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Research Article

Simultaneous Determination of Polyethylene Glycol-Conjugated Liposome Components by Using Reversed-Phase High-Performance Liquid Chromatography with UV and Evaporative Light Scattering Detection

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Abstract. Liposomes incorporating polyethylene glycol (PEG)-conjugated lipids (PEGylated liposomes) have attracted attention as drug delivery carriers because they show good *in vivo* stability. The lipid component of PEGylated liposomal formulations needs to be quantified for quality control. In this study, a simple reversed-phase high-performance liquid chromatography (HPLC) method with an evaporative light-scattering detector (ELSD) was established for simultaneous determination of hydrogenated soy phosphatidylcholine, cholesterol, PEG-conjugated lipid, and hydrolysis products of phospholipid in PEGylated liposomal formulations. These lipids were separated using a C18 column with a gradient mobile phase consisting of ammonium acetate buffer and ammonium acetate in methanol at a flow rate of 1.0 ml/min. This method provided sufficient repeatability, linearity, and recovery rate for all lipids. However, the linearity and recovery rates of cholesterol achieved using a ultraviolet (UV) detector were better than those achieved using an ELSD. This validated method can be applied to assess the composition change during the preparation process of liposomes and to quantify lipid components and hydrolysis products contained in a commercially available liposomal formulation DOXIL®. Taken together, this reversed-phase HPLC-UV/ELSD method may be useful for the rapid or routine analysis of liposomal lipid components in process development and quality control.

KEYWORDS: component analysis; evaporative light scattering; liposome; reversed-phase HPLC.

INTRODUCTION

Liposomes, which are closed vesicles consisting of a lipid bilayer, have been studied as drug delivery carriers, and have been applied in clinical treatments. It is well known that liposomes incorporating polyethylene glycol (PEG)-conjugated lipid (PEGylated liposomes) can escape uptake by the reticuloendothelial system (RES) and circulate in the blood stream for a prolonged period of time (1,2). Moreover, PEGylated liposomal doxorubicin—DOXIL®—has been marketed and is available commercially. Ambisome®, which contains amphotericin B in the lipid bilayer, demonstrates properties that tend to be uptaken by RES because of anionic lipids in the bilayer and exhibits anti-fungal effects within the RES (3). In gene delivery, cationic liposomes are widely used and numerous attempts have been made to increase the gene transfection efficiency by using ligand-modifying and functional lipids (4). Thus, the "liposome" does not exist, and the lipid component of liposomes is dependent on the encapsulated drug or the objective product performance. Therefore, the lipid and polymeric modifier composition is one of the important physicochemical properties to ensure the quality/safety/efficacy of liposomal products.

Hydrolysis is the primary chemical degradation process of phospholipids. The hydrolysis of ester functionalities is unavoidable in the presence of water, which results in the production of lysophospholipids and free fatty acids. Lysophospholipids may also be further degraded into glycerophosphorylcholine and free fatty acids. It has been reported that increased concentrations of degradation products in liposomal formulations enhance permeability and cause destabilization of the lipid bilayer (5), which significantly affects particle size and can change the structure of the lipid assembly from lamellar to micellar (6). Thus, lipid hydrolysis is considered a critical parameter for the chemical stability of liposomal products. A considerable amount of research has been conducted on the factors that affect the hydrolysis rate, including pH and ionic strength of the storage solution (7–9). Because of these factors, the "assay of lipid components" and "degradation products related to the lipids" are recommended for pharmaceutical specifications in the draft guidance presented by the US FDA for liposomal products (10). This guidance also recommends the development of a stability test which would help evaluate the chemical stability of lipids in liposomal formulations as well as the stability of the

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encapsulated drug, by measuring degradation products such as lysophospholipids and free fatty acids. Thus, appropriate methods for the quantification of lysophospholipids and free fatty acids as well as the liposomal components are required to ensure the efficacy and safety of liposomal products.

High-performance liquid chromatography (HPLC) is widely used to separate and analyze lipids of various origins into lipid classes or molecular species. While unsaturated lipids may be analyzed by ultraviolet (UV) detection, saturated lipids, which are commonly used in liposomal formulations, have no specific absorbance in the UV region, and conventional UV detection cannot be used unless derivatized. Thus, the refractive index detector (RID), evaporative light-scattering detector (ELSD), charged aerosol detector (CAD), and MS were applied for the simultaneous HPLC analysis of lipids (8,11–13). Since ELSDs are higher sensitivity than that exhibited by RIDs, compatible with gradient elution, more easily available than CAD, and simpler to maintain than LC-MS, they are widely used for lipid analyses. Several HPLC-ELSD methods for the analysis of lipids or the hydrolysis products of phospholipids have been previously reported; however, the normal-phase separation was often used, which resulted in the large consumption of chloroform (14–17). While the reversed-phase separation method for the analysis of lipids in cationic liposomes have been reported (18), the reversed-phase separation methods for the simultaneous analysis of PEG-conjugated lipid and the hydrolysis products in PEGylated liposomes have not been well studied. Thus, in this study, we attempted to develop a reversed-phase HPLC-ELSD system that could simultaneously analyze the lipid components and hydrolysis products in PEGylated liposomes.

MATERIALS AND METHODS

Materials and Buffer Solutions

Hydrogenated soy phosphatidylcholine (HSPC; C16:0, 11.4% and C18:0, 88.6%) and *N*-(carbonyl-methoxy polyethyleneglycol 2000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE-PEG), 1-palmitoyl-2-lyso-sn-glycero-3-phosphocholine (P-LysoPC), and 1-stearoyl-2-lyso-sn-glycero-3-phosphocholine (S-LysoPC) were purchased from Nippon Oil and Fat (Tokyo, Japan). Analytical-grade cholesterol (Chol), palmitic acid (PA), stearic acid (SA), HPLC-grade methanol, and ammonium acetate were purchased from Wako Pure Chemical (Osaka, Japan). Trifluoroacetic acid (TFA) was from Sigma-Aldrich Co (MO). Four lots (#011AFL, 012AGD, 029BJD, and 032BKA) of DOXIL® (JANSSEN PHARMACEUTICAL K.K, Tokyo, Japan) were purchased from a general sales agency for drugs, and the approximate elapsed time of each lot after manufacture was 68, 65, 29, and 26 months, respectively.

Standard stock solutions of PA, SA, P-LysoPC, S-LysoPC, HSPC, Chol, and DSPE-PEG were individually prepared by dissolving 3.0 mg of each lipid in 10 ml of methanol and stored at 4°C. The calibration standards were then prepared by diluting the standard stock solutions with methanol.

Instrumentation and Chromatographic Conditions

The apparatus used for the HPLC system consisted of two constant pumps (LC-10ADvp, Shimadzu, Kyoto, Japan), a degasser (DGU-14A, Shimadzu), an automated pretreatment system, an autoinjector (SIL-10ADvp, Shimadzu), a column oven (CTO-10ACvp, Shimadzu), a UV detector (SPC-20AV, Shimadzu), an ELSD (ELSD-LTII, Shimadzu), and a system controller (SCL-10Asp, Shimadzu). A nitrogen generator (SLP-221ED, ANEST IWATA, Yokohama, Japan) was used as the source for the nitrogen gas. Data analysis was performed with the LC Solution program (Shimadzu). The separation was performed at 45°C on a YMC-Triart C18 column (150×4.6 mm i.d., 5 μm) from the YMC Co. (Kyoto, Japan). The ELSD conditions were as follows: the drift tube temperature was set at 45°C, the nitrogen gas-pressure was set at 350 kPa, and the gain was set to 6. The flow rate was 1.0 ml/min for the mobile phases (mobile phase A, 4 mM ammonium acetate buffer (pH 4.0) and mobile phase B, 4 mM ammonium acetate in methanol). The binary linear gradient began from a mixture of 20% A and 80% B and ended at 100% B in 10 min. After the 10-min plateau at 100% B, the mobile phase composition changed back to its initial composition in 5 min. The liposome samples were directly diluted with methanol to the lipid concentration within the calibration range. DOXIL® was diluted 10- or 20-fold, and the liposomes under the preparation were 10-fold diluted. The sample injection volume was 20 μl.

Liposomal Preparation

The lipid composition of PEGylated liposome referred to DOXIL®. The liposome, which consisted of HSPC/Chol/DSPE-PEG, was prepared using a modified ethanol injection method (19). Briefly, 47.9 mg of HSPC, 15.95 mg of Chol, and 15.95 mg of DSPE-PEG were dissolved in approximately 10 ml of ethanol. The ethanol was then removed using a rotary evaporator, which left behind approximately 1 ml of ethanol solution. Next, 8 ml of 10% sucrose (pH 6.5) was added to the ethanol solution. Liposomes formed spontaneously after further evaporation of the residual ethanol, and were extruded through a series of polycarbonate filters (Nucleopore, CA) with pore sizes ranging from 0.4 to 0.1 μm. Following the extrusion, the liposome solution was placed in membrane tubing with a molecular weight cut-off of 50 kDa (Flat-A-Lyzer G2, Spectrum Laboratories, Inc., CA) and was dialyzed against fresh 10% sucrose solution to remove the free lipids or micelles.

Incubation of Liposome

Each 500 μl of liposome in glass tubes were incubated at 37°C or 57°C in a water bath without agitation, or at 4°C in a refrigerator for three days. After incubation, liposomes were diluted 10-fold with methanol, and 20 μl of aliquots were injected to HPLC system.

RESULTS AND DISCUSSION

Optimization of the Separation Conditions

The method for the quantification of lipids in a laboratory preparation of liposomes as well as liposomal formulation development and quality control should be simple, rapid, and safe. Thus, we aimed to develop an HPLC-ELSD system that would quickly and simultaneously separate all of the

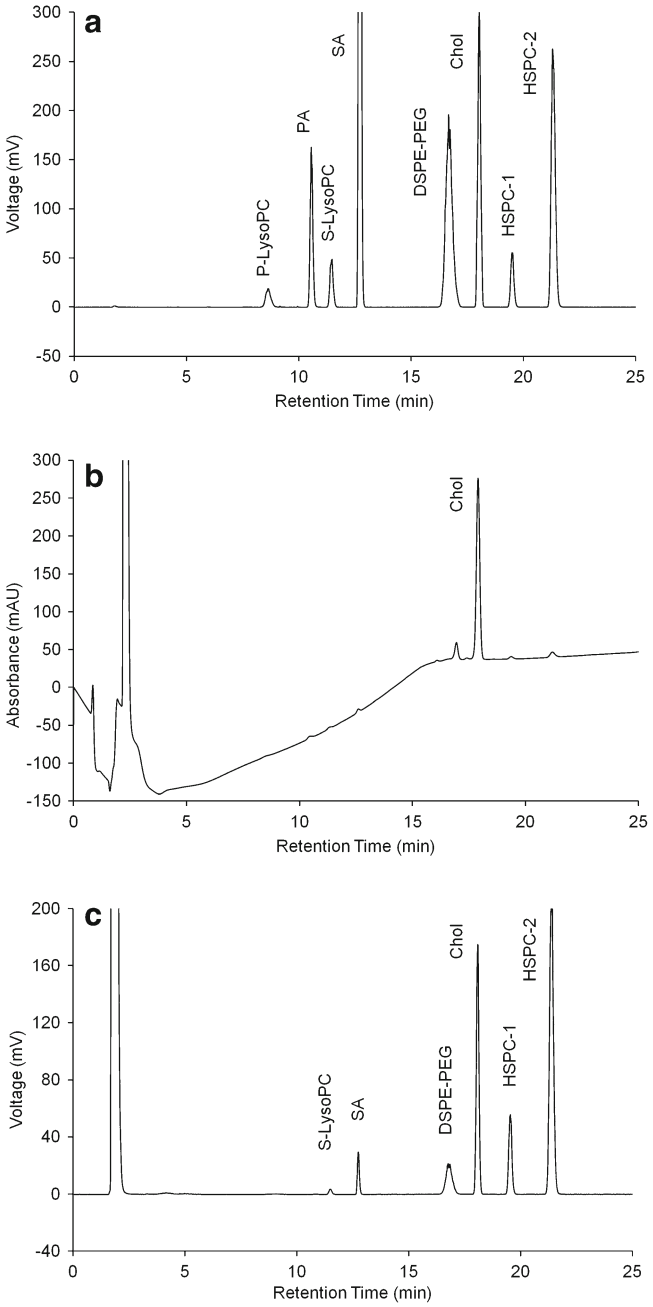

Fig. 1. Comparison of the chromatograms detected by ELSD (**a**) and UV (**b**). A standard mixture (20 µl) containing 150 µg/ml (each) of P-LysoPC, S-LysoPC, PA, and SA; 300 µg/ml of Chol; and 750 µg/ml each of HSPC and DSPE-PEG was injected. The chromatogram of DOXIL® detected by ELSD (**c**). DOXIL® was diluted 10-fold with methanol, and 20-µl aliquots of the diluted solution were injected

lipids in the PEGylated liposomal formulations, which are not only liposomal lipids, but also the hydrolyzed products (lysophospholipids and free fatty acids). It has been reported that the hydrolysis of phosphatidylcholine predominantly produces 1-acyl-2-lyso-phosphatidylcholine with free fatty acids (8). Thus, 1-acyl-2-lysoforms were analyzed. Consistent with previous reports describing reversed-phase HPLC methods for lipids (18), the HPLC separation was optimized using a C18 column with TFA or ammonium acetate in methanol. However, the mobile phase with TFA did not provide a reproducible retention time for DSPE-PEG, and thus the mobile phase with ammonium acetate was selected. Using an isocratic elution with methanol-ammonium acetate buffer, a stable separation of lipid components, lysoforms, and fatty acids was obtained after optimization of the volume ratio, concentration, and pH of the ammonium acetate buffer (Figs. 1, 2, 3, and 4 in the Electronic Supplementary Materials). The HSPC, which consists of fatty acids, C16 and C18, showed two peaks, HSPC-1 and HSPC-2. Since PEG has a distribution of molecular weight, the peak shape of DSPE-PEG was broad. While there were no effects on the concentration of ammonium acetate (25–400 mM) on the Chol and HSPC, the higher concentration of ammonium acetate was associated with the faster retention time of DSPE-PEG. Although the details remain unclear, a large amount of ammonium ions may ionically interact with the anionic portion of the DSPE-PEG and increase its polarity.

The separation of the lipid components and hydrolysis products was possible using an isocratic mobile phase with methanol–200 mM ammonium acetate buffer (pH 4.0) (98:2, *v*/*v*). To clearly separate the lysoforms and fatty acids from the highly polar materials in the liposomal formulations, such as the active ingredient and sucrose, gradient elution was optimized. To maintain a steady level of ammonium acetate, 4 mM

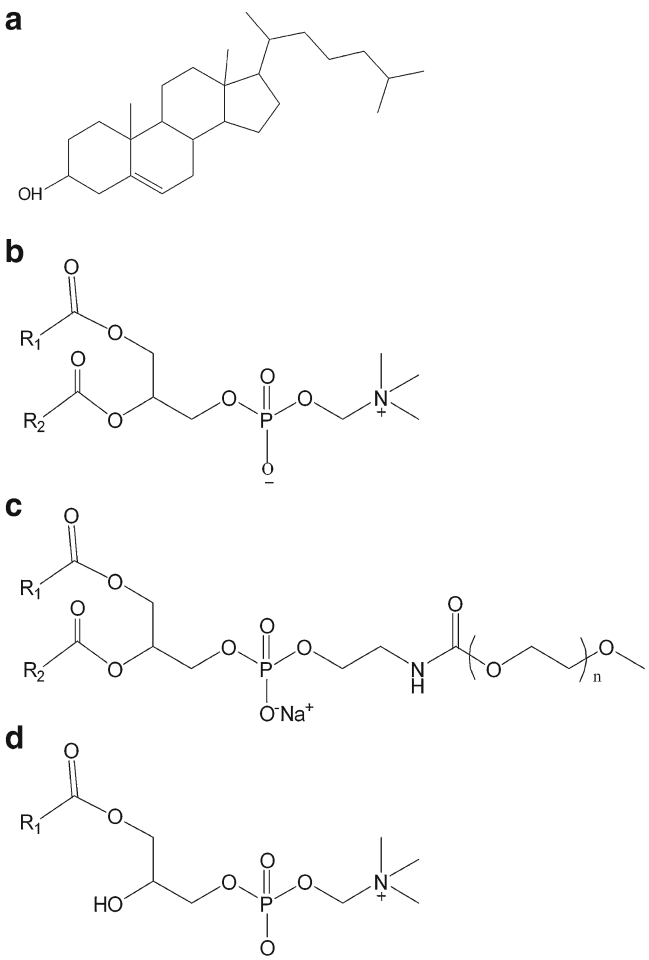

Fig. 2. Structure of **a** cholesterol, **b** HSPC, **c** DSPE-PEG, and **d** S-LysoPC)

Table I. Variability of Retention Times and Peak Areas of Standard Lipids (n=6), Limits of Detection (LOD), and Limits of Quantification (LOQ)

Lipid	Concentration (μg/ml)	Within-day		Between-day		LOD (μg/ml)	LOQ (μg/ml)
		Retention time (min, RSD)	Average area (mVs, RSD)	Retention time (min, RSD)	Average area (mVs, RSD)		
S-LysoPC	22.5	10.90 (0.33)	69.40 (3.62)	10.90 (0.13)	71.35 (4.20)	3.65	7.95
SA	12	12.33 (0.20)	316.22 (2.86)	12.33 (0.17)	334.65 (4.58)	1.31	2.36
DSPE-PEG	75	16.28 (0.16)	282.59 (1.68)	16.28 (0.08)	283.79 (1.55)	5.92	15.06
Chol	75	17.47 (0.06)	1,209.61 (2.81)	17.47 (0.05)	1,220.09 (2.64)	2.48	4.77
HSPC-1	240	18.75 (0.06)	267.60 (2.62)	18.76 (0.12)	257.80 (3.56)	12.42	34.35
HSPC-2	240	20.33 (0.07)	1,675.54 (2.41)	20.34 (0.13)	1,668.74 (2.95)	5.47	14.28

RSD relative standard deviation

ammonium acetate in methanol and 4 mM ammonium acetate buffer were used. The standard mixture which was diluted by methanol to obtain the target concentration was injected into the HPLC equipment, and sequentially monitored using a UV detector (205 nm) and the ELSD. A typical chromatogram is shown in Fig. 1a. Lipids were eluted in the order P-LysoPC, PA, S-LysoPC, SA, DSPE-PEG, Chol, HSPC-1, and HSPC-2 (Fig. 1a). Only the peak of the Chol was detectable using the UV detector; however, the other peaks could not be sufficiently obtained because of a lack of a C–C double bond (Fig. 1b). In contrast, higher and sharper peaks for all of the lipids were obtained using the ELSD (Fig. 1a). Next, to confirm the separation of the lipids from the additives in liposomal products, DOXIL® was diluted with methanol and injected into the HPLC equipment. A typical chromatogram is shown in Fig. 1c. Following the elution of highly polar materials such as doxorubicin and sucrose, the peaks of the hydrolysis products (S-LysoPC and SA) and lipid components in DOXIL® were obtained. Figure 2 shows the chemical structure of Chol and phospholipids detected in the sample of DOXIL®. Thus, the optimized analytical condition may be considered to exhibit sufficient sensitivity and separation for the quantification of lipid components and hydrolysis products in the liposomal products.

Method Validation

The developed HPLC-ELSD method for the analysis of lipids (S-LysoPC, SA, DSPE-PEG, Chol, HSPC-1, and HSPC-2) was validated with respect to repeatability, linearity, limits of detection (LOD) and quantification (LOQ), and accuracy (recovery rate). The within-day/between-day variability of the retention time and peak area of each lipid was evaluated (Table I). The between-day variability was determined on three different days by two different analysts. The relative standard deviation (RSD) values of the retention times were less than 0.33% for all lipids. For the peak areas, the RSD values using ELSD were higher. The LOD and LOQ for each lipid were evaluated (Table I). The LOD and LOQ values were calculated as three times the variation in the measured response (signal/noise ratio= 3) and ten times the variation (signal/noise=10), respectively. The values of the Chol and HSPC were the same number of digits as previously reported, which may be sufficient to determine the concentration of each lipid in the liposomal products.

Next, the linearity was tested for all lipids. Consistent with previous reports (20), the ELSD response was exponential rather than linear. The relationship between the peak area (A) and the concentration of each lipid (m) can be described as follows:

Table II. Standard Curves of Lipids in Methanol

Lipid		Regression equation	r^2
S-LysoPC (9.375–300 μg/ml)	Day 1	$y=98.049x^{1.6437}$	0.9985
	Day 2	$y=130.83x^{1.6063}$	0.9989
	Day 3	$y=124.16x^{1.6416}$	0.9988
SA (4.688–150 μg/ml)	Day 1	$y=493.39x^{1.9887}$	0.9992
	Day 2	$y=750.9x^{1.8791}$	0.9987
	Day 3	$y=525.74x^{2.027}$	0.9989
DSPE-PEG (18.75–600 μg/ml)	Day 1	$y=64.29x^{1.6745}$	0.9999
	Day 2	$y=64.848x^{1.6856}$	0.9999
	Day 3	$y=89.084x^{1.6294}$	0.9989
Chol (37.5–600 μg/ml)	Day 1	$y=5{,}759.3x^{1.0923}$	0.9957
	Day 2	$y=4{,}940.5x^{1.1139}$	0.9916
	Day 3	$y=4{,}722.9x^{1.1246}$	0.9950
HSPC-1 (46.875–1,500 μg/ml)	Day 1	$y=7.0282x^{1.7086}$	0.9995
	Day 2	$y=9.382x^{1.6658}$	0.9986
	Day 3	$y=9.2723x^{1.6605}$	0.9988
HSPC-2 (46.875–1,500 μg/ml)	Day 1	$y=31.233x^{1.7563}$	0.9980
	Day 2	$y=28.229x^{1.7698}$	0.9977
	Day 3	$y=21.056x^{1.8172}$	0.9973

Table III. The Recovery of the Lipid at Three Levels of Concentration: Accuracy of the HPLC-ELSD Method (n=3)

Lipid	Spiked concentration (μg/ml)	Recovered concentration (μg/ml)	Recovery (%)
S-LysoPC	15	15.02	100.10
	30	30.01	100.05
	60	58.57	97.62
SA	7.5	7.21	103.80
	15	14.76	98.42
	30	29.71	99.03
DSPE-PEG	75	74.77	99.69
	150	150.42	100.28
	300	302.67	100.89
Chol	75	85.38	113.83
	150	141.01	94.01
	300	285.06	95.02
HSPC-1	240	238.36	99.32
	480	468.95	98.59
	960	944.01	98.33
HSPC-2	240	238.99	99.58
	480	501.58	103.37
	960	964.63	100.48

$A=am^b$, where a and b are constants that are dependent on a variety of experimental conditions. A linear relationship was observed between the log (peak area) *versus* log (lipid concentration) for all lipids on three different days, where the correlation coefficients of S-LysoPC, SA, DSPE-PEG, HSPC-1, and HSPC-2 were greater than 0.997 (Table II). However, the correlation coefficient of Chol tended to be lower, from 0.9916 to 0.9957.

Recovery was assessed at low, medium, and high concentration levels of lipids. A stock standard solution for each lipid was diluted with methanol/10% sucrose (90/10, *v*/*v*) to obtain the theoretical concentration indicated in Table III, and three diluted solutions were individually injected. The average concentration was measured using the standard calibration curve of each lipid, and the average recovery rate was calculated based on the measured concentration against the theoretical concentration of each lipids. The recovery rates, except those for Chol, were 97.62–103.80%, indicating sufficient accuracy. The recovery rate of Chol was 94.01–113.83%, which did not suggest good accuracy. Because UV could sufficiently detect Chol as shown in Fig. 1, Chol was analyzed using the UV detector, which was placed in series with the ELSD, and the recovery rate for Chol was calculated (Table IV). The correlation coefficient for the calibration curve of Chol (*e.g.* y=7,774.4x+46,171) was 0.9999, and the recovery rate was 100.73–101.3%, indicating sufficient linearity and accuracy. These results indicated that the HPLC-ELSD method is applicable to the measurement of lipid components and hydrolysis products in liposomal formulation, while in the case of Chol, UV detection will be better using the same separation system. The low linearity of Chol, not additives in samples, can be considered a cause for the inadequate recovery rate because even the sample containing only Chol and methanol showed low recovery rate (data not shown). After an investigation, it was found that ammonium acetate in mobile phase affects the detection process of ELSD (droplet size/density/distribution by nebulization, and size/distribution of evaporated particles), and can cause a lower correlation coefficient between concentration and scattering intensity.

Table IV. The Recovery Rate of Chol at Three Levels of Concentration: Accuracy of the HPLC-UV Method (n=3)

Lipid	Spiked concentration (μg/ml)	Recovered concentration (μg/ml)	RSD (%)	Recovery (%)
Chol	75	75.55	0.10	100.73
	150	151.94	0.10	101.30
	300	303.56	0.03	101.19

Table V. Effect of Processing Stage on Liposome Component (n=3)

Processing stage	Calculated lipid molar ratio (mean±SD; %)		
	Chol[a]	HSPC[b]	DSPE-PEG
Dissolved in ethanol	40.48±0.32	54.13±0.32	5.39±0.13
Evapolation	40.72±0.04	53.99±0.11	5.29±0.10
Particle size reduction	40.73±0.45	53.99±0.54	5.29±0.12
Dialysis	42.47±0.74	53.02±0.55	4.51±0.33

[a] Chol was detected by a UV detector
[b] The value of HSPC was calculated from the peak of HSPC-1

Application of the Method

A validated HPLC-UV/ELSD method was first used for the quantitative analysis of liposomes prepared in our laboratory. To quantify the HSPC, the main HSPC-1 peak was evaluated based on the linearity of both peaks. Adequate quantification of the prepared liposomes is critical to maintain the attributes (such as encapsulation efficiency and lipid composition rate) of the liposomal formulation. Moreover, the evaluation of changes in lipid composition will be needed to properly design the preparation (manufacturing) process. Changes in the lipid composition rate were assessed in the liposome preparation process (Table V). Compared with the lipid composition when lipids were dissolved in ethanol, there were no significant changes after the evaporation and particle size reduction. However, after the dialysis for external solution exchange, the percentages of HSPC and DSPE-PEG slightly decreased, whereas the percentage of Chol was slightly increased. The total lipid amount decreased by 5–10% following dialysis (data not shown). Thus, changes in the lipid component may be caused by the removal of HSPC and DSPE-PEG, which is not incorporated into the liposome, from the dispersing solution by dialysis.

Next, DOXIL® was quantified. Four product lots that were stored for a long period of time were compared. Two lots were stored for over 65 months and two lots were stored for 26–29 months (Table VI). Compared with the lipid composition described in the package insert of DOXIL®, the ratio of Chol was slightly higher, and the ratios of HSPC and DSPE-PEG were slightly lower. These slight differences may have been caused by the liposomal preparation process because the lipid composition described in the package insert was the quantity of

Table VI. Determination of Lipid Concentration in Four Lots of DOXIL®

Lipid	Ingredient amount on labeling (μg/ml)	Calculated concentration (n=3; μg/ml, RSD)			
		Lot#011AFL	Lot#012AGD	Lot#029BJD	Lot#032BKA
Chol[a]	3,190	3,438.13 (0.29)	3,561.10 (0.41)	3,269.43 (0.24)	3,416.59 (0.26)
HSPC[b]	9,580	8,420.41 (1.18)	8,859.23 (0.48)	8,836.49 (1.53)	9,328.49 (1.22)
DSPE-PEG	3,190	2,435.90 (0.76)	2,552.80 (1.18)	2,686.19 (1.09)	2,757.95 (1.22)
Calculated molar ratio					
Chol/HSPC/DSPE-PEG		43.49/52.17/4.34	43.12/52.53/4.35	40.99/54.27/4.74	40.80/54.56/4.64

[a] Chol was detected by a UV detector
[b] The value of HSPC was calculated from the peak of HSPC-1

starting material. When compared lot-to-lot, while there were almost no differences between the two older lots or the two relatively newer lots, 0.4–2.4% differences were observed between the old and new lots. The ratio of Chol was higher in the old lots and those of HSPC and DSPE-PEG were lower. This may be due to the hydrolysis of phospholipids, which resulted in a reduced amount of HSPC and DSPC-PEG.

Analysis of Lipid Composition After Storage at Different Temperatures

Thus, the developed method was used to assess liposome stability. It is known that the hydrolysis of phospholipid follows a pseudo first-order kinetics model and that the hydrolysis rate correspondingly increases with temperature (7). First, the prepared liposomes were incubated for 3 days at 4, 37, or 57°C and the concentrations of the main hydrolysis products, S-LysoPC and SA, were measured. A significant increase in S-LysoPC and SA was observed with a rise in temperature (Fig. 3). Consistent with this observation, the concentrations of HSPC and DSPE-PEG decreased and that of Chol was stable (data not shown). Next, the concentration of the hydrolysis products in the four lots of DOXIL® were measured (Table VII). The concentrations of S-LysoPC and SA significantly increased in the two older lots (long past the expiration date). It was confirmed that even under storage at 4°C and solution of pH 6.5, *i.e.*, conditions in which the hydrolysis rate was the least (8), the lysophospholipid and free fatty acids were produced by hydrolysis during long-term storage.

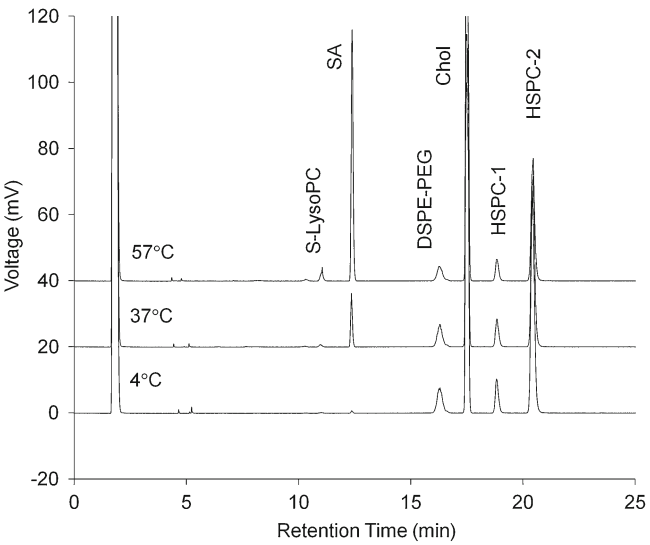

Fig. 3. A stability evaluation of the PEG liposome under each thermal condition (4°C, 37°C, and 57°C). The lipid composition of the PEG liposome, which was dialyzed against 10% sucrose, is presented in Table VI. The PEG liposome was incubated at each temperature for 3 days and diluted with methanol, and 20-μl aliquots of the diluted solution were injected

CONCLUSIONS

A simple reversed-phase HPLC-ELSD method was developed for the quantification of lipids in PEGylated liposomes. Although the linearity and accuracy obtained with UV detection of Chol were better those obtained with ELSD, the HPLC-ELSD method was validated to be linear, precise, accurate, and sensitive. Additionally, the HPLC-UV/ELSD method was found to be suitable for simultaneous determination of HSPC, DSPC-PEG, and Chol as well as their hydrolysis products in PEGylated liposomal products. It was also suggested that accurate quantification of the lipid component enables assessment of changes in lipid composition during the preparation process. In addition, the increase of hydrolysis products of phospholipids under a heat-accelerated condition may be observed. This method will be useful for quantifying the hydrolysis products in liposomal products in a stability test, such as a long-term storage test or an accelerated test, as well as for quantifying the lipid composition of liposomal products.

Table VII. Determination of S-LysoPC and SA Concentration in Four Lots of DOXIL by the HPLC-ELSD

	Calculated concentration (n=3; μg/ml, RSD)			
	Lot#011AFL	Lot#012AGD	Lot#029BJD	Lot#032BKA
	Approximate elapsed time after manufacture			
Lipid	68 months	65 months	29 months	26 months
S - LysoPC	442.83 (3.36)	425.00 (5.56)	238.06 (1.66)	202.61 (2.74)
SA	374.50 (2.10)	376.46 (3.04)	215.19 (2.30)	175.17 (4.59)

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