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Simple and Rapid Separation and Determination of Phospholipids by HPLC-UV System

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Abstract

Phospholipids are important class of complex molecules that provide structural and functional roles in the biological systems. Phospholipids anomalies and disorders are generalized membrane defects and so can be detected in a variety of tissues, including body fluids and blood elements. High-performance liquid chromatographic method for the analysis of major phospholipids is reported. Isocratic separation of phospholipids was achieved on silica gel column using elution solvent mixture of acetonitrile-methanol-phosphoric acid (100:10:1.8. v/v) within 9.5 min. Detection of phospholipids was attained by UV spectrometer at 203 nm with detection limit ~5 ng. The present method of HPLC hyphenated-UV system offers advantages of high speed and simplicity for the separation and detection of a variety of phospholipids including phosphatidylserine, lysophosphatidylserine, phosphatidylcholine , lysophosphatidylcholine, phosphatidylethanolamine, lysophosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol, phosphatidic acid and sphingomyelin in routine clinical studies pertaining to membrane/ cell disorders in health, toxicity, diseases, and biological systems, and in basic research, as well.

Keywords: Phospholipids analysis; High-performance liquid chromatography; HPLC-UV; Isocratic method

Introduction

Phospholipids are a group of complex lipids. They are essential building blocks of cell membranes in plants, animals, and microorganisms. They provide molecular strength for the construction and stability of membranes and other cellular constituents. Evidences have shown that phospholipids markedly control functions of proteins and enzymes [1,2] and their degradation causes lipid per oxidative damage in toxicity and diseases [3,4]. Besides the structural significance, their dynamic roles in the highly active process like methylation provide an important mechanism for the biological signal transduction through membranes [5]. Phospholipids also serve a source of arachidonic acid and other polyunsaturated fatty acids, metabolized into biologically active eisanoid and lipoids [6,7]. Phospholipids have received much attention in the area of biomembranes studies as biomarkers and in commercially making of skin-care formulations, and drug carrier system [8,9]. The membrane composition and defects of phospholipids in a number of diseases of brain, heart, lungs or in a variety of biological tissues and fluids including blood elements have been studied for years using solvent extraction, column chromatography, thin-layer chromatography, gas chromatography, and spectroscopic methods.

In recent years, however, the high performance liquid chromatography (HPLC) has emerged to be the most potent tool of phospholipids analysis in health, toxicity, and disease as well as in basic research and routine examination. The ultra-violet (UV) coupled HPLC system for the phospholipids detection has greater sensitivity over refractive index or flame-ionization detection. However, the UV detection restricts the use of common chromatographic solvents that are not transparent in 200 nm to 210 nm regions wherein phospholipids are competent of absorbing the light energy. The choice of the solvent in the HPLC-UV system studies has been limited as such. The HPLC-UV system however can successfully engage solvents such as acetonitrile, ethanol, methanol, n-hexane, iso-propanol and water [10,11]. The significance of UV detection is that it has multiple choices of compositions of mobile phase to advance the isocratic or gradient elution. The available gradient methods are complex, laborious and time-consuming [12-14], while the isocratic elution methods are simple [15,16]. Earlier we found promising results of the HPLC method of isocratic analysis of major phospholipids classes that constitute biological membranes [17]. So, we extended the application of the HPLC-UV system to offer a simple and rapid separation and detection

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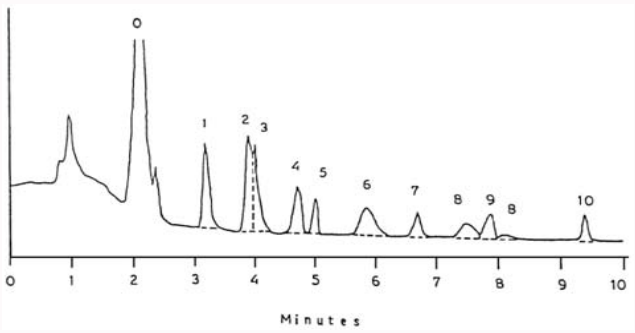

Figure 1: HPLC-UV chromatogram demonstrates separation and detection of phospholipids at 203 nm. The order of elution and phospholipids peaks are 0=SF, 1=PS, 2=PE, 3=LPS, 4=PI, 5=LPE, 6=PC, 7=PG, 8=SM, 9=LPC, and 10=PS.

method for more phospholipids including phosphatidylserine (PS), lysophosphatidylserine (LPS), phosphatidylcholine (PC), lysophosphatidylcholine (LPC), phosphatidylethanolamine (PE), lysophosphatidylethanolamine (LPE), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidic acid (PA) and sphingomyelin (SM).

Materials and Methods

Chemicals

The phospholipids were obtained from Sigma (Munich, Germany): PC, LPC, PS, LPS, PE, LPE, PG, PI, and SM (soybean, egg or bovine source). The chemicals and reagents used in this study were of HPLC or high-purified grade. Double distilled deionized water was used.

Analysis and detection of phospholipids by HPLC-UV system

The HPLC hyphenated-UV system used in this study was a waters liquid chromatography system (Milford, MA, USA) that incorporated a solvent-delivery system (Model 510), auto-sampler-cum-processor (Model WISP 710 B), and a system interface module with a variable UV-VIS wavelength absorbance detector (Model 481 Spectrophotometer). A work station (Model Professional 350) coupled with a printer-plotter (Model LA 50) from the Digital Equipment (Marlboro, MA, USA) was used for integration, calibration, and report information as well as to achieve optimum control and data acquisition.

The analytical column used in the separation was a stainless-steel Beckman Ultra sphere SI 250 mm × 4.6 mm ID packed with spherical silica particles of 5 µm bearing 80 Å pores. The isocratic separation of the phospholipids was achieved with a mobile phase of acetonitril-methanol 85% phosphoric acid (100:10:1.8, v/v/v). The mobile solvent was degassed in an ultrasonic bath prior to pumping. The flow rate of the mobile phase was maintained to 1.5 ml/min with the pressure of ca. 75 bar at room temperature of 25°C. For the greatest sensitivity of the UV-detection of eluting peaks in the present separation, the detector wavelength was set at 203 nm. The phospholipids samples in chloroform-methanol mixture were dried under a stream of nitrogen gas. The residues thus obtained were immediately dissolved in required volume of n-hexane and 2-propanol (3:1, v/v), as these solvents are transparent at the selected UV wavelength of 203 nm. As such, we finally worked out with the experimental conditions for the present HPLC analysis as described in Table 1.

Results and Discussion

The HPLC technique is a most versatile form of system of adsorption, partition, or ion exchange liquid chromatography, which owes its efficiency to the use of very uniform, finely divided micro spherical (3 µm to 10 µm diameter) supports. These supports have a controlled porosity and degree of saturation. The support is packed under high pressure into a stainless steel column of the length 100 mm to 250 mm and the internal diameter 2 mm to 10 mm. The most common type of packing is finely divided silica gel or silicic acid. Since it adsorbs water very strongly, the stationary phase is often aqueous. Polar solvents, such as aliphatic alcohols or glycols, alone or mixed with water, also behave as stationary phase on silica gel. The mobile may be a pure solvent or a mixture of solvents. Therefore, its purity must be markedly different from that of stationary phase so that the two are immiscible.

The HPLC detection of phospholipids is accomplished by ultra violet refractive index or flame ionization detector. The last two detection systems lack sensitivity. The UV detection system has greater sensitivity hence is most versatile form. For maximum sensitivity of the UV detection in the present HPLC separation, we chose the mobile phase having least absorbance at the monitoring wavelength to achieve high signal-to-noise ratio and lowest baseline noise. Moreover, the spectra are a function of the mobile phase components and the pH of the solution. The energy absorption by phospholipids ranges 200 nm to 210 nm (λ, under UV). Therefore, solvents that do not absorb energy or are transparent in this region of radiation were the apparent choice for the analysis that summoned acetonitrile, ethanol, methanol, n-hexane, 2-propanol and water as the most successful analytical solvents as mobile phase in the present separation of phospholipids by HPLC-UV System. Therefore, in the present study we utilized solvents acetonitrile, methanol, and water as mobile phase, which were transparent at the carefully tested and chosen wavelength of 203 nm (Table 1).

Table 1: Conditions and resolution of phospholipids separation by HPLC hyphenated-UV system.

Column	250 mm L × 4.6 mm ID, 5 µm silica, 80 Å pore (Beckman Ultra sphere SI)
Isocratic solvent system	Acetonitrile-methanol-phosphoric acid (100:10:1.8, v/v)
Sample injection solvent	n-Hexane and 2-propenol (3:1, v/v) (Waters Auto Sampler WISP 710 B)
Sample injection volume	10 µL
Flow rate	1.5 ml/min, 10 min separation time (Waters 510 Solvent Delivery System)
Detection	203 nm UV absorption (Waters Lamb-Max LC 481 Spectrophotometer)
Elution order	PS, PE, LPS, PI, LPE, PC, PG, SM, LPC, PA (Digital LA 50 Printer-Plotter)
Resolution time	9.5 min

PS: Phosphatidylserine, PE: Phosphatidylethanolamine, LPS: Lysophosphatidylserine, PI: Phosphatidylinositol, LPE: Lysophosphatidylethanolamine, PC: Phosphatidylcholine, PG: Phosphatidylglycerol, SM: Sphingomyelin, LPC: Lysophosphatidylcholine, PA: Phosphatidic acid.

The commercial phospholipids samples were in chloroform-methanol mixture. Injection of the sample in the original solvent mixture produced a broad tail of the SF peak, which subsequently interfered with the later eluting phospholipids peaks. To avoid this situation, the chloroform-methanol solvent-mixture was removed by drying the sample under a stream of nitrogen gas. The dried samples were immediately dissolved in required volume of n-hexane and 2-propanol (3:1, v/v), so injection of sample in this solvent into the HPLC resolved a sharp SF peak with a zero baseline projection of the later-eluting peaks of phospholipids. Therefore, we achieved optimum resolution (separation and detection) of the phospholipids within 9.5 min. The elution order of PS, PE, LPS, PI, LPE, PC, PG, SM, LPC and PA claimed, respectively, the retention times of 3.16, 3.89, 4.00, 4.70, 5.00, 5.83, 6.70, 7.50, 7.84 and 9.35 min of the phospholipids (Figure 1). The recovery of the phospholipids was found to be 98% ± 2% (relative standard deviation). The individual phospholipid can be quantitatively analyzed on the basis of peak area integration of chromatogram.

In conclusion, the present method of HPLC hyphenated-UV system offers advantages of high speed and simplicity for the separation and detection of a variety of phospholipids and its classes in routine clinical studies pertaining to membrane/cell disorders in health, toxicity, diseases, and biological systems, and in basis research, as well.

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