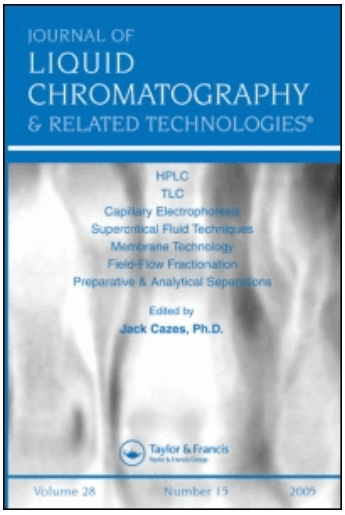

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SEPARATION OF INTACT PHOSPHATIDYLCHOLINE MOLECULAR SPECIES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Identification of the molecular species of phosphatidylcholines (PCs) is important for studies of lipid metabolism and the structure of cell membranes. The high performance liquid chromatography (HPLC) separations of the intact molecular species of PCs used earlier were mostly on reversed-phase C_{18} (octadecyl silica) columns, usually with isocratic elution. The eluent used contained a silanol suppressing agent. Volatile silanol suppressing agents are preferred for the purification of PCs because they can be removed from the HPLC fractions easily by nitrogen stream. They must be used when liquid chromatography-mass spectrometry (LC-MS) and evaporative light scattering detector (ELSD) are used because non-volatile silanol suppressing agents cause high background. For the identification of radioactive metabolites, using both the UV detector at 205 nm and radioactivity flow detector sequentially, we recommend the separation on a C_8 (octyl silica) column, eluted with a gradient of methanol-water containing conc. NH_4OH as silanol suppressing agent.

INTRODUCTION

Phosphatidylcholines (PCs) are important components of cell membranes and key intermediates of lipid biosynthetic pathways. Cell membranes contain a broad array of lipid classes such as PC and phosphatidylethanolamine (PE) acylated with different fatty acids (FAs). The structures, physical properties, and functions of cell membranes are affected to a large extent by their lipid components in terms of both lipid class and molecular composition of each lipid class. Identification of lipid class and the molecular composition of lipid classes is not only important for studies of the structure, physical properties, and functions of cell membranes, it is also important for elucidating lipid biochemistry. PCs are important intermediates in lipid biosynthetic pathways for the FA modifications, such as desaturation and hydroxylation of FAs.[1,2] PC (but also PE) also has been recognized as an important precursor in signal transduction pathways,[3] as the action of phospholipases C and D on PC releases the mediators, diacylglycerol, and phosphatidic acid.

Gas-liquid chromatography (GLC) is not suitable for the separation of intact molecular species of PCs because the high-temperature of the column would cause the decomposition of PCs. In contrast, intact PCs can be separated by HPLC at room temperature. There are some advantages of the separation of intact PCs by HPLC. Intact PCs in HPLC fractions can be easily collected for further analysis such as hydrolysis by phospholipase A_2 for the identification of the FA at sn-2 position.[2] HPLC co-chromatography with standards simplifies the identification of radioactive metabolites.[2] For quantitative studies of PCs, using HPLC for intact PCs instead of their derivatives can avoid their losses during derivatization and hydrolysis. Also, it is necessary to use intact PCs if radiolabeled with ^{32}P-phosphate or ^{14}C-choline. The HPLC systems for the separations of PCs may also be used for the separations of the intact molecular species of other lipid classes.[4,5]

For biological studies, the PC lipid class should be isolated and purified before the HPLC run of the molecular species of PCs. Recently, Christie[6] reviewed the separation of lipid classes by HPLC. Bell[7] also reviewed the separation of molecular species of phospholipids including PCs by HPLC. However, the later review included mostly the separation and identification of derivatives of diacylglycerols derived from phospholipase C hydrolysis and phosphatidic acids from phospholipase D hydrolysis of phospholipids using ultraviolet (UV) and fluorescence detection. It also included the derivatives of aminophospholipids (PEs and phosphatidylserines) for UV detection. Earlier, Patton and Robins[8] reviewed the extraction of phospholipids and analysis of phospholipid molecular species including some HPLC of the molecular species of PCs. Olsson and Salem[9] recently reviewed the analysis of molecular species of phospholipids by LC, LC-MS, GC, including separations of plasmalogen

phospholipids. We limit this review to the separation of intact molecular species of PCs by HPLC.

ELUENT AND DETECTOR

While C_{18} and C_8 columns were the primary choice for HPLC separations of the intact molecular species of PCs, the eluents used varied. The eluents used, either isocratic or gradient, were solutions composed of up to four of the following solvents: methanol, acetonitrile, isopropanol, dichloromethane, chloroform, hexane, isooctane, acetic acid, and water, with the addition of a silanol suppressing agent, as described in the next section. PCs with positive and negative charges elute as broad peaks (tailing) on reversed-phase HPLC as shown in Figure 1A, when the eluent without silanol suppressing agent was used.[10] Figure 1B shows the sharp peaks when silanol suppressing agent was added to the eluent. Similarly, the HPLC chromatograms of intact PCs using eluents with and without silanol suppressing agent were also shown.[5] The usual problem is with positively charged species which interact by ion exchange with ionized silanols in the stationary phase. Many silanol suppressing agents such as choline chloride,[4] ammonium acetate,[11] 1-ethylpropylamine,[12] trifluoroacetic acid ammonium salt,[13] ammonium hydroxide,[14,15] triethylamine,[10] tetraalkylammonium phosphates (ion-pair reagent),[5] and potassium phosphate buffer (pH 7.4)[16] have been used previously in the separation of intact PCs. We have previously added small amounts of acetic acid to improve peak shape for the separations of free fatty acids[17] and gibberellic acids,[18] a group of plant hormones, in C_{18} HPLC.

UV detection at 205 nm is sensitive and is the most common means of detection. The UV at 205 nm detects double bonds on the FA chain of PCs; PCs without double bond are also detected at 205 nm but the detection is not as sensitive.[9,14] The ratio of UV responses of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine is about 5.5 to 1 (peak areas). Eluents containing dichloromethane or chloroform can not be used for the detection of PCs by UV detector at 205 nm because of the absorption of these solvents at this wavelength.

Evaporative light scattering detector (ELSD) and flame ionization LC detector (FID) are sensitive universal mass detectors that overcome the problems associated with using UV detector at 205 nm. However, the reagents added to the eluent e.g. triethylamine,[10] ammonium acetate,[19] and 1-ethylpropylamine,[12] must be volatile, because non-volatile reagents add to the detected mass.

Liquid chromatography-mass spectrometry (LC-MS) also requires the use of volatile silanol suppressing agent e.g. ammonium hydroxide[15] or ammonium

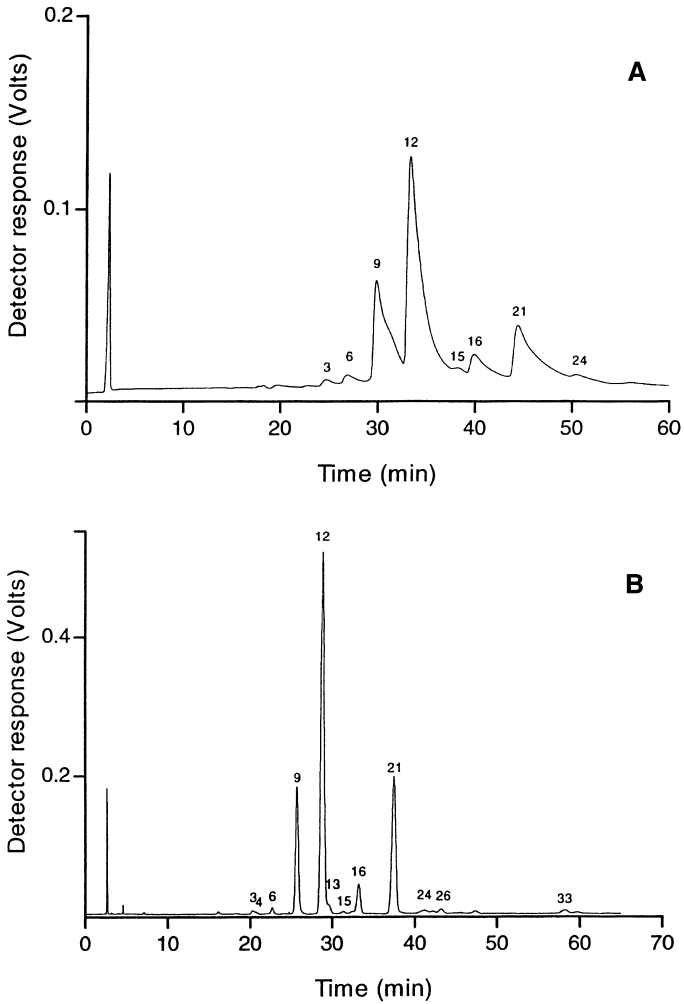

Figure 1. Silanol suppression in the separation of PC molecular species with the stationary phase of two Lichrosphere 100 RP18 columns in series by the addition of 1% (v/v) of triethylamine to the mobile phase consisting of methanol-acetonitrile (3:2). Flow-rate was 1 mL/min. Shown is the separation of PC molecular species derived from boar sperm cells as measured by ELSD. Figure A: without triethylamine, B: with triethylamine. PC component identification: 3 = 18:2-20:4; 4 = 18:2-18:2; 6 = 16:0-22:6; 9 = 16:0-22:5; 12 = (16:0-22:6)PlasCho; 13 = 16:0-18:2; 15 = 18:0-22:6; 16 = (16:0-22:6)AlkCho; 21 = (16:0-22:5)PlasCho; 24 = 16:0-18:1; 26 = 16:0-16:0; 33 = 18:0-18:1. PlasCho = alkenylacyl PC; AlkCho = alkylacyl PC. Alkenyl and alkyl chains are at sn-1 position. Reproduced from Ref. [10] with permission.

acetate.[11] It is a powerful detection method. Since it detects compounds on the basis of molecular weight or by fragmentation patterns (e.g. APCI, atmospheric pressure chemical ionization), it can detect individual PC species in a mixed peak of PC species. Detection by post-column fluorescence is used for its high sensitivity,[20] but requires post-column derivatization resulting in loss resolution.

Refractive index detection is also a universal mass detector; however it is insensitive compared to other detectors and only isocratic eluents can be used,[21] This detector is generally avoided at this time.

We have recently used both UV detector at 205 nm and radioactivity flow detector in sequence to identify PCs and quantify the radioactivity of PCs after resolution on a C_8 column.[2] The identification was performed by matching the retention times of UV peaks of PC standards added to the HPLC sample (co-chromatography) and the retention times of the radioactive peaks from the radioactive metabolites. Solvents, such as dichloromethane, absorb significantly at 205 nm and solvents, such as acetonitrile, which quench significantly in liquid scintillation counting must be avoided. We use a linear gradient of methanol-water-conc. NH_4OH (90:10:0.1) to methanol-conc. NH_4OH (99.9:0.1) in 40 minutes. The eluent can be removed easily by nitrogen stream, after the HPLC fraction collection, for further studies if necessary.

The flow-rate of the HPLC used is 1 mL/min while the flow-rate for liquid scintillation fluid is 3 mL/min. The ratio of the flow-rates, 1:3, is ideal for flow scintillation counting. We have recently also developed HPLC systems suitable for the identifications of radioactive free fatty acids (FFAs),[17] fatty acid methyl esters (FAMEs),[17] and triacylglycerols (TAGs)[22,23] using both UV and radioactive flow detectors. The solvents used for the eluents were methanol and isopropanol which are among the least toxic solvents and the least quenching solvents in liquid scintillation counting.

SEPARATION OF THE MOLECULAR SPECIES OF INTACT PHOSPHATIDYLCHOLINES

Isocratic C_{18} HPLC

Isocratic C_{18} HPLC has been the most commonly used HPLC method for the separation of intact molecular species of PCs. In 1981, Smith and Jungalwala reported the separation of molecular species of PCs by C_{18} HPLC.[16] Molecular species of PCs from egg, bovine brain, and porcine liver were resolved into 11-13 peaks on a Nucleosil-5-C_{18} column with an eluent of methanol-1 mM potassium phosphate buffer, pH 7.4 (95:5) at a flow-rate of 1 mL/min. Detection was at 205 nm.

The linear and parallel lines in the semilogarithmic plots of total hydrophobic carbons versus retention times (RTs) of PCs were shown. The RT increases logarithmically as the number of carbon atoms in the homologous series increases, whereas the RT decreases as the number of double bonds in the same FA chains increases. Introduction of the first double bond in the side chain reduced the RT to the greatest extent.

In 1982, Patton et al.[4] reported the separation of molecular species of PCs, PEs, phophatidylinositols (PIs), and phosphatidylserines (PSs) from rat liver. The lipid classes of PC, PE, and PI were separated into molecular species on a Ultrasphere ODS column eluted with methanol-water-acetonitrile (90.5:7:2.5) containing 20 mM choline chloride. PS was separated with the eluent of methanol-25 mM KH_2PO_4-acetonitrile-acetic acid (90.5:7:2.5:0.8) containing 30 mM choline chloride. The flow-rate was 2 mL/min. The RTs of the molecular species of phospholipids depends on both the polar head group and on FA chains. PI eluted earliest among the four lipid classes followed by PS, PC, and then PE. The relative retention time (RRT) of any particular molecular species relative to 16:0-22:6 was the same in all of the phospholipid classes studied. Patton et al. also improved the graphic relationship between the RRT and the total FA carbons shown[16] to the differentiation of the FA chains at both sn-1 and sn-2 positions as shown in Figure 2. It is possible to tentatively identify the molecular species of phospholipid classes by this graphic relationship.

The HPLC method developed by Patton et al.[4] for the separation of PCs, PEs, and PIs was later used for separations of the molecular species of monogal-actosyldiacylglycerols (MGDGs), digalactosyldiacylglycerols (DGDGs), phosphatidylglycerols (PGs), PCs, and PEs.[24] The HPLC system developed for the separation of molecular species of PCs can be used (or modified) for the separation of molecular species of other lipid classes.

Norman and St. John[12] separated the molecular species of PCs from spinach leaf on a Microsorb C_{18} column using an eluent of acetonitrile-methanol-acetic acid-water-1-ethylpropylamine (89.8:6.8:1.5:1.0:0.9) at a flow-rate of 1.1 mL/min. The PCs were directly quantitated by FID. The PC peaks were well separated and sharp.

Brouwers et al. reported in 1998[10] using two HPLC columns connected in series to separate intact molecular species of PCs (Figure 1) and using ELSD to quantitate intact PCs due to insensitivity of UV-detection for saturated species. The column was Lichrosphere 100-RP18. The isocratic eluent was methanol-acetonitrile (3:2) containing 1% of triethylamine at a flow-rate of 1 mL/min. The use of volatile triethylamine as silanol suppressing agent instead of choline chloride allowed the use of ELSD. The HPLC peaks in Figure 1B are sharp and well separated.

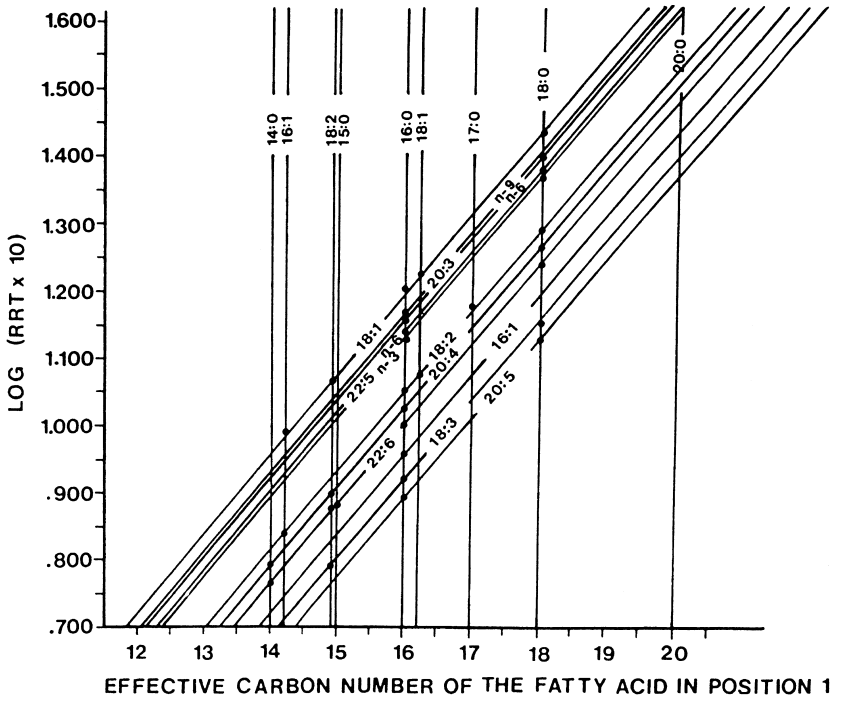

Figure 2. The effect of FA composition on the elution pattern of molecular species of phospholipids. The RRT calculated for each molecule was plotted as the log of the RRT × 10 against the carbon number of the FA in position-1. The points represent the molecular species identified by GLC. Oblique lines connect molecules that have the same FA in position-1. The intersection of oblique and perpendicular lines provides the RRT of individual molecules. Reproduced from Ref. [4] with permission.

All PCs displayed an exponential relation between RT and the percentage of acetonitrile or triethylamine. The response of the detector was invariant for the molecular species and allowed quantification of as little as 50 pmoles. The calibration curves of molecular species of PCs, saturated as well as polyunsaturated, were almost identical. Thus, for quantification of PCs, ELSD is a better choice than UV detector .

Gradient C_{18} HPLC

In 1993, Kaufmann and Olsson[19] reported a gradient HPLC method to separate PCs (Figure 3) and PEs from bovine milk in about 40 min as compared to the isocratic separation of egg PCs in 60 min[16] and rat liver PCs in 150 min.[4] An

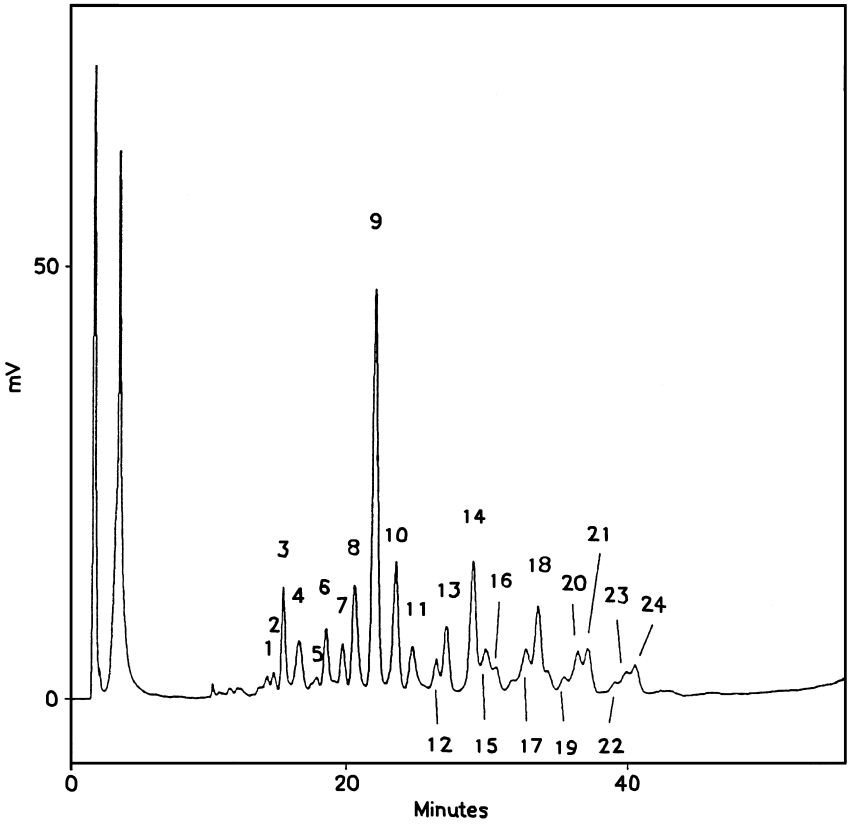

Figure 3. HPLC chromatogram of bovine milk PCs. An ODS column (Superspher RP-100) and ELSD were used. The eluent was from 1-propanol-water-isooctane (52:47:1) to 1-propanol-water-isooctane (58:33:9) both with 1.56 mmol ammonium acetate in 55 min using a positive exponential gradient and a flow-rate of 0.9 mL/min, at 75°C. PC component identification: 3 = 18:2-18:2; 4 = 18:1-18:3; 6 = 16:0-18:2; 7 = 18:1-18:2; 8 = 16:0-16:0; 9 = 16:0-18:1; 10 = 18:1-18:1; 11 = 18:0-18:2; 12 = 16:0-18:0; 13 = 18:1-18:0; 14 = 18:0-18:0. Reproduced from Ref. [19] with permission.

ODS column (Superspher RP-100) and ELSD were used. The eluent was from 2-propanol-water-isooctane (52:47:1) to 1-propanol-water-isooctane (58:33:9) each containing 1.56 mM ammonium acetate, in 55 min with a positive exponential gradient at a flow-rate of 0.9 mL/min at 75°C. This HPLC system can separate so-called "critical pairs" of molecular species such as 16:0-18:2 and 18:1-18:2. Critical pairs are usually difficult to separate and contain the same partition number (PN), the carbon number minus twice the number of double bonds.

McHowat et al.[25] used UV (203 nm) on-line absorption to quantitate molecular species of PCs. UV absorbance response factors of 37 synthetic PCs included diacyl, alkenylacyl, and alkylacyl species were given and were used for the quantification of PCs. The HPLC system used was a modification of Patton et al.[4] The eluent was methanol-water-acetonitrile (87:6:7) containing 20 mM choline chloride (solvent A) for 30 min, followed by a linear increase over 60 min to methanol-water-acetonitrile (76:4:20) containing 20 mM choline chloride (solvent B) and then solvent B for additional 30 min. The column used was Ultrasphere-ODS. Figure 4 is a HPLC chromatogram of plasmalogen PC standards.

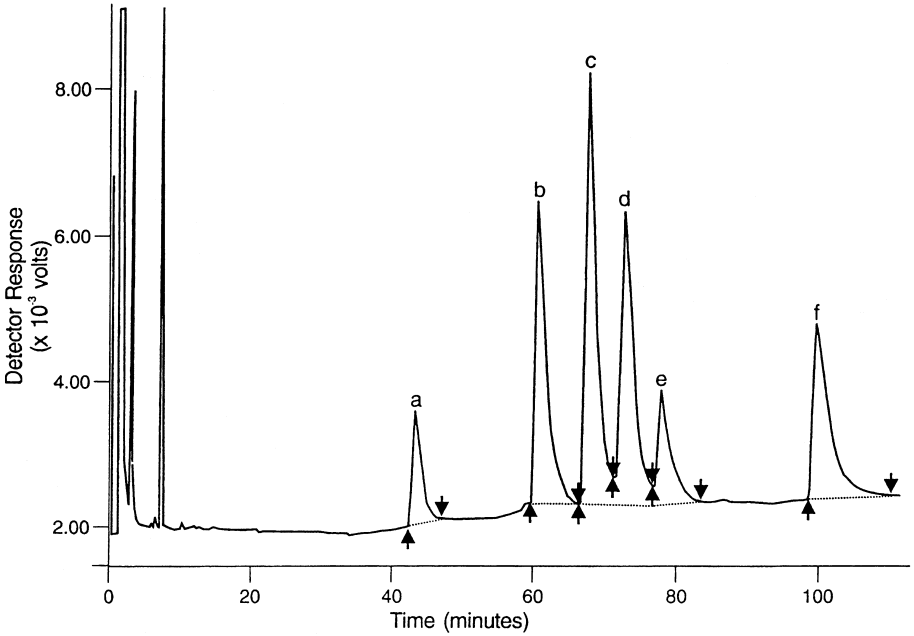

Figure 4. A C_{18} HPLC chromatogram for a mixture of synthetic plasmalogen PCs. The eluent was methanol-water-acetonitrile (87:6:7) containing 20 mM choline chloride (solvent A) for 30 min, followed by a linear increase over 60 min to methanol-water-acetonitrile (76:4:20) containing 20 mM choline chloride (solvent B) and then solvent B for additional 30 min. The column used was Ultrasphere-ODS. PC component identification: a = (16:0-18:2)PC; b = (18:0-18:3)PlasCho; c = (18:0-22:6)PlasCho; d = (18:0-20:4)PlasCho; e = (18:0-18:2)PlasCho; f = (18:0-18:1)PlasCho. Reproduced from Ref. [25] with permission.

LC-MS for the Separation and Identification of PCs

LC-MS is an efficient method to identify the molecular species of lipid classes from natural sources.[12,26] Kim and Salem[11] used thermospray LC-MS for the separation and identification of intact molecular species of PCs and PEs. A short column (Ultrasphere-ODS, 3 μm, 75 × 4.6 mm) and an eluent of methanol-hexane-0.1 M ammonium acetate (71:5:7) at a flow-rate of 1 mL/min were used. The separation of some PC standards was shown. The detection limit is in the 10-100 ng range. Positive ion spectra of (16:0-18:1) PC and (16:0-18:1) PE using thermospray shown were simple with extremely low background. The proposed structures of the fragment ions of PCs and PEs were shown. The principal fragment ions are the head group and the mono- and diacylglycerols. The positional isomers could not be distinguished from the spectra alone. LC-MS is valuable for the analysis of complex mixtures such as egg PCs. Although some molecular species of PCs were not resolved by HPLC, selective ion recording of the diacylglycerol ions allowed deconvolution of the HPLC peaks and the quantitative data of the unresolved PCs can be obtained.

Ma and Kim later in 1995[15] developed a LC-MS method to separate and identify lipid class (PC, PE, PS, and PI) and molecular species of phospholipids in a single HPLC run. A narrow-bore Hypersil C18 column (5 μm, 200 × 2.1 mm, Hewlett-Packard) was used. The initial eluent was methanol-hexane-water (88:0:12) containing 0.5% (v/v) ammonium hydroxide for 3 min at the flow rate of 0.4 mL/min. The eluent was then changed by linear gradient to (88:12:0) in 17 min. The elution order was PS, PI, PE, and PC for the species containing the same fatty acids. Within the same class, for the same acyl group in the sn-1 position, the elution order for the acyl group in the sn-2 position was 22:6, 20:4, 22:5, 18:1, and 18:0. By monitoring characteristic fragment ions (diacylglycerol ions) formed in the filament-on thermospray process and according to the retention time, individual molecular species in each phospholipid class can be identified. The detection limit in the range of 20-50 ng can be achieved using selective ion monitoring (SIM).

Recently Singleton et al.[27] used a narrow-bore C_8 column (150 × 2.1 mm, MacMod Analytical, Inc., Chadds Ford, PA, USA) and fast atom bombardment (FAB) MS to separate and characterize peanut phospholipid molecular species. The eluent consists of CH_3OH-hexane-0.05 M NH_4Oac-glycerol (84.3:7:8:0.7) isocratically at a flow-rate of 300 μL/min. Molecular and fragment ions were characterized by FAB MS.

Reversed-Phase Ion-pair HPLC

Abidi and Mounts[5] used ion-pair HPLC to separate molecular species of PCs and PEs, and compared reversed-phase C_{18} and C_8 columns from different

sources. For elution they used acetonitrile-methanol-water (70:22:8) containing 5 mM tetraalkylammonium phosphates as ion-pair reagent at a flow rate of 2 mL/min. HPLC on C_{18} column (Waters Resolve C_{18}) tended to give broader analyte peaks than with C_8 column (Brownlee C_8). When the mobile phase contained no ion-pair reagent, the peaks were too broad to be of any analytical value.

HPLC Separation of PCs Containing Oxygenated Fatty Acid

Bernstrom et al.[13] separated the molecular species of PCs and PEs containing 5,6-, 8,9-, 11,12-, and 14,15-epoxyeicosatrienoic acid (EET) at the sn-2 position. The EETs can be formed by cytochrome P-450 oxidation of arachidonic acid and have attracted attention because of their effects on endocrine, renal, ocular, and secretory cell function. The HPLC of PCs and PEs was performed essentially the same as Patton et al.[4] on an Ultrasphere ODS column. The eluent consisted of methanol-water-acetonitrile (90.5:7:2.5) containing 1 mM of the volatile buffer NH_4OOCCF_3, pH 7.4, at a flow-rate of 2 mL/min. The eluent contained a volatile buffer rather than that previously described. The PC and PE containing EET at the sn-2 position obtained from the incubation of EET with murine mast cells were purified and identified by MS. PC containing oxygenated FA is eluted earlier than the corresponding unoxidized PC.[14,28]

The importance of free radicals in causing alterations in various biological systems has led to numerous attempts to determine lipid peroxides in biological systems. Therond et al.[29] separated, simultaneously, molecular species of soy bean PCs or PEs together with their corresponding hydroperoxides obtained by lipoxygenase treatment. The separation was on RP18 Spherisorb at 40°C. The eluent was methanol-10mM ammonium acetate, pH 5 (95:5) at a flow-rate of 1 mL/min. The molecular species of PCs and PEs were detected at 205 nm, while the corresponding hydroperoxides were detected by fluorometer after derivertization with chemiluminescence reagent.

Later, Therond et al.[30] modified the method and separated the molecular species of human erythrocyte membrane PCs and PEs and their corresponding hydroperoxides after lipoxygenase treatment. The separation was on two analytical columns in series: a C_8 and C_{18} Kromasil at 40°C. The eluent was methanol-10mM ammonium acetate, pH 5 (94:6) at a flow rate of 1.5 mL/min. The HPLC chromatograms are shown as Figure 5.

Gradient C_8 HPLC

Recently we[14] developed a gradient C_8 HPLC system to separate molecular species of PCs and reported the RRTs of 32 synthetic PCs as shown in Table 1. The eluent was a linear gradient of 90-100% methanol (containing 0.1% of

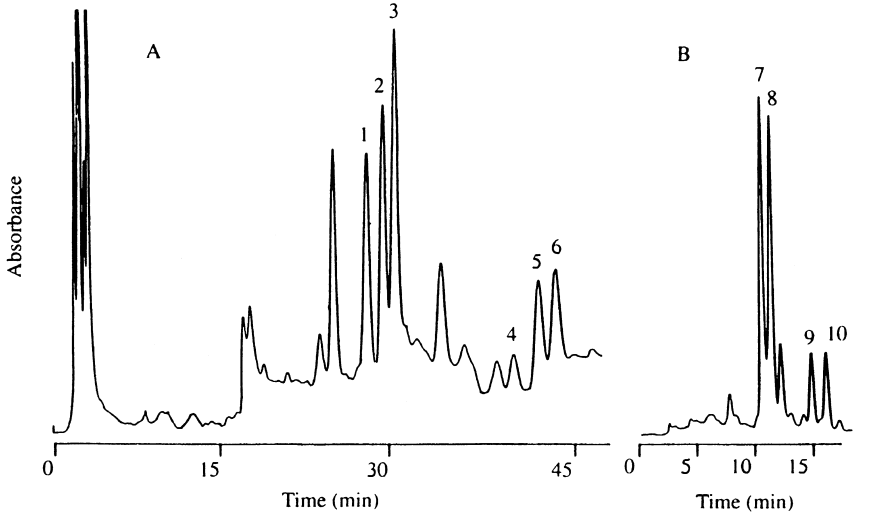

Figure 5. Separation of molecular species of PCs in human erythrocyte membrane before lipoxygenase treatment (A) and separation of the corresponding hydroperoxides after lipoxygenase treatment (B) using two serial Kromasil C_8 and C_{18} analytical columns at 40°C. The eluent was methanol-10mM ammonium acetate, pH 5 (94:6) at a flow rate of 1.5 mL/min. FA molecular species were detected at 205 nm, and hydroperoxides were detected with the chemiluminescence reagent at a flow rate 1.5 mL/min. PC component identification: A, 1= PC 16:0-22:6, 2 = PC 16:0-20:4, 3 = PC 16:0-18:2, 4 = PC 18:0-22:6, 5 = PC 18:0-20:4, 6 = PC 18:0-18:2. B, 7 = phosphatidylcholine hydroperoxide (PCOOH) 16:0-18:2, 8 = PCOOH 16:0-20:4 + 16:0-22:6, 9 = PCOOH 18:0-18:2, 10 = PCOOH 18:0-20:4 + 18:0-22:6. Hydroperoxide is at sn-2 position. Reproduced from Ref. [30] with permission.

conc. NH_4OH) in 40 min, then 100% methanol (containing 0.1% of conc. NH_4OH) at a flow-rate of 1 mL/min. A C_8 Luna column (silica gel based material) was used, and this column can withstand an eluent of pH 9.5 as used here. Another C_8 HPLC system was developed for the separation of lysophosphatidylcholines (lysoPCs) using a linear gradient eluent of 80-100% methanol (containing 0.1% of conc. NH_4OH) in 40 min. These HPLC systems are suitable for metabolic studies using both UV detection at 205 nm and radioactivity flow detection sequentially. The peak (column plate number N=59000) of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine from a C_8 column (Luna, Phenomenex, Torrance, CA, USA, stable at pH 9.5) is sharper than that (N=6700) from a C_{18} column (Ultrasphere, Beckman Instrument, Fullerton, CA, USA, unstable at pH 9.5) using a gradient of methanol-water containing NH_4OH.

Table 1

Relative Retention Times of Synthetic Phosphatidylcholines and Lysophosphatidylchloines in C_8HPLC[a]

Phosphatidylcholines and Lysophosphatidylcholines	Relative Retention Times (min) System 1[b]	System 2[c]
1-Oleoyl-sn-glycero-3-phosphocholine	8.17	14.43
1-O-Hexadecyl-sn-glycero-3-phosphocholine	8.92	16.78
1-Stearoyl-sn-glycero-3-phosphocholine	9.90	18.72
1-Palmitoyl-2-ricinoleoyl-sn-glycero-3-phosphocholine	18.2	
1,2-Dilinolenoyl-sn-glycero-3-phosphocholine	22.59	
1,2-Dimyristoyl-sn-glycero-3-phosphocholine	22.74	
1,2-Dipalmitoleoyl-sn-glycero-3-phosphocholine	25.46	
1,2-Dipalmitelaidoyl-sn-glycero-3-phosphocholine	25.88	
1,2-Dilinoleoyl-sn-glycero-3-phosphocholine	27.84	
1-Palmitoyl-2-palmitoleoyl-sn-glycero-3-phosphocholine	28.19	
1-Myristoyl-2-oleoyl-sn-glycero-3-phosphocholine	28.25	
1-Palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine	29.29	
1-Palmitoyl-2-linoleoyl-sn-glycero-3-phosphocholine	29.42	
1-Oleoyl-2-linoleoyl-sn-glycero-3-phosphocholine	30.63	
1,2-dipalmitoyl-sn-glycero-3-phosphocholine	30.94	
1-O-Hexadecyl-2-arachidonoyl-sn-glycero-3-phosphocholine	31.97	
1-Oleoyl-2-palmitoyl-sn-glycero-3-phosphocholine	32.06	
1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine[d]	32.15	
1-Stearoyl-2-arachidonoyl-sn-glycero-3-phosphocholine	33.17	
1-Stearoyl-2-linoleoyl-sn-glycero-3-phosphocholine	33.37	
1,2-Dioleoyl-sn-glycero-3-phosphocholine	33.41	
1-O-Hexadecyl-2-palmitoyl-rac-glycero-3-phosphocholine	33.55	
1,2-Dielaidoyl-sn-glycero-3-phosphocholine	33.67	
1,2-Dipetroselinoyl-sn-glycero-3-phosphocholine	34.28	
1-Stearoyl-2-palmitoyl-sn-glycero-3-phosphocholine	34.71	
1-Palmitoyl-2-stearoyl-sn-glycero-3-phosphocholine	34.72	
1-O-Hexadecyl-2-oleoyl-rac-glycero-3-phosphocholine	34.81	
1-Stearyl-2-oleoyl-sn-glycero-3-phosphocholine	35.93	
1-Oleoyl-2-stearoyl-sn-glycero-3-phosphocholine	36.00	
1,2-Di-O-hexadecyl-rac-glycero-3-phosphocholine	36.41	
1,2-Distearoyl-sn-glycero-3-phosphocholine	38.24	
1,2-Diarachidoyl-sn-glycero-3-phosphocholine	44.68	

[a] For HPLC conditions, see Figure 6. [b] Linear gradient of 90-100% methanol containing 0.1% of conc. NH_4OH in 40 min. [c] Linear gradient of 80-100% methanol containing 0.1% of conc. NH_4OH in 40 min. [d] Used for normalization of retention times to get relative retention times for system 1. Reproduced from Ref. 14 with permission.

The elution order of a given PC is inversely related to the polarity of its FA constituents. For acyl chains with lower polarity, elution time increases as follows: ricinoleic acid < linolenic acid < myristic acid < palmitoleic acid < palmitelaidic acid < arachidonic acid < linoleic acid < palmitic acid < oleic acid < elaidic acid < petroselinic acid < hexadecyl ether < stearic acid < arachidic acid. This elution order is similar to those of FA[17] and triacylglycerols[22] separated by C_{18} HPLC methods we have previously reported. A PC containing a cis-FA elutes slightly earlier than its trans-FA isomer. A baseline separation of PCs of double-bond positional isomers was achieved as shown in Figure 6. The elution order of these two PCs is the same as those of FA[17] and triacylglycerols[22] separated by C_{18} HPLC methods. The polarity of the acyl chain in the sn-2 position of a PC has slightly more influence on elution order than the acyl chain in the sn-1. The elution orders of lysoPCs depend on their FA constituents and seem to be the same as the FA order of PC.

CONCLUSION

The isocratic C_{18} HPLC chromatogram of Figure 1B shows a good separation of PCs with sharp peaks. This HPLC system used two reversed-phase columns in series while other HPLC chromatograms shown in this report used a single column (except the HPLC system of Figure 5). As expected, the HPLC system using two columns in series can improve the separation of PCs from using one column.

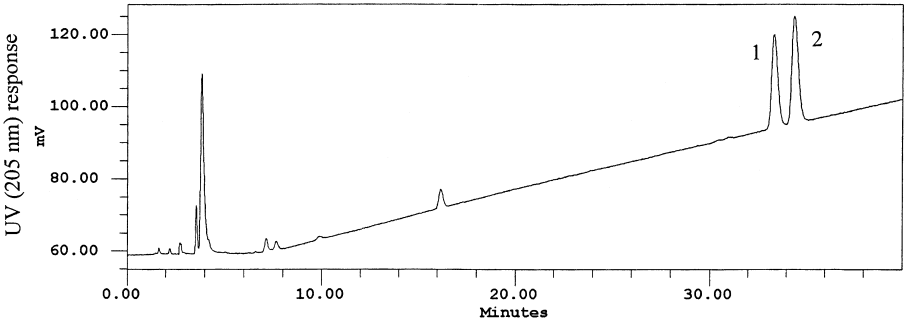

Figure 6. C_8 HPLC separation of PCs of double-bond positional isomers. (1) 1,2-dioleoyl-sn-glycero-3-phosphocholine (cis-9), retention time 33.33 min; and (2) 1,2-dipetroselinoyl-sn-glycero-3-phosphocholine (cis-6), 34.42 min. About 50 μg of the standard each in 10 μL of methanol were used. A C_8 Luna column was used with a linear gradient of 90-100% methanol (containing 0.1% of conc. NH_4OH) in 40 min at a flow-rate of 1 mL/min. Reproduced from Ref. [14] with permission.

The gradient C_{18} HPLC chromatogram of Figure 3 also shows a good separation with sharp peaks. The column was at 75°C which is different from other HPLC systems shown here. The use of a gradient eluent can save time and sharpen peaks, also column temperature can affect the separation.

Figure 6 also shows a good separation of PCs using a C_8 column at room temperature. We have included in this review a broad array of HPLC systems that have proven useful for the separation of molecular species of PCs. They should be useful to lipid researchers who can use one of them or modify one of them according to their needs.

For the identification of radioactive metabolites, the UV detector at 205 nm and radioactivity flow detector can be used sequentially. We have used this method and co-chromatography with lipid standards to identify the radioactive metabolites by matching the retention times of both detectors.[2] PC standards containing at least one unsaturated FA can be detected at 205 nm easily. The eluent of methanol-water for the separation of PCs is preferable for radioactive flow detector because acetonitrile, chloroform, and dichloromethane etc. quench significantly in liquid scintillation counting.

We recommend the use of a volatile silanol suppressing agent such as NH_4OH in the eluent, not only because LC-MS and ELSD can be used but also the eluent in HPLC fractions can be removed easily by nitrogen stream.

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