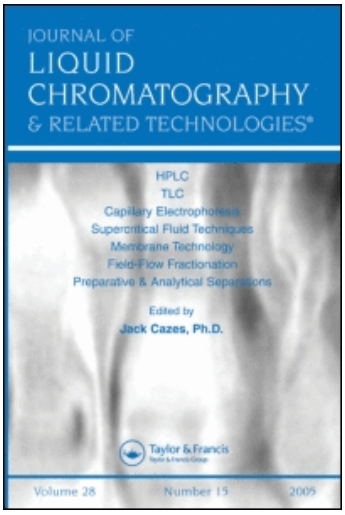

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IMPROVED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PHOSPHOLIPIDS

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ABSTRACT

The reversed-phase HPLC of phosphatidylcholine and di- and triglycerides is presented with particular emphasis on improving the efficiency of the phospholipid separations so that they are comparable to those attained for di- and triglycerides. Elevated column temperatures (60°C) and addition of strong mineral acids or ion-pairing agents to the mobile phase were found useful in accomplishing this goal.

INTRODUCTION

The utilization of normal-phase high-performance liquid chromatography (HPLC) for separating phospholipid classes has been demonstrated (1-5). The reversed-phase mode for resolving molecular species of phospholipids, however, has been given less attention (6,7,8). In addition, the separations demonstrated in this mobile-phase mode required, in one case (7), the use of chloroform as a major component. This limited the ultraviolet transparency of the mobile phase and

thus a refractive index detector was needed. We are interested here in demonstrating that phospholipids can be separated with high-efficiency, comparable to that attainable for triglycerides (9-12), and with mobile phases that are also compatible with ultra-violet detection at low wavelength (1-5). These separations are for phosphatidylcholine molecular species, and require mineral acid modifiers to aqueous-methanol mobile phases.

MATERIALS AND METHODS

The chromatographic system consisted of a 6000A solvent delivery system and U6K injector (Waters Scientific Ltd., Mississauga, Ontario), a SF770 variable wavelength detector (Schoffel Instrument Corp., Westwood, N.J.) and an in-laboratory packed 25x0.4 cm stainless steel column containing Sperisorb SS ODS (octyldecyl bonded phase, 5 μm, Phase Sep, 255 Oser Ave., Hauppage, N.Y.) or an Altex 25x0.32 cm LiChrosorb C_{18} column (10 μm, Aviation Electric Ltd., St. Laurent, Quebec). For column temperature control a laboratory-designed water jacket was employed.

The solvents used for the mobile phases were spectrograde methanol (American Chemicals Ltd., Montreal, Quebec), LiChrosolv acetonitrile (British Drug House, Montreal, Quebec) and double distilled (in glass) water. The acids utilized were from

Anachemia Chemicals Ltd. (Montreal, Quebec) while the diethylphosphate was purchased from Eastman Kodak Co. (Rochester, N.Y.).

The di- and triglycerides were from Sigma Chemical Co. (St. Louis, Missouri) while the synthetic phosphatidylcholines were from Serdary Research Laboratories (London, Ontario).

The lipid standards were dissolved in chloroform to give working standards from 1 to 10 mg/ml. Amounts injected, chromatographic solvent flow rates, and absorbance sensitivities (at 200 nm) are as given in each figure. Except when otherwise stated the column temperature was always 60°C. The one exception was when using temperature ramping of the column. This was accomplished by step ramping the column temperature from 30°C to 60°C at the time illustrated in the appropriate figure. Temperature changes of the solvent system were observed as a baseline shift in the detector. The post-column (to detector) connection was thus thermostated using an ice bath to minimize long-term baseline drift.

All mobile phases using polar modifiers, except for that involving triethylammonium sulfate, were made such that the modifier was added directly to 95% aqueous methanol to give an overall concentration of $1x10^{-2}$M modifier. In the case of triethylammonium

sulfate, a 0.1% (v/v) suspension of triethylammonia in water was acidified to pH 2.10 with concentrated sulfuric acid. This solution was used to make up the 95% aqueous methanol mobile phase.

The mobile phases were degassed using helium displacement and were maintained at the operating temperature of the column.

RESULTS

The separation of di- and triglycerides can be accomplished with high efficiency as shown in Figure 1,

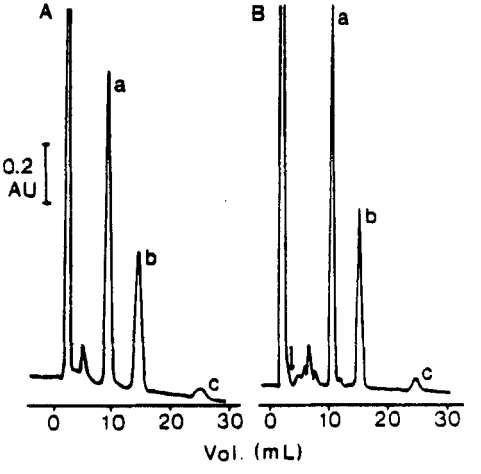

FIGURE 1. (A) Separation of (a) $C_{18:3}$, (b) $C_{18:2}$, and (c) $C_{18:1}$ triglycerides, 250 µgm of each injected. Methanol flow rate was 1 ml/min and using the Altex column. (B) Same as for (A) except the initial portion of the separation was run at 30°C and then temperature step programmed as indicated by (↓).

where equal amounts of trilinolenin (tri-$C_{18:3}$), trilinolein (tri-$C_{18:2}$), and triolein (tri-$C_{18:1}$) were injected into a mobile phase of methanol.

Figure 1B shows the same sample injected under identical conditions except that the initial portion of the separation is run at 30°C and then stepped to 60°C to increase both resolution and efficiency without loss of time.

As expected, a weaker eluent such as acetonitrile versus, for example, methanol gives better resolution for the degradation products of tri-$C_{18:3}$ (sample allowed to stand at room temperature for one week). The plate count realized for a typical 10-μm reversed-phase packing for tri-$C_{18:3}$ was measured to be as high as 16,000 plates/meter and typically greater than 6,000 plates/meter.

When using similar mobile phases for determination of phosphatidylcholines (PC), very low efficiencies were observed. This is illustrated in Figure 2 for acetonitrile and methanol mobile phases. The PC-$C_{18:3}$ is either totally retained on the column (A) or tails severely (B).

Figure 3 shows a more dramatic example of the problems often encountered with PC samples. In Figure 3A a mixture of the diglyceride diolein and PC dioleoyl was run using first 95% aqueous methanol-

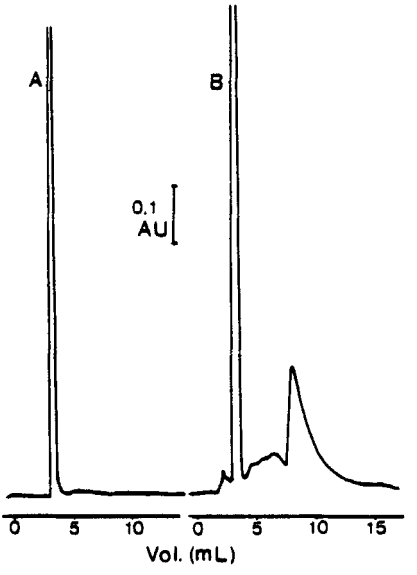

FIGURE 2. (A) Irreversible retention of 50 µgm PC-$C_{18:3}$ using acetonitrile as mobile phase. Flow rate was 1 ml/min using the Altex column. (B) Same as (A) but using methanol as mobile phase.

diethylphosphoric acid. Figure 3B shows the chromatogram for the same system except that the diethylphosphoric acid has been eliminated from the mobile phase. When comparing the two chromatograms it is apparent that peak-for-peak every component except for L-α-phosphatidylcholine dioleoyl is present in Figure 3B. When switching from the mobile phase containing diethylphosphoric acid to that lacking any mobile phase modifier, no baseline shift corresponding to an

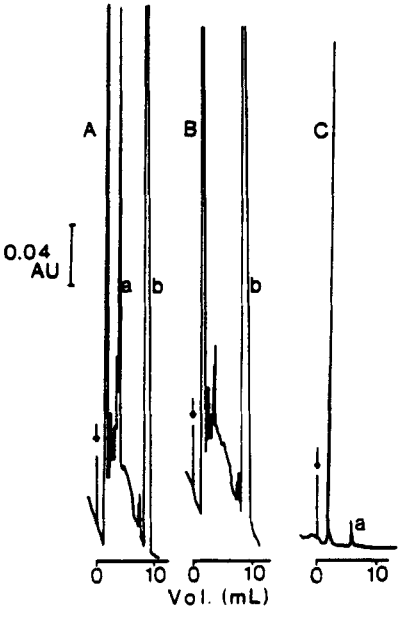

FIGURE 3. (A) Separation of a mixture of L-α-phosphatidyl choline dioleoyl (a) and diolein (b). All other peaks represent degradation products of diolein. Mobile phase 95% aqueous methanol-diethylphosphoric acid, 1 ml/min using the 5 μm ODS column. (B) Identical to (A) except phosphoric acid is eliminated from the mobile phase. (C) Chromatogram of the fraction collected when switching to 95% aqueous methanol-phosphoric acid, 1 ml/min. Band (a) corresponds to L-α-phosphatidyl-choline dioleoyl.

eluting band is seen. However, when changing solvent systems in the reversed order after injecting PC samples into the aqueous methanol mobile phase, an eluting band is observed. This band corresponds to the missing PC shown in Figure 3B, for upon collection and injection into a 95% aqueous methanol-phosphoric

acid mobile phase, the chromatogram Figure 3C shows the corresponding PC sample (highly diluted). In these studies diethylphosphoric acid and phosphoric acid were found to be interchangeable with respect to eluting off lost PC bands. The ratios of k' for diolein to that of PC dioleoyl were nearly identical for either orthophosphoric acid or diethylphosphoric acid as modifiers and were 0.39 versus 0.35, respectively.

To increase the reversed-phase chromatography of PC we have investigated other modifiers to the aqueous methanol mobile phase system. These results are summarized in Table I. The use of nitric acid was not attempted since it has high UV absorbance in the region (200 nm) of interest. Acetic acid was not useful for increasing efficiency even in concentrations

TABLE I: SUMMARY OF MOBILE PHASE MODIFIERS

Modifier Added[1]	N_{PC}/N_{DG}[2]	Effect
Nitric acid	-	Not useful due to UV cut-off
Acetic acid	-	Not useful at $5x10^{-2}M$
Sulfuric acid	0.60	Some tailing observed
Perchloric acid	0.34	Some tailing observed
Phosphoric acid	1.65	Very useful, no tailing
Diethylphosphoric acid	1.88	Very useful, no tailing
Triethylammonium sulfate	1.06	Very useful, no tailing

[1]See Methods and Materials Section for mobile phase composition

[2]Plate count for PC/Plate count for the analogous diglyceride

as high as $5x10^{-2}M$. Of the strong mineral acids, phosphoric acid consistently gave efficiencies comparable to the triglycerides. Tailing and general peak asymmetry were eliminated. The use of ion-pairing agents has been reported for phospholipids (8). We used one here, triethylammonium sulfate (pH 2.10), and as illustrated in Figure 4, PC-$C_{18:2}$ had similar chromatographic efficiency to di-$C_{18:2}$, the diglyceride analog to the phosphatidylcholine.

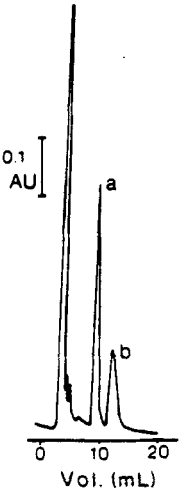

FIGURE 4. Comparison of band shapes of (a) PC-$C_{18:2}$ versus (b) $C_{18:2}$ diglyceride using t-ethylammonium sulfate (pH 2.10, 1% v/v in 95% methanol). Flow rate was 1 ml/min using the Altex column.

DISCUSSION

The potential of HPLC in the reversed-phase mode for the separation of molecular species of phosphatidylcholine was described by Porter et al. (7). The main problem we have encountered in attaining similar goals has been the adsorptive versus partition mechanisms (i.e. mixed-mode retention) encountered with a molecular species containing both a highly polar orthophosphate group and large hydrocarbon area. Thus, mixed or irreversible retention lead to the results given in Figure 2. The addition of ionic modifiers to the mobile phase has little effect on the k' of the phospholipid but greatly affects efficiency and band shape. Also, these modifiers have little effect on di- or triglycerides in terms of either retention or efficiency.

The main mode of action of the modifier is thought to be in masking highly adsorptive sites of the stationary phase. This is due to its relatively high concentration and matching polarity with respect to the solute of interest. Support for this mode of action is that the actual tailing and over-all inefficiency of PC chromatography has been observed by us to be a function of the course and history of the column as well as mobile phase composition. Generally, newer and reconditioned columns have given

worst chromatographic efficiency. One example is that for a Waters μBondapak C18 column, the adsorptive sites on the support could be 'titrated'. That is, PC bands start to elute off the column only after a number of injections have been made (11). Further support for this mode of action is that the k' ratios of PC dioleoyl over diolein were approximately the same for diethylphosphate and orthophosphate.

The use of modifiers not only gives greater chromatographic efficiency and enhanced sensitivity but also allows the employment of low wavelength UV detection. At the wavelength utilized in this study (200 nm), this detector is sensitive only to the hydrocarbon unsaturation. With the use of the UV detector in line with a more general response-type detector, such as the transport based Flame Ionization Detector (13,14) or mass spectroscopic (15) detector, one can generate much more information about the sample as well as place fewer demands on the separation system. Thus an overall improvement in resolution and detection of PC molecular species is possible.

ACKNOWLEDGEMENT

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