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Separation of synthetic phosphatidylcholine molecular species by high-performance liquid chromatography on a C_8 column

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Abstract

A C_8 high-performance liquid chromatography (HPLC) method for the separation of molecular species of phosphatidylcholines (PCs) was developed. This method uses a linear gradient of 90–100% methanol containing ammonium hydroxide as ion suppressor and is suitable for metabolic studies using both UV detection at 205 nm and radioactivity flow detection. The elution order of a given PC is inversely related to the polarity of its fatty acid 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 that of fatty acids separated by a C_{18} HPLC method we have previously reported. A PC containing a *cis*-fatty acid elutes slightly earlier than its *trans*-fatty acid isomer. 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. Another C_8 HPLC method was developed for the separation of lysophosphatidylcholines (*lyso*PCs) using a more polar eluent. The elution orders of *lyso*PCs depend on their fatty acid constituents and seem to be the same as the fatty acid order of the PC.

Keywords: Phosphatidylcholine; Fatty acids

1. Introduction

Phosphatidylcholines (PCs) are important components of cell membranes and intermediates of the lipid biosynthetic pathway. We have recently reported the biosynthetic pathway of triacylglycerols containing ricinoleate in castor microsomes [1]. In this previous study, we developed and used a reversed-phase C_8 high-performance liquid chromatography (HPLC) system to analyze molecular species of PCs and to purify the radioactive 1-palmitoyl-2-ricinoleoyl-*sn*-glycero-3-phosphocholine for use as the substrate for microsomal incubation. Here we report the relative retention times (RRTs) of various synthetic PCs and their elution characteristics in this reversed-phase C_8 HPLC system, to aid in identification of molecular species of PCs in analytical and metabolic studies.

The separation of lipid classes by HPLC was reviewed recently [2]. The separation of molecular species of phospholipids including PCs was also reviewed [3]. The HPLC separations of molecular

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species of PCs previously reported were mostly by isocratic elution of a C_{18} column and by the eluents containing acetonitrile [3]. Eluents containing acetonitrile quench the phosphor in radioactivity flow detection and limit the use of these eluents in biological studies. Patton et al. [4] used a C_{18} column, UV detection at 205 nm and isocratic eluent of methanol–water–acetonitrile (90.5:7:2.5) containing 20 m*M* choline chloride (2 ml/min, 150 min), to separate molecular species of PCs, phosphatidylethanolamines (PEs), phosphatidylserines and phosphatidylinositols from rat liver. A graphical relationship was developed to demonstrate that molecular species elute in a predictable sequence for a given solvent system. This HPLC method has been used in a wide variety of studies [3]. Norman and St. John [5] resolved PCs of spinach leaf using a C_{18} column and an isocratic eluent of acetonitrile–methanol–acetic acid–water–1-ethylpropylamine (89.8:6.8:1.5:1.0:0.9). Elution times were considerably shorter than those of Patton et al. [4] (43 min versus 89 min for dioleoyl-PC). Abidi and Mounts [6] used both C_{18} and C_8 columns and isocratic eluent of acetonitrile–methanol–water (70:22:8) containing 5 m*M* pentyltriacylammonium phosphate to resolve molecular species of soybean PCs and PEs. The addition of pentyltriacylammonium phosphate resulted in reduced retention and increased detection sensitivity. Kaufmann and Olsson [7] used a C_{18} column to separate molecular species of PCs and PEs from bovine milk and rape seed. The optimum conditions were from 1-propanol–water–isooctane (52:47:1) to (58:33:9) in 55 min with a positive exponential gradient and a flow-rate of 0.9 ml/min, 1.56 mmol ammonium acetate and at 75°C. Ma and Kim [8] developed an on-line thermospray liquid chromatography–mass spectrometry (LC–MS) method. Lipid classes and molecular species of phospholipids from rat brain were partially separated by using a C_{18} column and linear gradient elution with 0.5% ammonium hydroxide in methanol–hexane–water (88:0:12), changing to (88:12:0) from 3 to 17 min at a flow-rate of 0.4 ml/min. Molecular species of phospholipids containing epoxy and hydroxy fatty acids from murine mast cells were separated by Bernstrom et al. [9] using a C_{18} column and the eluent of Patton et al. [4] containing 1 m*M* ammonium trifluoroacetate (pH 7.4) instead of choline chloride to permit subsequent identification by negative ion fast atom bombardment MS. Molecular species of PCs and PE containing hydroperoxides of fatty acids from human erythrocyte membrane were separated by Therond et al. [10] using a C_8 and a C_{18} columns in series at 40°C and the eluent of 10 m*M* ammonium acetate, pH 5.0–methanol (6:94). Recently, Brouwers et al. [11] quantified the molecular species of PCs from rat liver, porcine pulmonary surfactant, bovine heart, boar sperm cells and the parasite *Schistosoma mansoni* by HPLC and light scattering detection. HPLC was performed on two C_{18} columns in series using isocratic elution of a mixture of acetonitrile–methanol–triethylamine in varying ratio. Since we rely on radioactivity flow detection of PC intermediates, we developed an eluent system that would not interfere with liquid scintillation counting and UV detection at 205 nm.

2. Experimental

HPLC was carried out on a liquid chromatograph consisting of a chromatography manager (Millenium V 2.15, Waters Associates, Milford, MA, USA) operated by a computer (PC 586), a pump (Waters 600 controller), an injector (7125, Rheodyne, Cotati, CA, USA) and a photodiode array detector (Waters 996) detecting at 205 nm. A C_8 Luna column (25 cm×0.46 cm, 5 μm, Phenomenex, Torrance, CA, USA) was used. In our previous report [1], a C_8 column of Ultrasphere C_8 (25 cm×0.46 cm, 5 μm; Beckman Instruments, Fullerton, CA, USA) was used. However the Luna column can withstand the eluent of pH 9.5 used here and it is not necessary to install a silica saturation column between the pump and injector to prevent the rapid deterioration of the C_8 column as we previously reported [1]. The eluent was a linear gradient of 90–100% methanol (containing 0.1% of conc. NH_4OH) in 40 min, then 100% methanol at a flow-rate of 1 ml/min (system 1 of Table 1). The retention times of PC can be varied by adjusting the initial methanol–water ratio. The back-pressure ranged from about 1900 p.s.i. (1 p.s.i.=6894.76 Pa) initially to 1400 p.s.i. Two columns in series at a flow-rate of 1 ml/min can be used to increase the resolution as we have shown in

Table 1
Relative retention times of synthetic phosphatidylcholines and lysophosphatidylcholines in C_8 HPLC[a]

Phosphatidylcholines and lysophosphatidylcholines	Relative retention times (min)	
	System 1[b]	System 2[c]
1-Oleoyl-*sn*-glycero-3-phosphocholine	8.17	15.43
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[d]	
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[e]	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-Stearoyl-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 Section 2.
[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] Obtained from Ref. [1].
[e] Used for normalization of retention times to get relative retention times for system 1.

the separation of fatty acids [12]. Another linear gradient of 80–100% methanol (containing 0.1% of conc. NH_4OH) was also used for the separation of lysophosphatidylcholines (*lyso*PCs) (system 2 of Table 1). An evaporative light scattering detection system (ELSD, MKIII, Varex, Rockville, MD, USA) was used to detect saturated-acyl PCs. The drift tube temperature of the ELSD was set at 85°C. Nitrogen gas flow of the nebulizer was set at about 1 l/min and N_2 gas pressure was about 22 p.s.i. The N_2 pressure on the regulator of the N_2 tank was set at 80 p.s.i. PCs containing only saturated fatty acids can also be detected by UV at 205 nm though not as sensitively as by ELSD. The PC standards (Table 1) were purchased from Sigma (St. Louis, MO, USA) and Avanti Polar Lipids (Alabaster, AL, USA). PC standards were dissolved in methanol at a concentration of about 1 mg/ml. About 5 μg of standards were injected on the HPLC column. HPLC was run at room temperature (22±2°C) in a central air conditioned laboratory.

Since retention times are not reproducible under

the same HPLC conditions, RRTs were used to correct for different HPLC runs, different columns, different instruments, different days, minor temperature variation and long-term column usage. The RRTs used here were described in the experimental section of our recent reports [13,14]. Briefly, the RRTs of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine in system 1 of Table 1 was the retention time of this PC in a HPLC run. The RRT of other PCs shown in system 1 of Table 1 were based on normalization to the retention time of this PC. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine was the PC we used most often in our metabolism studies and is the substrate of oleoyl-12-hydroxylase from castor bean that we have characterized [1,15]. The RRTs of *lyso*PCs shown in system 2 of Table 1 were the retention times of one HPLC run of the mixture of the three *lyso*PCs. The precision of the experimental RRTs as shown in Table 1 is ± 0.03 min.

3. Results and discussion

Our present studies on the metabolism of lipids containing oxygenated fatty acids in castor bean and other oil seeds require several HPLC systems to separate, identify and quantitate naturally occurring lipid classes, fatty acids, molecular species of triacylglycerols, diacylglycerols and phospholipids. Both a UV detector at 205 nm for the detection of the lipids containing unsaturated fatty acids and a radioactivity flow detector are often used simultaneously for the identification of radioactive metabolites from microsomal incubations. We have recently developed HPLC systems for the separations of free fatty acids [12], methyl esters of fatty acids [12] and molecular species of triacylglycerols [13,14], diacylglycerols [13], *lyso*PCs and PCs [1], in order to characterize the enzyme, oleoyl-12-hydroxylase, [15] and to establish the biosynthetic pathway of triacylglycerols containing ricinoleate in castor bean [1]. Non-radioactive lipid standards corresponding to the possible radioactive metabolites were added to the samples as carriers for HPLC runs. Identifications were made by matching the retention times of the standards by UV detection at 205 nm and the radioactive peaks by radioactivity flow detection [1,15]. These HPLC systems used only methanol, isopropanol and/or water for gradient eluents. These solvents were chosen because they do not absorb significantly at 205 nm and do not quench significantly when liquid scintillation counting is used for the radioactivity flow detection. The eluents used were the least toxic and hazardous environmentally among the eluents used previously. The eluents in the HPLC fractions included small amount of acetic acid [12] or conc. NH_4OH [1] which can be removed easily in a nitrogen stream to obtain the purified lipids. The flow-rate of HPLC was 1 ml/min while the flow-rate of liquid scintillation fluid was 3 ml/min, and the ratio of 1:3 was ideal for liquid scintillation counting by radioactivity flow detector. The HPLC run time was 40 min to accommodate both the resolution of the lipid standards and brevity. These HPLC systems could use underivatized intact lipids which can save time and avoid losses during derivatization and hydrolysis.

We report here the separation of numerous molecular species of synthetic PCs. The eluents used previously for the separation of PCs [4–11] can be used with UV detection at 205 nm, however, the eluents containing acetonitrile [4–6,9,11] quench significantly for liquid scintillation counting when the radioactivity flow detector is used. The eluent used in the present C_8 HPLC system, methanol–water, is the least toxic environmentally and one of the least quenching for liquid scintillation counting using a radioactivity flow detector among the eluents used previously [4–11] for the separation of molecular species of PCs. Because all components are volatile, the eluent also allows the coupling of the HPLC effluent to MS and the use of ELSD. In initial evaluations, we found that the PC peaks from a C_8 column were sharper than those from C_{18} column and thus C_8 column was chosen for the PC separation. We have used this HPLC system for the purification of radioactive 1-palmitoyl-2-ricinoleoyl-*sn*-glycero-3-phosphocholine as shown in Fig. 1 to be used in castor microsome metabolic studies [1].

The RRTs of synthetic PCs are shown in Table 1. The elution sequence of a given PC depends on its fatty acid components. According to the RRTs of PCs shown in Table 1, mostly from PCs containing identical fatty acids, the elution order of PCs in this HPLC system are ricinoleic acid<linolenic acid<myristic acid<palmitoleic acid<palmitelaidic acid<

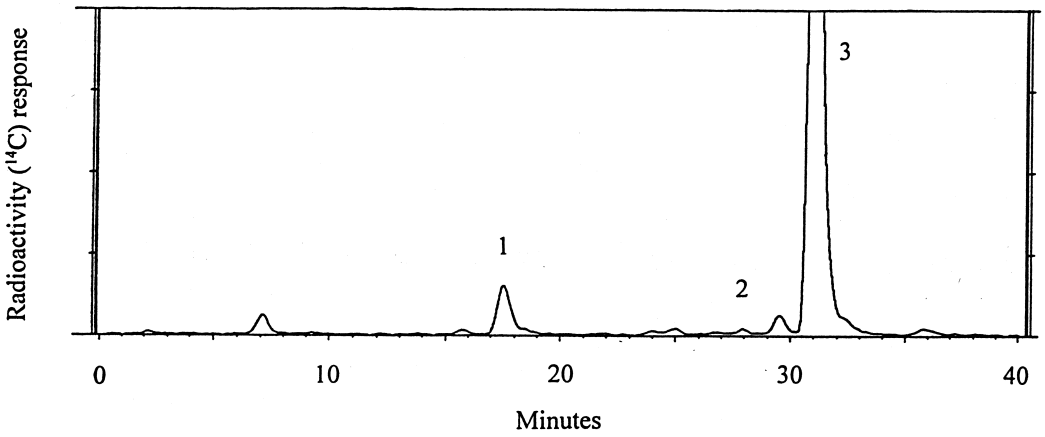

Fig. 1. Purification of 1-palmitoyl-2-[^{14}C]ricinoleoyl-*sn*-glycero-3-phosphocholine (peak 1, 17.6 min), using a C_8 HPLC system from the castor microsomal incubation of 1-palmitoyl-2-[^{14}C]oleoyl-*sn*-glycero-3-phosphocholine (peak 3, 31.0 min) [1]. Peak 2 was 1-palmitoyl-2-[^{14}C]linoleoyl-*sn*-glycero-3-phosphocholine (28.0 min). Linear gradient of 90–100% methanol containing 0.1% conc. NH_4OH in 40 min was used. For other HPLC conditions, see Section 2.

arachidonic acid<linoleic acid<palmitic acid<oleic acid<elaidic acid<petroselinic acid<hexadecyl ether<stearic acid<arachidic acid. This is the same elution order as those of fatty acids in the C_{18} HPLC we reported previously [12] except for the order of elaidic acid>petroselinic acid. The elution order is also the same as that of triacylglycerols [13] except for the order of myristic acid>palmitoleic acid and palmitic acid>oleic acid. Thus there are slight differences between the elution orders of molecular species of different lipid classes using different reversed-phase HPLC systems.

The elution of hexadecyl ether species of PCs are of interest because the alkyl chain is fixed to the glycerol backbone. Loss of the carbonyl oxygen results in a much less polar species, but the change is not as great as adding two methylene groups to an acyl chain. A PC containing a *cis*-fatty acid elutes slightly earlier than its isomer containing a *trans*-fatty acid similar to the elution characteristics of fatty acids [12] and triacylglycerols [13]. A PC containing oleic acid ($18{:}1^{\Delta 9}$) elutes slightly earlier than its isomer containing petroselinic acid ($18{:}1^{\Delta 6}$), which is also the same as the elution characteristics of fatty acids [12] and triacylglycerols [13]. The base-line separation of PCs containing these double-bond positional isomers of fatty acids is shown as Fig. 2. The elution orders shown in Table 1 are similar to that of PCs shown by Patton et al. [4] and Kaufmann and Olsson [7] using a C_{18} column but are not exactly the same. The PC peaks as shown in Fig. 2 are not as sharp as the HPLC peaks of fatty acids [12] and triacylglycerols [13] we reported earlier. The baseline separation of PCs as shown in Fig. 2 can be achieved when the difference of RRTs (Table 1) is equal to or more than 0.9 min. However, the baseline separation of 1-oleoyl-*sn*-glycero-3-phosphocholine and 1-*O*-hexadecyl-*sn*-glycero-3-phosphocholine (Table 1, System 1) eluted earlier can be achieved when the difference of RRTs is less than 0.9 min. Another C_8 HPLC method was developed for the separation of *lyso*PCs using more polar eluent as shown in Table 1, System 2. The elution orders of *lyso*PCs depend on their fatty acid constituents and seem to be the same as the fatty acid order of the PC elution.

We have recently reported a simple and accurate method to predict the RRTs of triacylglycerols [14] in a C_{18} HPLC system. The prediction is based on a simple calculation using the experimental RRT (min) of the triacylglycerol with the closest corresponding structure and takes into account the contribution (min) of functional groups present in the triacylglycerol for which the standard is not available in order to predict the RRT. We have tried to apply this method to predict the RRTs of PCs and have not been successful. Even though we can not predict the RRTs of PCs accurately for which a standard is not

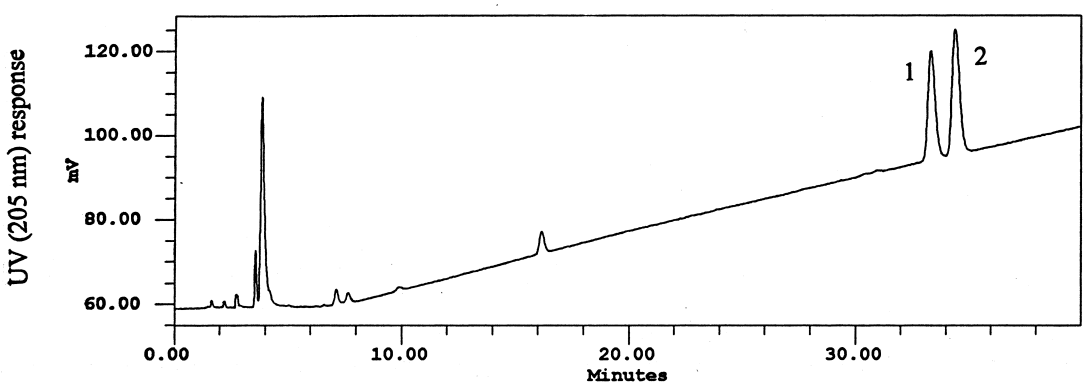

Fig. 2. C_8 HPLC separation of PCs of double-bond positional isomers. (1) 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (*cis*-9), retention time 33.33 min; and (2) 1,2-dipetroselinoleoyl-*sn*-glycero-3-phosphocholine (*cis*-6), 34.42 min. About 50 μg of the standard each in 10 μl of methanol were used. Linear gradient of 90–100% methanol containing 0.1% conc. NH_4OH in 40 min was used. For other HPLC conditions, see Section 2.

available, we can predict the elution order of PC species for which standards are not available. PCs containing a more polar acyl group of the two at *sn*-2 position elutes slightly earlier than its isomer at *sn*-1, as with triacylglycerols [13,14]. The examples are: 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine<1-oleoyl-2-stearoyl-*sn*-glycero-3-phosphocholine (because oleic acid<stearic acid); 1-oleoyl-2-palmitoyl-*sn*-glycero-3-phosphocholine<1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (because palmitic acid<oleic acid).

We have developed these HPLC systems to take advantage of radioactivity flow detection of PC metabolites. The resolution appears to be at least as good as that for HPLC systems that separate molecular species of PCs [4–11]. The elution characteristics reported here can be useful in identification of unknown PCs for which the standards are not available. We have previously given the elution characteristics of fatty acids [12], acylglycerols [13,14], and gibberellins, a group of plant hormone [16], and have used those elution characteristics to aid in the identification of unknown compounds. In the trace analysis of PCs in complex sample matrices, identification of unknown PCs based on elution characteristics or RRTs without other supporting evidence may lead to erroneous results.

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