*Journal of Chromatography*, 338 (1985) 61—69
*Biomedical Applications*
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2421

# REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF MOLECULAR SPECIES OF ALKYL ETHER, VINYL ETHER, AND MONOACYL LYSOPHOSPHOLIPIDS

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(First received July 11th, 1984; revised manuscript received September 24th, 1984)

## SUMMARY

An isocratic reversed-phase high-performance liquid chromatographic method was developed which resolved individual molecular species of choline and ethanolamine lysophospholipids utilizing a $C_{18}$ bonded porous silica stationary phase with a mobile phase comprised of methanol—water—acetonitrile (57:23:20) containing 20 m*M* choline chloride. Solute retention was primarily determined by hydrophobic interactions with the stationary phase permitting separation of individual molecular species of lysophospholipids according to the composition of the aliphatic chain and the nature of its covalent attachment to the *sn*-1 hydroxyl group. The elution profile of unsaturated monoacyllysophospholipid or lysoplasmalogen molecular species was readily obtained by measuring UV absorbance at 203 nm. Identification of column eluates containing saturated monoacyl and alkyl ether lysophospholipids was possible utilizing relative retention factors that were obtained for the majority of molecular species present in animal tissues.

## INTRODUCTION

Conventional approaches for preparing labeled homogeneous phospholipid probes required for studies of the metabolism and physicochemical properties of plasmalogens are based on the acylation of vinyl ether lysophospholipids. The total synthesis of homogeneous lysoplasmalogens with the appropriate *sn*-1 side-chain is, however, a laborious process accompanied by poor yields. Accordingly, the acylation reaction is routinely performed on mixtures of lysoplasmalogen molecular species obtained from biologic sources (typically alkaline hydrolysates of beef heart choline phosphoglycerides). This is an inefficient approach for the preparation of homogeneous plasmalogens or alkyl ethers and impractical when the desired species is a minor component of the

0378-4347/85/$03.30 

mixture (e.g., synthesis of phospholipid probes with an octadecyl side-chain at the *sn*-1 position).

To overcome these limitations, we developed a high-performance liquid chromatographic (HPLC) method for separating individual molecular species of lysophospholipids to provide milligram quantities of starting material for subsequent use in the synthesis of homogeneous diradylphospholipid probes. To achieve high-efficiency separation based on differences in the composition of the *sn*-1 side-chain, a reversed-phase approach was employed using octadecylsilane-derivatized porous silica as the stationary phase. Elution of individual components is accomplished isocratically with a solvent system that allows the measurement of UV absorbance to monitor the separation. The chromatographic procedure was evaluated using synthetic homogeneous lysophospholipid standards and mixtures of monoacyl, alkyl ether, and vinyl ether lysophospholipids derived from biologic sources.

## EXPERIMENTAL

### *High-performance liquid chromatography*

HPLC was performed using a liquid chromatographic system from Waters Assoc. consisting of a Model 720 system controller, a U6K injector, a Model 6000A pump, a Model 450 variable-wavelength UV detector with an 8-$\mu$l flow cell and a Model 730 printer—plotter integrator. Samples were injected in 10—200 $\mu$l vols. of chloroform—methanol and UV absorbance was monitored at a wavelength of 203 nm. All separations were performed at room temperature using a stainless-steel column (25 cm × 4.6 mm I.D.) packed with Ultrasphere ODS ($C_{18}$ bonded-phase on 5-$\mu$m porous silica microparticles, Altex) with a pre-column (8 cm × 3 mm I.D.) containing Co:Pell ODS pellicular packing ($C_{18}$ bonded-phase on 30-$\mu$m glass beads, Whatman). Sample components were eluted isocratically at a flow-rate of 2 ml/min with a mobile phase of methanol—water—acetonitrile (57:23:20, v/v/v) containing 20 m*M* choline chloride.

### *Solvent reagents and phosphate analysis*

Methanol and acetonitrile were obtained from Burdick & Jackson Labs. and doubly distilled water was obtained from J.T. Baker. Choline chloride was obtained from Sigma (St. Louis, MO, U.S.A.). All solvents had a UV cutoff below 210 nm and were filtered through a 0.45-$\mu$m filter (Millipore, Bedford, MA, U.S.A.) and thoroughly degassed under vacuum prior to use. The phosphate content of column eluates was determined by the microphosphate assay method of Chen et al. [1].

### *Lipids*

Lysophosphatidylcholine (LPC) and lysophosphatidylethanolamine (LPE) containing 14:0, 16:0, 18:0, and 18:1 fatty acids and beef heart lecithin were purchased from Avanti (Birmingham, AL, U.S.A.). 1-O-[Alkyl-1′,2′-$^3$H]glyceryl-3-phosphorylcholine was obtained from New England Nuclear (Boston, MA, U.S.A.). Bovine brain LPE, and soybean LPC were obtained from Sigma.

Purity of each of the lipids was verified by the presence of a single spot after thin-layer chromatographic (TLC) separation and iodine staining and scintillation spectrometry of the radiolabeled compounds.

Choline lysoplasmalogen was prepared as previously described [2]. The 1-alkenyl groups in the final product were 14:0 (6%), 15:0 (7%), 16:0 (68%), 17:0 (4%), 18:0 (10%) and 18:1 (4%) as determined by fast atom bombardment mass spectrometry (MS) and gas chromatography (GC) of the dimethylacetal derivatives.

*Derivatization, GC and GC—MS*

Column fractions were obtained from individual peaks in the UV absorbance profile. To each ml of effluent collected, 0.3 ml of water and 0.6 ml of chloroform were added to form a biphasic extraction mixture. Lysophospholipids were recovered in the lower chloroform layer following two successive extractions, dried under nitrogen, and derivatized by acid methanolysis as previously described [3]. The fatty acid methyl ester (FAME) and/or dimethylacetal (DMA) products were injected onto a 30 m × 0.25 mm fused-silica capillary GC column coated with SP 2330 (Supelco) utilizing a split ratio of 5 to 10:1 and eluted isothermally at 190°C utilizing a Varian Model 3700 chromatographic system (Varian, Walnut Creek, CA, U.S.A.) with helium as a carrier gas at a flow-rate of 2 ml/min. Components eluting from the column were detected and quantified with a flame-ionization detector and Varian Model 4270 integrator. Mass spectra were obtained by electron impact (70 eV) at a source temperature of 150°C following GC separation on a 1-m glass column packed with 10% SP 2330 on 100—120 mesh Chromosorb W AW (Supelco) using a Finnigan Model 3200 GC—MS system. Identification of lysoplasmalogen molecular species was established by comparison of the GC retention times of their DMA derivatives with those derived from myocardial sarcolemma (containing approximately 40% plasmalogens [3]) and by GC—MS of the DMA derivatives prepared from column eluates corresponding to each peak. The assignment of molecular species with methyl branched-chain substituents was based on comparison of the relative GC retention times of the branched-chain and straight-chain DMA derivatives containing equal numbers of carbon atoms [4], by comparison of the mass spectra of both isomers demonstrating parent ions of identical mass, and based on the previous demonstration of branched-chain isomers in choline plasmalogen from bovine heart in the same relative proportions that we observed [5]. Unsaturated monoacyllysophospholipids were identified by comparing GC retention times of their FAME derivatives with commercially available standards and by GC—MS of the FAME derivatives prepared from column eluates.

*Computation of relative retention time*

Relative retention time (RRT) values for individual molecular species were adjusted to account for the time required to displace the void volume using the following formula: RRT = (retention time of component species $- t_0$)/(retention time of 18:1 monoacyllysophosphatidylcholine $- t_0$), where $t_0$ = time to reach solvent front peak.

## RESULTS

For isocratic elution of lysophospholipid standards, a ternary mobile phase containing methanol, water and acetonitrile was found to provide greater separation selectivity than binary solvent mixtures of methanol—water or acetonitrile—water. An example of the separation of individual species of choline lysoplasmalogens and alkylglycerylphosphorylcholines is shown in Fig. 1. With the exception of fractions 3p and 8p, all other fractions were homogeneous as determined by the presence of a single component on GC analysis of acid methanolysates prepared from column eluates. As suggested by the HPLC chromatogram, there were three components in fraction 3p and two components in fraction 8p. In both cases, the predominant species were methyl branched-chain isomers. The identity of the minor constituents was not established.

For the alkyl ether choline lysophospholipids (which do not have appreciable UV absorbance), $^{3}$H-labeled 1-O-alkyl-2-lysophosphatidylcholine [lyso-platelet activating factor (lyso-PAF)] was used to establish the retention time of individual molecular species. Since lyso-PAF was prepared by hydrogenation of choline lysoplasmalogen with tritium gas the distribution of saturated $^{3}$H-labeled product species reflects that of the choline lysoplasmalogen starting material. Accordingly, the identification of lyso-PAF species was made by comparing the retention times and relative peak areas from the elution profile of the $^{3}$H-labeled components to that of their choline lysoplasmalogen precursors (Fig. 1).

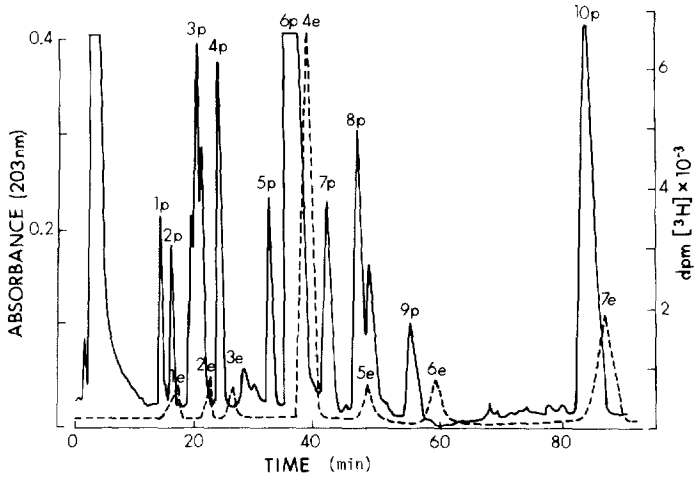


Fig. 1. Separation of molecular species of vinyl ether and $^{3}$H-labeled alkyl ether lysophosphatidylcholine. Bovine heart choline lysoplasmalogen (1.4 $\mu$mol) and 1-O-[alkyl-1',2'-$^{3}$H]-glyceryl-3-phosphorylcholine (4.5 nCi, specific activity = 45 mCi/$\mu$mol) dissolved in 150 $\mu$l of chloroform—methanol (2:1, v/v) were injected onto a 5-$\mu$m Ultrasphere ODS $C_{18}$ reversed-phase column. Lysophospholipids were eluted isocratically at room temperature (20—24° C) with a flow-rate of 2 ml/min using a solvent mixture of methanol—water—acetonitrile (57:23:20, v/v/v) containing 20 m*M* choline chloride. Individual molecular species were identified as described in the text. The numbers above each peak correspond to those in Table I. ——, UV absorbance at 203 nm; - - - -, $^{3}$H radioactivity.

The separation of monoacyllysophospholipids is illustrated in Fig. 2. For saturated species, detection was accomplished by measuring the phosphate content of column eluates collected at 2-min intervals following the injection of synthetic homogeneous lysophospholipid standards. The retention times of the 14:0, 16:0 and 18:0 monoacyl species were 13, 28, and 59 min, respectively. The 18:2 FAME derivatives prepared from column eluates corresponding to peaks 2a and 3a were indistinguishable by comparison of GC retention times or electron-impact mass spectra. Peak 3a represents 1-linoleoyl-*sn*-glycero-3-phosphocholine and peak 2a was tentatively identified as a positional isomer (i.e., differing with respect to location of the acyl chain or position of the double bonds) and was not characterized further.

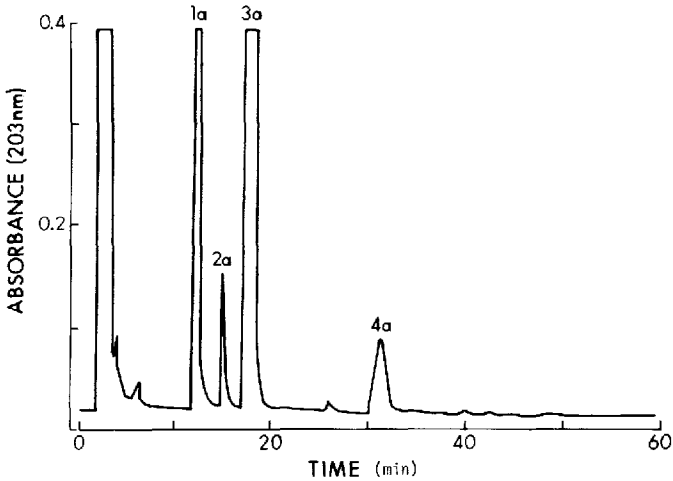


Fig. 2. Separation of molecular species of monoacyllysophosphatidylcholine. Soybean LPC (1.4 μmol, prepared by phospholipase $A_2$ catalyzed hydrolysis of soybean phosphatidylcholine) was dissolved in 50 μl of chloroform—methanol (2:1, v/v) and injected. Conditions for separation and peak detection and methods used for identification of individual molecular species are the same as those for the vinyl ether species given in Fig. 1. The numbers above each peak correspond to those in Table I.

Fig. 3 represents an example of the separation of bovine brain LPE. The UV absorbing fractions were homogeneous, however, the sample also contained saturated monoacyl LPE species that were not detected by measurement of UV absorbance. Accordingly, equal amounts of 14:0, 16:0 and 18:0 monoacyl LPE standards were injected separately and the phosphate content of fractions collected at 2-min intervals was determined to establish retention times of saturated monoacyl LPE species. The 14:0, 16:0 and 18:0 LPE molecular species eluted at 13, 28 and 58 min, respectively.

In order to compare the retention times of molecular species in different samples, relative retention factors were obtained in a separate series of injections by adding 18:1 monoacyl LPC as an internal reference standard to each sample. The relative retention time of individual molecular species was then calculated (Table I). Comparison of the relative retention factors demonstrated that substitution of ethanolamine for choline in the polar head group had no appreciable effect on the retention time of vinyl ether and monoacyllyso-

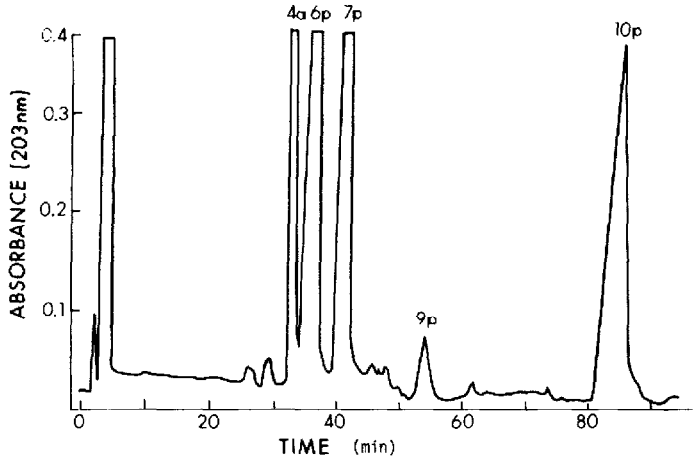


Fig. 3. Separation of molecular species of lysophosphatidylethanolamine from bovine brain. LPE (1.5 μmol, prepared by phospholipase $A_2$ catalyzed hydrolysis of bovine brain phosphatidylethanolamine) was dissolved in 50 μl of chloroform—methanol (1:1, v/v) prior to injection. Methods for separation, peak detection, and identification of individual molecular species are identical to those described for the choline lysoplasmalogen species in Fig. 1. The numbers above each peak correspond to those in Table I.

TABLE I

RELATIVE RETENTION TIMES FOR INDIVIDUAL LYSOPHOSPHOLIPID MOLECULAR SPECIES

RRT values were computed as described in the experimental section. Numbers in parentheses identify individual peaks in the elution profiles of Figs. 1—3.

| Species* | Alkyl ether | Vinyl ether | Monoacyl ester |
|---|---|---|---|
| 14:0 br | — | 0.478 (1p) | — |
| 14:0 | 0.533 (1e) | 0.532 (2p) | 0.366 |
| 18:3 | — | — | 0.371 (1a) |
| 18:2 | — | — | 0.559** (isomer 1) (2a) |
| | — | — | 0.589** (isomer 2) (3a) |
| 15:0 br | 0.733 (2e) | 0.682 (3p) | — |
| 15:0 | 0.867 (3e) | 0.799 (4p) | — |
| 16:0 br | — | 1.087 (5p) | — |
| 16:0 | 1.267 (4e) | 1.184 (6p) | 0.805 |
| 18:1 | — | 1.411 (7p) | 1.000*** (4a) |
| 17:0 br | 1.687 (5e) | 1.622 (8p) | — |
| 17:0 | 1.967 (6e) | 1.886 (9p) | — |
| 18:0 | 2.800 (7e) | 2.863 (10p) | 1.885 |

*Listed in order of elution from the column. Individual molecular species are designated in the form *a*:*b* where *a* is the number of carbon atoms and *b* is the number of double bonds in the aliphatic chain (br. indicates a methyl branched-chain isomer).
** Isomer 1 and 2 were tentatively identified as positional isomers (see text).
***Used as the internal standard.

phospholipids. Thus, under the conditions employed, separation selectivity is determined primarily on the basis of interactions of the stationary phase with the hydrophobic portion of the lysophospholipid molecule.

For all samples, optimal separation efficiency was achieved when the mobile phase contained 20—25% (v/v) water with typical plate counts of 20 000 to 35 000 plates per m for monooleoyllysophosphatidylcholine at a flow-rate of 2 ml/min (mobile phase velocity of 0.2 cm/sec). Varying the flow-rate from 1.0 to 2.5 ml/min resulted in a two-fold decrease in retention time but had no significant effect on the plate count. Operating pressures required to maintain a 2 ml/min flow-rate ranged from 170 to 310 bars. Routine use of a guard column reduced the rate at which operating pressure increased with age of the column without adversely affecting separation efficiency.

In order to reduce peak tailing that results from interaction of the polar head group with highly adsorptive sites on the porous silica, it was necessary to add an ionic modifying agent to the mobile phase. From previous reports of the reversed-phase separation of diacylphospholipid species, such mixed-mode retention was prevented by adding phosphoric acid [6] or choline chloride [7] to the mobile phase. Since plasmalogens are acid-labile, choline chloride was used as the modifying agent. With 20 m*M* choline chloride interactions of the polar head group with the stationary phase were effectively suppressed. This was demonstrated by an improvement in peak symmetry and by the identical retention times obtained for monooleoyl LPC and LPE standards. With an ionic modifier in the mobile phase, it was possible to inject up to 2 mg of lysophospholipid without overloading the column. When it was necessary to remove the excess choline from column eluates (e.g., prior to acylation of the lysophospholipid), a two-phase extraction was performed by adding 0.6 ml chloroform and 0.3 ml water to each ml of mobile phase, mixing, centrifuging, and finally collecting the lower chloroform layer to recover the lysophospholipids. Using this extraction procedure, recovery of lysophospholipids exceeded 93% based on results obtained with $^{3}$H-labeled alkyl ether lysophospholipids and phosphate measurements following separation of monoacyllysophospholipid standards.

## DISCUSSION

Reversed-phase chromatographic methods have recently been successfully applied to the separation of diacylphospholipid molecular species using a stationary phase of octadecylsilyl groups covalently bound to porous silica microparticles [6, 7]. In those studies, it was necessary to add an ionic modifying agent to the mobile phase to mask highly adsorptive sites that remained after derivatization in order to prevent the loss of resolution that arises from mixed-mode retention. Utilizing this information we developed the present method which, for the first time, permits separation of lysophospholipids into homogeneous fractions containing a single molecular species.

For the separation of a homologous series of compounds with long-chain aliphatic groups the step-wise addition of methylene ($-CH_2-$) units leads to a step-wise increase in the logarithm of the distribution constant, $K_D$ [8]. Since the relative retention time is directly proportional to $K_D$, by placing individual lysophospholipid species into classes based on similarities in the covalent attachment of the aliphatic chain to the polar head group, the degree of unsaturation, and the presence (or absence) of methyl branching, a plot of

log (RRT) versus chain length for each class should produce a series of parallel straight lines with different intercepts [9]. This is indeed the case as shown in Fig. 4. As expected, the values for the intercept increase in the observed order of elution: O-acyl esters < vinyl ethers < alkyl ethers since the polar character of these functional groups decreases accordingly. In addition to changes in solute polarity, retention time also appears to be dependent on steric interactions. Thus, while the introduction of methyl branching or carbon—carbon double bonds would not have a marked effect on solute polarity, these structural modifications which occur near the methyl end of the aliphatic chain also resulted in a significant decrease in the value of the intercept.

Since we have found that measurements of the retention time relative to the 18:1 monoacyl species are reproducible within ± 10% ($n$ = 10), it would be possible to make a tentative identification of an unknown lysophospholipid species based on measurement of the RRT value and using the information given in Fig. 4. This is illustrated by point A in Fig. 4 where if an unknown lysophospholipid detected by the UV monitor had an RRT value of 0.447 [log (10 × RRT) = 0.65] it would tentatively be identified as a 16:1 monoacyllysophospholipid.

By monitoring UV absorbance, the present method is ideally suited for separating mixtures of lysoplasmalogens since each individual species is readily detected by the UV absorbance which arises from the $\pi$—$\pi^*$ transition of electrons forming the double bond between the C-1 and C-2 carbon atoms of the vinyl ether group. For vinyl ethers with saturated aliphatic chains, the extinction coefficient is not affected by changes in chain length or methyl branching and therefore comparison of the peak areas provides a direct assessment of the

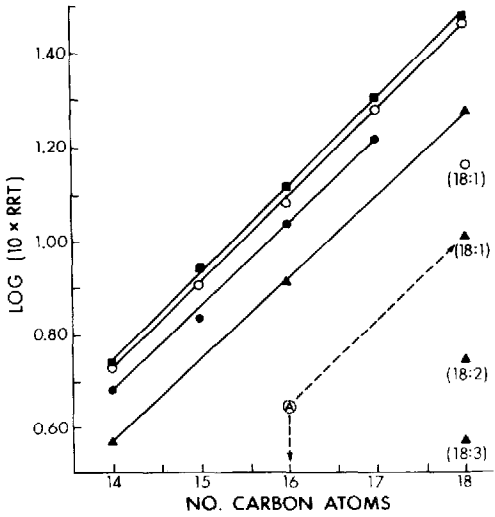


Fig. 4. Relationship between the relative retention time (RRT) and the number of carbon atoms in the acyl, alkyl, or alkenyl *sn*-1 side chain. Data for this figure were obtained from Table I. Individual molecular species were placed into one of the following groups: straight-chain 1-O-alkylglyceryllysophospholipids (■), straight-chain 1-O-alk-1′-enyllysophospholipids (○), branched-chain 1-O-alk-1′-enyllysophospholipids (●), and 1-O-acyllysophospholipids (▲). The linear regression correlation coefficient ($r$) had an average value of 0.9988 for the four groups shown.

relative amounts of each lysoplasmalogen present. For species with additional olefin groups, a better estimate of the relative amount of lysophospholipid contained in the corresponding fraction is obtained by dividing the area under each peak by the total number of unsaturated centers per molecule. This approach provides only an approximation, however, since the contribution of the vinyl ether double bond to the measured UV absorbance was substantially greater than that due to olefin groups.

ACKNOWLEDGEMENTS

This was supported in part by NIH Grant HL-17646, SCOR in Ischemic Heart Disease and an American Heart Association Grant-in-Aid award. R.W. Gross is the recipient of a Searle Scholar Award.

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