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Determination of phosphatidylcholine, phosphatidylglycerol and their lyso forms from liposome dispersions by high-performance liquid chromatography using high-sensitivity refractive index detection

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ABSTRACT

An assay for quantitative analysis of phosphatidylcholine and phosphatidylglycerol, and their corresponding hydrolysis products lysophosphatidylcholine and lysophosphatidylglycerol using high-performance liquid chromatography with high-sensitivity refractive index detection was developed. The separation of the phospholipids of interest was achieved on a Zorbax NH_2 column (25 cm × 4.6 mm I.D.) with a mobile phase consisting of acetonitrile–methanol–10 m*M* ammonium dihydrogenphosphate solution pH 4.8 (64:28:8, v/v/v) at a flow-rate of 1.5 ml/min. The response of the refractive index detector to different types of phosphatidylcholine with varying degrees of unsaturation was constant, while the ultraviolet detector response was strongly dependent on the degree of unsaturation. This makes refractive index detection suitable for the determination of natural phospholipids which show a wide variety of fatty acid composition. The method was validated for the determination of phosphatidylcholine, phosphatidylglycerol, lysophosphatidylcholine and lysophosphatidylglycerol in a model liposome dispersion. Synthetic phospholipids of high purity served as external standards and quantitation was based on peak areas. Calibration curves were linear over two orders of magnitude, and detection limits of phosphatidylcholine, phosphatidylglycerol, lysophosphatidylcholine and lysophosphatidylglycerol were 22, 29, 30 and 50 μg/ml, respectively. The method precision for a standard phospholipid mixture and for a phosphatidylcholine–phosphatidylglycerol containing liposome dispersion was in the range of 0.6–4.5% relative standard deviation.

INTRODUCTION

Liposomes, (phospho)lipid vesicles which form spontaneously in an aqueous environment, can be used as pharmaceutical drug carriers [1]. As a part of pharmaceutical formulation process, the long-term stability of liposomes has become an important issue. In an aqueous phospholipid liposome dispersion, the liposomal phospholipids can hydrolyse to free fatty acids and lysophospholipids [2], a process which destabilizes the liposome dispersions and limits the shelf life of liposome-based pharmaceuticals.

Traditionally, thin-layer chromatography (TLC) followed by phosphorus analysis has been the preferred technique for quantitative phospholipid analysis. The different TLC procedures used have been reviewed extensively [3]. TLC, however, is time-consuming, shows high variability and cannot be easily applied to the routine analysis of a large

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number of samples. High-performance liquid chromatographic (HPLC) methods have been used to overcome these limitations.

Phospolipid analysis using HPLC can be divided into two groups: separation of phopholipid classes (*i.e.* by the nature of the head group) and separation of the molecular species within a phospholipid classes (*i.e.* by the nature of the fatty aids). Separation of the phospholipid classes has been achieved on silica gel [4–10], cyano phase [11], diol phase [11,12], amino phase [12,13] and ion-exchange columns [14], while reversed-phase columns separate phospholipids primarily by the molecular species within a given phospholipid class [15–21]. Most of the available methods deal with the determination of phospholipids from biological sources and require solvent or flow gradients to elute the phospholipid classes with good peak shapes and reasonable retention times. Most commonly phospholipids are monitored by low-wavelength UV detection, which is compatible with most solvent systems. The UV response is highly dependent on the nature of the fatty acid residues and varies with the degree of unsaturation. The highly unsaturated phospholipids yield a good response, while the sensitivity for the fully saturated species is poor. This makes UV detection unsuitable for the quantitation of phospholipids with undefined or varying phospholipid composition such as natural phospholipids (phospholipids isolated from natual sources). Usually liposomes consist of more than one phospholipid class and are often made of natural phospholipid raw materials with a wide range of molecular species with varying degrees of unsaturation.

In this study, the usefulness of high-sensitivity refractive index (RI) detection as a mass-sensitive HPLC detector was investigated for the quantitative analysis of phosphatidylchloline (PC) and phosphatidylglycerol (PG), and the corresponding lysophospholipids, lysophosphatidylchloline (LPC) and lysophosphatidylglycerol (LPG), in a model liposome formulation. The column separation was developed from a previously described HPLC procedure [13]. Mobile phase composition was optimized for the separation of the phospholipids of interest. The HPLC–RI assay was validated for the quantiative determination of PC, PG, LPC and LPG.

EXPERIMENTAL

Materials

Dimyristoylphosphatidylcholine (DMPC), monomyristoylphosphatidylcholine (MPC, lysophosphatidylcholine), dimyristoylphosphatidylglycerol (DMPG) and monomyristoylphosphatidylglycerol (MPG, lysophosphatidylglycerol) were purchased from Avanti (Pelham, AL, USA). Dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), dipalmitoylphosphatidylglycerol (DPPG) and distearoylphosphatidylglycerol (DSPG) were purchased from KSV (Helsinki, Finland). Dioleylphosphatidylcholine (DOPC) and dilinoleylphosphatidylcholine (DLPC) were purchased from Sigma (St. Louis, MO, USA). Natural egg phosphatidylcholine (EPC, iodine value 65) and partially hydrogenated egg phosphatidylcholines with iodine values of 40, 30, 20, 10 and 1 were purchased from Asahi (Tokyo, Japan) through Austin (Rosemont, IL, USA). Natural egg phosphatidylglycerol (EPG) and Phospholin 100H were products of Nattermann (Cologne, Germany). Other chemicals used were of analytical grade.

HPLC system

The HPLC system consisted of a solvent delivery system (Autochrom M 500, Knauer, Berlin, Germany), a Rheodyne injection unit (loop volume 5 or 20 μl), a variable-wavelength detector (Model SF 773, Kratos, Ramsey, NJ, USA), a differential refractometer (Waters 410 RI detector, Waters Assoc., Milford, MA, USA) and a Turbochrom 2700 (PE-Nelson, Cupertino, CA, USA) data acquisition and processing system. In some experiments a Hewlett-Packard Type 3390A integrator (Avondale, PA, USA) was used. The separation of the phospholipids was carried out on a Zorbax amino phase column (25 cm × 4.6 mm I.D., 5 μm particle size, Du Pont, Wilmington, DE, USA) at ambient temperature at a flow-rate of 1.5 ml/min, unless otherwise stated. Detection was carried out both with an RI detector and with a UV detector at 206 nm. The detectors were set up in series with the column effluent first passing through the UV detector.

Preparation of the mobile phase

The mobile phase used in this study consisted of acetonitrile, methanol and a 10 m*M* ammonium dihydrogenphosphate solution pH 4.8 (64:28:8, v/v/v). The 10 m*M* ammonium dihydrogenphosphate solution, pH 4.8, was prepared by adjusting the pH of a 10 m*M* phosphoric acid solution to pH 4.8 with a dilute ammonium hydroxide solution. To prepare the mobile phase, acetonitrile and methanol were mixed first and finally the ammonium dihydrogenphosphate solution was added. Direct mixing of acetonitrile and the ammonium dihydrogenphosphate solution caused precipitation of the salt.

Preparation of (standard) phospholipid solutions

All solid phospholipid materials were dissolved in chloroform–methanol (6:4, v/v). This solvent mixture proved to be an acceptable solvent for all phospholipids tested and exhibited minimal interference of the solvent front with the relevant phospholipid peaks in the chromatogram. Pure chloroform was a superior solvent but resulted in a large broad solvent peak.

Preparation of samples from aqueous liposome dispersions

A model liposome dispersion consisting of EPC (iodine value 40) and EPG was prepared by the "film" method [22]. After formation of the phospholipid film in a round-bottom flask from a solution of phospholipids in chloroform in a rotary evaporator at ~50°C, the film was left overnight under reduced pressure. It was hydrated at ~50°C with 0.05 *M* acetate buffer (pH 4.0) containing 0.8% sodium chloride. The initial EPC and EPG concentrations were 24 and 8 mg/ml, respectively. The liposome dispersion was filled into 1-ml ampoules and stored at 70°C for up to 53 h.

The samples were prepared for HPLC analysis by ten-fold dilution of the liposome dispersion with the chloroform–methanol (6:4, v/v). In the diluted samples the salts and the buffer components of the liposome formulation formed a precipitate. These samples were made particle-free by centrifugation at 2700 *g* for 15 min and injected directly into the HPLC system.

RESULTS AND DISCUSSION

Optimization of mobile phase composition for the separation of the PC, PG, LPC and LPG

As RI detectors are sensitive to changes in flow and mobile phase composition, they require isocratic constant-flow conditions for the column separation. Of the numerous HPLC separations reported in the literature, only few can achieve a satisfactory separation of phospholipid classes under isocratic conditions and without flow gradients. HPLC conditions as described by Shimbo [13] for the analysis of rat liver phospholipids proved to be suitable for the isocratic separation of typical liposomal phospholipids PC and PG and their lyso products. Fig. 1 shows the separation of a mixture of synthetic phospholipid standards, DMPC, DMPG, MPC and MPG, under the conditions described by Shimbo. When the flow-rate increased to 1.2 and 1.5 ml/min, the peak shapes improved and the retention times were shorter without loss of resolution. A chromatogram representing the separation of a mixture of the phospholipids carried out at a flow-rate of 1.5 ml/min is presented in Fig. 2A.

Lysophospholipid standards, MPC and MPG, consistently eluted as two peaks. This is consistent

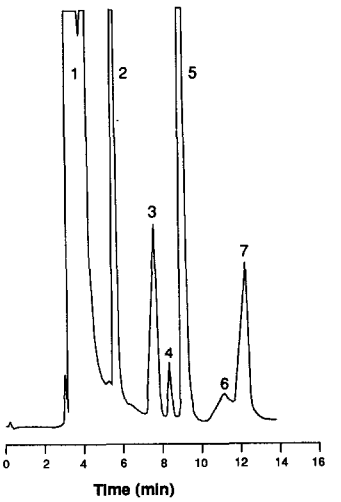

Fig. 1. HPLC profile of phospholipids. Peaks: 1 = solvent front; 2 = phosphatidylcholine (PC); 3 = phosphatidylglycerol (PG); 4 = 2-acyl lysophosphatidylcholine (LPC); 5 = 1-acyl LPC; 6 = 2-acyl lysophosphatidylglycerol (LPG); 7 = 1-acyl LPG. HPLC conditions: mobile phase, acetonitrile–methanol–10 m*M* ammonium dihydrogenphosphate solution pH 4.8 (61:29:10, v/v/v); flow-rate, 1.0 ml/min; detection, RI.

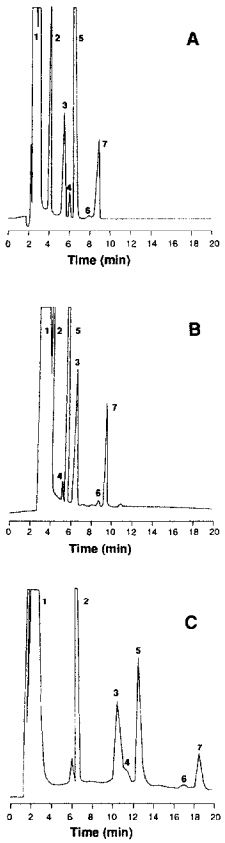

Fig. 2. HPLC profiles of phospholipids. Numbers refer to the same components as in Fig. 1. (A) Mobile phase, acetonitrile–methanol–10 m*M* ammonium dihydrogenphosphate solution pH 4.8 (61:29:10, v/v/v); flow rate, 1.5 ml/min. (B) Mobile phase, acetonitrile–methanol–10 m*M* ammonium dihydrogenphosphate solution pH 4.8 (50:40:10, v/v/v); flow rate, 1.5 ml/min. (C) Mobile phase, acetonitrile–methanol–10 m*M* ammonium dihydrogenphosphate solution pH 4.8 (64:31:5, v/v/v); flow-rate, 1.5 ml/min. Other conditions as in Fig. 1.

with a separation of the lysophospholipids into the two positional isomers which form via acyl migration during the formation of lysophospholipids by the action of phospholipase A_2 and upon subsequent storage; the equilibrium mixture contains approximately 10% of the 2-acyl isomer [23]. Presumably, the peaks eluting before the major lysophospholipids were the 2-acyl isomers of MPC and MPG.

The effect of the mobile phase composition on the separation of the phospholipids was studied by determination of the retention times of the phospholipids with different mobile phase compositions. Either the acetonitrile/methanol ratio (volume fraction of the ammonium dihydrogenphosphate solution kept constant) or the ammonium dihydrogenphosphate solution volume fraction (acetonitrile/methanol ratio kept constant) was varied. Typical examples of chromatograms are presented in Fig. 2B and C. Increasing the concentration of methanol and conversely decreasing the concentration of acetonitrile in the mobile phase resulted in changes in the elution order: from PC, PG, LPC and LPG to PC, LPC, PG and LPG (Fig. 2A and B), while a decrease in the volume fraction of the ammonium dihydrogenphosphate solution in the mobile phase caused longer retention times for all phospholipids (Fig. 2A and C).

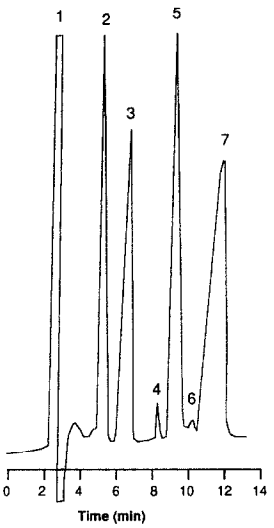

Fig. 3. HPLC profile of phospholipids. Numbers refer to the same components as in Fig. 1. HPLC conditions: mobile phase, acetonitrile–methanol–10 m*M* ammonium dihydrogenphosphate solution pH 4.8 (64:28:8, v/v/v); flow-rate, 1.5 ml/min; detection, RI.

On the basis of these chromatograms, the optimal mobile phase composition was modelled on the Drylab I [24] solvent optimization program. Maximal resolution for all phospholipids of interest in the shortest overall run time was obtained with a mobile phase composition of acetonitrile–methanol–10 m*M* ammonium dihydrogenphosphate solution pH 4.8 (64:28:8, v/v/v). Fig. 3 shows a typical chromatogram of the phospholipid standard mixture under these optimized conditions.

Separation of the other phospholipid classes such as phosphatidylethanolamine (PE), phosphatidic acid (PA), phosphatidylserine (PS), phosphatidylinositol (PI) and sphingomyelin (SPH) was investigated by the analysis of spiked mixtures. Except SPH, all other phospholipids were well separated from PC, PG, LPC and LPG and eluted after the LPG peak (results not shown). SPH, however, was eluted together with PG. The separation of α-tocopherol and cholesterol, which are often used in liposomal preparations, as well as fatty acids, which are hydrolysis products of phospholipids, was also investigated. Standard solutions of α-tocopherol, cholesterol or palmitic acid did not show peaks within a 20-min run time. α-Tocopherol and cholesterol were found in the solvent front when the collected solvent front fraction was analysed on another HPLC system [25].

RI and UV detector response to phospholipids

For both the RI and UV detectors the detection limit for the phospholipid of interest was defined as the concentration which resulted in a signal-to-noise ratio of 2. Detection limits obtained were $3 \cdot 10^{-5}$, $4 \cdot 10^{-5}$, $6 \cdot 10^{-5}$ and $1 \cdot 10^{-4}$ *M* by RI detection and $9 \cdot 10^{-5}$, $1 \cdot 10^{-4}$, $2 \cdot 10^{4}$ and $4 \cdot 10^{-4}$ *M* by UV detection for DMPC, DMPG, MPC (lyso) and MPG (lyso), respectively. The values were determined at an attenuation of 16× for the RI detector and 0.01 a.u.f.s. for the UV detector. It is clear that for saturated phospholipids the detection limit for RI detection is lower than that achievable by UV detection. The difference is slightly more pronounced for the determination of the lysophospholipid.

The RI and UV detector response to different types of phosphatidylcholine species was investigated by determination of the molar response of phosphatidylcholine with varying iodine value, varying chain length and varying number of unsaturated bonds. The iodine values for the synthetic phosphatidylcholine carrying unsaturated bonds, namely DOPC and DLPC, were calculated on the basis of the iodine values reported for oleic acid and linoleic acid. The results show that the UV detector response is strongly dependent on the degree of saturation, while the RI detector response is not significantly affected by the degree of saturation of phosphatidylcholine (Fig. 4). Neither of the two detectors showed a chain length-dependent response (results not shown).

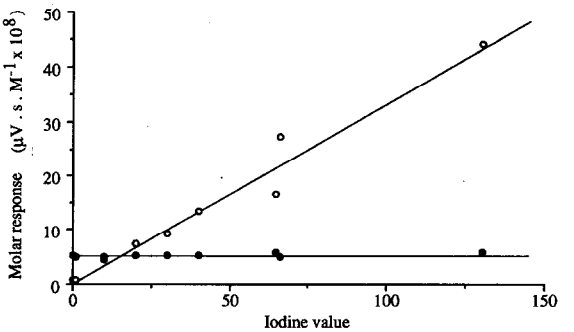

Fig. 4. Relationship between the molar response of different species of phosphatidylcholine with varying degrees of saturation with the RI (●) and UV (○) detectors.

Linearity of response

Fig. 5 shows the relationship between peak area and concentration for DMPC, DMPG, MPC and MPG. In all cases the RI detector response was linear with concentration (Fig. 5). Quantitation by peak area was chosen over peak heights because PG and LPG peaks were not symmetrical and the relationship between peak height and concentration was not linear.

Precision

The precision of the determination was tested by repetitive injection a mixture of phospholipids at different concentrations. The results indicated relative standard deviations (R.S.D.) between 0.6 and

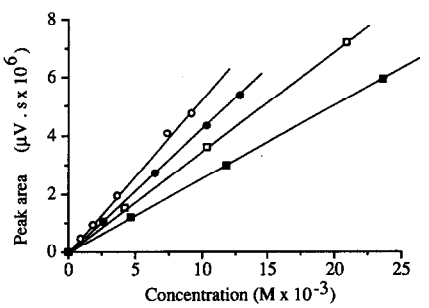

Fig. 5. Standard calibration curves of the phospholipids of interest: ○ = DMPC, ● = DMPG; □ = MPC; ■ = MPG. Lines were calculated by linear regression analysis. Each data point is the average of two determinations.

TABLE I

PRECISION OF THE DETERMINATION

HPLC conditions were the same as in Fig. 3. Injection volume, 20 μl; $n = 8$; C.V., coefficient of variation.

Phospholipid	Concentration (M)	Area (mean ± S.D.) (mV s)	C.V. (%)
Phospholipon 100H	$2.7 \cdot 10^{-4}$	710 ± 30	3.7
	$2.2 \cdot 10^{-3}$	5880 ± 40	0.7
Egg PG	$2.4 \cdot 10^{-4}$	560 ± 20	4.2
	$2.7 \cdot 10^{-3}$	5650 ± 140	2.6
MPC	$4.3 \cdot 10^{-4}$	700 ± 20	2.6
	$3.6 \cdot 10^{-3}$	5810 ± 40	0.6
MPG	$4.9 \cdot 10^{-4}$	720 ± 20	2.8
	$5.5 \cdot 10^{-3}$	8390 ± 140	1.6

4.2% (Table I). The precision of the analysis of a liposome sample was in the range of 1.7–4.5% R.S.D. (Table II).

Analysis of phospholipids in aqueous liposome dispersions

The liposome dispersion was analysed shortly after preparation and again after 30 and 53 h of storage at 70°C for its content of PC, PG and the hydrolysis products LPC and LPG. Representative chromatograms are presented in Fig. 6A C for fresh samples and liposomes aged for 30 and 53 h at 70°C, respectively. A quantitative analysis of the composition of the dispersion on storage is presented in Table II. The recovery of the phospholipids from the aqueous liposome dispersion was complete for the fresh liposome samples. For aged samples a substantial drop in PC and PG content was observed. Lower total lipid recovery of the aged samples is due to the further hydrolysis of lysophospholipids to glycerophospho compounds [26,27].

TABLE II

RESULTS OF THE ANALYSIS OF EPC–EPG-CONTAINING LIPOSOME DISPERSION

Composition: 24 mg/ml EPC and 8 mg/ml EPG; samples were diluted ten times and 20 μl were injected ($n = 8$). Other conditions are the same as in Fig. 3.

Compound	Concentration (mean ± S.D.) (mg/ml)	C.V. (%)	Recovery (mean ± S.D.) (%)
t = 0			
EPC	24.1 ± 0.44	1.9	100.4 ± 1.9
EPG	7.9 ± 0.17	2.2	98.8 ± 2.1
t = 30 h			
EPC	12.4 ± 0.21	1.7	
EPG	2.8 ± 0.08	3.0	
LPC	3.7 ± 0.07	1.9	
LPG	1.4 ± 0.06	4.5	
t = 53 h			
EPC	8.1 ± 0.24	3.0	
EPG	1.3 ± 0.05	3.9	
LPC	4.1 ± 0.06	1.5	
LPG	1.6 ± 0.07	4.0	

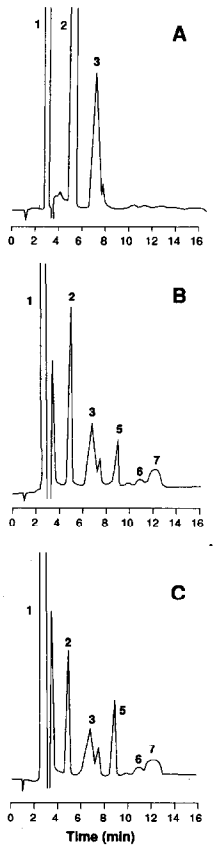

Fig. 6. HPLC profiles of egg phosphatidylcholine (EPC)–egg phosphatidylglycerol (EPG)-containing liposome samples. Numbers refer to the same components as in Fig. 1. HPLC conditions same as in Fig. 3. Composition of liposome dispersion: 24 mg/ml EPC and 8 mg/ml EPG dispersed in pH 4.0 acetate buffer (0.05 *M*); samples were diluted ten times and 20 μl of this solution injected into the HPLC system. (A) Fresh liposome dispersion, (B) 30 h aged at 70°C, (C) 53 h aged at 70°C.

CONCLUSIONS

The analysis of phospholipids by HPLC has advantages over TLC, *.e.g* improved sensitivity, precision and resolution and the possibility of automation. Most of the methods for phospholipid analysis by HPLC available in the literature require either a solvent or a flow gradient to elute the phospholipids with a reasonable retention time, acceptable resolution and a good peak shape. Gradient elution, however, is not compatible with RI detection. Therefore, an isocratic method is essential for the analysis of phospholipids by RI detection.

In this study low-wavelength UV and RI detection systems were compared in terms of sensitivity and changes in the molar response of the phospholipids as a function of phospholipid type, degree of unsaturation and chain length of fatty acid components. As shown in Fig. 4, the UV response is mainly dependent on the degree of unsaturation. The highly unsaturated phospholipids yield a good response while the sensitivity for fully saturated species is poor. The RI detector response, however, is not affected by the degree of unsaturation. This is an advantage of the RI detection over UV detection in the quantitative analysis of phospholipids. Also, RI detection is more sensitive than UV detection for fully saturated phospholipids. PC can be detected at a concentration as low as $3 \cdot 10^{-5}$ *M*. Upon hydrolysis of natural phospholipids, lysophospholipids with a variety of saturated and unsaturated fatty acids are produced. The molar extinction coefficients of these lysophospholipids depends on their fatty acid composition. These lipids cannot be accurately quantified by UV detection. This study shows that within a phospholipid class, the molar response on the RI detector is not significantly affected by either the degree of saturation or the length of the fatty acyl chain; thus, a response factor can be determined once for all molecular species of a phospholipid class using a well defined (synthetic) model lipid as a reference standard. Such a reference standard was used for quantitative analysis of a phospholipid sample with a qualitatively known composition. We found highly purified DMPC, DMPG, MPC and MPG to be good external standards to quantitate PC, PG, LPC and LPG from liposome dispersions.

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