Separation and Determination of Phospholipids in Biological Samples by High-Performance Liquid Chromatography

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Abstract

An isocratic high-performance liquid chromatographic method is developed for the determination of phospholipids in biological samples using a µPorasil silica column and a mobile phase of acetonitrile–methanol–85% phosphoric acid (90:3:1, v/v/v) at a flow rate of 0.80 mL/min. The effluent is monitored by a UV detector at 203 nm. With the method reported in this paper, phosphatidylinostol, phosphatidylserine, phosphatidylethnolamine, and phosphatidylcholine in biological samples are separated and detected successfully. The method is simple, rapid, and has excellent precision.

Introduction

Phospholipids are the major component parts of the biological membranes. Changes of the phospholipid content, to a certain extent, might affect cell functions. Cellular signal conductance is also closely related to phospholipids. For example, only with the participation of phosphatidylserine (PS) can the protein kinase C be activated. In addition, phospholipids are the main constituent of the pulmonary surfactant, which is closely related to the pulmonary functions. Therefore, the determination of phospholipids is of great significance. Thin-layer chromatography, now commonly adopted to separate and determine phospholipids, is not simple and sensitive. Thus, a high-performance liquid chromatographic (HPLC) method was established to separate and determine phospholipids in biological samples.

Experimental

Materials

Apparatus

A Gilson HPLC system (Gilson Medical Electronics, Villiers le Bel, France) consisting of a computerized solvent delivery system, injector, variable-wavelength UV detector, and strip-chart recorder was used. A Model DL-800 (Dalian Elite Analytical Instruments Corporation, Dalian, China) chromatographic work station was supplied by the Institute of Chemical Physics, Chinese Academy of Sciences (Dalian, China).

Chemicals

The phospholipid standards were phosphatidylinostol (PI), PS, phosphatidylethnolamine (PE), and phosphatidylcholine (PC), and they were purchased from Sigma Chemical Company (St. Louis, MO). Each standard solution, at a concentration of 1 mg/mL, was prepared in chloroform–methanol (2:1, v/v) and stored at –20°C. Methanol and acetonitrile were chromatographic grade and were obtained from the Wujing Chemical Plant (Shanghai, China) and the Shanghai Institute of Neuroscience (Chinese Academy of Sciences, Shanghai, China), respectively. All other chemicals were of analytical-reagent grade.

Methods

Phospholipid extracts

The methods reported by Folch et al. (1,2) were modified to extract phospholipid from biological samples. Briefly, tissue homogenate, or bronchoalvelor lavage fluid (BALF), was transferred to a graduated glass tube. Subsequently, chloroform–methanol (2:1, v/v) was added to the glass tube at twice the volume as that of the used samples. It was then strongly oscillated for 1 min and centrifuged at 2500 g for 10 min. After centrifugation the supernatant was discarded, but the boundary layer was not. The methanol–water solution (1:1, v/v) was added to the glass tube at a quarter of the volume as that of the subnatant. It was also subsequently oscillated strongly for 1 min and centrifuged at 2500 g for 10 min. The supernatant and the boundary layer were then discarded. Finally, the subnatant was transferred to another glass tube, dried under a stream of the nitrogen, and stored at –20°C. The extracted phospholipid was dissolved in a mobile phase solvent containing 20% chloroform before HPLC analysis.

Chromatographic conditions

The chromatographic column was a 300- × 4-mm-i.d.

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prepacked stainless steel µPorasil silica column (Institute of Chemical Physics, the Chinese Academy of Science, Dalian, China), which contained silica gel (10-µm particle size). The mobile phase solvent [acetonitrile–methanol–85% phosphoric acid (90:3:1, v/v/v)] was thoroughly mixed in advance, filtered through a microporous membrane (0.2 µm) and degassed, and delivered to the column by a computerized solvent delivery system at the flow rate of 0.80 mL/min. The sample volume injected for HPLC analysis was 20 µL. The effluent was detected by a UV detector at 203 nm. The data were analyzed by the Model DL-800 chromatographic workstation.

Results and Discussion

Chromatographic conditions and chromatograms

Phospholipids have a glycerol backbone. A fatty acid is esterified to two of the hydroxyl groups of the glycerol. The third is esterified by a phosphate group and a nitrogenous compound (choline, ethanolamine, or serine) except that one family of phospholipid contains inositol, a 6-C sugar alcohol. The nitrogenous moiety is often referred to as a "base", and the phosphate and base together create the "polar head group", which is the hydrophilic domain that contains phosphate and other charged or polar groups. According to the methods in the literature (3), a silica-gel column (which was usually used to separate phospholipids) was chosen as the stationary phase. Furthermore, several mobile phase solvent systems (including isocratic and gradient mobile phase) were respectively tested. Their chromatographic behavior was simultaneously compared. It was found that isocratic mobile phase containing acetonitrile–methanol–85% phosphoric acid (90:3:1, v/v/v) was successful in separating the four major phospholipid components present in the biological samples with less baseline drift. Figures 1 and 2 show the representative chromatograms of phospholipid standards and extracts from the biological samples. It is worthy to note that of the four phospholipids, acidic phospholipids, PI, and PS were eluted before the neutral phospholipids (PE and PC). The retention times of the four phospholipids were 4.40, 8.16, 16.60, and 19.30 min, respectively. The analysis time was only 25 min, which was much shorter than that reported in the literature (4). PI was an exception because it was somewhat affected by the interference from the nonretained fraction (such as the solvent); the other three phospholipids in both the standard solution and biological samples were separated successfully. In the biological samples there were always two unknown fractions between PI and PS that did not interfere with their separations. These two unknown fractions were probably the other phospholipids, which could not be confirmed for lack of other phospholipid standards.

Linear correlation and range

Based on the data that resulted from analyzing the phospholipid standards under the previously-mentioned chromatographic conditions, the regression equation and correlation coefficient between the content (*Y*-axis, µg) and the peak area (*X*-axis, µV/s) were calculated for each phospholipid fraction. The results appear in Table I, which shows that for each phospholipid the content was significantly linear with the peak area within the wide linear range.

Detection limit and sensitivity

Under the previously-mentioned chromatographic conditions, the detection limit when the signal-to-noise ratio was equal to 2 was calculated from the baseline noise data. The results listed in Table I indicate that the method was sensitive. With the increase

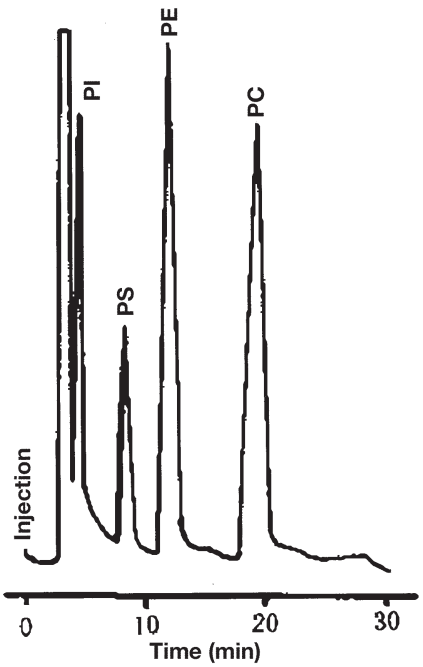

Figure 1. Representative chromatogram of phospholipids standards.

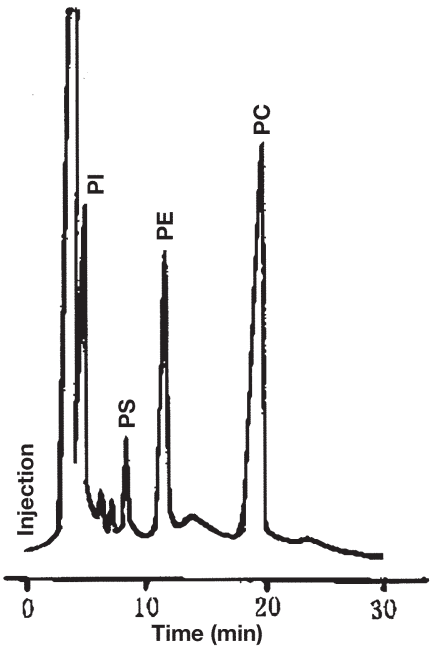

Figure 2. Representative chromatogram of phospholipids extracted from biological samples.

Table I. Linear Correlation, Range, and Detection Limit

Standard	Regression equation	Coefficient	Range (µg)	Limit (µg)
PI	$Y = 0.374 + 2.551 \times 10^{-6}X$	0.9973	0.2–16	0.012
PS	$Y = 0.125 + 1.375 \times 10^{-6}X$	0.9991	0.2–16	0.014
PE	$Y = -0.218 + 6.707 \times 10^{-7}X$	0.9974	0.4–32	0.011
PC	$Y = -0.731 + 9.453 \times 10^{-7}X$	0.9988	0.8–64	0.031

Table II. RSD and Recovery

Standard	Intraday RSD (%)	Interday RSD (%)	Recovery (%)
PI	2.68	2.89	83.58 ± 7.58
PS	2.41	2.82	87.83 ± 6.25
PE	1.84	3.83	87.73 ± 7.77
PC	1.32	2.82	96.67 ± 5.93

Table III. Phospholipid Contents in Lung, BALF, and Hepatocytic Mitochondria in Normal Rats

Phospholipid fraction	Lung (mg/g)	BALF (mg/g tissue)	Mitochondria (µg/mg protein)
PI	4.04 ± 0.88	0.48 ± 0.09	8.12 ± 0.77
PS	3.26 ± 0.87	0.32 ± 0.16	4.27 ± 0.63
PE	4.81 ± 1.22	0.64 ± 0.26	94.30 ± 8.40
PC	38.95 ± 6.93	6.72 ± 1.29	106.85 ± 9.63

of the detector sensitivity, the method would be more sensitive. But when judged by hundreds of analyzed samples, the sensitivity was high enough to detect phospholipids in biological samples.

Precision and repeatability

Experiments were performed to determine the precision and repeatability of the method. In order to estimate the precision, five measurements of the same biological sample were studied in the same assay to calculate intraday relative standard deviation (RSD). Additionally, seven measurements of the same biological sample (each prepared separately from the same homogenized sample) were studied in seven different assays to calculate interday RSD. The results are shown in Table II. Both inter- and intraday RSD were less than 5%, which indicates that the stability and repeatability of the method are excellent.

Recovery

The recovery processing of the endogenous substances in biological body fluids or tissues is quite complicated. A standard addition assay is usually adopted to evaluate the recovery. In the present method, a standard addition assay was also adopted to calculate the recovery for each phospholipid. PI, PS, PE, and PC standards at the amounts of 20, 20, 40, and 80 µg were respectively added to the sample of lung tissue and extracted according to the mentioned procedure. The recovery results are listed in Table II. The average recoveries for PI, PS, PE, and PC were all greater than 80%, which indicates that the accuracy of the method is high enough.

Application

With the method reported in this article, the phospholipid contents in lung, BALF, liver, and hepatocyte mitochondria were determined in ten normal rats. The results are listed in Table III. After the pretreatment process, the phospholipid in all of the biological samples could be rapidly and simply separated and determined by the HPLC method.

References

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