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Phosphatidylcholine isolation from egg yolk phospholipids by high-performance liquid chromatography

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Abstract

Cell membrane components have been increasingly recognized as important biochemicals in the fields of biochemistry and pharmacy due to their relationship with metabolite transport in the cells. Among the components, phosphatidylcholine (PC) is considered a valuable biochemical, because it is difficult to commercialize. PC demand has been largely increased in the fields of the nutrient, cosmetic and pharmacy industries, and so the development of a preparative chromatography process is critical to supply a low-cost PC. In this study, we investigated the HPLC separation of phospholipid originated from egg yolk, which contains 80% (w/w) PC and 15% (w/w) phosphatidylethanolamine. Column temperature, mobile phase composition and its flow-rate and kinds of stationary phase were varied to understand the effectiveness of PC separation. For studying the relationship between recovery yield and sample loading amount in HPLC, we performed overloading experiments. In this way, we successfully separated PC with over 99% purity and with 98% yield with the following HPLC operating conditions; pure methanol as a mobile phase, 2.0 ml/min flow-rate and 1000 mg/ml feed concentration in a KR-100-10SIL column.

Keywords: Phospholipids; Phosphatidylcholine; Phosphatidylethanolamine

1. Introduction

Since phospholipids of cell membranes are involved in the mass transport of metabolites in living cells, many researchers in biochemistry have studied them [1]. The phospholipids inhibit crystallization of fatty acids, so that they ascend the usefulness of bioactivate components in vivo [2,3]. Among phosaphatidylcholine (PC), phosphatidylethanolamine (PE) and SM (*N*-acyl-D-sphingosine-1-phosphocholine), PC has gained the interest of researchers because of its pharmaceutical usefulness which promotes metabolism through the cell membrane. However, it was not largely consumed due to high price in a stable form [4,5].

Phospholipids are produced from by-products of vegetable oil manufacturing, and are utilized as emulsification agents, stabilization agents and wetting agents due to their amphoteric characteristics which have hydrophilic and hydrophobic functional groups [6]. Recently, the demand for phospholipids has increased in the fields of nourishment, cosmetic and pharmaceutical industries. Phospholipids used in the pharmaceutical industry should be highly purified, so that they are synthesized artificially and purified with a high-level purification method such as preparative high-performance liquid chromatography (HPLC) [7,8].

As the importance of phospholipids increased, many studies on the separation and purification of

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phospholipids have been done. In the first stage, phospholipids were separated from the tissue or serum of animals. Identification and characterization possibilities of phospholipids improved, and various separation techniques were perused to separate phospholipids [9,10].

Several papers on the separation of phospholipids are published. Amari et al. [11] obtained PC from egg yolk phospholipids using a 200×50 mm preparative HPLC column, and a gram-scale phospholipid fractionation was performed by Meeren et al. [12]. Furthermore, to reduce solvent consumption, the purification procedure was optimized using a method-development column by Meulenaer et al. [13].

Most researchers were interested in the mobile phase design, however there was difficulty in generating mobile phase from purified sample solution. Therefore, a simpler mobile phase design is necessary to develop an economic HPLC process. To this end, we attempted to gain high purity PC from a phospholipid mixture containing 80% (w/w) PC and 15% (w/w) PE by using analytical HPLC with a single component mobile phase. To optimize HPLC operating conditions, various packing materials were tested, and overloading experiments were performed.

2. Materials and methods

2.1. Chemicals

Raw phospholipid compound was provided by the Doosan Institute, Yong-in, South Korea. The source compound was separated from egg yolk, and freeze–dried in a powder form. It contains 80% (w/w) PC and 15% (w/w) PE. Raw sample was dissolved in methanol to concentration of 10 mg/ml and passed through a glass column packed with silica powder that was dried at 200°C [14]. Standard PC (L-α-phosphatidylcholine), PE (L-α-phosphatidylethanolamine) and SM were obtained from Sigma (USA).

The general molecular formula of phospholipids with fatty acid and phosphate groups is given in Fig. 1A. There are various alkyl groups containing different numbers of carbons. As shown in Fig. 1B, phospholipids are subdivided by functional group in phosphate groups. When X is assigned by choline, it is called phosphatidylcholine [15,16].

2.2. HPLC system

The HPLC apparatus was a Model 244 HPLC system from Waters. Absorbance was measured at 206 nm using an M-720 UV monitor (Young-in, South Korea), and the detector signal was recorded and peak area calculated by an integrator (PU4810, Philips, USA). A column heater (CH-30, Eppendorf, USA), and various sample injection loops (Rheodyne 4995, USA) were equipped in the HPLC system.

2.3. Selection of stationary phase

Normal-, reversed- and bonded-phase columns utilized in this experiment were purchased from Eka-Nobel (Sweden) (column dimensions: 250×4.6 mm). A normal-phase stationary phase column

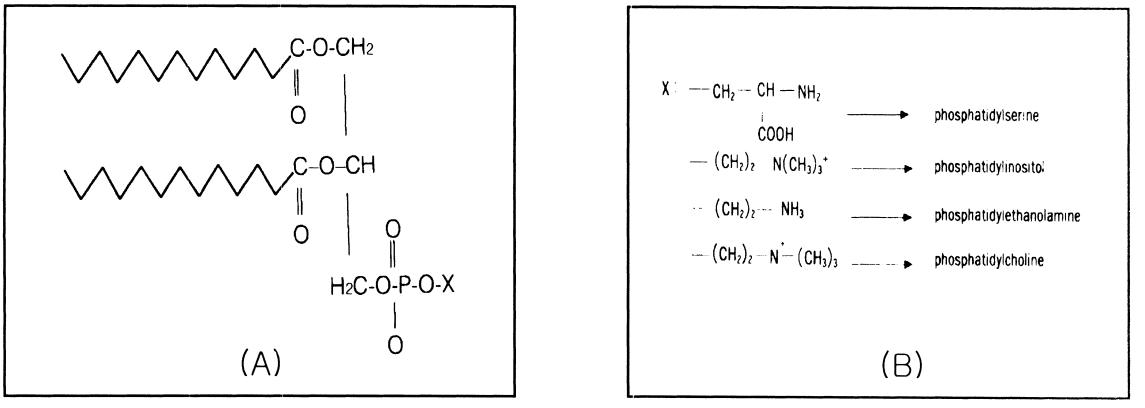

Fig. 1. Structure (A) and classification (B) of phospholipid.

Table 1
Mobile phase compositions

Column selection	Mobile phase selection	
	Analytical condition	Overloading condition
MeOH–water (93:7, v/v)	Isopropanol–hexane–MeOH (55:36:9, v/v) Isopropanol–hexane–water (55:36:9, v/v) Isopropanol–hexane–water (41:34:25, v/v)	MeOH–water (90:10, v/v) MeOH–water (95:5, v/v) MeOH–water (100:0, v/v)

packed with silica was preferred to separate PC because of easy solvent recovery and cheaper price than bonded phase. Particle sizes were 5, 7, 10 and 13 μm. Derivative bonded stationary phases like C_1, NH_2 and CN were also used to accomplish good separation of phospholipids.

2.4. Selection of mobile phase

Mobile phase components such as methanol, hexane, isopropanol, and water were purchased from Merck. All solvents were filtered through a Millipore filter (Durapore membrane filters, 0.22 μm GV, USA), and degassed by a sonicater (Bransonic, Branson, USA), before use in the HPLC system. Mobile phase was prepared at various ratios of each solvent shown in Table 1, and mobile phase composition was maintained in an isocratic condition.

2.5. HPLC operating conditions

Phospholipids were separated at different temperaturess (30, 40 and 45°C), and at the various compositions of solvent mentioned previously. The flow-rate of the mobile phase was varied from 1.0 to 2.0 ml/min. Moreover, overloading experiments were done to investigate the relationship between sample loading amount and recovery yield.

3. Results and discussion

3.1. Phosphatidylcholine separation on different stationary phases

Typical chromatograms of phospholipids are shown in Fig. 2. The sample quantity was 20 μl and methanol and water were mixed in the ratio 93:7 (v/v). All sample components were eluted within 10 min. Fig. 2A shows the chromatogram from the C_1 bonded column, the peaks of PC and SM were not well separated. In the cases of the NH_2-bonded and Diol columns, the peaks of PC and SM were overlapped (Fig. 2B and C). When the silica packed column is used, better separation between PC and SM than obtained by the above mentioned derivative bonded columns was attained. Fig. 2D shows the chromatogram from the silica column packed with 10 μm particles. Other silica columns packed with 5 μm and 7 μm particles were tested to expect good elution profiles, but there was no remarkable difference except higher back-pressure than that of 10 μm particle silica. The column packed with 10 μm particles was selected in order to separate PC from the phospholipid mixture.

3.2. Effects of mobile phase composition on the resolution of PC

Modification of mobile phase composition was performed for better resolution. Fig. 3A shows that PC and SM were not separated with a composition of isopropanol–hexane–methanol (55:36:9, v/v/v). Baseline increases by the residual effects were caused by adsorbed materials in column. Although we obtained a better separation of PC and SM in Fig. 3B with isopropanol–hexane–water (55:36:9, v/v/v), with isopropanol–hexane–water (41:34:25, v/v/v), peak broadening and an increase of retention time occurred due to high water content (Fig. 3C). Therefore, we used the lower water content (10%, v/v), for phospholipid separation in the silica packed column. The effect of high water content was also

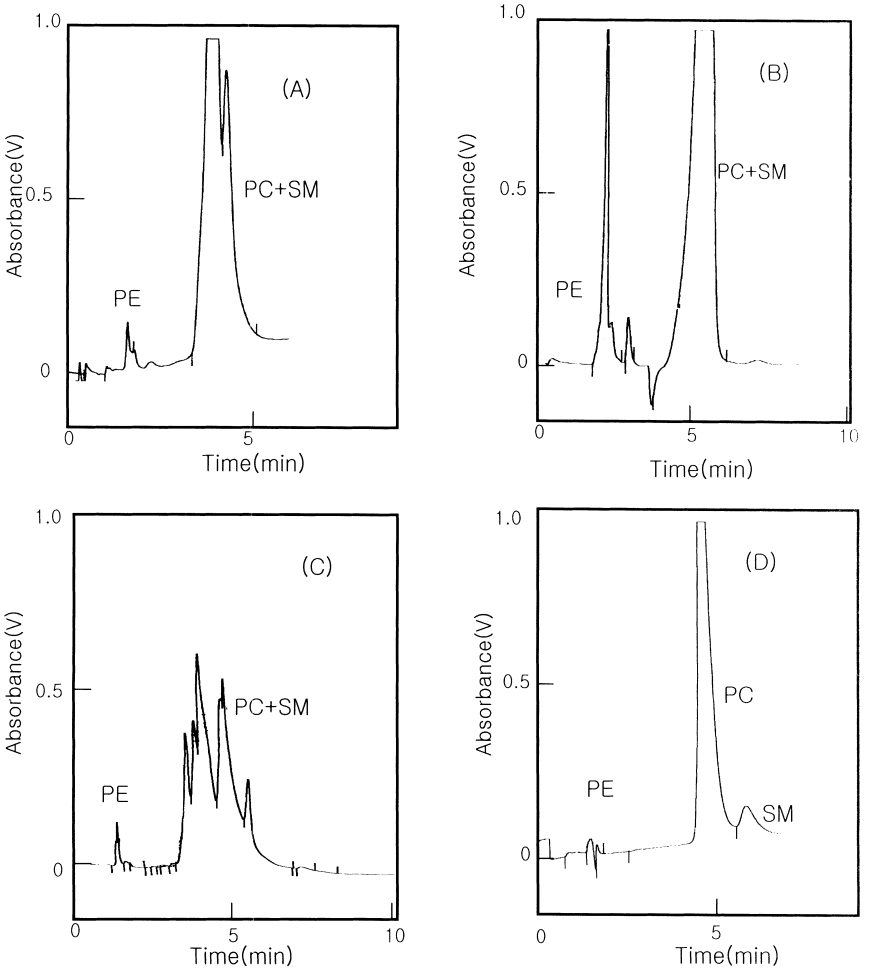

Fig. 2. Elution profiles of phospholipid mixture in various columns; mobile phase: MeOH–water (93:7, v/v); flow-rate, 2.0 ml/min; injection volume, 20 μl; wavelength, 206 nm; sample concentration, 10 mg/ml (in MeOH). (A) C_1 bonded column; (B) NH_2 bonded column; (C) Diol column; (D) silica-10 μm size particle packed column.

reported in Blank and Snyder's paper on PC and SM [17].

Binary isocratic mobile phase is usually utilized in preparative chromatography because solvent can be easily recovered. Therefore, we changed the isopropanol–hexane–water system to a methanol–water system. Fig. 4A shows the elution profile under the condition of methanol–water (90:10, v/v). Retention times of PC and SM decreased, so we varied water content to increase the retention time. In Fig. 4B, the retention time of PC is increased with a lower percentage of water, i.e., 5% (v/v). When pure methanol was used as mobile phase, the PC peak was isolated from the SM peak in the elution profile in Fig. 4C. Therefore, pure methanol was finally selected as a mobile phase to perform overloading experiments.

3.3. Relationship between sample loading amount and recovery ratio

Fig. 5 shows the relationship between sample loading amount and recovery ratio. When the sample loading amount based on the PC exceeds 10 mg (sample concentration 125 mg/ml), the recovery decreased from 95 to 80%. Purity of separated PC

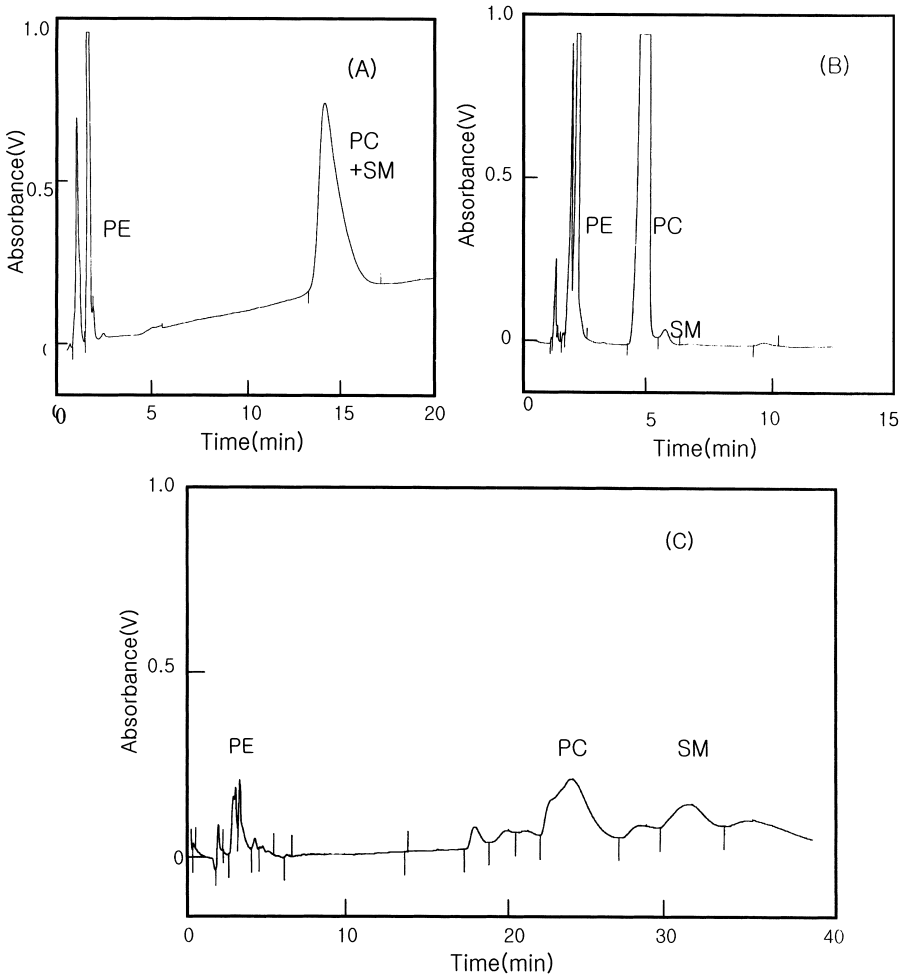

Fig. 3. Chromatograms of KR100-10SIL at various water contents. Flow-rate, 2.0 ml/min; injection volume, 20 μl; wavelength, 206 nm; sample concentration, 10 mg/ml (in MeOH); (A) isopropanol–hexane–MeOH (55:36:9, v/v/v); (B) isopropanol–hexane–water (55:36:9, v/v/v); (C) isopropanol–hexane–water (41:34:25, v/v/v).

was 99% (v/v) which was measured by an ELSD (evaporative light scattering detection) method from the Doosan Institute (Fig. 6).

Considering that the total volume of the column and the density of the stationary phase is 4.15 ml and 0.5 g/l, respectively, we can maintain a recovery above 85% per unit gram of stationary phase if the sample loading amount is set below 4.8 mg per unit gram stationary phase. This means that the concentration of the phospholipid mixture can be kept below 100 mg/ml (PC 80 mg/ml) with a 100 μl injection volume and then 7.8 mg PC can be obtained.

3.4. Recovery of PC through fractionation experiments

Fig. 7 shows an elution profile from overloading experiments (sample concentration=600 mg/ml, loading volume=16 μl). The quantity of PC loaded in the column was 8 mg, based on 80% content in egg yolk. The eluted sample was collected in 0.5 ml volumes after 5.33 min, and then 0.3 ml of sample fractionated after 7.33 min when the PC peak and SM peak were fused. In this case, we collected 2.3 ml of purified sample with 3.13 mg/ml PC as shown in Table 2. Therefore, 7.2 mg PC was recovered

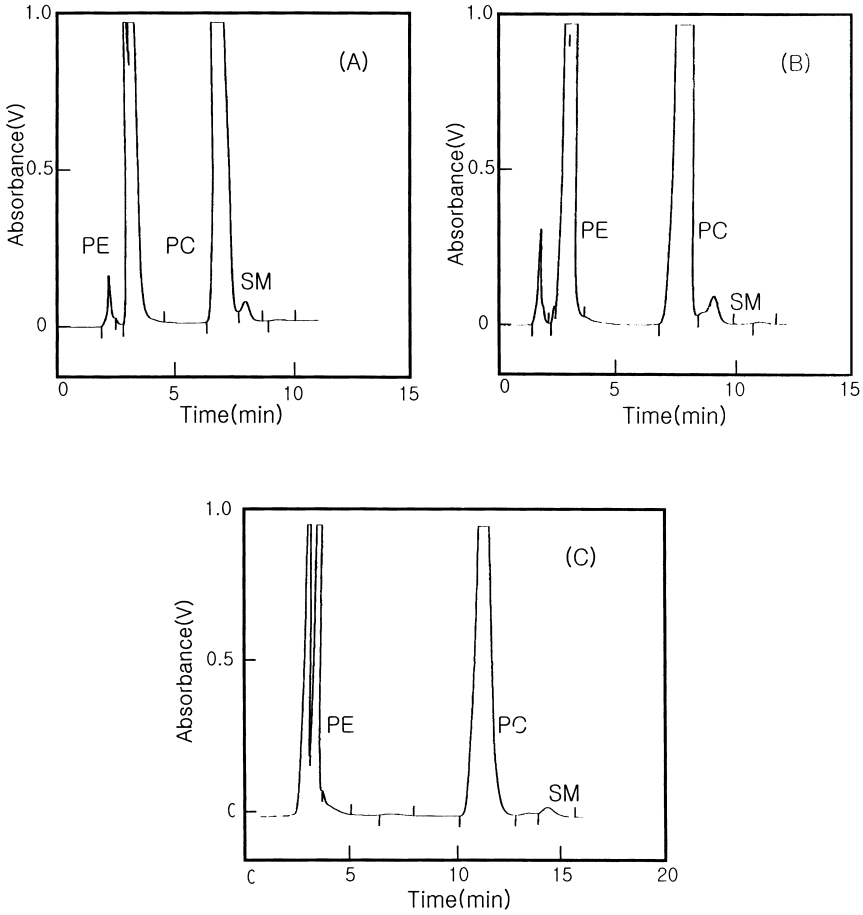

Fig. 4. Chromatograms with different methanol–water ratios. Column, KR100-10-SIL; injection volume, 20 μl; sample concentration, 10 mg/ml (in MeOH); flow-rate, 1.0 ml/min; wavelength, 206 nm, (A) MeOH–water (90:10, v/v); (B) (95:5, v/v); (C) pure MeOH.

from an 8 mg loaded sample by a one-batch overloading experiment with 90% yield. To achieve higher yield, we changed HPLC conditions such as column temperature, flow-rate and sample concentration.

The results of other overloading experiments under various HPLC operating conditions are listed in Table 3. When sample concentration was increased to 1000 mg/ml and sample volume decreased to 12 μl, the total collected sample volume and recovered PC was 4.1 ml and 85%, respectively, due to contamination of SM in the collected sample. In experiment No. 3, yield increased up to 95% by a temperature change of 45°C. This temperature rise is very effective to diminish SM contamination. Sample concentration in experiment No. 4 was adjusted to 1000 mg/ml so as to obtain high yield. When temperature and sample loading amount were increased to 45°C and 12 mg, respectively, recovery increased to 98%. The reason of phenomena is decrease of collected volume due to shortening of elution time that affected by decrement of mobile phase viscosity.

4. Conclusion

A normal-phase column packed with 10 μm silica

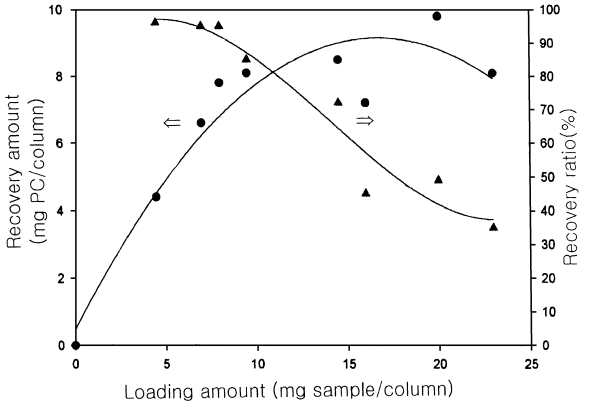

Fig. 5. Recovery amount and recovery ratio at different sample loading amounts. Column, KR100-10-SIL; flow-rate, 2.0 ml/min; sample volume, 100 μl; sample concentration varied from 75 to 300 mg/ml (in MeOH).

was selected from various kinds of HPLC conditions to separate PC from natural phospholipids. For analytical PC separation, we determined mobile phase composition as isopropanol–hexane–water (55:36:9, v/v/v) for separating PC and SM. In overloading experiments, 100% methanol was selected as a mobile phase to simplify the HPLC preparative operation. The PC loading amount is needed to be set below 4.8 mg per unit gram stationary phase for maintaining 90% yield. Sample concentration and loading volume were increased to 600 mg/ml and 16 μl, respectively, in order to obtain 7.2 mg of PC by collecting fractions of column eluents as well as by pooling appropriate fractions. Further increase in sample loading amount to 1000 mg/ml concentration with a 45°C column temperature resulted in a higher yield, i.e., 98%.

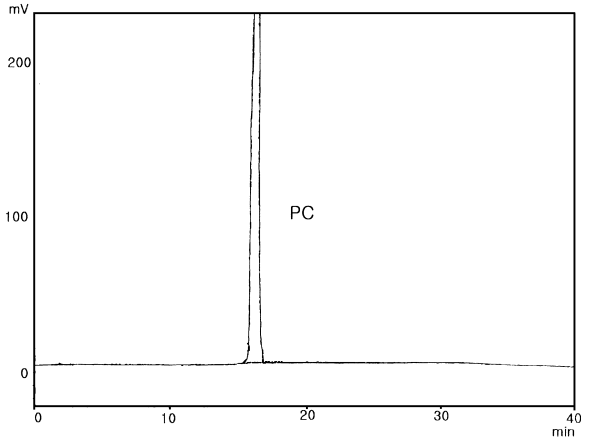

Fig. 6. ELSD chromatogram for separated PC (provided by the Doosan Institute).

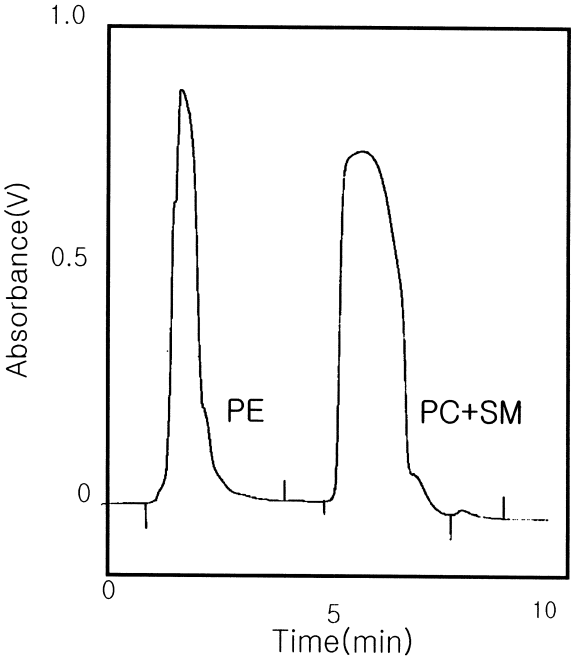

Fig. 7. Overloading experiment. Column, KR100-10-SIL; flow-rate, 2.0 ml/min; sample volume, 16 μl; sample concentration, 600 mg/ml (in MeOH).

Table 2
Fraction analyses of the mobile phase eluted

Fraction No.	Volume (ml)	PC peak area ($\cdot 10^4$)	SM peak area ($\cdot 10^4$)
1	0.5	148	0
2	0.5	340	0
3	0.5	278	0
4	0.5	150	0
5	0.3	53	2
Total	2.3		

Mobile phase, MeOH; column, KR-100-10-SIL (250×4.6 mm); flow-rate, 2.0 ml/min; sample concentration, 600 mg/ml; sample volume, 16 μl; column temperature, 40°C.

Table 3
Results of fractionation experiment under various HPLC conditions

Experiment No.	HPLC operating conditions			Total collected Volume (ml) (recovered PC, mg)	Yield (%)
	Column temperature (°C)	Feed concentration (mg/ml)	Loading amount (PC, mg)		
1	40	600	8	2.3 (7.2)	90
2	40	1000	9.5	4.1 (8.1)	85
3	45	600	9.5	2.5 (9.0)	95
4	45	1000	12.0	3.9 (11.8)	98

Mobile phase, MeOH; column, KR-100-10-SIL (250×4.6 mm); flow-rate, 2.0 ml/min.

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