Molecular Species Analysis of Phosphatidylcholine by Reversed-Phase Ion-Pair High-Performance Liquid Chromatography

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Abstract

The retention behavior of molecular species of phosphatidylcholine (PC) is studied by reversed-phase (RP) ion-pair high-performance liquid chromatography (HPLC). Mobile phases contain tetraalkyl ammonium phosphates (TAAPs) in methanol–acetonitrile–water. The stationary phase is alkyl-bonded silica. Competitive interactions of TAAPs, analyte solutes, and an RP-HPLC column result in reduced retention of PC molecular species. PC molecular species are eluted at longer retention times with a larger size of TAAP in the mobile phase, and an increase in the TAAP concentration invariably causes a decrease in PC molecular species retention times. There is a linear correlation between the logarithmic retention factors (*k*) of PC molecular species and the total number of carbon atoms of TAAP, and the logarithm of *k* values of PC molecular species can be approximated as a linear function of the logarithm of the counter-ion concentration. There is found to be no distinct dependence between *k* values of PC molecular species and the mobile phase pH.

Introduction

Structures of phosphalipids (PLs) generally consist of nonpolar tail groups of fatty acid chains and polar head groups of phosphoric acid derivatives. There are three structural types of PLs that naturally occur in plants and animals: (*a*) PLs with neutral charges, (*b*) negatively-charged PLs, and (*c*) a combination of (*a*) and (*b*) with a net negative charge in the molecule. As a prototype of the first structural type, phosphatidylcholine (PC) is widely spread and its abundant constituents are found in the cell membranes of animals and plants. The presence of R_1 and R_2 groups in PC (Figure 1) is responsible for the existence of molecular species. It is known that variations in the distribution of PC molecular species alter chemical and physical properties of cell membranes and have significant influence on cellular biochemical processes. In addition, rapid and accurate analytical methods for PC molecular species measurements have been in continuous demand by lipid scientists. The primary purpose of our work is to explore the separation potential of PC molecular species.

In recent years, there have been a few publications in the literature dealing with reversed-phase (RP) high-performance liquid chromatography (HPLC) of PC molecular species. According to Sotirhos (1), it was difficult to obtain a good chromatogram for the later-eluting components because of severe peak broadening and tailing. In contrast, Abidi (2) developed a method to separate six kinds of PC molecular species by adding tetraalkyl ammonium phosphate (TAAP) or alkyltriethyl ammonium phosphate (ATAP) to the mobile phases. In this procedure, PC molecular species in the presence of TAAP were less retained and had better shaped peaks than in the absence of the ammonium salt in the mobile phases, and a significant improvement in detector response was observed. However, baseline separation was not easily established, and the peaks of later-eluting components were still broadening and tailing. In this study, we modified Abidi's method of separating seven kinds of PC molecular species in less than 50 min. At the same time, we discussed the effects of size and concentration of TAAPs, mobile phase solvent compositions, and mobile phase pH on the retention characteristics of PC molecular species.

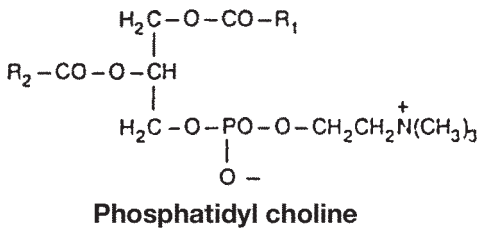

Figure 1. Structure of phosphatidylcholine. R_1 and R_2 represent alkyl or alkenyl groups of fatty acids.

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Experimental

Materials

PC molecular species were purchased from Sigma (St. Louis, MO). Tetramethyl ammonium phosphate (TMAP), tetraethyl ammonium phosphate (TEAP), and tetrabutyl ammonium phosphate (TBAP) were prepared from the corresponding tetraalkyl ammonium hydroxide (Shanghai No. 1 reagent factory, Shanghai, China) by titration with phosphoric acid (Shanghai No. 1 reagent factory) until reaching a desired pH value. HPLC-grade acetonitrile and methanol were obtained from Fisher Chemicals (Fair Lawn, NJ). Ultrapure water for HPLC was obtained by filtering Sparklin distilled water through a Millipore Milli-Q water purification system (Millipore, Bedford, MA).

Table I. Effect of TAAPs on *k* Values of PC Molecular Species

Mobile phase counter ion	Retention factor (*k*) Component 1	2	3	4	5	6	7
TMAP	1.376	2.608	5.667	9.247	28.220	43.575	73.522
TEAP	2.091	3.129	6.258	11.210	32.016	50.903	89.753
TBAP	2.243	3.290	6.489	11.441	35.194	57.828	101.075

* Component identification, PCs: 18:2–16:0 (1), 22:6–18:0 (2), 16:1–16:0 (3), 18:2–18:2 (4), 16:1–16:1 (5), 18:1–14:0 (6), and 18:1–18:1 (7).

Table II. Effect of TMAP Concentration on *k* Values of PC Molecular Species

TMP concentration (mM)	Retention factor (*k*) Component 1	2	3	4	5	6	7
0	2.909	5.651	6.876	16.145	59.581	111.387	134.290
1.25	1.919	3.172	6.906	11.849	36.704	62.489	105.117
2.50	1.903	3.075	6.667	11.435	35.630	60.038	101.457
5	1.882	3.038	6.575	10.855	33.704	53.382	91.102
10	1.301	2.925	6.527	10.306	32.156	50.758	85.586
15	1.376	2.608	5.667	9.247	28.220	43.575	73.522
20	1.586	2.543	6.183	9.172	28.473	44.753	73.269

* For peak identification, see Table I.

Table III. Effect of Mobile Phase Solvent Compositions on the *k* Values of PC Molecular Species

Mobile phase Ratio	TMAP (mM)	Retention factor (*k*) 1	2	3	4	5	6	7
70:22:8		1.376	2.608	5.667	9.247	28.220	43.575	73.522
65:22:13	15	1.468	2.925	6.075	10.505	31.839	52.505	87.925
80:12:8		1.586	2.543	6.183	9.172	28.473	44.753	73.269

* For peak identification, see Table I.

HPLC

In all HPLC analyses, a PerkinElmer (Shelton, CT) series 300 liquid chromatograph interfaced with a 235C diode array detector was used. Mobile phases were composed of various concentrations (1.25–20.00mM) of different types of ion-pair (IP) reagents, acetonitrile, methanol, and water. All mobile phases were prepared daily before analyses were run. Aliquots of freshly prepared samples (5–10 µL of 5–10-mg/mL solutions) were injected via a Rheodyne (Cotati, CA) Model 7125 injector (25-µL loop) onto an RP-HPLC column. In some experiments, relatively old samples that had been standing in the freezer for 20–30 days were also used. The column was a PerkinElmer Brownlee validated C_{18} column (150 × 4.6 mm). In typical HPLC analyses, mobile phases were filtered, degassed, and pumped through the column at a flow rate of 2.0 mL/min. HPLC column effluents were monitored at 208 nm, and band width was 5 nm. Retention factors (*k*) were determined from the following equation:

$$k = t/t_0 \qquad \text{Eq. 1}$$

where t and t_0 are the retention times of an analyte and an unretained solute (uracil), respectively. The retention times were the average values obtained from three replicate injections. HPLC peaks were identified by peak matching using standards.

Results and Discussion

PC consisted of molecular species that differ in the R_1 and R_2 groups of fatty acid, as shown in Figure 1. Notwithstanding the existence of polar head groups of ammonium moiety and phosphoryl moiety, RP-HPLC of PC in mobile phases containing no IP agents produced unusual peaks with long retention caused by the influence of nonpolar tail groups in the fatty acid moieties. HPLC chromatograms obtained under various conditions are presented in Figure 2 to illustrate the beneficial effect of mobile phase TAAPs on the peak characteristics of PC molecular species. It was evident that adding TAAPs to the mobile phase led to significant shortening of retention times of the PC molecular species. Therefore, it was logical to study the effect of IP agents on the chromatographic behavior of PC molecular species.

Effect of the molecular masses of TAAPs on *k* values of PC molecular species

Retention data for seven kinds of PC molecular species are summarized in Table I to show the effect of the molecular masses of TAAPs on the *k* values of PC molecular species. In general, the *k* values of the PC molecular species increased with

an increase in the size of the TAAPs in the mobile phases. The logarithmic *k* values of PC molecular species can be linearly related to the total number of carbon atoms in TAAPs. As illustrated in Figure 3, with TAAPs in the mobile phases, higher members of ion pairing in the series gave rise to higher *k* values of the components (TBAP > TEAP > TMAP); thus, in order to get shorter analytical times, it was reasonable to choose TMAP to serve as a counter ion in the mobile phase.

Concentration effect of TMAP on *k* values of PC molecular species

Table II shows the concentration effect of TMAP on *k* values of PC molecular species. The *k* values decreased with increasing TMAP concentrations, and the logarithm of molecular species *k* values could be approximated as a linear function of the logarithm of the counter ion concentration at a concentration of ≤ 15mM, as illustrated in Figure 4. The observations are similar to those found by Bartha (3). Whereas *k* values obtained from mobile phases containing 15mM TMAP were compared with those from mobile phases containing 20mM of the same salt, few differences were noticeable between the corresponding retention data of PC molecular species. The results suggest that the degree of ion interactions between PC components and TMAP in the HPLC system processes reached a maximum at a concentration of ≤ 15mM.

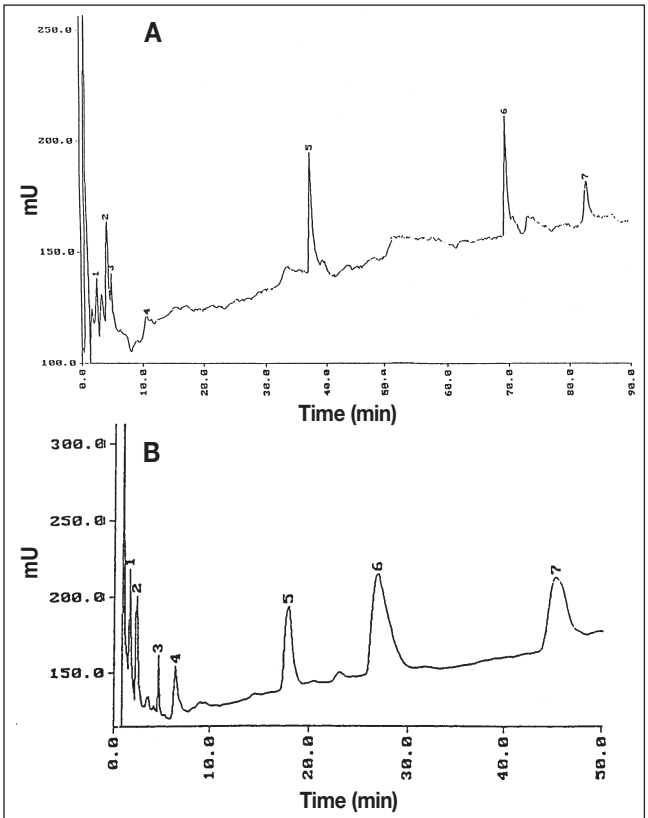

Figure 2. HPLC separation of molecular species of PC in the absence of mobile phase salts (A) and in the presence of mobile phase salts (B). For peak identification, see Table I.

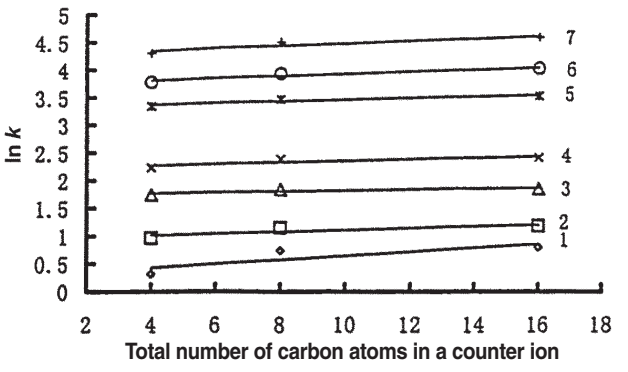

Figure 3. Linear correlation between ln *k* of PC molecular species and the total number of carbon atoms in TAAP. See Table I for component identification.

Influence of mobile phase compositions on *k* values of PC molecular species

The influence of mobile phase solvent compositions on the retention characteristics of PC molecular species is shown in Table III. As is usually observed in RP-HPLC, higher *k* values were obtained when the mobile phases contained higher percentages of water. On the other hand, with constant percentages of water, *k* values of PC molecular species were less sensitive to changes in relative percentages of methanol versus acetonitrile.

Influence of mobile phase pH on *k* values of PC molecular species

As depicted in Figure 1, PC contains an ammonium moiety along with a negative charge in the phosphoryl moiety, and its ionic charges were internally neutralized within the molecules. This could have affected the ionization degree of phosphoryl moiety by varying the mobile phase pH. Therefore, it is probable that retention characteristics of PC molecular species were anticipated to be sensitive to variations in mobile phase pH. Table IV presents the retention data of PC molecular species studied under three different mobile phase pHs. Obviously, it indicates that there was no marked change in the *k* values of PC molecular species after adjusting mobile phase pHs. In view of the lower *k* values of component 6,7 in mobile phase pH 7, it was reasonable to choose mobile phase pH 7 to shorten the analytical time.

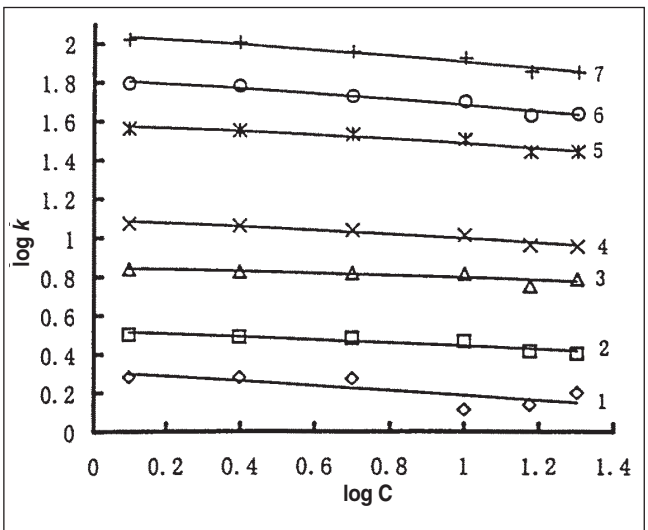

Figure 4. Log *k* versus log C relationships for PC molecular species and TMAP as the pairing ion. See Table I for component identification.

Separation of molecular species of PC from bovine brain

HPLC separations of molecular species of PC from bovine brain are shown in Figure 5. HPLC experiments were carried out under optimized conditions: the mobile phase was methanol–acetonitrile–water (70:22:8) containing 15mM TMAP, a pH of 7, a flow rate of 2 mL/min, and UV detection at 208 nm. HPLC peaks were identified by peak matching using standards; five of them were known, and the others were unknown. The baseline was not stable, which may be the result of impurities in the IP agents.

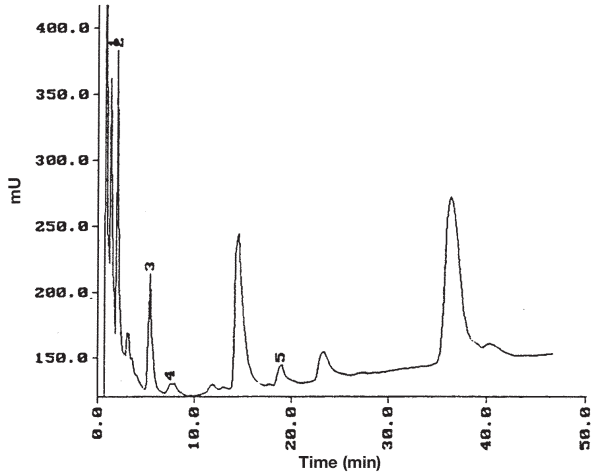

Figure 5. HPLC separation of molecular species of PC from bovine brain. The HPLC conditions are the same as in Figure 2. See Table I for component identification.

Table IV. Effect of Mobile Phase pH on *k* Values of PC Molecular Species

Mobile phase pH	*k* Component 1	2	3	4	5	6	7
7.00	1.376	2.608	6.258	9.247	28.220	43.575	73.522
5.00	1.720	2.548	5.624	8.930	27.263	44.973	75.081
2.50	2.314	2.661	5.747	9.269	29.011	48.538	78.280

* For peak identification, see Table I.

Conclusion

Molecular species of PC, an abundant constituent in phospholipids, can be separated by RP-IP-HPLC. The advantage of this approach is that it does not require additional steps to derivatize PC components with a strong UV chromophore, and thus this method is simplified by eliminating the need for chemical derivatization. However, this method can only separate several PC components, which may cause problems in the analysis of biological samples in which each chromatographic peak may contain different molecular species of PC, or when other molecular species are not eluted from the column. In order to solve this problem, we would optimize more reasonable HPLC conditions by separating molecular species that may be eluted together.

Acknowledgment

The authors thank Ms. Karen Cawrse for her comments on language.

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Manuscript accepted April 10, 2003.