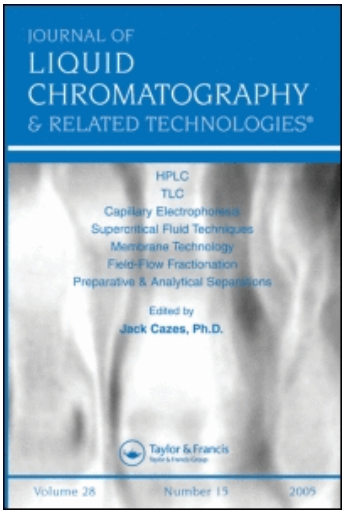

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HPLC PHOSPHOLIPID SEPARATION MECHANISMS ON SILICA, AMINO, AND DIOL COLUMNS

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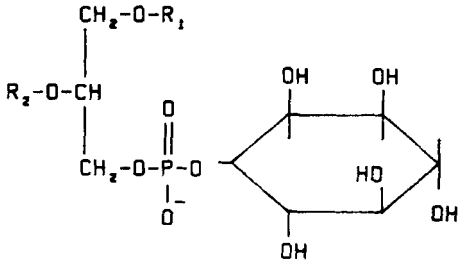
ABSTRACT

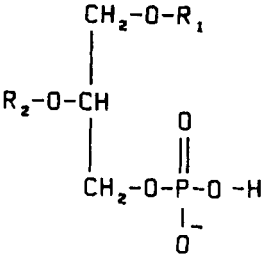
The HPLC separation of phospholipid mixtures was investigated on silica, amino, and diol columns, using mobile phases consisting of acetonitrile, methanol, and phosphoric or trifluoroacetic acids in varying proportions. An explanation of the mechanism of these separations is given with regard to the individual column types.

INTRODUCTION

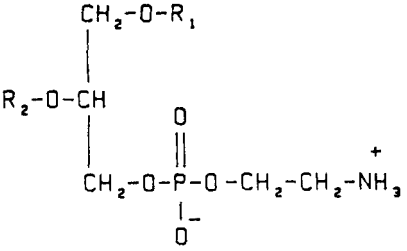
The preparative HPLC isolation of the phosphatides of inositol, serine, ethanolamine, and choline (Figure 1), (abbreviated herein as PI, PS, PE, and PC, respectively), was recently reported from soy lecithin (1) using a mobile phase consisting of acetonitrile, methanol, and phosphoric acid on a silica column. The low column loading limits experienced in that work (10 to 20 percent of that



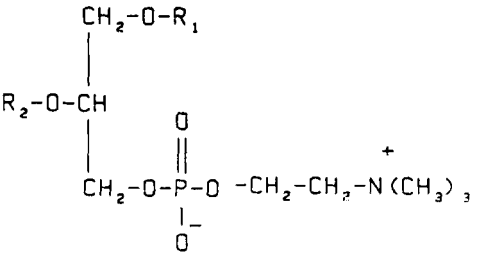
Phosphatidyl Inositol



Phosphatidic Acid



Phosphatidyl Ethanloamine



Phosphatidyl Choline

Figure 1

Structures of Phospholipid Species

normally expected for HPLC) suggested that the column's activity may possibly be due to a solvent-generated phosphate coating on the silica surface.

In continuing our studies of the mechanism of the preparative system, we investigated the analytical system originally used for this work in addition to two other column types, amino and diol, using variations of the same mobile phase and standards. These studies gave additional practical and mechanistic information. Subsequent substitution of trifluoroacetic acid for phosphoric acid in the respective mobile phases was carried out to test several hypotheses.

EXPERIMENTAL

Instrumentation

All experimental work was done using a Model 6000A Solvent Delivery System (Waters), a Model 7125 Loop Injection Valve (Rheodyne), a Spectrophotometer Flow Cell (Altex) installed in an Model 100-40 Ultraviolet-Visible Spectrophotometer (Hitachi) @ 205 nm, a Model 3390A Integrator (Hewlett-Packard), and an RCM 100 Radial Compression Module (Waters). The columns used are described in Tables II and III.

The mobile phases used consisted of various combinations of HPLC grade acetonitrile and methanol, and reagent grade phosphoric acid (85 percent) and trifluoroacetic acid. These combinations are described in Table II.

Standards

All standards were obtained from Sigma Chemical Company, and consisted of L-α-phosphatidylinositol (soybean), L-α-phosphatidylserine (bovine brain), Lα-phosphatidylethanolamine (soybean), and L-α-phosphatidylcholine (soybean). The solutions as received in methanol and/or chloroform were evaporated to dryness under an argon stream with very slight warming to prevent water condensation, weighed by difference, and made up to a concentration of 0.1 mg/ml in Spectrograde chloroform (Fisher). The solutions were injected and chromatographed as equivolume mixtures or single components.

Samples

Commercial soy lecithin was obtained from Staley (Sta-Sol), prepared and injected similarly to the standards. All phospholipid and lecithin solutions were stored at 4°C throughout the work. Even though phosphatidylserine is not a major component of soy lecithin it was included with the standards, since it is a different phospholipid with a different structure and would provide useful information on the mechanism.

All standards and samples were allowed to equilibrate to room temperature prior to injection. Continued opening and closing of the containers subjects the phospholipids to oxidative degradation, and it was suggested that a valve fitting

Table I[9]

Composition of a Typical Natural Lecithin

Phospholipid Class	Percent
Phosphatidylcholine	29.5
Phosphatidylethanolamine	29.5
Phosphatidylinositol	31.6
Miscellaneous phosphatides (Including Phosphatidylserine)	6.3

be used on such containers rather than a simple screw cap to prevent repeated oxygen renewal of the head space.

RESULTS AND DISCUSSION

Column behaviors

As expected, the behaviors of the phospholipids in the standard mixture varied with the column type. Duplicate runs using a commercially available soy lecithin gave the same patterns as the standard mixture.

When 85 percent phosphoric acid was used as the acidic component of the mobile phase, the phospholipids (phosphatidylinositol, phosphatidylserine, phosphatidylethanolamine, and phosphatidylcholine), were eluted from both silica columns (μPorasil and the radial compression cartridge) in the same order: PI, PS, PE, and PC. The retention times were found to be dependent on both acid and methanol concentrations. However, only if methanol was eliminated from the mobile phase could PI be detected using the radial compression cartridge. It is suspected that PI is carried with the front at high flow rates when methanol is present because of

TABLE II

Summary of Experimental Data

Column Type and Specifications	Mobile Phase Comp., % P=Phosphoric Acid TFA = Trifluoroacetic acid CH_3CN	CH_3OH	Acid	Flow Rate ml/minute	Retention Time in Minutes Monitored at 205 nm 0	2	4	6	8	10	12	14	16	18	20
Waters, μ-Porosil, 10 3.9 x 300 mm	97.6	1.3	1.1 P	2.0			PI		PS			PE			PC
Waters, Silica, Radial Compression Cartridge, 10 μm	99.8	0	0.2 P	4.0	PI			PS		PE		PC			
Waters, Silica, Radial Compression Cartridge 10 μm	98.0	0	2.0 P	4.0	PI	PS		PE	PC						
Waters, Silica, Radial Compression Cartridge 10 μm	39.9	59.5	0.2 TFA	4.0	PI		PS		PE	PC					
Alltech/Applied Science Econosil NH_2, 10 μm 250 mm x 4.6 mm	78.4	19.6	2.0 P	1.0	PI							PS	PE		PC
Alltech/Applied Science Econosil NH_2, 10 μm 250 mm x 4.6 mm	77.7	19.4	2.9 P	1.0	PI				PS	PE	PC				
Alltech/Applied Science Econosil NH_2, 10 μm 250 mm x 4.6 mm	76.9	19.2	3.9 P	1.0	PI			PS	PE	PC					
E M Hibar, Lichrosorb Diol, 10 μm 250 mm x 4.0 mm	91.8	8.0	0.2 P	2.0			PC	PI		PE	PS				
E M Hibar, Lichrosorb Diol, 10 μm 250 mm x 4.0 mm	91.8	8.0	0.2 TFA	2.0		PE		PS		PI					

TABLE III[4]

Structures of Active Sites on Various Column Types

Column Type	Active Site
Silica	Si — OH
Amino	Si — CH_2 — CH_2 — CH_2 — NH_2
Diol	Si -$(CH_2)_3$ -O- CH_2 - CH(OH) - CH_2(OH)

the inositol moiety. The absence of acid precluded any separation of phospholipid components.

Substitution of trifluoroacetic acid (TFA) increased the retention times to impractical values on the μ-Porosil column, and although with the radial compression cartridge elution occurred in the same order, the methanol content of the mobile phase had to be increased drastically to allow efficient separation and retention times. Again, PI appeared as a shoulder on the chloroform front, due to the large proportion of methanol.

These data suggest that the separation of phosphatides on silica is not dependent on the presence of phosphate in the mobile phase, and consequently is not necessarily due to the formation of a solvent-generated phosphate layer on the silica particles of the stationary phase as previously suggested (1). Rather, it appears that the mechanism is one of initial adsorption of the phosphatides on the silica, with subsequent displacement by the acid concommitant to their elution from the column. Phosphoric acid appears to be the more efficient displacing acid, as indicated by the lowered retention times with increased phosphoric acid concentration (Table II).

The amine column acted similarly upon the PL mixture. The elution sequence was the same, except for the invariable persistent early elution of PI with the chloroform front, probably due to the necessarily high concentration of methanol

needed in the mobile phase to move the other components significantly. As in the silica columns, increased phosphoric acid concentration in the mobile phase decreases the retention times. Replacement of the phosphoric acid with TFA allowed elution, but with no effective separation of the injected mixture. This phenomenon is still under investigation.

With the diol column an interesting variation in the elution sequence was observed (Table II). When phosphoric acid was used in the mobile phase, the elution sequence became PC, PI, PE, PS as opposed to the PI, PS, PE, PC sequence seen with the silica column. Substitution of TFA for the phosphoric acid effected still another elution sequence, wherein the PI, which usually has a very low retention time, trailed all the other components. This may be explained by the affinity of the hydroxy-rich inositol moiety (Figure 1) of the PI for the diol system of the column (Table III).

Proposed mechanism of separation

The mechanism underlying the behavior of the phospholipids on the silica columns can be postulated as follows: Since the important differences among the standard phospholipids lie in the head groups (Figure 1), one would expect them to be attracted to the active sites of the columns in direct relation to the charge intensity of those groups. The positive charge on the quaternary choline nitrogen of PC would be expected to retain its intensity in the acidic environment of the mobile phases, and consequently be attracted to the silica sites rather strongly. Considering the fact that it does not depend on the mobile phase acid to generate this positive site, it would not be as subject to the acid concentration, as would the other three components, and hence would tend to have retention times of high values, in this case the highest of the four phospholipids used.

The behavior of the phospholipids on the amine column can be explained in a similar manner. Assuming that there is an equilibrium between protonated and nonprotonated amino groups on the column, which would leave a reasonable percentage of them in the free amino form at any time, the positively charged phosphlipids would be retained by the electron-rich amino groups to a degree relative to their charge intensities. The phosphlipids would then be expected to be eluted in the same order as with the silica column, which is exactly what occurs. Increased

phosphoric acid or methanol concentration produced the same results as were noted with the silica columns (Table III). Again, PI ran essentially with the front, since it would be expected to have even less interest in the amino groups than in the silica surface.

The diol column did not present a picture as obvious as that of the silica and amino columns. With phosphoric acid in the mobile phase, the order of elution was PC, PI, PE, and PS, very different from the other columns. The most feasible explanation of this behavior is that one would expect molecules such as PI and PS, that possess a hydroxy group, to be highly attracted to the 1,2-diol system of such a column. This is more fully evident with the TFA acidified mobile phase, where the elution order is PC, PS, and PI. In this work PC has not been able to be located with this diol system, but from the elution patterns of the mobile phase in which phosphoric acid was used, it is suspected that in this case PC is moving with the front. The unusual elution patterns with the diol column have prompted further work of a nature not as pertinent to this research, which will be included in a later paper.

On the other hand, PI, having no basic nitrogen to be protonated by the acidic mobile phase, has only the weakly protonated inositol moiety to attract it to the silica, hence it becomes more closely associated with the methanol of the mobile phase and is eluted early - so quickly in fact that it often appears as a shoulder on the chloroform front.

This leaves PE and PS, which are moderately attracted to the column due to their protonated amino groups. Since ethanolamine has a higher pKa than serine 9.50 vs. 9.21, respectively (5), one would expect the PE to be more strongly protonated, hence more positively charged, than PS in the acidic mobile phase, and consequently held more tightly to the column. PE then would be expected to have a higher retention time than PS on a silica column, which indeed it does.

Apparently, as the mobile phase moves past the phospholipids on the column, an equilibrium is set up between the phosphoric acid and each PL, with the phosphoric being preferentially adsorbed. Thus, each PL is gradually moved along the column in direct relation to the differences in their affinities for the column, with the consequential order of elution, PI, PS, PE, and PC, determined by the intensity of the positive charge on the molecule. Methanol appears to act as a simple solvent,

decreasing retention time by increasing the polarity of the mobile phase, but not altering the order of elution (Table II).

These studies, in addition to offering an explanation of the mechanism of the separation of phospholipids by the admittedly unorthodox system previously reported, have offered two systems that may be useful for selective determination of specific phospholipids.

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