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Short communication

Separation of the molecular species of intact phosphatidylethanolamines and their *N*-monomethyl and *N*,*N*-dimethyl derivatives by high-performance liquid chromatography on a C_8 column

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Abstract

We have developed a gradient reversed-phase C_8 high-performance liquid chromatography method for the separation of molecular species of phosphatidylethanolamines (PEs) and their *N*-monomethyl and *N*,*N*-dimethyl derivatives. This method uses a 40-min linear gradient of 88–100% methanol, containing ammonium hydroxide as silanol suppressing agent, and is suitable for metabolic studies using both UV detection at 205 nm and radioactivity flow detection. The elution order of a given PE is inversely related to the polarity of its fatty acid constituents. Lipid classes studied here containing the same fatty acyl chains elute in the order: PE-*N*,*N*-dimethyl<PE<PE-*N*-monomethyl<phosphatidylcholine, indicating that elution order is not simply a function of the numbers of methyl groups on the nitrogen atom of PE. © 2000 Published by Elsevier Science B.V.

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1. Introduction

Phosphatidylcholines (PCs) and phosphatidylethanolamines (PEs) are important components of cell membranes and intermediates of the lipid biosynthetic pathways. We previously developed a high-performance liquid chromatography (HPLC) method to separate the molecular species of PCs [1] to study the biosynthetic pathway of triacylglycerols (TAGs) containing ricinoleate in castor microsomes [2]. In order to evaluate the role of PEs in the biosynthesis of TAGs containing ricinoleate in castor microsomes, we developed an HPLC method to separate the molecular species of PEs as well as the corresponding *N*-methyl and *N*-dimethyl derivatives. Here we report a HPLC method for the separation of molecular species of PE-based lipids, the relative retention times (RRTs) of these molecular species and their elution characteristics. Several other HPLC systems have been developed to separate PEs and

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related phospholipids [3–8]. However, we rely on radioflow and UV (205 nm) detection, so we used 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 Waters liquid chromatograph system, previously described [1] using a C_8 Luna column (25 cm×0.46 cm, 5 μm, Phenomenex, Torrance, CA, USA) eluted with a linear gradient of 88–100% methanol (in water, containing 0.1% of conc. NH_4OH in both) in 40 min, then 100% methanol at a flow-rate of 1 ml/min. This HPLC method is similar to that for PCs as reported previously [1] except the method for PCs uses a linear gradient of 90–100% methanol (containing 0.1% of conc. NH_4OH) in 40 min. The back pressure ranged from 1900 p.s.i. initially to 1400 p.s.i. (1 p.s.i.=6894.76 Pa). UV at 205 nm was used for the detection of unsaturated-acyl PE. Evaporative light scattering detection (ELSD) was also used to detect both saturated-acyl PE and unsaturated-acyl PE as previously described [1]. The PE, PE-*N*-monomethyl and PE-*N*-dimethyl standards (Table 1) were purchased from Sigma (St. Louis, MO, USA) and Avanti Polar Lipids (Alabaster, AL, USA). The standards were dissolved in methanol at a concentration of about 2 mg/ml and about 20 μg was injected onto the HPLC column. No hydrolysis of PE in this basic eluent was detected.

The 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-monomethyl and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine-*N,N*-dimethyl were prepared according to the method of Paltauf

Table 1
Relative retention times of synthetic PEs, *lyso*PEs, PE-*N*-monomethyl derivatives, PE-*N,N*-dimethyl derivatives and PCs in a C_8 HPLC[a,b]

Phosphatidylethanolamines and lysophosphatidylethanolamines	RRT (min)
1-Myristoyl-*sn*-glycero-3-phosphoethanolamine	5.39
1-Palmitoyl-*sn*-glycero-3-phosphoethanolamine	6.16
1-Oleoyl-*sn*-glycero-3-phosphoethanolamine	6.61
1,2-Dilauroyl-*sn*-glycero-3-phosphoethanolamine	11.70
1,2-Dimyristoyl-*sn*-glycero-3-phosphoethanolamine	20.66
1,2-Dipalmitoyl-*rac*-glycero-3-phosphoethanolamine-*N,N*-dimethyl	22.87
1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine-*N,N*-dimethyl	24.57
1,2-Dipalmitoleoyl-*sn*-glycero-3-phosphoethanolamine	25.36
1,2-Dioleoyl-*rac*-glycero-3-phosphoethanolamine-*N,N*-dimethyl	26.86
1,2-Dilinoleoyl-*sn*-glycero-3-phosphoethanolamine	27.81
1-Palmitoyl-1,2-linoleoyl-*sn*-glycero-3-phosphoethanolamine	29.02
1,2-Dipalmitoyl-*sn*-glycero-3-phosphoethanolamine	30.38
1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine[c]	31.63
1-Oleoyl-2--palmitoyl-*sn*-glycero-3-phosphoethanolamine	31.68
1,2-Dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-monomethyl	31.76
1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-monomethyl	32.21
1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine	32.74
1,2-Dielaidoyl-*sn*-glycero-3-phosphoethanolamine	33.12
1,2-Dioleoyl-*rac*-glycero-3-phosphoethanolamine-*N*-monomethyl	33.29
1,2-Dipetroselinoyl-*sn*-glycero-3-phosphoethanolamine	34.61
1,2-Diheptadecanoyl-*sn*-glycero-3-phosphoethanolamine	35.03
1-Stearoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine	36.52
1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine	38.16
1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine	38.80
1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine	39.36
1,2-Dioleoyl-*sn*-glycero-3-phosphocholine	42.56

[a] For HPLC conditions, see Section 2.
[b] Linear gradient of 88–100% methanol containing 0.1% of conc. NH_4OH in 40 min.
[c] Used for normalization of retention times to get relative retention times.

and Hermetter [9]. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine was incubated with phospholipase D in a buffer containing 2-(methylamino)ethanol or 2-(dimethylamino)ethanol to produce the standards which were then purified by HPLC. We used the *Streptomyces antibioticus* phospholipase D as prepared from recombinant *Escherichia coli* [10], generously provided by Professor Iwasaki, Nagoya University, Japan.

Since retention times (t_R values) are not reproducible under the same HPLC conditions, RRTs were used in Table 1 to correct for different HPLC runs as previously described [1], normalizing against the t_R of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine. The precision of the experimental RRT as shown in Table 1 is ± 0.03 min for all and provides very accurate assessment of elution order.

3. Results and discussion

We have developed a gradient HPLC system to separate the molecular species of PEs and their *N*-monomethyl and *N*,*N*-dimethyl derivatives for metabolic studies. The radioactive metabolites can be identified by co-chromatography with non-radioactive standards by matching the retention times from UV (205 nm) and radioactivity flow detectors sequentially as we previously reported in the identification of radioactive TAGs [2]. The eluent used does not absorb significantly at 205 nm and does not significantly quench radioflow detection. The eluents used were the least toxic and hazardous environmentally among the eluents used previously for the separation of PEs [3–8]. The eluent contains small amounts of NH_4OH as silanol suppressing agent [13] to prevent peak broadening. NH_4OH can be removed easily in a nitrogen stream to obtain the purified lipids. This HPLC system is readily adapted to the coupling of the eluent to a mass spectrometric (MS) system or an ELSD system, since all components of the eluent are volatile. We have also developed HPLC systems previously for the separation of fatty acids (FAs) [14], molecular species of TAGs [11,12] and PCs [1] to conduct metabolic studies of lipid biosynthesis [2].

In earlier reports [3–8] on the separation of molecular species of PEs, C_{18} columns were used. In this report, we choose to use a C_8 column, while a C_8 and a C_{18} column in series were also used earlier [7]. As for PC separations [13], the peak of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine from a C_8 column (column plate number $N=44\,000$) is also sharper than that from a C_{18} column ($N=15\,000$) using a linear gradient of 88–100% methanol (containing 0.1% of conc. NH_4OH). The peaks in Figs. 1 and 2 are sharper than those in the chromatograms reported earlier [3–8].

The RRTs of synthetic PEs, *lyso*PEs and PE-*N*-monomethyl, PE-*N*,*N*-dimethyl derivatives and PCs for this HPLC system are shown in Table 1, arranged in the order of RRT. The elution orders shown in Table 1 are consistent with those of earlier reports [3–8]. The elution order of the molecular species of a given lipid class depends on its FA components. According to the RRTs of PEs containing identical FAs shown in Table 1, the elution order of PEs in this HPLC system is: lauric acid<myristic acid<palmitoleic acid<linoleic acid<palmitic acid<oleic acid<elaidic acid<petroselinic acid<heptadecanoic acid<stearic acid. This is the same as the elution orders of FAs [14] and PCs [1], and is similar to the elution order of TAGs [11] in the reversed-phase HPLC systems as we reported earlier. Fig. 1 shows the separation of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine and 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphoethanolamine which were used in our metabolic study. The band widths of the peaks shown in Fig. 1 are about 0.7 min. Any two PEs in Table 1 with RRTs of more than about 0.7 min apart can be baseline separated. Both PE-*N*-monomethyl derivatives and PE-*N*,*N*-dimethyl derivatives show the elution order of palmitic acid<oleic acid and the elution orders for other molecular species of each are likely to correspond to those of FAs [14], PCs [1] and PEs in reversed-phase HPLC. Lipid classes studied here containing the same fatty acyl chains are eluted in the order: PE-*N*,*N*-dimethyl<PE<PE-*N*-monomethyl<PC. This order is not related simply to the number of methyl groups on the nitrogen atom of PE. The separation of the molecular species of PE-*N*-monomethyl and PE-*N*,*N*-dimethyl derivatives has not been previously reported.

We have recently studied the metabolism of 1-palmitoyl-2-[^{14}C]oleoyl-*sn*-glycero-3-phosphoethanolamine in castor microsomes [15]. We have

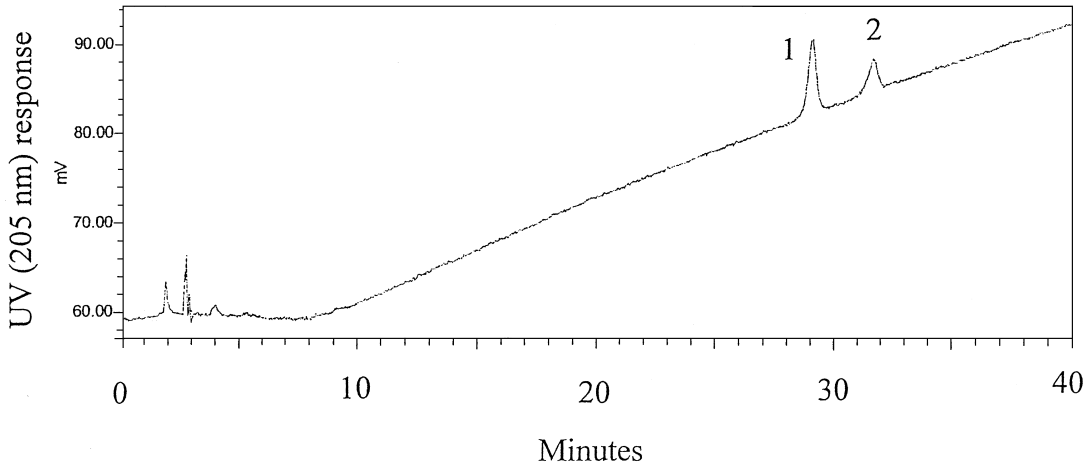

Fig. 1. C_8 HPLC separation of (1) 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphoethanolamine (t_R, 29.02 min; detection limit, 2 μg) and (2) 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (t_R, 31.63 min; detection limit, 5 μg). Linear gradient of 88–100% methanol containing 0.1% conc. NH_4OH in 40 min was used.

used this HPLC system (Fig. 2) to prove that the substrate is not hydroxylated to 2-[^{14}C]ricinoleoyl-PE or desaturated to 2-[^{14}C]linoleoyl-PE [15] while 1-palmitoyl-2-[^{14}C]oleoyl-*sn*-glycero-3-phosphocholine can be hydroxylated and desaturated [2].

We have developed a HPLC method for the separation of molecular species of PEs suitable for the identification of radioactive metabolite when the PE standards are available for co-chromatography. The elution characteristics and RRTs reported here

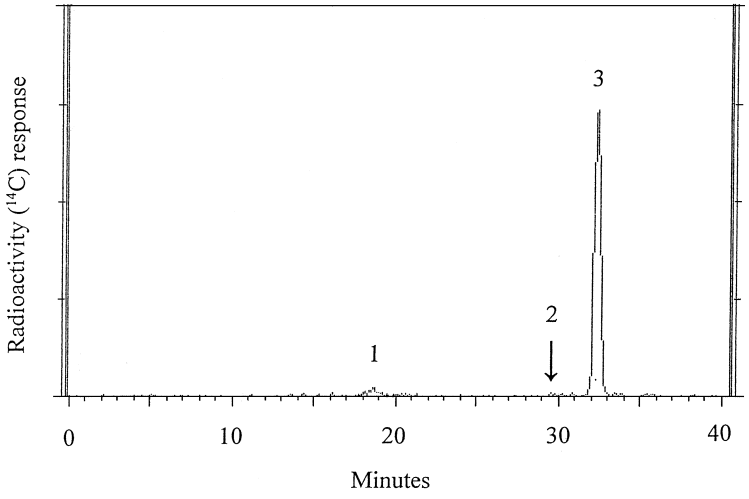

Fig. 2. HPLC radiochromatogram of PE fraction from the castor microsomal incubation with 2-[^{14}C]oleoyl-PE using the C_8 HPLC system (see Experimental). Peaks: 1=unknown (t_R, 18.6 min which is close to that of 1-palmitoyl-2-ricinoleoyl-*sn*-glycero-3-phosphoethanolamine); 2 (arrow)=1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphoethanolamine (t_R, 29.7 min); 3=2-oleoyl-PE (t_R, 32.4 min). These compounds were not detected by UV at 205 nm, because no carriers were added for co-chromatography.

as well as those of PCs [1] and FAs [14] can be useful in the identification of unknown PEs for which the standards are not available.

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