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Liquid chromatography determination of liposome components using a light-scattering evaporative detector*

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Abstract: Analysis of liposomal components is important in stability testing of formulations. An LC method for the analysis of liposomal components cholesterol, phosphatidylcholine, phosphatidylglycerol, phosphatidylethanolamine and their lyso-forms was developed. The method uses a light-scattering evaporative detector and isocratic mobile phase. In addition, components of pH-sensitive liposomes, cholesterylhemisuccinate and cationic lipid dimethyldioctadecylammonium bromide used in transfections were determined by the method. The separations were carried out on a Spherisorb S5 NH_2 cartridge column or Zorbax NH_2 column (25 cm × 4.6 mm, 5 μm particle size). The mobile phase consisted of acetonitrile–methanol–ammonium acetate solution (pH 4.8, 0.1 M) (52:32:16, v/v/v) at a flow rate of 2 ml min^{-1}. Detection limits were 1.3–8.0 μg ml^{-1} depending on the lipid. The precision (RSD) of the method was 1.5–3.3% for lipid standard solutions at 50 μg ml^{-1} concentration and 2.0–11.8% for lipids analysed from liposome suspensions.

Keywords: *Liposomes; phospholipids; LC analysis; stability.*

Introduction

Liposomes can be made from a variety of materials, which makes them versatile as drug carrier systems [1]. Phosphatidylcholine (PC) is the most common constituent of liposomes and cholesterol (Chol) is often used as a bilayer stabilizing agent. To obtain liposomes with net negative charge, acidic phospholipids like phosphatidylglycerol (PG) are used.

After cellular uptake by endocytosis, liposomes are entrapped in acidic endosomes and finally degraded in lysosomes [2]. Cytoplasmic delivery of the drug can increase with pH-sensitive liposomes [3]. These liposomes can be made of dioleoylphosphatidylethanolamine (DOPE) and cholesterylhemisuccinate (CHEMS) [4, 5]. With cationic liposomes DNA and possibly other negatively charged molecules can be effectively delivered into cells by fusion mechanism [6]. For example cationic dimethyldioctadecylammonium bromide (DDAB) and neutral DOPE are used for the transfection of animal cells [7].

Purity and stability of the raw materials as well as the stability of liposomes (e.g. hydrolysis of phospholipids) are important. In addition to lipid peroxidation of unsaturated acyl chains of phospholipids, formation of free fatty acids and lysophospholipids by hydrolytic cleavage may destabilize liposomal membranes and limit the utility of liposomes. Storage of liposomes in aqueous solution at room temperature for several months, especially with oxygen present, enhances the formation of lysophospholipids, whereas use of lower storage temperatures, oxygen-free atmosphere and antioxidants slows down this process [8]. Lysophospholipid formation is dependent upon pH, lipid charge and liposome composition. At acidic and basic side of the optimal pH, hydrolysis of phospholipids to lyso-forms and fatty acids is accelerated [10]. Since lysophospholipids may cause morphological changes and haemolysis in erythrocytes [11, 12] or induce cell fusion [13] their formation in liposomes during shelf-life is not clinically acceptable.

TLC followed by phosphorus determination has been widely used for quantitation of phospholipids and phosphorus assay alone to measure total phospholipid in liposome dispersions. Recently an LC method with refractive index detection and separation with amino

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column was developed for analysis of PC and PG and their hydrolysis products, LPC and LPG [9, 10]. Furthermore, isocratic analysis of PC, LPC, PE, LPE and sphingomyelin (SPH) from rat brain extracts was performed using an amino column and UV detection [14].

Evaporative light-scattering detectors have been used for quantitation of lipid fractions by separation with silica columns with binary [15–17] and ternary [18, 19] gradient elution systems. This paper describes an LC method with an isocratic eluent system using a mass evaporative light-scattering detector for quantitative determination of several different lipids.

Experimental

Materials

Dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylglycerol (DPPG) were from Orion Corp. Farmos (Turku, Finland). Lysophosphatidylcholine (LPC), lysophosphatidylglycerol (LPG), lysophosphatidylethanolamine (LPE), dimethyldioctadecylammonium bromide (DDAB), cholesterylhemisuccinate (CHEMS), and cholesterol (Chol) were purchased from Sigma (St Louis, MO, USA). Phosphatidylethanolamine (PE, egg transphosphatidylated) and dioleoylphosphatidylethanolamine (DOPE) were obtained from Avanti Polar Lipids (Pelham, AL, USA). PG and LPG were dissolved in chloroform–methanol (1:9 v/v) and other lipids in chloroform. Lipids were stored in a freezer under nitrogen until use. LC grade acetonitrile and methanol were from LAB-SCAN.

Hepes buffer (pH 7.4, 20 mM Hepes, 150 mM NaCl) was prepared by weighing 4.77 g of Hepes and 8.77 g of NaCl for 1 l of buffer. pH was adjusted to 7.4 by 5 M NaOH. Ammonium acetate solution (pH 4.8, 0.1 M) was prepared by weighing 6 g of acetic acid for 1 l of the solution. pH was adjusted to 4.8 with 25% NH_3.

LC system

The LC system consisted of a SP8810 precision isocratic pump (Spectra–Physics, San Jose, CA, USA), 7125 Rheodyne injector with a 50 μl loop (Rheodyne, Cotati, CA, USA), ACS model 750/14 evaporative analyser (Applied Chromatography Systems, Macclesfield, Cheshire, UK), and Hitachi D-200 Chromato-Integrator (Hitachi Ltd, Tokyo, Japan). The separations were carried out on a Zorbax NH_2 column (Rockland Technologies Inc., Newport, DE, USA) or a Spherisorb S5 NH_2 cartridge column (Phase Separations Inc., Norwalk, CT, USA). Both columns had similar dimensions (25 cm × 4.6 mm, 5 μm particle size). Temperature of the evaporative analyser was set at 75°C, time constant at 10 s and photomultiplier sensitivity at 2.

The mobile phase consisted of acetonitrile, methanol and ammonium acetate solution (pH 4.8, 0.1 M) usually 52:32:16, v/v/v. The mobile phase was prepared by adding 520 ml of LC grade acetonitrile to 160 ml of ammonium acetate solution. Finally 320 ml of HPLC grade methanol was added to make a total of 1 l of the mobile phase. For the preparation of 8 (80 ml) and 12 (120 ml) vol% ammonium acetate mobile phases the ratio of acetonitrile to methanol was kept constant. Flow rate was 2 ml min^{-1}. Separations were carried out at ambient temperature.

Liposomes

Negatively charged liposomes (37.5 μmol of lipid in 1 ml of 20 mM Hepes buffer, pH 7.4) composed of DPPC, DPPG and Chol (8:2:5 by mol) and pH-sensitive DOPE–CHEMS (6:4 by mol, 37.5 μmol of lipid in 1 ml 20 mM Hepes buffer, pH 8.0) liposomes [5] were prepared by the reversed-phase evaporation (REV) method [20]. Cationic liposomes composed of DDAB–DOPE (8:15 by mol) were prepared by the thin lipid layer hydration method [21] followed by sonication.

For the LC analysis liposome suspension was either dissolved directly (DDAB–DOPE liposomes) into chloroform–methanol, 1:9 by vol., or extracted [22] to avoid interfering peaks from the buffer. Lipids were extracted from liposome dispersions by adding 375 μl methanol–chloroform (2:1, v/v) to 100 μl of liposome suspension. After vigorous vortexing 125 μl of 0.1 M HCl and chloroform were added and after vortexing, the samples were centrifuged for 5 min at 4000 rpm. The lower phase was diluted with the mobile phase and injected into the HPLC system.

Calibration curves and extraction efficiency

Calibration curves in the range of 3.85–125 μg ml^{-1} of each lipid were prepared by diluting lipid stock solutions with the mobile phase and injected into the chromatographic system. Calibration curves were made by plot-

ting peak area (mV) against lipid concentration ($\mu g\ ml^{-1}$).

To determine the extraction efficiency from the liposome suspensions (PC–PG–Chol, DOPE–CHEMS) standard lipids were diluted to equal concentrations with the lower phase of the final extraction stage of liposomes and diluted accordingly with the mobile phase before injection into the chromatographic system.

Results and Discussion

Figure 1 shows the separation of pure lipid standards (250 $\mu g\ ml^{-1}$) of DPPC, DPPG, Chol, LPC and LPG (Fig. 1a) and DDAB, PE, LPE and CHEMS (Fig. 1b). In addition to clear separation of standard lipids, partial separation of 1- and 2-acyl forms was achieved, especially in case of LPG (Fig. 1a). Other lysophospholipid standards (LPC, LPE) did not contain high enough concentrations of 2-acyl form for the separation.

Figure 2 shows the separation of lipids from liposome suspensions (Fig. 2a: DPPC–DPPG–Chol 8:2:5 by mol, total lipid 232 $\mu g\ ml^{-1}$, Fig. 2b: DDAB–DOPE 8:15 by mol, total lipid 200 $\mu g\ ml^{-1}$, Fig. 2c: DOPE–CHEMS 6:4 by mol, total lipid 241 $\mu g\ ml^{-1}$).

Figure 3 shows the effect of the amount (% vol) of 0.1 M ammonium acetate solution in mobile phase on the retention times of Chol, PC, LPC, PG and LPG. pH of the solution was always 4.8. The decrease from 16% to 8% caused significantly longer retention times for PG and LPG with only a small effect on the retention times of Chol, PC and LPC (Fig. 3). A similar phenomenon, with a more pronounced effect on PC and LPC, has been observed earlier with ammonium dihydrogenphosphate (from 10% to 5%) in mobile phase [9]. The subsequent decrease in the solubility of PC with longer saturated acyl chains than in DPPC, (distearoylphosphatidylcholine, DSPC), on the mobile phase has to be taken in account when the amount of ammonium

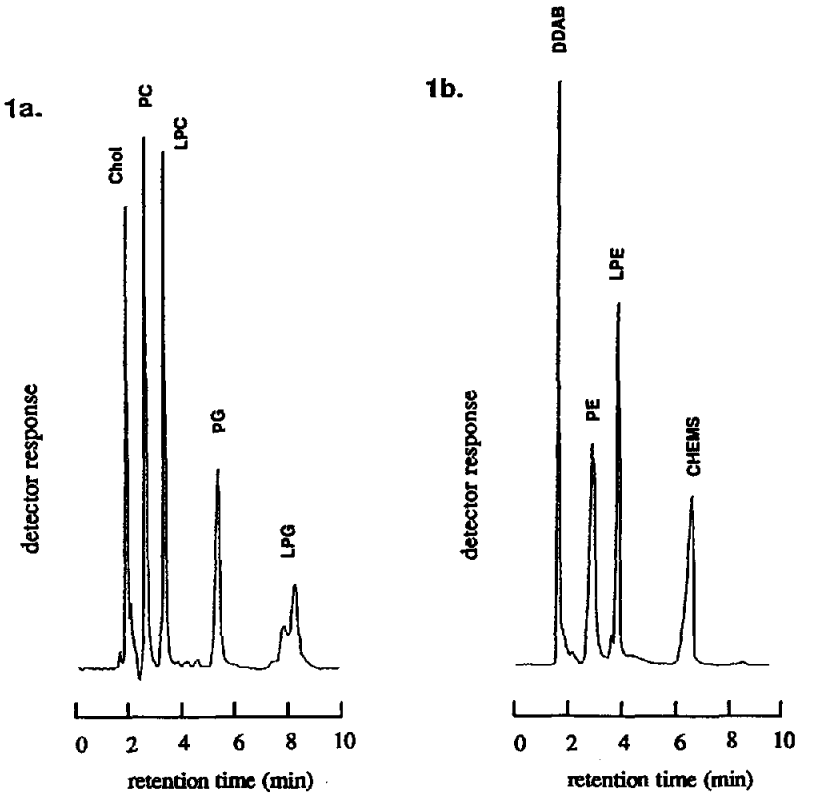

Figure 1
LC separation of phosphatidylcholine (PC), phosphatidylglycerol (PG), cholesterol (Chol), lysophosphatidylcholine (LPC) and lysophosphatidylglycerol (LPG) (Fig. 1a) and dimethyldioctadecylammonium bromide (DDAB), phosphatidylethanolamine (PE), lysophosphatidylethanolamine (LPE) and cholesterylhemisuccinate (CHEMS) (Fig. 1b) from lipid standard mixtures on a Zorbax NH_2 column.

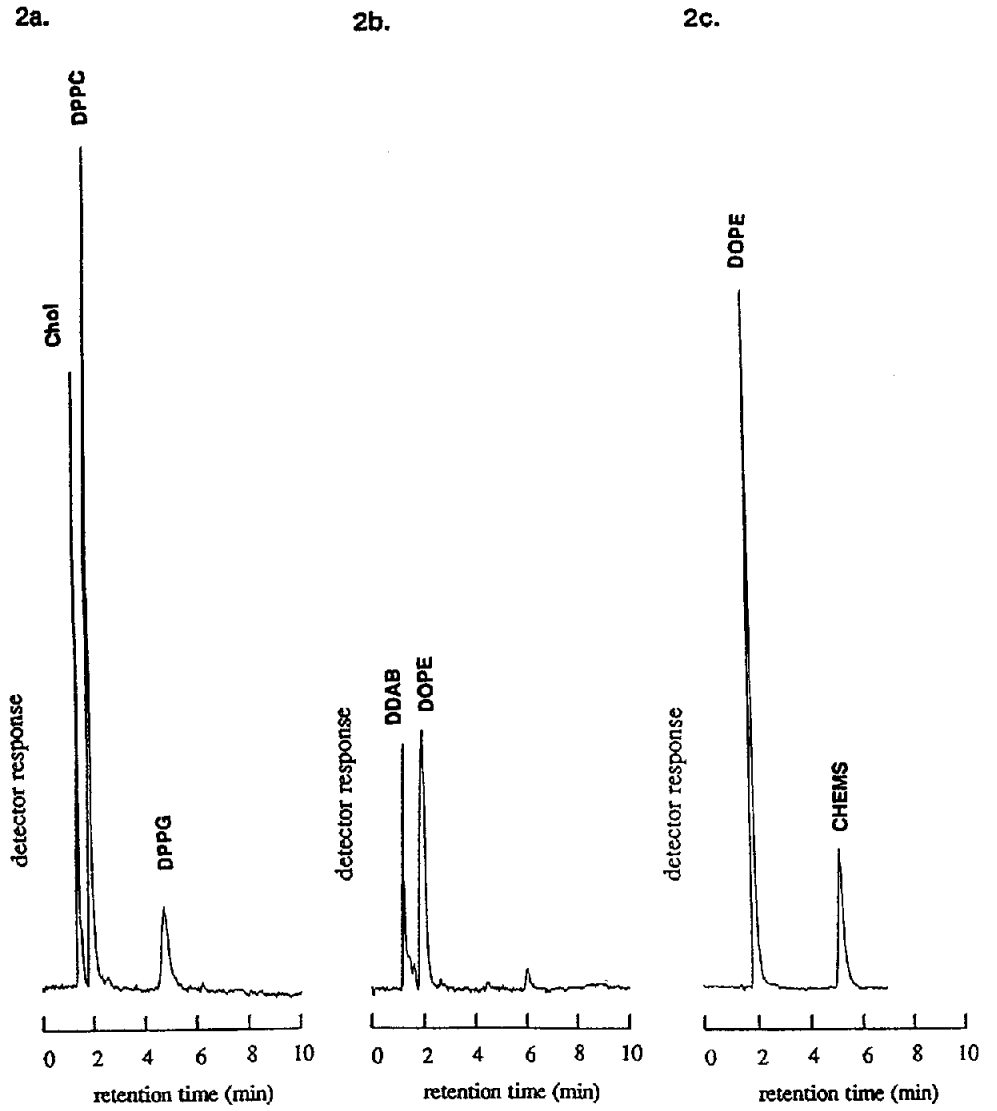

Figure 2
LC separation of lipids from liposome suspensions on a Spherisorb S5 NH_2 cartridge column, (a) DPPC–DPPG–Chol 8:2:5 by mol, (b) DDAB–DOPE 8:15 by mol, (c) DOPE–CHEMS 6:4 by mol.

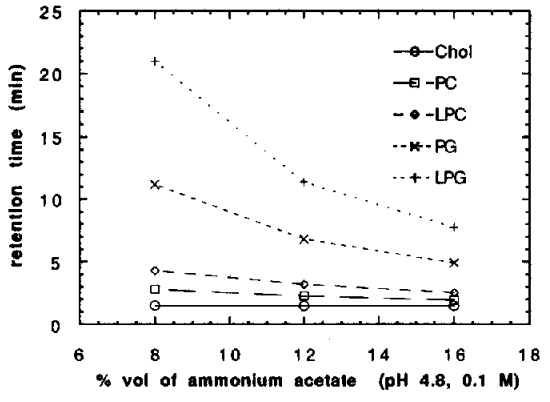

Figure 3
The effect of the amount (% volume) of 0.1 M ammonium acetate in the mobile phase on the retention times of Chol, PC, LPC, PG and LPG.

acetate is chosen. Decreasing the amount of ammonium acetate to 8% gave good peak shapes also for DSPC. For lipids DDAB, DOPE, CHEMS and LPE there were no significant changes in retention times at different amounts of ammonium acetate solution in mobile phase (data not shown).

The effect of ammonium acetate concentration, 0.01–0.1 M, on the retention times is shown in Fig. 4. The retention times of neutral lipids did not change significantly with concentration of ammonium acetate, whereas the retention times of acidic lipids PG, LPG and CHEMS significantly decreased, when the concentration of ammonium acetate was increased. In 0.01 M ammonium acetate acidic

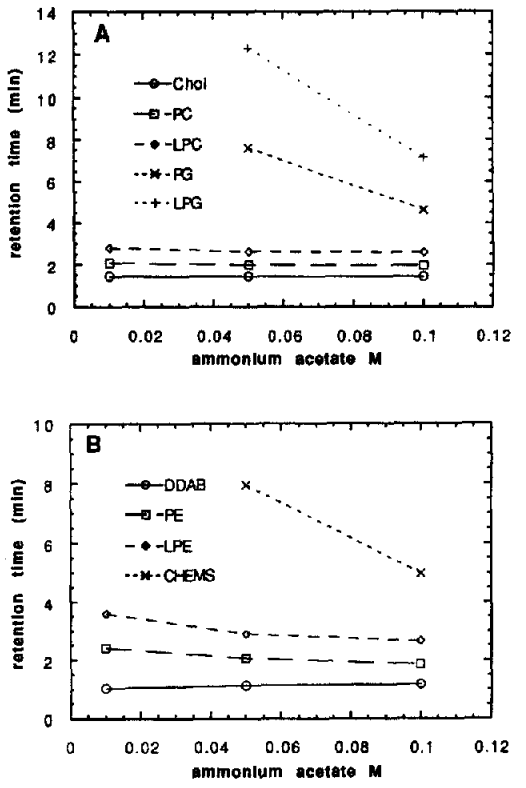

Figure 4
The effect of ammonium acetate at concentrations 0.01–0.1 M on the retention times of Chol, PC, LPC, PG and LPG (a) and DDAB, PE, LPE and CHEMS (b).

lipids either did not elute at all or had too long retention times for proper detection. Ionic bonds between negatively charged lipids and positively charged amino groups of the column are the probable explanation [23]. Increase in ammonium acetate concentration (Fig. 4) or per cent volume fraction (Fig. 3) in the mobile phase helps the elution from the column probably by displacement of lipids by negative carboxylic groups of acetate.

These results were reproducible in all three Spherisorb S5 NH_2 cartridge columns tested. Comparable results were obtained with Zorbax NH_2 column.

Relatively good linearity was observed in the range of 3.85–125 μg ml^{-1} for each lipid (Table 1), although the mass responses, especially at wider concentration ranges, have been shown to be nonlinear [15, 18]. In many cases, this linear range is sufficient for the method to be applied to various stability tests.

Detection limits (μg ml^{-1}) with signal to noise ratio of 5–1 were 1.3 (PC), 4.9 (PG), 2.9 (PE), 1.4 (LPC), 6.7 (LPG), 2.9 (LPE), 1.4 (Chol), 8.0 (CHEMS) and 4.0 (DDAB).

Table 1
Assay performance data from the mean of three calibration curves for different lipids at concentration range of 3.85–125 μg ml^{-1}

Lipid	Slope	Intercept (mV)	Correlation coefficient
Chol	1.190	1.431	0.99819
PC	0.812	2.745	0.99633
PG	0.491	4.086	0.99883
LPC	0.536	5.717	0.99870
LPG	0.206	4.119	0.99781
DDAB	0.767	−3.116	0.99751
PE	0.576	2.176	0.99928
LPE	0.602	6.007	0.99943
CHEMS	0.664	1.980	0.99765

The precision of the method (RSDs) for components from liposome dispersions (n = 10) was 11.8 (Chol), 2.0 (PC), 4.6 (PG), 5.4 (DOPE), 3.5 (CHEMS) and 4.2% (DDAB). For pure lipid standard solutions the values were 0.8–2.5% (200 μg ml^{-1}) and 1.5–3.3% (50 μg ml^{-1}).

Extraction of lipids from liposomes prepared in buffer solutions was necessary due to interfering peaks from the buffer. All other lipids, except cholesterol and lysophosphatidylglycerol could be analysed without extraction (data not shown). Cationic liposomes are usually prepared in H_2O making the extraction step unnecessary.

Extraction efficiencies (%) of lipids from liposome suspensions (mean ± SD, n = 6) were 86.2 ± 7.8 (Chol), 106.1 ± 6.0 (PC), 99.9 ± 5.4 (PG), 93.3 ± 2.7 (DOPE) and 96.1 ± 9.3 (CHEMS).

Adequate chromatographic separation of PC, PG and PE from the hydrolysis products LPC, LPG and LPE (lysophospholipids) makes this method useful in stability tests of liposome dispersions. The method is also applicable for pH-sensitive and cationic liposomes using the same mobile phase.

The response of the evaporative light scattering detector is not affected by the degree of saturation of the phospholipid acyl groups, thus enabling quantitation solely by polar head group. This is an advantage compared with UV-detection, where the molar response is proportional to the degree of saturation. This is a major obstacle especially in the case liposomes, which often contain fully saturated phospholipid acyl chains with very low absorbance in the UV range [9, 23].

Previously an LC method using refractive index (RI) detection was developed for

analysis of PC, PG, LPC and LPG with similar independence of phospholipid or lysophospholipid fatty acid composition [9]. That method, however, gave a relatively large peak from the solvent front making detection of early eluting compounds like cholesterol impossible. The method described here can separate cholesterol from typical liposome formulations due to lack of a large solvent peak with appropriate mobile phase. Furthermore, the method described here is more sensitive than the RI method. Detection limits (PC, PG, LPC, LPG) were 1.3–6.7 $\mu g\ ml^{-1}$ compared to 22–50 $\mu g\ ml^{-1}$ of RI detection [9], and the method can be made even more sensitive by using more advanced light-scattering detectors [17].

Evaporative light-scattering detection is also applicable for gradient analysis unlike RI detection. RI detection is also more sensitive to changes in ambient or mobile phase temperature and small variations in the flow rate of the eluent [23]. Major drawbacks of this type of detection compared to RI detection are the inability to detect small volatile compounds, the necessity to use sufficiently volatile solvents and limited amount of salts or ionic materials in the mobile phase [23].

Amino columns in phospholipid analyses have the disadvantage of variable column performance during time. Frequent cleaning of the column and the use of a guard column [14] significantly enhances the column performance.

Conclusions

The described LC method is relatively simple, fast and sensitive for the analysis of many commonly used liposome components and it can be applied to routine analysis of different types of liposomes and raw materials.

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