High-Purity Separation of Phospholipids
by Preparative High-Performance Liquid Chromatography

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Abstract: Soybean lecithin (phospholipids), mainly phosphatidylethanolamine(PE), phosphatidylinositol(PI), and phosphatidylcholine(PC), is a commercial by-product of vegetable oil production. The purpose of this work was to investigate the effects of packing size in a chromatographic column, and to obtain the optimum mobile phase composition for preparative work. Normal-phase High-Performance Liquid Chromatography (HPLC) was applied. Hexane, methanol, and isopropanol(IPA) were used as the ternary mobile phases. With the commercially available Nova-Pak column (4 m packing size, 3.9 mm i.d., 15 cm length), the gradient condition of mobile phase composition was that hexane linearly decreased from 85 to 0 vol.% and IPA and methanol, linearly increased 10 to 30 and 5 to 70 vol.% in the following two steps during the total run time of 25 min at the mobile phase flow rate of 1.5 mL/min. For the three other preparative packing sizes experimented were 15, 5/20, and 25/40 μm, the optimum mobile phases were experimentally obtained. The resolutions between PE and PI, PI and PC with 15 μm packings was better over the other two preparative packings. A slight change in mobile phase composition was observed to increase the resolution at the same mobile phase flow rate.

Keywords: Phospholipid, Preparative HPLC, Gradient condition, Packing size

Introduction

Phospholipids are found in all biological membranes and they contain extremely complex mixture of different classes, such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylinositol (PI) [1]. Phospholipids are functioning phosphatides in cell membranes and important substances in biomedical, pharmaceutical, and nutrition research [2,3]. Phospholipids are commonly found in plant and animal tissue and serve as structural components in membranes in addition to playing a role in enzyme activation [4]. For this reason, they are widely based in the food and cosmetic industries, as well as industrial manufacturing [2,4]. For example, phospholipids are key factors in determining phase-transition temperature and membrane fluidity, and play important roles in events such as resistance to the freeze-thaw process, motility, acrosomal exocytosis, and fusiogenic properties of the sperm [5].

Phospholipids separations have been studied by many researchers [4-6] and performed with numerous methods. Previous quantitation and separation of phospholipids have been almost performed with thin layer chromatography (TLC) [5,7]. This method has several disadvantages, such as quantitative separation of individual species is very difficult [8] and incorrect. Recently, several reports have been published that describe successful separation of individual phospholipid species by HPLC [5-7,9-11], especially normal-phase HPLC (NP-HPLC) [10] due to its better reproducibility and shorter analysis time. It is known that a simple isocratic elution for the separation of phospholipids takes long analysis time because of various polarity of each phospholipids [10] and the resolutions between neighbor peaks are also affected by the polarity. Consequently, in order to achieve the proper separation of phospholipids with NP-HPLC, a gradient elution of mobile phase is necessary. During the gradient elution, the polarity of mobile phase is changed, which helps that (phospholipids) with

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different polarities can be partitioned on the silica surface simultaneously. The packing sizes also have great effects on the resolution of the phospholipids. As the size is smaller, the resolution of solutes shows better [12]. Few works have been reported for the separation of phospholipids on various packing materials.

The purpose of this work is to find the optimum operating conditions of the mobile phase with the preparative packing and separate phospholipids from lecithin on a large-scale process. Hexane, methanol, and IPA were used as the ternary mobile phases. The optimum composition of mobile phase for the separation of phospholipids from lecithin was based on the column efficiency and the resolution of phospholipids.

Experimental

Reagents

HPLC grade solvent, hexane, isopropanol (IPA), and methanol were from J. T. Baker (Philipsburg NJ, USA). Lecithin, labeled PERCEPT 8140, the major mixture of phospholipids, was supplied from Central Soya (Fort Wayne, Indiana, USA). Phospholipid standards, PC, PE, and PI from soybean, were purchased from Sigma (St. Louis, MO, USA).

Sample Preparation

The powder of PERCEPT 8140 was dissolved in chloroform and the concentration of solution was adjusted to 5000 ppm. The concentrations of PC, PE, and PI were 5000, 1000, and 1265 ppm, respectively. The injection volume of phospholipid solution, was maintained in 10 mL for all the packings in this work.

Apparatus and Method

HPLC was performed with Waters 600S solvent delivery system and 2487 UV dual channel detector (Waters, Milford, MA, U.S.A.). Data acquisition system was Millennium 3.2 installed in HP Vectra 500 PC. The flow rate of mobile phase was fixed at 1.5 mL/min. Dual UV wavelengths were set at 208 nm and 210 nm.

The mobile phases were degassed with helium. The mobile phases were hexane, IPA and methanol. For the stationary phase, we used three different sizes of preparative packing materials, 5 ~ 20 μm, 15 μm, and 25 ~ 40 μm purchased from Merck (Darmstadt, Germany). They were in-house packed with an aspirator (Tokyo RIKAKIKAI, Tokyo, Japan). The performance of preparative packings was compared with the commercially available Nova-Pak column (4 μm, Waters Co.). The column size was fixed as 0.39 x 15 cm for all of the preparative packings including Nova-Pak column. According to the mobile phase composition, the total elution times were adjusted between 25 and 50 min. This experiment was carried out in an ambient temperature.

Results and Discussion

Separation

The optimum conditions of the mobile phases were investigated to separate phospholipids from lecithin on the three preparative packing and one commercial Nova-Pak column. The polarities of phospholipids, PE, PI, and PC are very different. PE is nonpolar, while PC is very polar, and PI is in the middle range. In a single column, a gradient mode should be required to separate these materials. One linear gradient of hexane/ IPA/methanol (80/10/10 to 0/10/90) step was tried to separate and identify phospholipids from lecithin with Nova-Pak column. In the gradient, the composition of methanol increased, but that of hexane decreased at constant composition of IPA during 25 min. Although PC was eluted as a single peak, PE and PI were not resolved by the linear gradient step. To increase the efficiency of PC and PE and to isolate PI from PC, one or more gradient steps were required [13]. Resolution and separation times were considered to determine the optimum condition. The optimum gradient condition of hexane, IPA, and methanol were listed in Table 1. The experimental conditions were changed as the preparative packing materials were varied. The void volume in a

Table 1. Linear Gradient Conditions with Various Analytical and Preparative Packings

<table>
<thead>
<tr>
<th>Packings (particle size)</th>
<th>Flow rate (mL/min)</th>
<th>Gradient time (min)</th>
<th>Hexane vol.%</th>
<th>IPA vol.%</th>
<th>Methanol vol.%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nova-Pak (4 μm)</td>
<td>1.5</td>
<td>0</td>
<td>85</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15</td>
<td>50</td>
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<td>30</td>
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<td></td>
<td></td>
<td>25</td>
<td>0</td>
<td>30</td>
<td>70</td>
</tr>
<tr>
<td>Lichrosphere Si 60 (15 μm)</td>
<td>1.5</td>
<td>0</td>
<td>85</td>
<td>10</td>
<td>5</td>
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<td>20</td>
<td>0</td>
<td>30</td>
<td>70</td>
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<td></td>
<td></td>
<td>25</td>
<td>0</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td>Lichroprep Si 60 (5/20 μm)</td>
<td>1.5</td>
<td>0</td>
<td>75</td>
<td>15</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>20</td>
<td>30</td>
<td>50</td>
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<td></td>
<td></td>
<td>30</td>
<td>0</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>Lichroprep Si 60 (25/40 m)</td>
<td>1.5</td>
<td>0</td>
<td>85</td>
<td>10</td>
<td>5</td>
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<td>0</td>
<td>100</td>
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</table>
chromatographic column is changed with packing sizes, which can be observed on others works [7,9]. The experimental variables were the gradient time and the number of gradient steps (stepwise or linear) as well as the composition of mobile phases. At the constant column size and mobile phase flow rate, the identification of PE, PI, and PC in a sample were performed by the comparison with standard chemicals.

The chromatogram of phospholipids on the commercial 4 μm Nova-Pak column was shown in Figure 1. The components of PE, PI, and PC were well resolved, although the baseline was not flat due to the gradient mode applied in this work. As shown in Table 1, two or three linear gradients were used with the packing sizes. In Figures 2, 3 and 4, phospholipids separated on 15 μm, 5/20 μm and 25/40 μm preparative packing, respectively were shown. The resolutions of between PE and PI, PI and PC on 5/20 μm and 15m preparative packings were relatively better than on the larger 25/40 μm packing. Especially, PC was always eluted as a single peak for all the packing materials. Band broadening of a peak was serious with larger packing size. The peak height of PC was gradually lower with the larger packings. The peak appearance and the polarity-dependent retention time of PC were also highly reproducible. It implies that PC could be easily separated even in commercial scale by controlling various packing sizes and solvent compositions. PE and PI were relatively well separated on the 15 μm preparative packing as shown in Figure 2, while they were almost coeluted on the larger packings, 5/20 and 25/40 μm, as shown in Figures 3 and 4. This tendency can be explained by the polarity-dependent retention time. That is, the elution of phospholipids having different polarity can be affected by changing polarity of mobile phase [9,12].

![Figure 1](image1.png) **Figure 1.** Separation of phospholipids from lecithin with 4 μm packings. (refer to Table 1 for the mobile phase composition)

![Figure 2](image2.png) **Figure 2.** Separation of phospholipids from lecithin with 15 μm packings. (refer to Table 1 for the mobile phase composition)

![Figure 3](image3.png) **Figure 3.** Separation of phospholipids from lecithin with 5/20 μm packings. (refer to Table 1 for the mobile phase composition)

The particle size of packing material has a great influence on elution of phospholipids.

**Column Efficiency and Resolution**

Column efficiency of a peak is expressed as the number of theoretical plates, N, and it is calculated from the following relationship,

\[ N = 5.54 \left( \frac{t_R}{w_{1/2}} \right)^2 \]  \hspace{1cm} (1)

where \( t_R \) denotes the retention time, and \( w_{1/2} \) is the peak width at the half height. The larger number of theoretical plates, the sharper shape of the peak. Many factors affect the column efficiency, and in this work, it is used as a standard to confirm the effects of packing size on the number of theoretical plates. The results were shown in Table 2. Generally the numbers are closely related to the
physical properties such as viscosity and density of carriers. It amounts to 100000 in gas chromatography, but 1000 in liquid chromatography. The column efficiency of the analytical packings of 4 μm has the largest numbers of theoretical plates. The efficiency was deteriorated by larger packings. It was interesting that even in larger packing of 15 μm, the number of theoretical plates was increased due to the mobile phase composition and gradient conditions.

In order to investigate the resolutions of PE, PI, and PC with the packing materials, the following equation was used,

$$ R = \frac{t_{R2} - t_{R1}}{(W_1 + W_2)/2} $$

where $t_{R1}$ and $t_{R2}$ are retention times of the eluted peaks, and $W_1$ and $W_2$ are the width of the peaks. Larger values of $R$ mean better separation, and smaller values of $R$ poorer separation [12]. Resolution depends on two factors, the narrowness of the peaks and the distance between the highest points of two peaks. Therefore, it is a function of column selectivity, that is, the nature of the stationary and mobile phases [14]. The separation at the baseline is usually designated as the value above 1. The calculated resolutions with the packings are shown in Table 3. The resolutions with 15 μm packing were calculated as about 1.2 for PE and PI, and 7.3 for PI and PC. Those values were larger compared to the other preparative packings. As the packing is smaller and more uniform, the axial dispersion is reduced and the paths of samples are shortened. Consequently, from the experimental results, it suggested the proper size of packings used for the preparative-scale operation should be 15 μm.

### Conclusion

The optimum experimental conditions of the mobile phase compositions and gradient conditions were investigated to separate phospholipid from lecithin with the different particle sizes. The ternary mobile phase of hexane, methanol, and IPA were used as mobile phases. The optimum composition of mobile phase for the separation of phospholipids from lecithin was determined on the basis of resolutions and separation times. The better resolutions were obtained when the particle size of packing material is smaller and more uniform. Among the three preparative packings, 15 μm packings is recommended for the preparative work. The experimental result of the operating condition might be extended to commercial scale. As the higher pressure drop was accompanied by larger packings, a pump with high-pressure rating should be necessary.

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References