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papers and notes on methodology

Analysis of aminophospholipid molecular species by high performance liquid chromatography

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Abstract A new method is described for the separation of individual molecular species of the aminophospholipids, phosphatidylethanolamine and phosphatidylserine. Trinitrobenzenesulfonic acid was used to derivatize both aminophospholipids and the derivatives were purified by thin-layer chromatography. A reversed-phase high performance liquid chromatography technique was developed to separate and quantify individual molecular species based upon ultraviolet detection of the attached chromophore. The retention times of the molecular species on the C_{18} reversed-phase column were longer with increasing carbon chain length and decreasing degree of unsaturation of fatty acyl chain. The overall procedure allowed a quantitative recovery of the aminophospholipid species. The lower limit of detection was about 10 pmol and a linear response was observed in the range of 0.1-10 nmol of phospholipid. Using this method, we were able to separate and quantify trinitrophenyl-phosphatidylethanolamine molecular species of both subclasses (diacyl and alkenyl) from human red blood cells and rat brains. Separation of species was confirmed by gas-liquid chromatographic analysis of the fatty acid content of each peak and by thermospray liquid chromatography-mass spectrometry. This new method provides a convenient and sensitive technique for studies of aminophospholipid molecular species composition. Furthermore, it appears to be a useful tool for the analysis of asymmetric distribution of these species in biological membranes. —**Hullin, F., H-Y. Kim, and N. Salem, Jr.** Analysis of aminophospholipid molecular species by high performance liquid chromatography. *J. Lipid Res.* 1989. **30:** 1963-1975.

Supplementary key words trinitrobenzenesulfonic acid • thermospray liquid chromatography-mass spectrometry • membrane lipid asymmetry • phosphatidylethanolamine • phosphatidylserine

Biological features of membranes including their physical state, enzymatic activity, and permeability are influenced by the fatty acyl profiles of component phospholipids (1). Precise control of membrane lipid composition appears important for proper cell functioning as, for example, erythrocytes have been shown to undergo morphological changes and osmotic fragility after modification of their molecular species composition (2, 3). Therefore, techniques for analyzing the various molecular species of phospholipids are of great relevance to studies of membrane properties. Recently, individual molecular species of glycero-phospholipids have been separated by reversed-phase high performance liquid chromatography (RP-HPLC) as intact lipids (4-7) or as benzoate derivatives of diradylglycerols produced after phospholipase C hydrolysis (8-10). Detection methods used have been based on measurement of ultraviolet absorption at about 205 nm for intact phospholipids or 230-250 nm for the benzoate derivatives. In addition, in our laboratory (7, 11, 12), mass spectrometric detection using the thermospray interface has provided a powerful technique for identification of molecular species of the major phospholipid classes.

The amino group modifying reagents, 1-fluoro-2,4-dinitrobenzene (FDNB) and 2,4,6-trinitrobenzenesulfonic acid (TNBS), have been used previously for amino-lipid derivatization (13). Studies using the membrane impermeant reagent TNBS have shown that aminophospholipids are primarily localized on the plasma membrane interior in a variety of cells (14-17), although some of the PE is found on the cell surface. The asymmetric distribution of aminophospholipids was confirmed by the use of purified phospholipases (18-20). By contrast, the membrane permeant reagent FDNB is able to react with a much greater proportion of the PE as well as a substantial amount of PS in intact cells (21, 22). In addition to the transbilayer asymmetry of a phospholipid class, some studies have indicated a non-random distribution of phospholipid subclasses. Record et al. (23) found a preferential localization of alkyl species of PE in the inner leaflet of the Krebs II ascite cell plasma membrane. However, diacyl PE displayed an external localization and

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Abbreviations: BHT, 2,6-di-tert-butyl-cresol; EDTA, ethylenediamine tetraacetic acid; FDNB, fluorodinitrobenzene; HPLC, high performance liquid chromatography; PE, phosphatidylethanolamine; PS, phosphatidylserine; RRT, relative retention time; TLC, thin-layer chromatography; TNBS, trinitrobenzenesulfonic acid; LC-MS, liquid chromatography-mass spectrometry; RP, reversed phase; GLC, gas-liquid chromatography.

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alkenyl PE was randomly distributed in these membranes. Asymmetric localization of plasmalogen PE was observed in lymphocytes (24). In this case, the degree of asymmetry was not constant but depended on the cell cycle. Furthermore, molecular species asymmetry has also been observed in that a higher degree of unsaturation on the cytoplasmic leaflet of the plasma membrane was apparent (25-27). The functional significance of this asymmetry is still unknown, but we may expect important consequences for the membrane shape, as has been suggested for echinocyte or stomatocyte formation of erythrocytes (3), and for the subsequent metabolism of the polyunsaturated species by the lipoxygenase and/or cyclooxygenase pathways.

We now report an HPLC separation of trinitrophenyl (TNP) derivatives of the aminophospholipids, phosphatidylethanolamine (PE) and phosphatidylserine (PS), using an RP-HPLC method that permits effective separation, predictable retention times (according to differences in carbon chain length and degree of unsaturation), and quantification using a sensitive ultraviolet method for detection of various molecular species. Separation and quantification by HPLC of individual PE or PS species modified with TNBS can provide a useful method not only for the analysis of molecular species composition of these phospholipids, but also might be applied to the study of molecular species asymmetry in biological membranes.

MATERIALS AND METHODS

Synthetic and natural PE and PS were purchased from Avanti Polar Lipids (Birmingham, AL) and from Sigma (St. Louis, MO). Their purity was tested by thin-layer chromatography (TLC) using the solvent system of chloroform-methanol-water-acetic acid 65:43:3:1 (v/v) (28) and with iodine vapor as the detection reagent. All of the PE and PS preparations were pure; they ran as one spot when 100 μg was analyzed, with the exception of the soybean PE. Thus, the latter was purified by TLC using PLK5 plates (Whatman Inc., Clifton, NJ) and the above solvent system. It was eluted from the gel with a mixture of chloroform-methanol-acetic acid-water 50:39:1:10 (v/v) as described by Arvidson (29). After removal of the gel by centrifugation, sufficient volumes of methanol and water were added in order to bring the solvent ratios of chloroform-methanol-water to 2:2:1.8 as described by Bligh and Dyer (30) in order to form a two-phase system.

TNBS and FDNB derivatization of natural and synthetic aminophospholipids

2,4,6-Trinitrobenzenesulfonic acid and 1-fluoro-2,4-dinitrobenzene were obtained from Pierce Chemical Co. (Rockford, IL). The derivatization was similar to that described by Gordesky and Marinetti (31) for TNBS labeling of PE and PS in organic solution but used chloroform-methanol-water in the proportion of 1:2:0.8 as described by Bligh and Dyer (30). Up to 2 mg of synthetic or natural PE or PS was dissolved in 1 ml of chloroform in screw-capped glass tubes. Two ml of methanol (containing 50 μg of 2,6-di-tert-butyl-cresol), 19 μl of 500 mM TNBS or FDNB in methanol, and 0.4 ml 5% $NaHCO_3$ were added successively with vortex agitation. The tubes were then flushed with nitrogen and incubated at 30°C in a shaking water bath. After 30 min, another aliquot of $NaHCO_3$ and reagent (5 mM final concentration) was added and incubation was continued for an additional 60 min. For complete derivatization, a sufficient amount of $NaHCO_3$ was necessary to reach the 8.0-8.5 pH range (14). The reaction was stopped by cooling on ice. To each tube, 2 ml of methanol, 1 ml of chloroform, and 1 ml of 0.5N HCl (to bring the pH to 5.0) were added; then the solutions were made into two phases by addition of 2 ml of chloroform and 1.8 ml of water using the solvent ratios described by Bligh and Dyer (30). The lower (chloroform) phase containing the yellow lipid derivatives was concentrated with a stream of nitrogen, redissolved in 200 μl of chloroform-methanol 2:1 and spotted on preparative silica gel plates (PLK5, Whatman Inc.). TNP and DNP derivatives were separated using the solvent system of chloroform-acetone-methanol (containing 100 μg BHT)-acetic acid-water 5:2:1:1:0.5 (v/v) (32). The TLC plates were developed in a light-shielded tank to avoid photodecomposition. TNP- or DNP-labeled PE and PS were easily visualized by their yellow color and were well separated from underivatized PE and PS. The R_f values of PS, PE, DNP-PS, TNP-PS, DNP-PE, and TNP-PE were 0.33, 0.50, 0.55, 0.57, 0.85, and 0.85, respectively. The plates were briefly dried under nitrogen and the yellow bands were scraped and extracted twice with 4 ml of methanol containing 50 μg of BHT. The extracts were centrifuged at 3000 rpm at 4°C for 20 min in order to remove fine particles of silica gel. After evaporation under nitrogen, the extracts were dissolved in methanol and the absorbances of TNP-PE and TNP-PS were measured at 338 and 342 nm, respectively, whereas the DNP derivatives of PE and PS were measured at their absorbance maxima of 348 nm. An HP 8451A UV-visible spectrophotometer was used (Hewlett-Packard, Avondale, PA). The phospholipid phosphorus was determined according to Nelson, using H_2SO_4 as the digestion acid (33).

Rat brain preparation

Five adult rats (Sprague-Dawley) fed a standard laboratory chow diet were decapitated and the brains were removed. They were freed from adhering connective tissue and processed immediately for lipid extraction.

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They were homogenized in chloroform–methanol and processed according to the method of Bligh and Dyer (30).

Human red blood cell (RBC) preparation

Ten ml of blood was withdrawn by venipuncture from six human volunteers (25 to 35 years old) using acid citrate dextrose as anticoagulant. After centrifugation at 250 *g* for 15 min at room temperature, the platelet-rich plasma (PRP), the buffy coat, and a small amount of the top layer of erythrocytes were removed. The cells were washed three times in cold buffer containing 154 mM NaCl, 25 mM glucose, and 10 mM Tris-HCl, pH 7.4, by centrifuging at 1000 *g* for 15 min at 4°C. A small portion of the top of the erythrocyte layer was removed after each centrifugation to avoid leukocyte contamination. Packed red blood cells were then extracted according to the procedure of Reed et al. (34). One ml of packed red blood cells was added dropwise into 4 ml of methanol containing 50 μg of BHT and 100 μl of 0.2 M EDTA with continuous stirring. Extraction was performed for 5 min a 4°C. Then 4 ml of chloroform containing dipalmitoyl-PE as internal standard was added and extraction was continued for another 5 min. After centrifugation, the supernatant was retained and the pellet was reextracted. The two chloroform–methanol extracts were pooled and then separated into two phases by addition of 6.6 ml of water (30).

TNBS labeling of rat brain and human red blood cell total lipid extracts

An aliquot of the total lipid extracts of the red blood cell or rat brain samples containing about 4 μmol of lipid phosphorus was evaporated with a stream of nitrogen and derivatized with TNBS as described above. The yellow TNP-labeled PE and PS were separated by TLC as described above, except that LK5 plates were used (Whatman Inc.). Molecular species of TNP-PE were then analyzed by HPLC by duplicate injection for each sample.

Hydrolysis of TNP derivatives of plasmalogen PE

Aliquots of TNP-labeled PE from human red blood cells (about 300 nmol) were subjected to acidolysis according to the procedure of Pugh, Kates, and Hanahan (35). After evaporating the solvent under nitrogen, they were redissolved in 2 ml of chloroform; 0.2 ml of 4 N HCl was added and the mixture was vortexed thoroughly for 2 min at room temperature. In order to separate the mixture into two phases, 4 ml of cold methanol containing 50 μg of BHT and 4 ml of water were added. After centrifugation at 4°C, the chloroform phase was washed with 4 ml methanol–water 1:1 (v/v) in order to eliminate the remaining HCl. The chloroform phase was spotted on an LK5 silica gel plate and separated using the TLC system described above (32). The yellow TNP-PE spots were separated from the yellow TNP-lyso-PE spots (R_f 0.85 and 0.70, respectively) and were eluted twice with 3 ml of methanol containing 50 μg of BHT. Molecular species of TNP-PE were then analyzed by HPLC.

HPLC analysis

Separation of molecular species of TNP and DNP derivatives of PE and PS was accomplished on an HP 1090 liquid chromatograph equipped with an HP 1040 diode array detector and an HP 79995A analytical workstation. The chromatographic column was an Axxi-chrom ODS (5 μ, 4.6 × 250 mm) (Thomson Instrument Co., Springfield, VA) coupled to a 2.0 × 20 mm precolumn (Upchurch Scientific, Inc., Oak Harbor, WA) packed with 10 μ Adsorbosphere C_{18}. PS and PE derivatives were separated using a mobile phase consisting of 10 mM ammonium acetate, pH 5.0, and methanol. Since the polarity of PS and PE derivatives differed, two different elution schemes were used. The analysis of TNP- and DNP-PS began with a 5-min isocratic elution at 84% methanol, followed by a linear increase to 87% methanol in 15 min; this proportion was then maintained for 40 min. For TNP- and DNP-PE, the same program as described above was used during the first 20 min. This was followed by an isocratic elution at 87% methanol for a period of 70 min and finally by a linear gradient increasing to 93% methanol in 20 min (total analysis time of 110 min). Trinitrophenyl derivatives of PE and PS were quantified by their absorption at 338 and 342 nm, respectively. The DNP derivatives were quantified at their absorbance maxima which was 348 nm. The flow rate was 1 ml/min and the column temperature was maintained at 40°C for all analyses. Samples were filtered through a 0.45-μm type HV filters (Millipore Co., Bedford, MA), evaporated to dryness under nitrogen, then dissolved in methanol prior to injection. HPLC grade organic solvents were purchased from Burdick and Jackson (Muskegon, MI). Ammonium acetate (ACS grade) was obtained from Mallinckrodt (Paris, KY). Distilled water was purified using an RO-5 and a Milli-Q system which included an Organex cartridge (Millipore).

Fatty acid analysis

Fractions collected after HPLC purification were separated into two phases (30) and dried with a stream of nitrogen. Fatty acids were transmethylated with 1 ml of boron trifluoride in methanol (14% w/v, Sigma) at 100°C for 90 min according to Morrison and Smith (36) and extracted three times with hexane (Burdick and Jackson, distilled in glass grade). The combined extracts were evaporated to dryness under nitrogen, redissolved in a minimal volume of hexane and injected into an HP 5880 gas chromatograph equipped with a flame ionization detector. Helium was used as carrier gas with a linear velocity of 35 cm/sec and nitrogen as make-up gas. A 50

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m × 0.25 mm i.d. OV-351 capillary column with a 0.25 μm film thickness (Analabs, Foxboro, MA) was used with an oven temperature program from 200° to 225°C at 1 degree/min and held at 225°C thereafter. Fatty acid methyl ester peaks were identified by their retention times based on a comparison to commercial standard mixtures (Nu-Chek-Prep, Elysian, MN).

Thermospray liquid chromatography–mass spectrometry analysis (LC–MS)

The thermospray LC–MS system was an Extrel ELQ-400 quadrupole mass spectrometer equipped with a Vestec interface as previously described (7, 11, 12). Samples were injected into an Ultrasphere-ODS column (5 μ, 4.6 mm × 7.5 cm) and eluted with a solvent mixture of methanol–0.1 M ammonium acetate–hexane 500:25:5 with a flow rate of 1 ml/min. Thermospray spectra were obtained with the electron-emitting filament on and with a source and vaporizer temperature of 300° and 146°C, respectively.

RESULTS AND DISCUSSION

Introduction of a nitrophenyl chromophore into the polar head group of aminophospholipid molecules presented several advantages for molecular species analysis. The decreased polarity of the aminophospholipid derivatives facilitated their separation by RP-HPLC. It allowed ultraviolet detection in a wavelength range that was far removed from solvent and other interfering absorbances. Moreover, it permitted a substantial increase in sensitivity in comparison with UV detection in the 200-nm region.

In preliminary experiments, we used commercial synthetic and natural mixtures of PE and PS containing fewer molecular species in order to establish the HPLC method. TNP derivatives of soybean phosphatidylethanolamine were resolved into 11 components by this method (**Fig. 1**). Each chromatographic peak was collected and the fatty acid composition was determined by GLC analysis of the methyl esters. Molecular species were then assigned as shown in Fig. 1.

Molecular species assignments were further confirmed by thermospray LC–MS. Typical thermospray spectra of phospholipids contain mono- and diglyceryl ion peaks as well as fragments derived from the head group (7, 11, 12). TNP derivatives also fragmented in a similar manner as is shown in **Fig. 2** for the chromatographic peak assigned to 16:0,18:2 TNP-PE in Fig. 1. The peak at *m/z* 576 is derived from the diglyceryl fragment for the 16:0,18:2 species and was the base peak in the spectrum. The ions at *m/z* 313 and 337 arose from the monoglyceryl ions containing either the 16:0 or 18:2 fatty acyl chain, respectively. Ions characteristic for the head group were also detected at *m/z* 239 and 255; the latter corresponds to $C_6H_2(NO_2)_3$-NH-CH_2-CH_2^+; the loss of an oxygen atom from it is presumably responsible for the peak at *m/z* 239. The head group ions characteristic for PE (at *m/z* 142 or 124) were not observed for the derivatized phospholipid. Although molecular ion species were not detected in the spectra, the identity of each chromatographic peak could be unambiguously determined from the observation of these head group, mono- and diglyceryl fragment ions.

Under these HPLC conditions, the retention times of the TNP derivatives depended both on the polar head

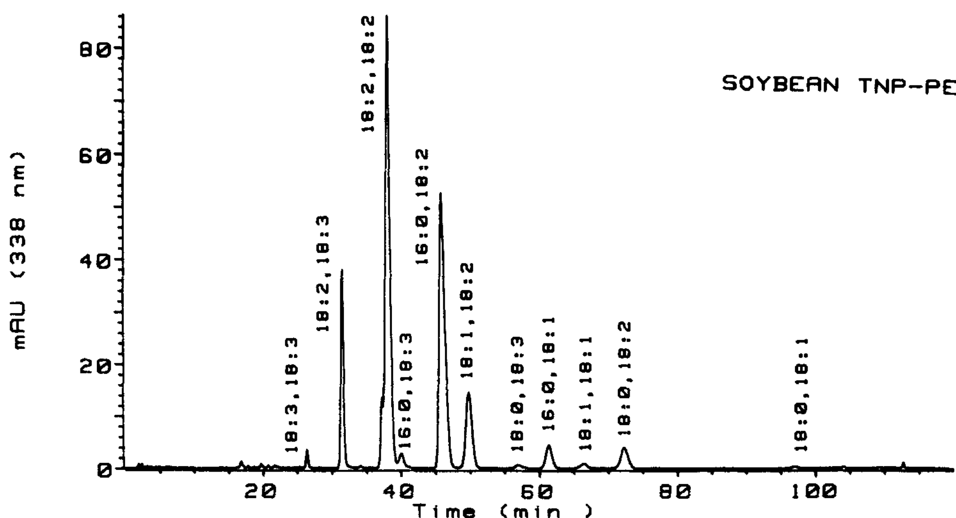

Fig. 1. Molecular species separation of trinitrophenyl derivatives of soybean PE by HPLC. Sixteen nmol of TNP-PE was chromatographed on an Axxi-chrom ODS column at a flow rate 1 ml/min. The solvent mixture was 10 mM ammonium acetate, pH 5.0, and methanol, and gradient conditions were as descirbed under Methods. Column temperature was 40°C and detection was by absorption at 338 nm. Molecular species of TNP-PE were assigned after GLC analysis.

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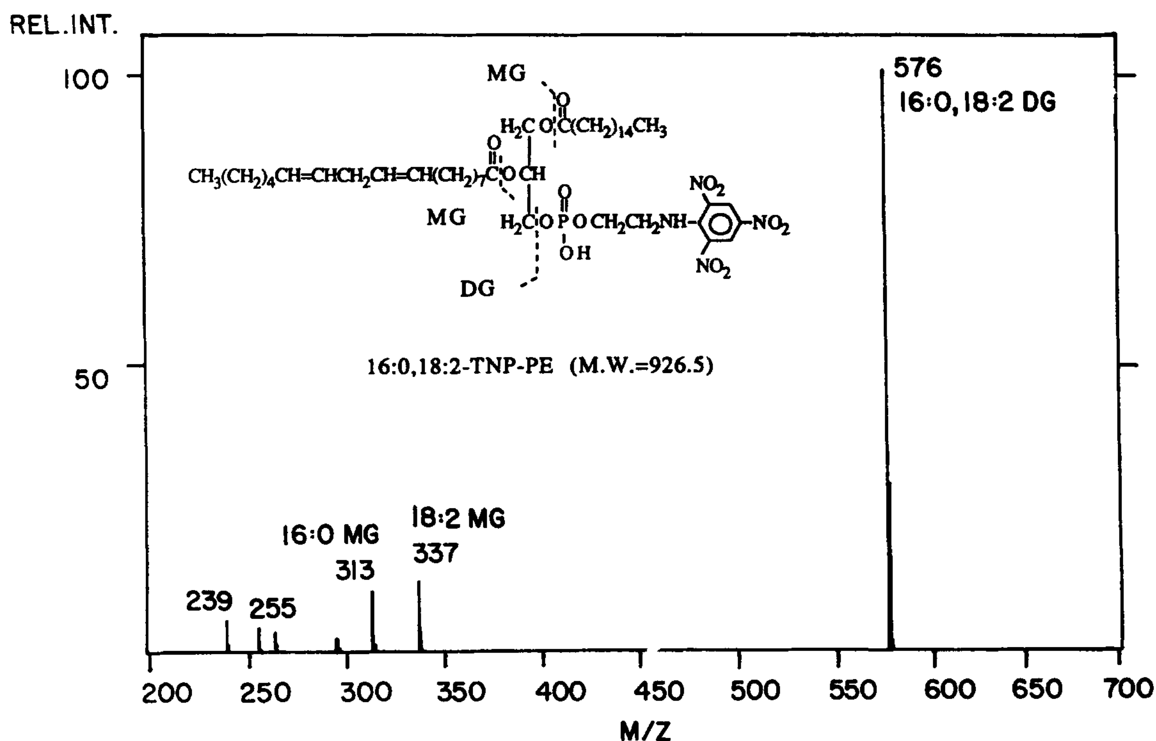

Fig. 2. Positive ion spectrum obtained from thermospray LC-MS analysis of the peak assigned as the 16:0, 18:2 species in Fig. 1. See Methods for mass spectral conditions. Abbreviations: DG, diglyceryl; MG, monoglyceryl fragments.

group and on the fatty acid composition of the phospholipid. The TNP derivatives of PS eluted at a lower methanol concentration than the corresponding species of PE. This difference in chromatographic behavior between the two lipid classes could be due to the ionization of their polar head groups since, at pH 5.0, serine (pK_{a1} = 2.21) remains mostly ionized and this increases mobility. The ionic strength of the mobile phase was also an important variable as increasing the ammonium acetate concentration from 1 to 10 mM resulted in greater retention and allowed the separation of PS derivatives from reagent-derived peaks. Increasing the column temperature to 40°C decreased the viscosity of the mobile phase and produced higher peak efficiency. **Fig. 3** shows chromatograms of the separation of TNP derivatives of synthetic and bovine brain PS into individual molecular species. The bovine brain TNP-PS preparation was resolved into 11 components. Fractions were collected and each was analyzed by GLC; assignments of major molecular species are indicated. The 18:0,22:6(n−3) and 18:0,18:1 species were the two major components of this preparation (25.4 and 44.2 mol%, respectively). This is consistent with previous results based on argentation thin-layer chromatgraphy (37, 38) and thermospray LC-MS (11), taking into account the variations in fatty acid composition of commercial preparations. Other minor species were also detected, e.g., 18:1,18:1; 18:0,22:5(n−6); and 18:0,20:1(n−9) species.

This chromatographic method is capable of yielding a separation of many molecular species of both PE and PS in a single run. However, TLC purification still appeared to be useful for biological samples. This step removed both yellow decomposition products (due to reagent side reactions) that could overlap with PS derivatives, and also other lipid classes. Moreover, it is possible that some PS molecular species could overlap with PE species in a particular biological tissue. For example, in brain PS (Fig. 3B) the 18:0,18:1 species may overlap with dipolyunsaturated PE species.

Within a phospholipid class, the elution order of molecular species was dependent on the fatty acid composition as the hydrophobic effect was the dominant interaction. Molecular species eluted from the column in order of decreasing degree of unsaturation and increasing chain length as was shown for a mixture of synthetic molecular species of TNP-PE (**Fig. 4A and B**). While molecular species with longer chain lengths were retained longer, introducing double bonds increased polarity and reduced the retention time. Thus an 18:3,18:3 species had almost the same retention time as a saturated species with eight fewer carbon atoms, i.e., the 14:0,14:0 species.

When we compared the effects of chain length, number, and configuration of double bonds on the chromatographic behavior (Figs. 1 and 4B) we observed the following. *1*) Different molecular species containing the same total number of carbon atoms and double bonds were

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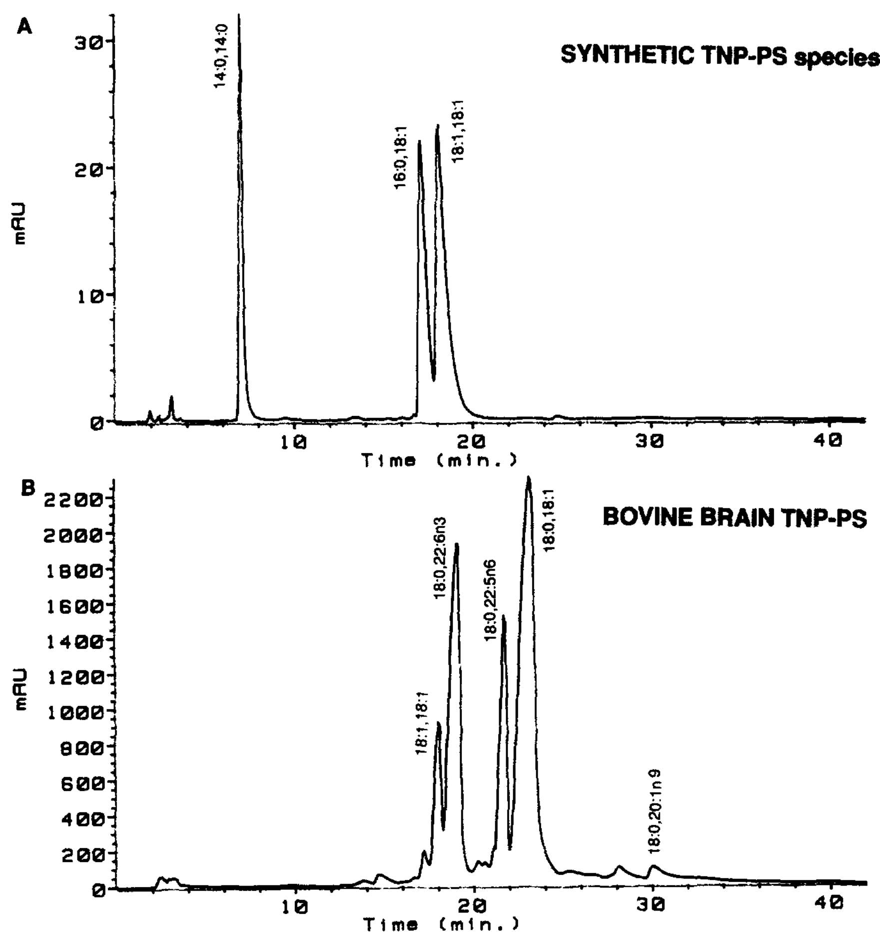

Fig. 3. HPLC separation of a mixture of three molecular species of synthetic trinitrophenyl-PS (A) (3 nmol) and bovine brain trinitrophenyl-PS (B) (600 nmol). HPLC conditions were as described in Fig. 1; UV detection was at 342 nm. For bovine brain TNP-PS (B), the major molecular species were assigned after the fatty acids were analyzed by GLC.

separated. Furthermore, addition of a double bond at the *sn*-1 position seemed to have a greater effect on retention time than at the *sn*-2 position. As examples, 18:1,18:1 eluted before 18:0,18:2 and 18:1,18:2 before 18:0,18:3. *2*) The presence of a *trans* double bond increased the retention more than did a *cis* double bond as expected on the basis of their conformational differences (39). Thus our chromatographic system could completely separate 9-*trans*,di-18:1 from 9-*cis*,di-18:1.

When DNP derivatives were analyzed, similar chromatographic behavior was observed except that they had slightly lower retention times than the corresponding TNP species (data not shown). This suggested that the trinitrophenyl group could provide a more nonpolar interaction with the C_{18} stationary phase.

The quantitative aspects of this analysis were also investigated so that peak areas could be related to absolute amounts of a phospholipid species. When UV absorption and phosphate analysis were compared, it was observed that the molar extinction coefficients of TNP-PE, TNP-PS, and DNP-derivatives in methanol at their optimal wavelengths were essentially the same: 17140 ± 750. As expected, different species within one lipid class all had the same extinction coefficient

In order to determine the relationship of peak area to molar amounts, duplicate injections were made for increasing amounts of various synthetic species of TNP-PE containing 0, 1, 2, 4 or 6 double bonds and those of TNP-PS containing 0, 1, or 2 double bonds and up to 36 carbons. Duplicate injections of the same sample yielded area values that agreed to within 0.6%. The response remained linear in the range of 0.1-10 nmol for all the species studied. The calculated response factor was 1.74 ± 0.02 (pmol per area unit) for TNP-PE species (n = 65) and 1.62 ± 0.03 for TNP-PS species (n = 23). The lower limit of detection (i.e., producing a 0.17 mAU response in the presence of baseline noise of 0.05 mAU) was 10 pmol. Thus, the method is very sensitive.

In order to evaluate the overall recovery for the entire procedure, standard mixtures containing different

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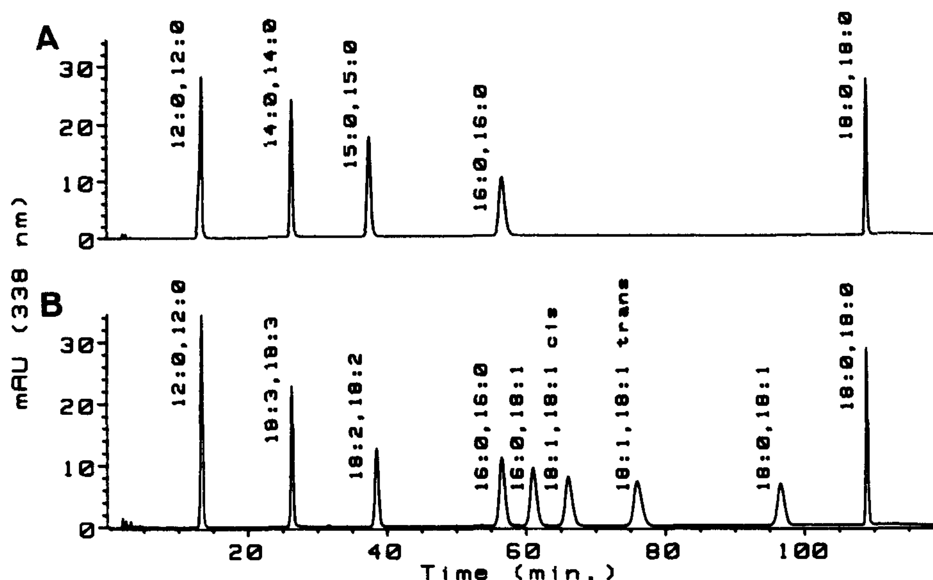

Fig. 4. Molecular species separation of purified synthetic TNP-PE by HPLC; (A) disaturated species (1 nmol/peak) and (B) both saturated and unsaturated species (1 nmol/peak). Separation conditions were as described in Fig. 1.

amounts of eight molecular species of PE and of two species of PS were derivatized, separated by TLC, eluted, and analyzed by HPLC. The overall recovery was 82.0 ± 1.5% (n = 24) for TNP-PE species and 77.6 ± 2.2% (n = 6) for TNP-PS species. Furthermore, the percentage of individual molecular species in the standard mixture as determined by phosphorus analysis was compared with the area percent of each peak obtained after HPLC separation (**Table 1**). The relative distributions of the molecular species of TNP-PE and TNP-PS as determined by these two methods were very similar, indicating that this technique yields results that reflect the molar composition of the original sample; any losses that occur are nonselective. It was therefore valid to convert the area percentage reports to mole percentages. The quantification capability of this method was further assessed by comparing the fatty acid composition of soybean PE as determined by GLC with that calculated from the HPLC mole percentage report. **Table 2** shows that these distributions are in good agreement and confirms the quantitative

TABLE 1. Distribution of a standard mixture of PE and PS molecular species before and after derivatization and chromatographic analysis

Molecular Species	Standard Mixture % of Total P[a]	HPLC Analysis % ot Total Peak Area[b]
I. PE species		
di-12:0	14.0	14.3 ± 0.2
di-16:0	23.7	26.3 ± 0.1
di-18:0	9.1	9.1 ± 0.1
16:0,18:1	10.8	10.4 ± 0.1
18:0,18:1	11.0	10.0 ± 0.1
di-18:1, *cis*	10.7	9.8 ± 0.1
di-18:1, *trans*	10.7	9.2 ± 0.1
di-18:2	10.0	10.9 ± 0.1
II. PS species		
16:0,18:1	48.8	45.4 ± 0.1
di-18:1	51.2	54.6 ± 0.1

[a]Percentages were determined by phosphorus analysis (mean of duplicate analyses).
[b]Samples were subjected to derivatization with TNBS; the TNP-PE derivatives were separated by TLC, eluted with methanol, and analyzed by HPLC. These values correspond to mole % and are the mean ± SEM of three duplicate experiments.

TABLE 2. Comparison of the fatty acid composition of soybean PE before and after derivatization and chromatographic analysis

Fatty Acid	Total Sample[a]	HPLC Analysis[b]
	mol %	
16:0	19.4	17.0
18:0	2.8	1.9
18:1	6.3	6.7
18:2	64.8	67.2
18:3	6.7	7.2

[a]The fatty acyl distributon was determined by GLC analysis of the soybean PE. Weight % reports were converted to mole % in order to facilitate comparison to the results obtained by HPLC.
[b]The theoretical fatty acid composition was calculated from the mole percentage of the molecular species is assigned in Fig. 1. Values are expressed as mol %.

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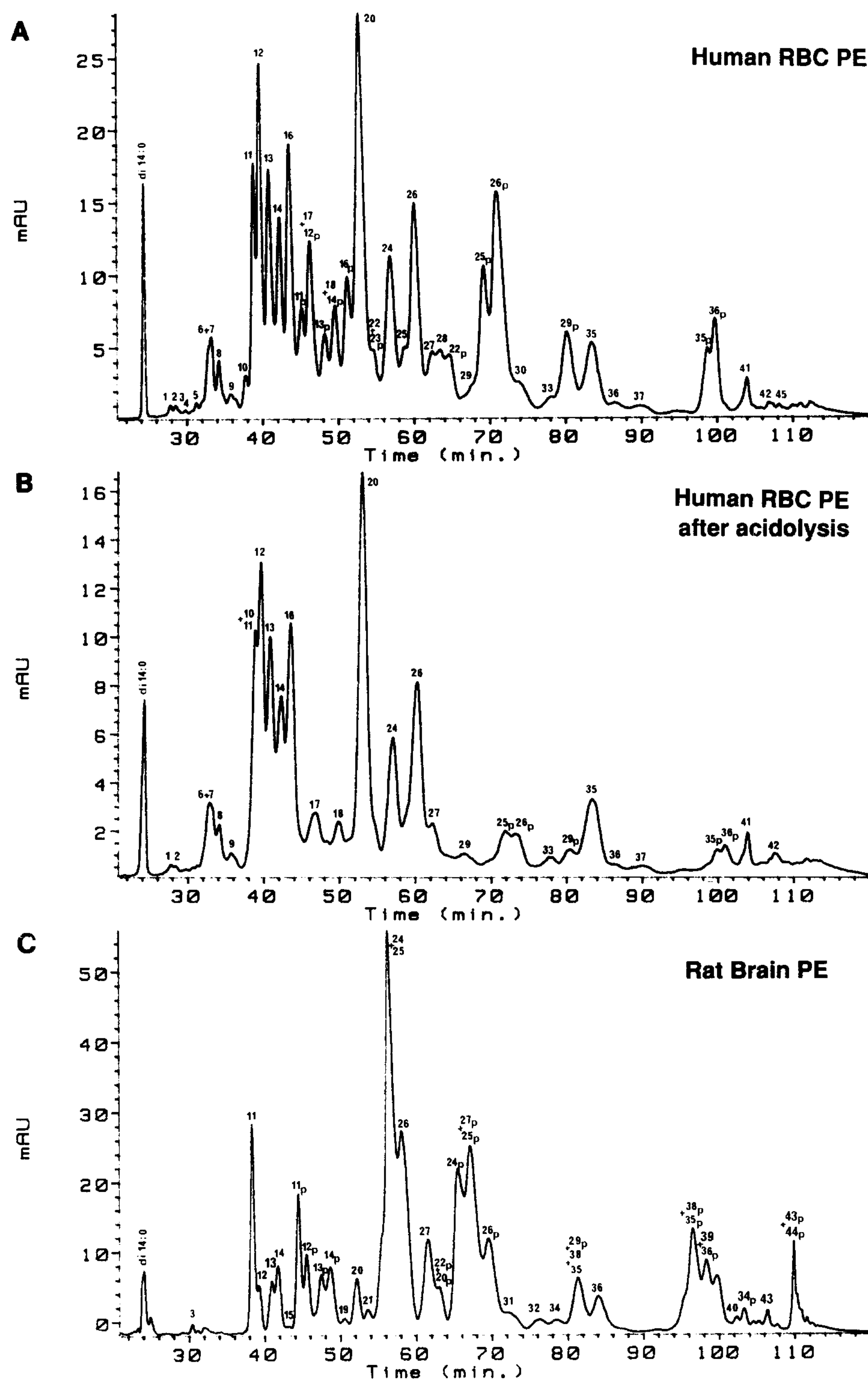

Fig. 5. Molecular species separation of trinitrophenyl derivatives of human red blood cell PE (A) and rat brain PE (C) by HPLC. Trinitrophenyl derivatives of human red blood cell PE were analyzed after acidolysis (B) as described in Methods. Separation conditions were as in Fig. 1 and UV detection was at 338 nm. Molecular species were assigned after GLC analysis. Peak numbers correspond to those listed in Table 3. The letter p beside the number indicates the plasmalogen species, Dipalmitoyl-PE, di-14:0 was used as internal standard.

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nature of the analysis of TNP-molecular species by this procedure.

This HPLC method was applied to the analysis of the phosphatidylethanolamine species composition of biological membrane samples. **Fig. 5** shows the separation of TNP derivatives of human red blood cell and rat brain PE. Molecular species were assigned after fatty acid and fatty aldehyde determination by GLC analysis of each peak after collection and transmethylation. The identity of the alkenyl species was further confirmed by their selective acidolysis according to the procedure of Pugh et al. (35) as shown in Fig. 5B. We identified a total of 42 molecular species in human red blood cell PE and 36 in rat brain PE (**Table 3**). The greater standard errors for human red blood cells compared to rat brain might be explained by the greater variability of the human diet and the greater resistance of the brain to dietary influences. The recovery of the internal standard (di-14:0 PE) was

TABLE 3. Molecular species composition of human red blood cell (RBC) and rat brain trinitrophenyl-phosphatidylethanolamines

Peak[a]	Molecular Species	Human RBC PE		Rat Brain PE	
		Diacyl	Alkenyl	Diacyl	Alkenyl
1	18:2,20:5(n-3) + 18:3(n-3),20:4	0.20 ± 0.01			
2	18:2,18:3(n-3)	0.22 ± 0.01			
3	Un	0.25 ± 0.02		0.90 ± 0.07	
4	20:4,22:6(n-3) + 16:1,18:2(n-6)	0.21 ± 0.02			
5	20:4,20:4(n-6)	0.28 ± 0.03			
6	18:2,22:6(n-3)	} 2.28 ± 0.15			
7	18:2,20:4(n-6) + 16:0,20:5(n-3)				
8	18:2,18:2(n-6)	1.06 ± 0.07			
9	18:2,22:5(n-3)	0.87 ± 0.02			
10	18:1,20:5(n-3)	0.83 ± 0.14			
11	16:0,22:6(n-3)	4.50 ± 0.54	6.10 ± 0.37	7.59 ± 0.05	6.98 ± 0.20
12	16:0,20:4(n-6)	10.23 ± 0.64	7.07 ± 0.32	1.65 ± 0.05	4.56 ± 0.09
13	18:1,22:6(n-3) + 16:0,18:2(n-6)[b]	6.46 ± 0.28	4.94 ± 0.31	2.18 ± 0.02	4.13 ± 0.07
14	18:1,20:4(n-6)	5.86 ± 0.21	4.47 ± 0.29	3.23 ± 0.07	5.51 ± 0.13
15	Un			0.37 ± 0.02	
16	18:1,18:2(n-6)[b] + 16:0,22:5(n-3)	7.36 ± 0.21	7.24 ± 0.37		
17	16:0,20:3(n-6)	3.77 ± 0.22			
18	18:1,22:5(n-6)	2.38 ± 0.16			
19	Un			0.97 ± 0.02	
20	16:0,18:1	16.86 ± 0.18		3.38 ± 0.05	1.67 ± 0.01
21	Un			1.38 ± 0.02	
22	16:0,22:4(n-6)	1.88 ± 0.28	7.64 ± 0.71		1.67 ± 0.01
23	18:1,22:5(n-6)		3.54 ± 0.42		
24	18:1,18:1	6.20 ± 0.30		10.32 ± 0.16	15.36 ± 0.22
25	18:0,22:6(n-3)	1.42 ± 0.25	10.27 ± 0.76	20.67 ± 0.32	17.69 ± 0.28
26	18:0,20:4(n-6)	9.33 ± 0.40	27.76 ± 0.92	16.92 ± 0.53	7.33 ± 17
27	18:1,22:4(n-6)	} 4.40 ± 0.17		8.41 ± 0.24	4.43 ± 0.07
28	18:0,18:2(n-6)				2.76 ± 0.04
29	18:0,22:5(n-3)	1.04 ± 0.07	8.06 ± 0.41		2.06 ± 0.09
30	Un	2.32 ± 0.48			
31	Un			6.12 ± 0.15	
32	18:0,22:5(n-6)			1.84 ± 0.07	
33	Un	1.00 ± 0.08			
34	18:0,20:2(n-6)			1.32 ± 0.05	1.49 ± 0.2
35	18:0,18:1	4.13 ± 0.29	6.45 ± 0.36	1.73 ± 0.20	6.39 ± 0.13
36	18:0,22:4(n-6)	1.37 ± 0.08	6.46 ± 0.35	4.74 ± 0.07	3.16 ± 0.11
37	Un	0.92 ± 0.11			
38	16:0,20:1(n-9)			1.73 ± 0.07	6.39 ± 0.15
39	18:1,20:1(n-9)			2.63 ± 0.09	2.74 ± 0.04
40	Un			0.84 ± 0.02	
41	Un	1.79 ± 0.08			
42	Un	0.24 ± 0.04			
43	18:0,20:1(n-9)			1.08 ± 0.02	2.85 ± 0.07
44	18:1,22:1(n-9)				2.83 ± 0.09
45	Un	0.34 ± 0.07			
mole % of total PE		60.78 ± 0.51	34.73 ± 0.57	48.68 ± 0.26	45.58 ± 0.24
Unidentified		4.49 ± 0.32		5.74 ± 0.04	

Values are expressed as the mole percentage and are the mean and standard error of n = 6 samples for human red blood cells and n = 5 for rat brains.
[a]Peak numbers correspond to those shown in Fig. 5; peaks were identified by GLC analysis; Un, unidentified.
[b]These species were not found in the alkenyl fractions.

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81.1 ± 1.5% and the total amount of TNP-PE was 1096 ± 17 nmol (n = 6) from 1 ml of packed RBC. This is in good agreement with the value obtained by Dodge and Phillips (40). With the present HPLC system, we were able to separate molecular species of diacyl and alkenyl-acyl PE in a single run. Alkyl species were not identified by our analytical procedures. This subclass represents a relatively small proportion of ethanolamine glycerophospholipids, i.e, about 2% of the human erythrocyte PE (41) and 3 to 4% of the rat brain PE (41, 42). Some of the unidentified peaks may be alkyl species, as the former comprised about 4.5% and 5.7% of the human erythrocyte and rat brain TNP-PE, respectively (Table 3). The proportion of diacyl and alkenyl subclasses in our HPLC analysis was in agreement with other published results (41-43). Diacyl and alkenyl species represented 60.8% and 34.7%, respectively, of the total TNP-PE molecular species in human erythrocytes; whereas they accounted for 48.7% and 45.6%, respectively, in rat brain TNP-PE. In the latter case, it should be emphasized that the analysis was performed on the whole brain. In this organ, the proportion of plasmalogen PE depends upon the proportion of white and grey matter; plasmalogen PE occurs at higher levels in white matter fractions (42).

Since isocratic HPLC conditions were used during most of the analysis period (from 20 to 90 min) for the TNP-PE species, we were able to apply relative retention time (RRT) calculations (6, 8). The RRTs of the main molecular species shown in Fig. 5 were calculated relative to that of the 16:0,18:1 (diacyl) species (**Table 4**). We chose this molecular species as a reference compound for both diacyl and alkenyl species since it is a commonly occurring species in biological samples and it elutes in the middle of the chromatogram. It may also be used to compensate for small shifts in retention times that may occur, for example, when a column is changed.

Fig. 6 shows a graphical representation of the RRTs of the PE species (plotted as the log of the RRT × 10) against the carbon number of the acyl or alkenyl chain in the *sn*-1 position, using the method as previously reported by Patton, Fasulo, and Robins (6). We did not include molecular species eluting after 90 min, since a gradient was begun at this time. In this way, parallel lines were constructed from the retention time data of species containing the same unsaturated fatty acid in the *sn*-2 position. The intercept of these points with the x-axis gave the effective carbon cahin length for the unsaturated chain in the *sn*-1 position. Thus a quite predicable elution pattern appears for all the molecular species tested. This relationship helps to predict the identification of molecular species that may exist in quantities that are insufficient for fatty acid analysis by GLC. Such predictions should prove useful in the design of analytical procedures used to verify the identity of a particular peak. For example, thermospray LC-MS in the selected ion monitoring mode may be used to verify the presence of a diglyceryl ion at a particular mass value. This representation also showed the diffierence in the elution profile of molecular species of diacyl and alkenyl analogues. Alkenyl moieties present in the *sn*-1 position elute after the corresponding ester under our RP-HPLC conditions. This was expected due to the more lipophilic character of an ether in comparison to the more polar ester (44). In addition there was a linear correlation (r = 0.995) between the log (RRT × 10) of molecular species of diacyl and alkenyl analogues. The regression equation was Y = 0.981X ± 0.086 with Y and X representing the log (RRT × 10) of the corresponding alkenyl and diacyl species, respectively (n = 14 different molecular species). This relation may also help either to predict or confirm the identity of peaks.

TABLE 4. Relative retention time (RRT) values of the molecular species of TNP derivatives of human red blood cell PE

Peak[a]	Molecular Species	Diacyl	Alkenyl
1	18:2,20:5 (n-3) + 18:3(n-3),20:4	0.52	
2	18:2,18:3(n-3)	0.55	
3	Un	0.57	
4	20:4,22:6(n-3) + 16:1,18:2(n-6)	0.59	
5	20:4,20:4(n-6)	0.60	
6	18:2,22:6(n-3)	0.61	
7	18:2,20:4(n-6) + 16:0,20:5(n-3)	0.64	
8	18:2,18:2(n-6)	0.65	
9	18:2,22:5(n-3)	0.68	
10	18:1,20:5(n-3)	0.72	
11	16:0,22:6(n-3)	0.74	0.86
12	16:0,20:4(n-6)	0.75	0.88
13	18:1,22:6(n-3) + 16:0,18:2(n-6)[b]	0.78	0.92
14	18:1,20:4(n-6)	0.80	0.94
16	18:1,18:2(n-6)[b] + 16:0,22:5(n-3)	0.83	0.97
17	16:0,20:3(n-6)	0.88	
18	18:1,22:5(n-6)	0.94	
20	16:0,18:1	1.00	
22	16:0,22:4(n-6)	1.04	1.03
23	18:1,22:5(n-3)		1.04
24	18:1,18:1	1.08	
25	18:0,22:6(n-3)	1.12	1.13
26	18:0,20:4(n-6)	1.14	1.35
27	18:1,22:4(n-6)	1.18	
28	18:0,18:2(n-6)	1.20	
29	18:0,22:5(n-3)	1.31	1.52
30	Un	1.40	
33	Un	1.48	
35	18:0,18:1	1.54	1.89
36	18:0,22:4(n-6)	1.64	1.92
37	Un	1.70	
41	Un	2.01	
42	Un	2.10	
45	Un	2.12	

RRT values are calculated relative to the 16:0,18:1 (diacyl) molecular species.
[a]Peak numbers correspond to those shown in Fig. 5; peaks were identified by GLC analysis; Un, unidentified.
[b]These species were not found in the alkenyl fractions.

The quantitative distribution of the various molecular species was different for the diacyl and alkenyl subclasses (Table 3). The principal alkenyl chains were 16:0, 18:0,

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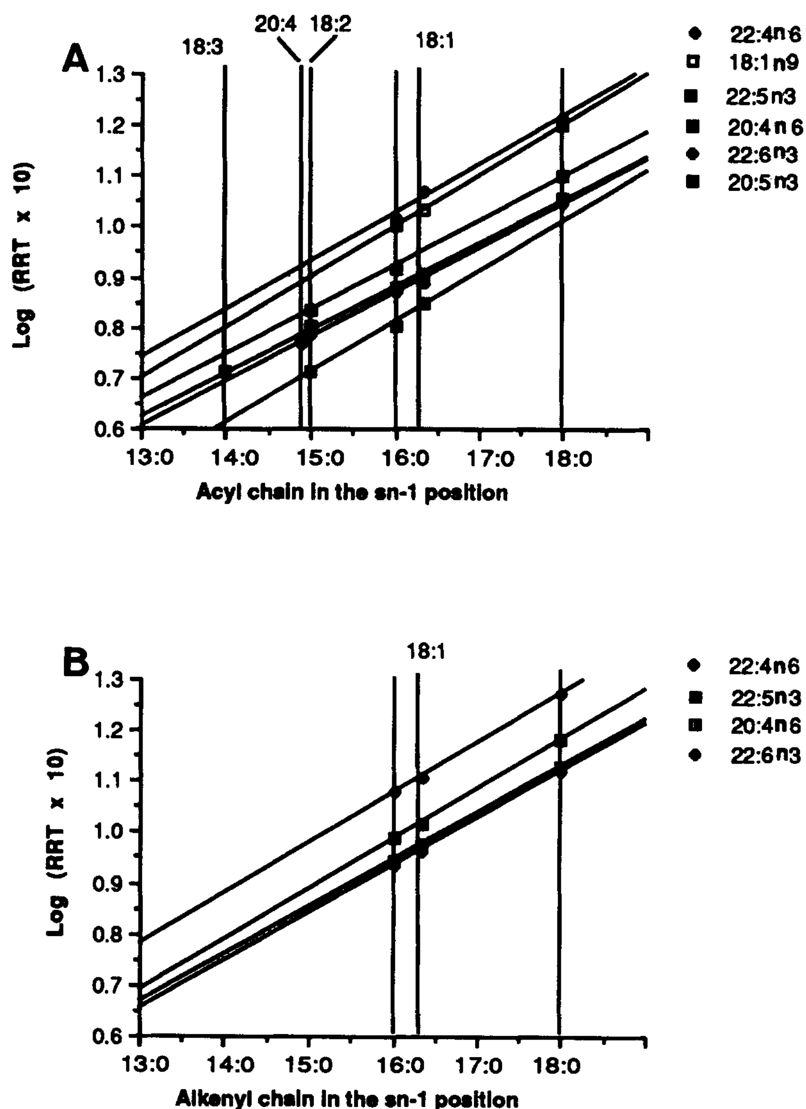

Fig. 6. Relationship between relative retention time (RRT) and fatty acid composition on the elution of diacyl (A) and alkenyl (B) molecular species of TNP-PE by HPLC. The log (RRT × 10) of molecular species of TNP-PE shown in Fig. 5 was plotted against the acyl or alkenyl chain length in the *sn*-1 position. All RRTs were calculated relative to the 16:0, 18:1 species. Vertical lines represent the molecular species with the same acyl or alkenyl chain in the *sn*-1 position, whereas the oblique lines represent species with the unsaturated fatty acid indicated.

and 18:1; 18:0 predominated as it accounted for 59.0% and 43.7% of the plasmalogen PE species in human erythocytes and rat brain, respectively. This subclass was enriched in polyunsaturated fatty acids as they comprised 93.5% and 61.8% of the acyl chains in the *sn*-2 position of human erythrocytes and rat brain, respectively. In human erythrocyte plasma membranes, 20:4(n-6), 22:6(n-3), and 22:5(n-3) predominated accounting for 39.4%, 21.2%, and 18:8% of the fatty acids in the *sn*-2 position of plasmalogen PE, respectively. About 55% of the total n-3 polyunsaturates was found in plasmalogen PE and 45% in diacyl species in the human RBC. This localization may be of importance for their oxidative metabolism; a lipoxygenase activity has been detected in mammalian erythrocytes (45).

In rat brain alkenyl PE, 22:6(n-3) was the major fatty acid representing 28.8% of the fatty acids in the *sn*-2 position, whereas 20:4(n-6) accounted for only 17.4%. We also noted a high proportion of monoenes in brain PE plasmalogens in comparison to that of red blood cells; 18:1(n-9) and 20:1(n-9) represented 23.4% and 12.0%, respectively, of the fatty acids in the *sn*-2 position. These results are consistent with those of Nakagawa and Horrocks (43), except that 18:1(n-9) accounted for 29-32% of the fatty aicds in the *sn*-2 position in their study. However, the content of 18:1(n-9) in the *sn*-2 position is also a func-

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tion of the proportion of white and grey matter in the brain samples as higher values are obtained in white matter (42).

The diacyl PE of rat brain contained a molecular species profile that was similar to that of alkenyl PE but the quantitative distribution was different. Polyunsaturated fatty acids were found in a higher proportion in acyl species (77%), whereas the proportion of monoenes (23%) was lower than in the alkenyl (38%) species.

In contrast, in the human erythrocyte membrane, diacyl PE displayed a lower amount of polyunsaturates (66%) in comparison to that of the plasmalogen PE (94%). Correspondingly, the proportion of monoene species (27%) was higher in the diacyl species than in the plasmalogen (6%) PE. Interestingly, we also found several dipolyunsaturated species in the red blood cell membrane among which were: 20:4(n-6),20:4(n-6); 20:4(n-6),22:6(n-3); 18:2,20:4(n-6); 18:2,22:6(n-3). Although they represented only a small proportion of the diacyl species, these species may have highly specialized functions and metabolic behavior (46).

In conclusion, the HPLC method described provides a new analytical procedure for the separation of molecular species of aminophospholipids. The HPLC systems are capable of good species resolution, are stable and reproducible, and the overall method recovery is good. Also, the chromophore introduced by derivatization leads to good sensitivity as it allows for determination of picomolar amounts of aminophospholipids. Finally, this analytical technique could be a useful tool for the study of the asymmetrical localization of aminophospholipid species in plasma membranes.

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