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Simultaneous quantification of components of neoglycolipid-coated liposomes using high-performance liquid chromatography with evaporative light scattering detection

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Abstract

Liposomes composed of dipalmitoylphosphatidylcholine (DPPC), cholesterol and a neoglycolipid, mannopentaose-conjugated dipalmitoylphosphatidylethanolamine (Man5-DPPE), have been shown to have a strong adjuvant effect in inducing the antigen-specific cellular immunity. In this study, a rapid and simple analytical method using a HPLC system with an evaporative light scattering detector was developed for simultaneous quantification of the liposome components Man5-DPPE, cholesterol and DPPC. The chromatographic separation of these components was performed using a trimethylsilane column with an isocratic mobile phase of chloroform–methanol–water (1:33:6, v/v) after disrupting the liposomes with chloroform–methanol–water (10:10:3, v/v). This HPLC method provided sufficient reproducibility and linearity of calibration curves for the quantification of the liposome constituents. In addition, this method can be used for the quantification of various neoglycolipids with different carbohydrate structures.

Keywords: Liposomes; Neoglycolipids; Dipalmitoylphosphatidylcholine; Cholesterol

1. Introduction

Liposomes, lipid vesicles prepared by dispersing phosphatidylcholine and cholesterol in an aqueous phase, have been used as carriers of drugs or antigens [1,2]. Several modifications of liposome surfaces have been attempted to improve targeting efficiency to specific cells or organs [2,3]. One possible approach is the modification of the liposome surface using carbohydrates which target carbohydrate receptors on specific cell surfaces. We have synthesized a neoglycolipid, mannopentaose-conjugated dipalmitoylphosphatidylethanolamine (Man5-DPPE), and constructed liposomes coated with this moiety on their surfaces (Man5-liposomes) in order to improve targeting directed at antigen-presenting cells such as macrophages and dendritic cells [4,5], which express mannose receptor on their cell surfaces [6,7]. Using a mouse model, we were able to demonstrate that Man5-liposomes can induce a strong cellular immune response against an antigen encapsulated into the liposome without causing any detectable toxicity.

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Man5-liposomes are composed of Man5-DPPE, cholesterol and dipalmitoylphosphatidylcholine (DPPC). Quantitative analysis of the components is important for the quality control of the generated liposomes and the level of Man5-DPPE is critical in terms of the ability of these liposomes to exert the adjuvant activity [4]. To date, liposomal DPPC and cholesterol have been quantitatively determined using two separate enzymatic methods [8,9]. However, there has been no reliable method for quantification of neoglycolipids. In an effort to simultaneously quantify all components of Man5-liposomes, a rapid and reliable method is needed.

In this report, we describe a method for the separation and quantitative detection Man5-liposome components by means of high-performance liquid chromatography (HPLC) using a trimethylsilane (TMS) column and evaporative light scattering detection (ELSD). This method is suitable for the simultaneous quantification of components of Man5-liposomes.

2. Experimental

2.1. Chemicals

DPPC, cholesterol and dipalmitoylphosphatidylethanolamine (DPPE) were purchased from Sigma–Aldrich Japan (Tokyo, Japan). Mannopentaose (Man5), mannotriose (Man3) and mannobiose (Man2) with the structures Manα1–6(Manα1–3)Manα1–6(Manα1–3)Man, Manα1–6(Manα1–3)Man and Manα1–3Man, respectively, were from Dextra Labs. (UK). Maltopentaose (Mal5), isomaltopentaose (IsoMal5), cellopentaose (Cel5) and *N*-acetylchitopentaose (GlcNAc5) were from Seikagaku Kogyo (Tokyo, Japan). Lactose (Lac) was from Nacalai Tesque (Kyoto, Japan). Neoglycolipids were prepared by conjugation of carbohydrates with DPPE as described previously [10,11]. HPLC-grade chloroform and methanol were obtained from Nacalai Tesque. All other chemicals were of analytical-reagent grade.

2.2. Instrumentation

The HPLC system was equipped with a Showa Denko KT-27 degasser (Tokyo, Japan), a Reodyne Model 7125 injector (Cotati, CA, USA) and a Shimadzu LC-6A solvent delivery pump, a SCL-6A system controller, a CTO-6A column oven (Kyoto, Japan), and a SEDERE SEDEX-55 evaporative light scattering detector (Alfortville, France). The detector was set at 50°C for evaporation and at 2.0 bar for the nebulization gas pressure using compressed air. The chromatograms were recorded with a Shimadzu C-R7A integrator.

2.3. Chromatographic conditions

Chromatographic analysis was performed at 50°C using a Wakosil 5TMS column (25 cm×4.6 mm I.D., 5 μm particle size) protected by a guard column (1 cm×4.6 mm I.D.) packed with Wakosil 5TMS (Wako, Osaka, Japan). The prefiltered isocratic mobile phase, chloroform–methanol–water (1:33:6, v/v), was used at a flow-rate of 0.8 ml/min. The injection volume was 20 μl.

2.4. Preparation of standard solutions

Stock solutions of neoglycolipids, cholesterol and DPPC were separately prepared by dissolving them in a solvent system of chloroform–methanol–water (10:10:3, v/v) to yield final concentrations of 0.4, 4.0 and 4.0 m*M*, respectively. Appropriate volumes of the stock solutions of Man5-DPPE, cholesterol and DPPC were mixed and diluted with the same solvent. A 250-μl aliquot of the diluted mixture was added to a glass vial in which 250 μl of phosphate-buffered saline (PBS) had been evaporated to dryness. The mixture was then vortex-mixed vigorously, passed through a 0.5-μm filter (Millipore, Bedford, MA, USA), and used as the standard solution. When neoglycolipids other than Man5-DPPE were subjected to HPLC, they were dissolved in the solvent at appropriate concentrations without adding cholesterol, DPPC, or PBS.

2.5. Linearity

To prepare calibration curves, HPLC was carried out with 20-μl aliquots of the standard solutions containing 0.15, 0.3, 0.6, 1.0, 1.5, 2.0 and 3.0 nmol of neoglycolipids and 0.5, 1.0, 2.0, 5.0, 10, 20 and 30 nmol of cholesterol and DPPC. With evaporative light scattering, the peak area (*A*) can be related to

the sample mass (m) by means of the following relationship: $A=am^b$, where a and b are constants dependent on a variety of experimental conditions [12]. Therefore, calibration curves were prepared by plotting the peak area (μV s) versus the amount of a compound injected (nmol) using double logarithmic coordinates. The resulting plots described a linear relationship as follows: log $A=b$log $m+$log a. Linearity was determined by calculation of a regression line using the method of least-squares.

2.6. Preparation of liposomes and sample solutions for HPLC analysis

Man5-liposomes and noncoated liposomes were prepared by dispersion of a lipid film consisting of cholesterol and DPPC (1:2, mol/mol) into PBS, followed by incubation with or without Man5-DPPE as described previously [5]. For HPLC analysis, an appropriate volume of each liposome suspension was mixed with PBS in a glass vial to a final volume of 250 μl and then evaporated in vacuo. The residue was redissolved in 250 μl of chloroform–methanol–water (10:10:3, v/v), filtered, and subjected to HPLC as described above.

2.7. Enzymatic quantification of cholesterol and DPPC

Aliquots of liposome suspensions were heated in boiling water for 5 min in the presence of 2% (w/v) sodium dodecyl sulfate and then subjected to enzymatic determination of cholesterol and DPPC. Cholesterol was quantified with a total cholesterol assay kit (Wako) based on a series of reactions with cholesterol oxidase and peroxidase [8]. DPPC was measured with a choline-containing phospholipid assay kit (Wako) based on a series of reactions with phospholipase D, choline oxidase and peroxidase [9].

3. Results

3.1. HPLC separation

Since liposomes are used as adjuvants, they are usually prepared and kept in PBS to insure that the pH and salt concentration are at physiological conditions. In a preliminary experiment, we found that the retention time of each liposome component varied depending on the amount of salt in the injected samples. Therefore, a consistent amount of PBS was added to each standard sample.

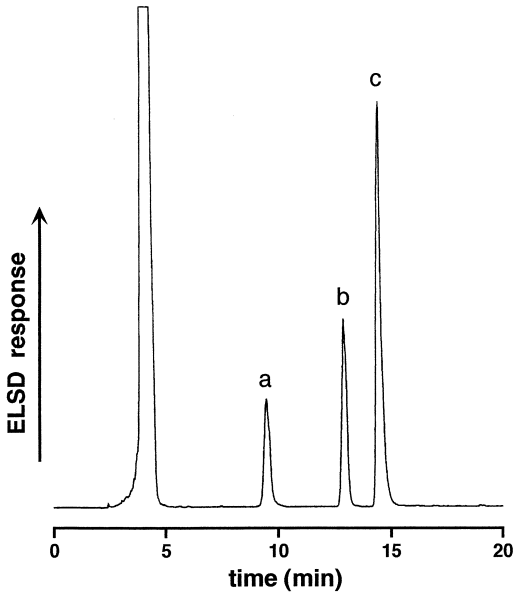

Fig. 1. HPLC chromatogram showing resolution of Man5-DPPE (a), cholesterol (b) and DPPC (c). A standard mixture (20 μl) containing 2 nmol of Man5-DPPE and 5 nmol each of cholesterol and DPPC was injected.

Fig. 1 shows a typical chromatogram obtained by analysis of a standard solution containing 2 nmol of Man5-DPPE and 5 nmol each of cholesterol and DPPC. The peaks of Man5-DPPE, cholesterol and DPPC were well defined and easily distinguished from each other and from the PBS-salt which eluted at the solvent front. Reproducibility was determined with seven subsequent injections. As shown in Table 1, relative standard deviation (RSD) values for the

Table 1
Reproducibility of retention times and peak areas of standard compounds[a]

Compound	Retention time (min)	Peak area (10^3 μV s)
Man5-DPPE	9.46±0.04 (0.42)	523±12 (2.3)
Cholesterol	12.86±0.02 (0.16)	716±17 (2.4)
DPPC	14.39±0.04 (0.28)	1596±43 (2.7)

[a] Values are means±SD of seven estimations. Values in parentheses are relative standard deviations (%).

retention time of each compound that eluted from the TMS column were less than 0.5% and those for the peak area of each compound obtained with ELSD were less than 2.7%, indicating sufficient reproducibility with this method.

3.2. Linearity

The standard calibration curves were linear in the range of 0.15–3.0 nmol for Man5-DPPE and 0.5–30.0 nmol for cholesterol and DPPC. Least-squares regression data of calibration curves of the respective components are summarized in Table 2. Correlation constants of 0.995 or greater were obtained for all analytes. On the other hand, the ELSD response for each compound was different. Therefore, individual calibration curves are needed for accurate quantitative analysis of each compound.

The detection limit for the compounds ranged from 0.05 to 0.1 nmol, since the detector showed a reduced sensitivity and a lower signal/background height ratio in this range.

3.3. Quantitative analyses of liposome components

Three Man5-liposome samples and a sample of noncoated liposomes were separately prepared, and their components were analyzed by the present HPLC method. Fig. 2 shows typical chromatograms obtained with a Man5-coated sample and with a noncoated sample. In analysis of the Man5-liposomes, three peaks representing Man5-DPPE, cholesterol and DPPC were clearly detected, and the degree of response was sufficient for their simultaneous quantification. On the other hand, in the analysis of the noncoated liposomes, only two peaks representing cholesterol and DPPC were detected.

Quantitative results obtained with the HPLC method were compared with those obtained by conventional enzymatic methods. As shown in Table 3, in the analyses of cholesterol and DPPC, the values obtained with the two methods were in close agreement, confirming the reliability of the HPLC method employed here. In addition, Man5-DPPE of Man5-liposomes was quantified by HPLC. Molar ratios of liposome cholesterol, DPPC and Man5-DPPE were calculated from the results obtained with HPLC and were as follows: Man5-liposome sample 1, 1:2.11:0.14; Man5-liposome sample 2, 1:2.10:0.16; Man5-liposome sample 3, 1:2.12:0.17; and noncoated liposomes, 1:2.03:0.

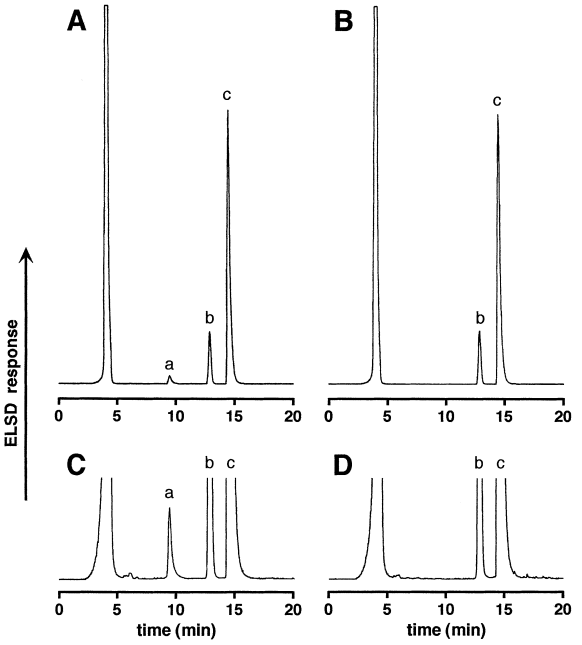

Fig. 2. HPLC chromatograms of extracts from a Man5-liposome sample (A and C) and a noncoated liposome sample (B and D). Peaks a–c are the same as those in Fig. 1. C and D show profiles obtained by amplifying the detector sensitivity at eight-times higher than in the case of A and B, respectively.

Table 2
Least-squares regression analyses of standard calibration curves[a]

Compound	Slope, b	Intercept, log a	Correlation coefficient
Man5-DPPE	1.115±0.091	5.269±0.175	0.995±0.010
Cholesterol	1.283±0.041	4.953±0.034	0.998±0.003
DPPC	1.372±0.086	5.173±0.137	0.997±0.003

[a] Values are means±SD of eight estimations. The linear regression equation is as follows: log $A = b\log m + \log a$; A=peak area in μV s; m=nmol injected.

Table 3
Quantitative analyses of components in liposome samples by the HPLC method and conventional enzymatic methods

Liposomes and components	Concentration (μmol/ml)[a]		%[b]
	HPLC method	Conventional methods	
Man5-liposome sample 1			
Cholesterol	3.81	3.92	97.2
DPPC	8.03	7.91	101.5
Man5-DPPE	0.53	ND[c]	ND
Man5-liposome sample 2			
Cholesterol	3.82	3.94	96.9
DPPC	8.01	8.15	98.3
Man5-DPPE	0.62	ND	ND
Man5-liposome sample 3			
Cholesterol	3.78	3.92	96.3
DPPC	8.02	8.06	99.5
Man5-DPPE	0.64	ND	ND
Noncoated liposome			
Cholesterol	3.98	3.99	99.6
DPPC	8.09	8.05	100.3
Man5-DPPE	NF[d]	ND	ND

[a] Values are the means of two estimations.
[b] Relative percentages of the values obtained with the HPLC method compared with those using the conventional methods.
[c] ND, Not determined.
[d] NF, Not found.

3.4. Application of the method to quantification of various neoglycolipids

We then attempted to apply this HPLC method to quantitative analyses of various neoglycolipids with different carbohydrate structures. Neoglycolipids tested eluted with retention times between 7.89 and 11.00 min as shown in Table 4. The standard calibration curve of each neoglycolipid showed good linearity from 0.15 to 3.0 nmol with a coefficient constant greater than 0.99 (data not shown). These results indicate that the HPLC method can be applied to the quantification of various neoglycolipids.

Table 4
Retention times of various neoglycolipids

Neoglycolipid[a]	Retention time[b] (min)
Man3-DPPE	10.25±0.02
Man2-DPPE	10.98±0.02
Mal5-DPPE	9.32±0.08
IsoMal5-DPPE	9.26±0.17
Cel5-DPPE	8.76±0.01
GlcNAc5-DPPE	7.89±0.03
Lac-DPPE	11.00±0.15

[a] Abbreviations are described in the text.
[b] Values are means±SD of five estimations.

4. Discussion

ELSD is useful for highly sensitive detection of nonvolatile compounds which do not possess a chromophore or fluorophore [12]. Recently, HPLC systems equipped with ELSD systems have been evaluated in terms of the separation and quantification of lipid compounds, such as neutral lipids, phospholipids, glycolipids, and glycated aminophospholipids, all of which are found in organic extracts of biological materials [13–16]. HPLC in combination with on-line mass spectrometry was also used for this purpose [16–18]. In this study, we attempted to establish a method for simultaneous quantification of components of Man5-liposomes (i.e. Man5-DPPE,

cholesterol and DPPC) using a HPLC system. Several columns, different packing materials, and several mobile phase solvent systems were employed in an attempt to evaluate their usefulness in chromatographic analysis (data not shown). Our results indicated that the combination of a TMS column and an isocratic mobile phase consisting of chloroform–methanol–water (1:33:6, v/v) gave the best resolution within a short period of analysis. We have been able to show here that the reproducibility and reliability of the results and the linearity of standard calibration curves obtained using our HPLC method were satisfactory. We also showed that the method is suitable for simultaneous quantification of components of Man5-liposomes.

The present HPLC method has three major advantages. Firstly, the method can be used to directly detect and quantify components of Man5-liposomes rapidly and simultaneously. The speed in obtaining results indicates that it is ideal for routine assays of liposome composition. The method is also cost-effective since tedious procedures involving two separate enzymatic assays for cholesterol and DPPC are no longer needed. We also showed here that the cholesterol and DPPC values obtained by the present method were in good agreement with those obtained by enzymatic methods.

Secondly, this method can be used to specifically detect and quantify the Man5-DPPE of Man5-liposomes. Man5-DPPE possesses both a carbohydrate moiety and a phospholipid moiety. It is well known that the phenol–sulfuric acid method is useful for quantification of carbohydrates and related substances [19]. However, this method is not appropriate for the quantification of Man5-DPPE of Man5-liposomes because insoluble material is formed in the reaction mixture and interferes with the subsequent spectrometric analysis. An inorganic phosphate assay based on the Fiske and Subbarow method [20], which is often used to quantify phospholipids, is also not appropriate because of the coexistence of DPPC and large amounts of phosphate from the PBS used in the liposome suspensions. Thin-layer chromatography followed by sequential staining with primulin and orcinol is often used to detect neoglycolipids [21]. However, this method is not applicable to the quantification of Man5-DPPE of Man5-liposomes because PBS-salt in the liposome suspension disturbs the normal separation of Man5-DPPE on the chromatography. On the other hand, our findings showed that the HPLC method provides a highly reliable and sensitive means for quantifying Man5-DPPE in Man5-liposomes.

Thirdly, the HPLC method is useful for quantification of various neoglycolipids having different carbohydrate structures. Recently, neoglycolipids with various carbohydrates have been used to examine interactions of lectins or microorganisms with oligosaccharides and to clarify their specificity in carbohydrate recognition [22–24]. However, until now, there has been no highly sensitive method for quantifying neoglycolipids. Therefore, this is the first report to describe such a method which we used here to quantify neoglycolipids synthesized by conjugation of carbohydrates with DPPE.

Carbohydrate receptor-mediated targeting of specific cells or organs has been considered to be a useful approach for drug delivery [2,3]. It has been demonstrated in mice that Man5-liposomes act as a capable useful adjuvant in inducing a strong and antigen-specific cell-mediated immune response without any detectable toxicity [4,5]. The adjuvant effect of Man5-liposomes is considered to be due to their facilitation of antigen delivery to antigen-presenting cells such as macrophages and dendritic cells through interaction between the mannose receptor on cell surfaces and oligomannose exposed on the Man5-liposomes. Therefore, the standardization of Man5-liposome composition is essential in studies to elucidate the significance of Man5-liposomes in the induction of cell-mediated immunity and to construct Man5-liposomes with improved targeting efficiency and adjuvant activity.

Man5-liposomes usually encapsulate an antigen protein when they are used as an adjuvant. Even when samples derived from these liposomes were subjected to HPLC, no protein peak was detected on the chromatograms, and the shape or retention time of peaks of Man5-DPPE, cholesterol, or DPPC was not affected (data not shown). The proteins incorporated into the liposomes were probably precipitated by the addition of the chloroform–methanol–water solvent and removed by the subsequent filtration. The amount of encapsulated antigen protein could be determined, if necessary, by a protein assay after lysis of the liposomes.

5. Conclusions

In the present report, a method for quantitative analysis of the components of Man5-liposomes is described. The resolution, reproducibility and linearity of standard calibration curves indicates that the present method using HPLC with ELSD is well suited to the simultaneous quantification of Man5-DPPE, cholesterol and DPPC, the three components that constitute Man5-liposomes. The same chromatographic procedure can also be applied to quantification of various neoglycolipids with different carbohydrate structures. This method will be useful for quality control of neoglycolipid-coated liposomes in studies on drug delivery targeting of carbohydrate receptors.

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