PEDA	8.65	1.19	10.3	1.13	11.6	1.41	16.4	1.05	17.3	1.10	19.0
HTAP (10 m*M*)											
PE	4.76	1.19	5.68	1.16	6.57	1.36	8.92	1.07	9.57	1.08	10.3
PEMM	5.58	1.20	6.67	1.17	7.83	1.37	10.7	1.07	11.5	1.08	12.4
PEDM	5.68	1.21	6.85	1.16	7.94	1.36	10.8	1.07	11.6	1.09	12.7
PC	15.3	1.18	18.1	1.15	20.9	1.39	29.1	1.07	31.1	1.06	33.0
PECA	6.66	1.20	8.00	1.18	9.43	1.38	13.0	1.08	14.1	1.07	15.1
PEDC	1.83	1.22	2.23	1.13	2.52	1.39	3.51	1.05	3.68	1.07	3.92
PEDA	11.2	1.19	13.3	1.14	15.1	1.40	21.2	1.05	22.3	1.09	24.4
DTAP (10 m*M*)											
PE	8.07	1.19	9.58	1.17	11.2	1.37	15.3	1.10	16.8	1.07	17.9
PEMM	9.00	1.21	10.9	1.16	12.6	1.37	17.3	1.10	19.0	1.07	20.4
PEDM	10.1	1.22	12.3	1.15	14.2	1.39	19.8	1.10	21.7	1.07	23.3
PECA	9.43	1.20	11.3	1.17	13.2	1.41	18.6	1.09	20.3	1.09	22.1
PEDC	5.83	1.20	7.00	1.17	8.18	1.38	11.3	1.09	12.3	1.07	13.2

For HPLC conditions, see footnote to Table 1. PE=phosphatidylethanolamine, PEMM=monomethyl-PE, PEDM=dimethyl-PE, PC=phosphatidylcholine, PECA=N-caproylamine-PE, PEDC=N-dodecanyl-PE and PEDA=N-dodecanylamine-PE. For identification of molecular species, see Fig. 2.

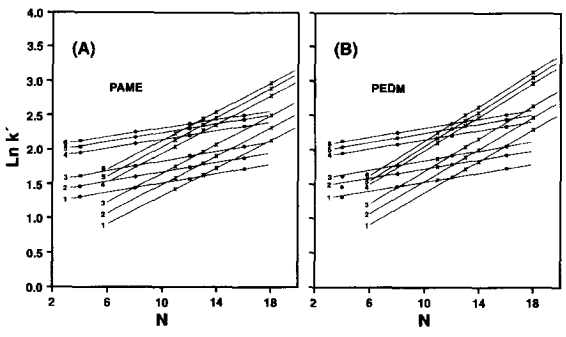

Fig. 4. Linear relation between ln k' of selected PL derivatives and the total number (N) of carbon atoms in QAP. (A) PA-monomethyl ester, PAME; (B) dimethyl-PE, PEDM. Symbols: (×) asymmetrical QAP; (●) symmetrical QAP. Column: Beckman ODS. Mobile phase: CH_3CN–CH_3OH–H_2O (49:49:2) containing 10 m*M* QAP at pH 6.5.

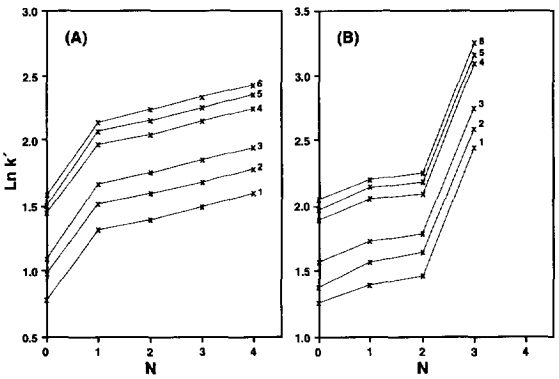

Fig. 5. Nonlinear relation between ln k' of PA and PE derivatives and the number (N) of carbon atoms in respective O-alkyl (A) and N-methyl (B) groups. Column: Beckman ODS. Mobile phase: CH_3CN–CH_3OH–H_2O (49:49:2) containing 10 m*M* PTAP at pH 6.5.

Table 3
Effects of the concentration of QAPs on capacity factors, k', of molecular species of monoalkyl esters of PA derived from egg PC

QAP concentration	Capacity factor, k'					
	Molecular species					
	1	2	3	4	5	6
TMAP (10 m*M*)						
PAMM	3.40	4.15	4.82	6.66	7.31	7.74
PAME	3.67	4.33	5.08	7.07	7.74	8.32
PAMP	4.15	5.00	5.82	7.92	7.84	9.42
PAMB	4.66	5.68	6.50	8.91	9.84	10.5
TMAP (50 m*M*)						
PAMM	7.15	8.56	10.1	13.7	15.1	16.3
PAME	7.83	9.50	11.0	15.0	16.6	17.8
PAMP	9.33	11.3	13.2	18.3	20.2	21.8
PAMB	10.3	12.6	14.9	20.3	22.3	24.1
HTAP (5.0 m*M*)						
PAMM	3.92	4.67	5.40	7.34	8.08	8.58
PAME	4.09	5.00	5.84	7.92	8.56	9.15
PAMP	4.50	5.42	6.41	8.58	9.44	10.1
PAMB	5.08	6.05	6.75	9.59	10.5	11.3
HTAP (10 m*M*)						
PAMM	4.74	5.67	6.58	9.08	9.84	10.6
PAME	5.16	6.15	7.26	9.83	10.7	11.5
PAMP	5.59	6.75	7.92	10.9	11.8	12.6
PAMB	6.00	7.32	8.67	11.9	13.0	14.0

For HPLC conditions and PL abbreviations, see footnote to Table 1. For identification of molecular species, see Fig. 2.

Table 4
Effects of the concentration of QAPs on the capacity factors, k', of molecular species of PE derivatives derived from egg PC

QAP concentration	Capacity factor, k'					
	Molecular species					
	1	2	3	4	5	6
TMAP (10 m*M*)						
PE	3.33	4.00	4.57	6.33	6.92	7.42
PEMM	3.67	4.41	5.07	7.00	7.58	8.17
PEDM	3.83	4.50	5.17	7.06	7.76	8.34
PC	10.2	12.3	14.3	20.1	21.8	23.4
PECA	4.05	4.92	5.65	7.91	8.75	9.33
PEDC	1.02	1.17	1.33	1.92	2.08	2.25
PEDA	7.24	8.66	10.0	13.8	14.9	16.2
TMAP (50 m*M*)						
PE	8.10	9.68	11.4	15.7	17.1	18.5
PEMM	8.81	10.9	12.7	17.2	18.8	20.3
PEDM	9.24	11.2	13.1	17.9	19.7	21.4
PC	10.0	11.9	14.0	19.4	21.1	22.8
PECA	9.00	10.8	12.6	17.8	19.3	20.7
PEDC	4.17	4.93	5.83	7.84	8.50	9.08
PEDA	12.2	14.5	15.8	23.2	24.7	26.9
HTAP (5.0 m*M*)						
PE	4.30	5.23	6.30	8.30	9.06	9.65
PEMM	4.59	5.42	6.33	8.50	9.17	9.82
PEDM	5.17	6.34	7.35	10.0	11.0	11.8
PECA	7.51	9.03	10.6	14.5	15.8	16.8
PEDC	1.08	1.25	1.52	1.92	2.02	3.17
PEDA	10.7	12.6	14.3	20.2	21.3	23.2
HTAP (10 m*M*)						
PE	4.76	5.68	6.57	8.92	9.57	10.3
PEMM	5.58	6.67	7.83	10.7	11.5	12.4
PEDM	5.68	6.85	7.94	10.8	11.6	12.7
PECA	6.66	8.00	9.43	13.0	14.1	15.1
PEDC	1.83	2.23	2.52	3.51	3.68	3.92
PEDA	11.2	13.3	15.1	21.2	22.3	24.4

For HPLC conditions, see footnote to Table 1. For PL abbreviations, see footnote to Table 2. For identification of molecular species, see Fig. 2.

with other neutrally charged PE compounds in the PE series investigated. There is a common feature in the structures of the latter group of polar lipids that all contain at least one N–H bond. For these compounds, the mode of QAP concentration effects changed from positive to negative as the QAP concentrations were lowered correspondingly from high levels to low levels (Fig. 6). While the exact nature of retention mechanisms [21] for compounds in PE series is unclear at the present time, the positive QAP concentration effects observed at high QAP concentrations were suggestive of predominant ion-pairing processes [22] much like the situation in reversed-phase HPLC of negatively charged PL (e.g., PA derivatives).

In conclusion, the reversed-phase HPLC techniques developed can be used to improve separations of complex mixtures of PL molecular species as their PA or PE derivatives in various sample matrices. The use of PL derivatives of enhanced hydrophobicity in the reversed-phase HPLC systems evaluated is advantageous, because the methods facilitate the analysis of low UV absorbing minor species permitting the sensitive analysis of PLs at trace levels. Molecular species of both PA and PE derivatives can be resolved with higher efficiency than the underiva-

Table 5
Effects of low level HTAP concentrations on the capacity factors, k', of molecular species of PE derivatives derived from egg PC

HTAP concentration	Capacity factor, k'					
	Molecular species					
	1	2	3	4	5	6
0.32 m*M*						
PE	7.00	7.28	9.88	13.1	15.0	15.8
PEMM	9.32	11.2	13.4	17.6	20.0	21.1
PEDM	10.4	12.4	14.7	19.5	22.0	23.2
PC	11.4	13.6	15.8	21.4	23.5	24.9
PECA	13.1	15.3	18.0	23.8	26.7	28.0
PEDC	0.76	0.76	0.76	0.76	0.76	0.76
PEDA	15.0	16.9	19.9	26.8	30.0	31.6
0.83 m*M*						
PE	5.16	6.04	7.32	9.40	11.0	11.4
PEMM	7.24	8.60	10.5	13.4	15.5	16.1
PEDM	9.00	10.8	12.8	16.8	18.8	19.6
PEDC	0.83	0.83	0.83	0.83	0.83	0.83
PEDA	13.4	16.3	19.0	25.4	28.4	29.9
1.67 m*M*						
PE	4.76	5.72	6.76	8.92	9.96	10.5
PEMM	6.68	7.96	9.56	12.4	14.4	15.2
PEDM	7.00	8.44	10.0	13.2	14.8	15.6
PC	11.3	13.6	15.6	21.2	23.2	24.6
PECA	11.8	14.0	16.6	22.0	24.6	25.9
PEDC	0.92	1.16	1.24	1.48	1.83	1.83
PEDA	9.88	11.8	14.0	18.7	21.1	21.8
5.00 m*M*						
PE	5.72	6.76	7.96	10.5	11.5	12.2
PC	11.2	13.2	15.3	20.0	22.7	24.0
PECA	8.36	10.0	11.9	16.0	17.4	18.2
10.0 m*M*						
PE	6.04	7.40	8.60	11.4	12,5	13.3
PC	10.5	12.6	14.6	19.6	21.4	22.7
PECA	8.04	9.64	11.2	15.6	16.4	17.4

Column, Phenomenex Prodigy ODS-2; mobile phase solvents, as in Table 1. For PL abbreviations, see footnote to Table 2. For identification of molecular species, see Fig. 2.

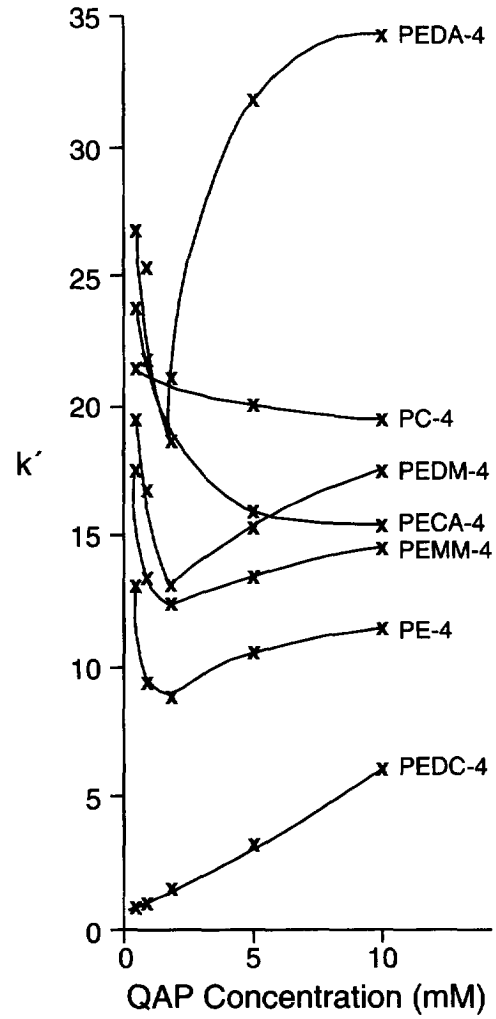

Fig. 6. Variation of k' values of investigated compounds in PE series with QAP concentrations. The numerical "4" following each abbreviated compound name represents molecular species "4" (16:0–18:1) of each lipid species in the series. For HPLC conditions, see footnote to Table 5.

tized parent compounds in HPLC with QAP mobile phase modifiers. Alkanol ester derivatives of PA exhibited HPLC characteristics predictable by linear correlation with the number of carbon atoms in QAP or in O-alkyl groups. Furthermore, the degree of component resolution can be controlled by selecting the type of QAP or alcohols used in the derivatization of the parent polar lipid. The linear $\ln k'$–N (QAP) correlation lines for PE derivatives can be used for the prediction of retention times of their analyte species. However, the $\ln k'$–N (N-methyl) correlation curves are not linear partially due to the polarity effect of N–H bonding on solute retention. In reversed-phase HPLC, the N-protonated analogues PE, PEMM, and PEDM in the PE structural series are considerably less hydrophobic than the permethylated compound, PC (trimethyl-PE).

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