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Short communication

Analysis of cationic liposomes by reversed-phase HPLC with evaporative light-scattering detection

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ABSTRACT

Cationic lipid-mediated drug delivery of small pharmaceutical molecules and biological molecules, such as proteins and DNA, has gained increasing popularity for many in vitro and in vivo applications. For this purpose, 1,2-dioleoyl-3-trimethylammonium propane (DOTAP) is one of the most widely used and efficient cationic lipids. In this work, a simple and rapid reversed-phase HPLC method was developed for the simultaneous determination of cationic lipid DOTAP and neutral co-lipids cholesterol and phosphatidylcholine (DPPC or DSPC) as well as their degradation products in liposome-based drug formulations. Due to the poor UV absorbance of the lipids and their degradation products, an evaporative light-scattering detector (ELSD) was used to monitor the separation. The HPLC separation was achieved using a Phenomenex Luna C18 column at 50 °C by a linear gradient elution with methanol–water mobile phase at a flow rate of 2.0 mL/min. 0.1% (v/v) trifluoroacetic acid (TFA) was added into the mobile phase to enhance the retaining of the cationic lipid DOTAP. This newly developed method enabled direct analysis of liposomes without solvent lipid extraction, and was validated to be linear, precise, accurate, specific and sensitive. The limit of detection (LOD) and limit of quantitation (LOQ) were determined to be 0.15 and 0.30 μg, respectively, for all the four lipids. The method has been successfully employed in a wide range of lipid-based formulation screening, process development and stability testing. Studies of liposome samples under accelerated thermal conditions revealed that the hydrolysis of DOTAP, DPPC and DSPC followed pseudo-first-order kinetics.

1. Introduction

Liposomes are spherical vesicles composed of a lipid bilayer with the polar headgroups of the lipids oriented towards the extravesicular solution and inner core. They are widely studied as an effective drug delivery system due to their similarity to cell membranes [1–5]. Based on the molecular make-up of polar head groups of the amphiphilic lipids, the liposomes can be classified into cationic, anionic, zwitterionic and non-ionic subgroups. Cationic liposomes are commonly composed of a cationic lipid (e.g. 1,2-dioleoyl-3-trimethylammonium propane (DOTAP), or N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA)) and an electrically neutral ("helper" or "co-") lipid (e.g. cholesterol, or phosphatidylcholine), resulting in positively charged amphiphile systems. They can mediate the entry of drug molecules into the negatively charged biological cell surfaces and are viewed as one of the most promising non-viral vectors for delivering negatively charged small pharmaceutical molecules or biological molecules, such as nucleic acids, into body cells [4–8].

With the increasing use of cationic liposomes as drug carriers for many in vitro and in vivo applications, it is imperative to accurately assess the lipid compositions in the pharmaceutical liposomal formulations. It has been reported that liposome characteristics of cationic lipid/co-lipid molar ratio and positive/negative charge ratio of the liposomes both affect the in vivo drug activities [9–12]. Furthermore, liposomes that consist of cationic lipids and phospholipids are prone to hydrolysis and the formation of their lyso- (monoacyl-) forms and free fatty acids by hydrolytic cleavage of the ester functionalities in presence of water is virtually unavoidable [13–15]. The evaluation of liposome stability and its degradation products is clinically important.

High-performance liquid chromatography (HPLC) is a technique of choice for separating lipids from various origins (e.g. rat liver, bovine milk, or bacterial cell membranes) into different classes or species based on their relative hydrophobicity, such as alkyl chain lengths and head group polarity [16–21]. Although both normal- and reversed-phase HPLC modes have been previously studied for separation and quantitation of lipid components in pharmaceutical liposome formulations, some methods require the tedious and time-consuming lipid extraction or sample manipulation prior to HPLC analysis [13,22–24], whereas others have detection limitation to unsaturated lipids with ultraviolet (UV) detection at 205 nm for

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carbon–carbon double bonds found in the fatty acid tails [25,26]. These methods are not effective in detecting most lipids that are lack of a chromophore, such as saturated aliphatic chain lipids. Moreover, HPLC separation of lipid hydrolysis products from liposomal components was not investigated.

In this work, we describe a simple and rapid reversed-phase HPLC method for the simultaneous determination of the most commonly used cationic lipid DOTAP and neutral co-lipids cholesterol and/or phosphatidylcholine (1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC) or 1,2-distearoyl-sn-glycero-3-phosphatidylcholine (DSPC)) in various liposome-based drug formulations developed in the lab. An evaporative light-scattering detector (ELSD) was used to monitor separation and sensitively detect the lipids as well as their degradation products. The ELSD is a quasi-universal mass detector that can detect any analyte less volatile than the mobile phase and has been proven to be effective in detecting poor UV-absorbers, such as most lipids [22,23,27–30]. Unlike refractive index detector (RID), another commonly used mass-detection method, ELSD is compatible with gradient elution and insensitive to ambient temperature variation, making it apparently the detection method of choice for lipid analysis.

2. Experimental

2.1. Materials and solutions

Neutral lipid of cholesterol (catalog #700000P) and cationic lipid of DOTAP (catalog #890890P) were purchased from Avanti Polar Lipids (Alabaster, AL) and used as received. Phosphatidylcholines of DPPC (catalog #MC-6060) and DSPC (catalog #MC-8080) were purchased from NOF America Corporation (White Plains, NY). HPLC grade methanol and methylene chloride were obtained from Fisher Scientific (Fair Lawn, NJ). Trifluoroacetic acid (TFA) was from Fluka (St. Louis, MO). Water was purified by a Millipore Milli-Q purification system (Bedford, MA).

Individual standard stock solutions of cholesterol (1.0 mg/mL), DOTAP (2.0 mg/mL), DSPC (2.5 mg/mL) and DPPC (5.0 mg/mL) were prepared using methanol. Calibration standards were then prepared by mixing the standard stock solutions in desired volume ratios and by further diluting the mixtures with methanol to the calibration range.

2.2. Liposome preparation

Cationic liposomes consisted of different molar amounts of DOTAP were prepared by thin film hydration method. Briefly, lipids were weighed and mixed in methylene chloride at specific molar ratios in a suitable round bottom flask. Solvent removal was performed by connecting the flask to a Buchi R110 rotary evaporator with water bath B-465 (Flawil, Switzerland) until a dry thin film layer was formed on the flask wall. The resulted lipid film was then rehydrated with 10% sucrose solution containing targeted concentration of drug molecules and vortexed until lipids were completely resuspended. Liposomes with desired particle sizes were produced by sequential extrusion through different pore diameter polycarbonate filters using a Lipex Biomembranes extruder (Vancouver, Canada). Preformed liposomes were stored under refrigerated condition before use.

2.3. High-performance liquid chromatography

A Waters 2695 Alliance module equipped with quaternary pump, mobile phase degasser, temperature controlled autosampler and column thermostat was used for HPLC analysis. The separation was carried out on a Luna C18(2) column (150 mm × 4.6 mm i.d., 3 μm particle size, 100 Å pore size) from Phenomenex (Torrance, CA) at column temperature of 50 °C and sample temperature of 20 °C. Lipids were eluted using binary linear gradients starting from a mixture of 15% A and 85% B to 100% B in 10 min followed by a 3-min plateau at 100% B, where A is 0.1% (v/v) TFA in water and B is 0.1% (v/v) TFA in methanol. The mobile phase composition was then changed back to initial solvent mixture and the column was equilibrated for 7 min before every subsequent run. The flow rate of the mobile phase was set to 2.0 mL/min. The samples for HPLC injections were prepared by direct dilution of the liposome formulations with methanol to bring the concentrations of lipid components into the calibration range. Typically, a 50-fold dilution was necessary for liposome formulations, resulting in clear lipid solutions for direct injection onto the HPLC column. Sample injection volume of 30 μL was used.

The separation of lipids was detected using a PL-ELS1000 ELSD from Polymer Laboratories (Amherst, MA) operated with 0.2 s response time setting. The ELSD nebulizer and evaporator temperatures were set at 50 and 75 °C, respectively, while the high-purity nitrogen gas flow rate was set at 1.5 standard liters per minute. The data acquisition and processing was performed using the Empower chromatographic software through Waters SAT/IN module. Liquid chromatography–mass spectrometry (LC–MS) identification of lipid degradation products was conducted on an Alliance ZQ mass detector system with electrospray ionization and cone voltage of 30 V.

3. Results and discussion

3.1. Method development and optimization

An effective HPLC method that can simultaneously quantitate cationic lipid of DOTAP and other neutral co-lipids of cholesterol, DPPC or DSPC within a short period of analysis was developed for the evaluation of various cationic liposome drug formulations in the lab. Chromatographic conditions of different column types, various column temperatures, addition of mobile phase modifier, as well as the flow rate were systematically evaluated. The optimum HPLC separation was achieved using a Phenomenex Luna C18 column at 50 °C by a linear gradient elution with methanol–water mobile phase at a flow rate of 2.0 mL/min. Cationic lipid of DOTAP is highly polar as compared to other neutral co-lipids. 0.1% (v/v) trifluoroacetic acid (TFA), a volatile pH modifier that is compatible with ELSD, was added into the mobile phase to significantly enhance the retaining of DOTAP on the C18 stationary phase through ion-pair formation. The addition of TFA to the mobile phase was also noted to improve the peak shape of DOTAP by suppressing the ion-exchange interaction between the positively charged head group of cationic lipid and residual silanol groups in the stationary phase.

Fig. 1 shows the chemical structures of the four lipids used in various liposome-based drug formulations. Due to the absence of carbon–carbon double bond in the fatty acid chains of DPPC and DSPC, ultraviolet detection of DPPC and DSPC poses more of a challenge than that of DOTAP and cholesterol, as illustrated in Fig. 2. A standard lipid mixture of cholesterol (6 μg), DOTAP (6 μg), DPPC (15 μg) and DSPC (15 μg) was injected into the chromatographic column and sequentially monitored by ultraviolet spectrophotometric detector and ELSD. The four lipids were baseline separated in the order of increasing retention time of 7.7, 9.9, 10.5 and 12.0 min for DOTAP, cholesterol, DPPC, and DSPC, respectively. It is shown that accurate quantitation of the saturated aliphatic chain lipids of DPPC and DSPC using absorbance at 205 nm could not be achieved despite the presence of a large injection amount. Contrary to UV detection, the response from ELSD is a function of the mass of the analyte, and therefore, ELSD is able to overcome the problem of

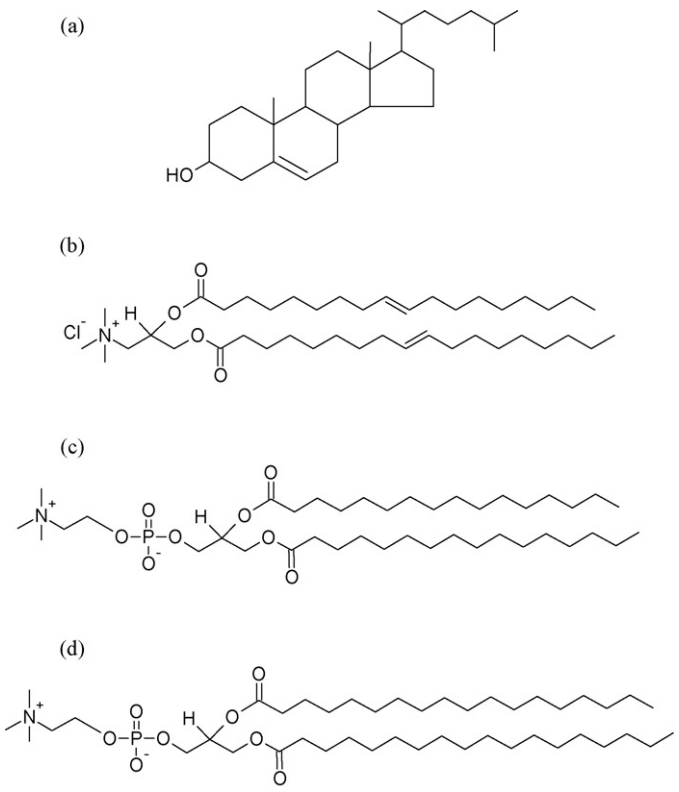

Fig. 1. Structures of (a) cholesterol, (b) 1,2-dioleoyl-3-trimethylammonium propane (DOTAP), (c) 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC), and (d) 1,2-distearoyl-sn-glycero-3-phosphatidylcholine (DSPC).

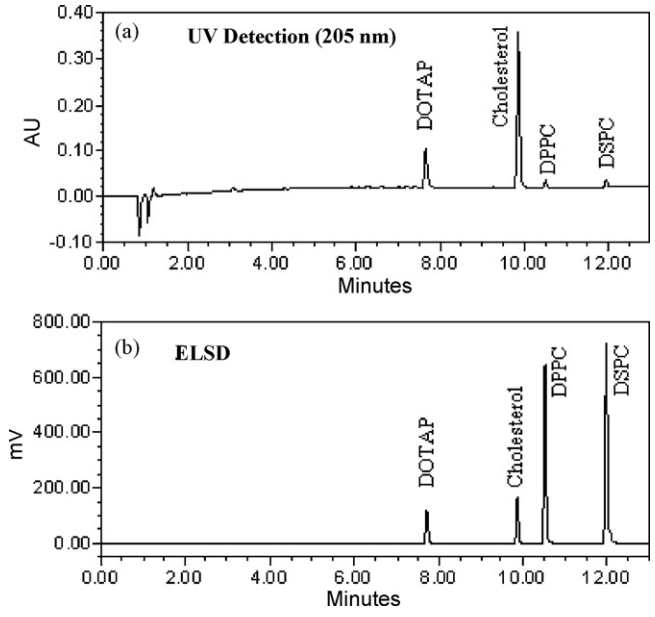

Fig. 2. Chromatograms of a standard mixture detected by (a) ultraviolet detector at 205 nm, and (b) evaporative light-scattering detector in a tandem arrangement. The amount of each injected lipid was 6 μg for cholesterol and DOTAP, and 15 μg for DPPC and DSPC.

lack of a chromophore in lipid molecules, and consequently lower sensitivity associated with using UV detection at 205 nm. The ELSD showed great sensitivity for all the four lipids with DPPC and DSPC displaying larger signal responses than DOTAP and cholesterol due to their corresponding larger injected mass amounts.

Table 1
Study of recovery at low and high levels to bracket the concentrations used in liposome formulations.

Lipid	Spiked conc. (μg/mL)	Recovered average conc. (μg/mL)	R.S.D. (%, $n = 2$)	Recovery (%)
DOTAP	41.04	41.43	1.28	100.95
	164.16	163.24	0.45	99.44
Cholesterol	40.48	39.62	0.76	97.88
	161.92	158.59	0.54	97.94
DPPC	200.22	206.24	0.23	103.01
	800.90	800.57	0.02	99.96
DSPC	200.62	208.13	0.25	103.74
	802.50	802.03	0.20	99.94

3.2. Method validation

Linearity, accuracy, specificity, sensitivity and system reproducibility of the developed HPLC-ELSD method were validated for its intended purpose. Although ELSD involves the processes of nebulization and evaporation of eluent prior to subsequent light-scattering detection of analyte, the reproducibility of the ELSD was found to be comparable with that of UV detection once physical conditions of the system were optimized and stabilized. The relative standard deviation (R.S.D.) values of the retention times and peak areas from six repeated injections of a standard solution were less than 1.0% for all the four lipid compounds. Slight variation in retention time of DOTAP (difference within 0.5 min) was observed from day to day operation, probably due to a minor change of TFA concentration in the mobile phase. The retention times for cholesterol, DPPC and DSPC, however, remained almost the same between inter-day runs.

The linearity for the four lipids was tested in the concentration ranges of 20–200 μg/mL for DOTAP and cholesterol and 100–1000 μg/mL for DPPC and DSPC based on the lipid ratio in the liposome formulations. As expected, the ELSD response of peak area (A) and the lipid concentration (C) follow a non-linear empirical exponential relationship described by the equation: $A = aC^b$, where a and b are constants dependent on specific experimental conditions. A linear relationship was observed between log (peak area) versus log (lipid concentration) with correlation coefficient greater than 0.999 for each of the four lipid compounds.

Study of recovery for the four lipid compounds was individually performed at two concentration levels to bracket all the concentrations used in different liposome formulations. In brief, a known amount of each lipid was spiked into the excipient containing the other three lipids, sucrose, as well as the active drug component, and the sample was analyzed by HPLC method using the standard calibration curve of that lipid. The recovery was calculated based on the assay value against the known concentration (spiked) of the lipid. As shown in Table 1, the percentages of recovery for all the four lipids were within the range from 97.9 to 103.7%, indicating sufficient accuracy of this method.

Sensitivity of the detection system for DOTAP, cholesterol, DPPC and DSPC was also studied. A series of progressively diluted standard lipid mixtures were injected into the column and signal-to-noise ratios were determined for each lipid compound. The limit of quantitation (LOQ) was found to be near 0.30 μg for each lipid that showed a signal-to-noise ratio of at least 10. The limit of detection (LOD) was found to be 0.15 μg for each lipid that displayed 3 times the noise of the detector. Although the LOQ values of 1 μg for DOTAP and 0.5 μg for cholesterol were reported previously using UV detection [26], the achieved high sensitivity for saturated aliphatic chain lipids of DPPC and DSPC using ELSD is great advantage of this method.

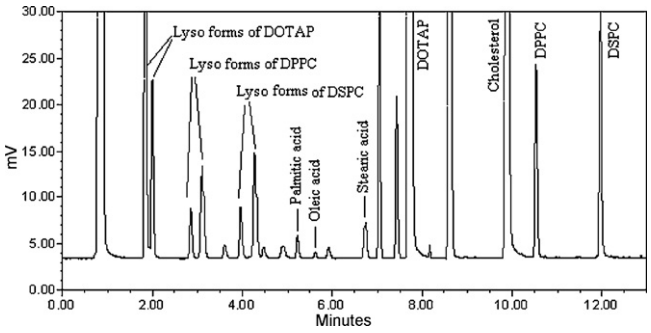

Fig. 3. Example chromatogram of forced degradation of a standard mixture using 0.1N HCl at room temperature for 4 days.

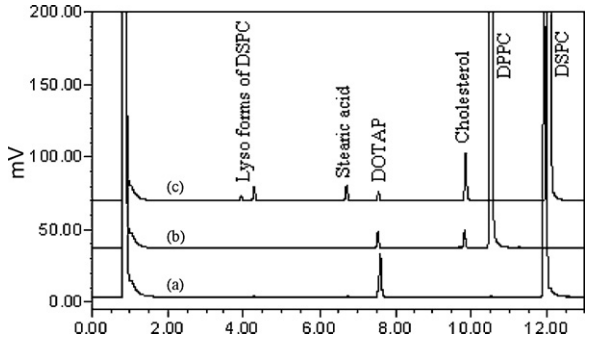

Fig. 4. Example chromatograms of cationic liposome samples: (a) DOTAP/DSPC, (b) DOTAP/Cholesterol/DPPC, and (c) DOTAP/Cholesterol/DSPC as presented in Table 2.

The specificity of the method was tested by conducting forced degradation of standard lipid mixtures under various stress conditions. Forced degradation was carried out using 3% H_2O_2, 0.1N NaOH, 0.1N HCl and heat treatment at 50 °C. It was observed that all the lipid components were sufficiently separated from their degradation products and were free of interference from stressed samples under the above mentioned stress conditions. Fig. 3 shows an example chromatogram for a standard mixture undergoing stress condition of 0.1N HCl at room temperature for 4 days. Peak identification of degradation products was achieved by matching retention times by injecting individual components, as well as using on-line mass spectrometry. Hydrolysis products of lyso-(monoacyl-) forms and free fatty acids from DOTAP, DPPC and DSPC were all well separated from each other. It was also noticeable that the method completely resolves the 1- and 2-acyl lyso-forms of DOTAP, DPPC and DSPC, with 2-acyl lyso-form eluted slightly ahead of 1-acyl lyso-form.

3.3. Analytical application

The validated HPLC-ELSD system was applied to quantitative analysis of various cationic liposomes prepared in the lab. An accurate lipid quantitation of prepared cationic liposomes is essential in the determination of cationic lipid/co-lipid molar ratio, positive/negative charge ratio of liposome formulations and drug entrapment efficiency. Analysis of lipid components is also needed for stability testing of liposome-based formulations. Table 2 shows example quantitative results obtained with the HPLC method for three cationic liposome samples of various lipid compositions. The corresponding sample chromatograms are shown in Fig. 4. For all the liposome samples, the active drug molecules and sucrose were elated at the solvent front. The HPLC-calculated and targeted lipid molar ratios were in close agreement for the first two tested liposome samples which were newly prepared, suggesting a good control of liposome preparation procedure. For the third tested sample that had been prepared and stored as an aqueous dispersion at refrigerated condition for about 2 months, there was a slight discrepancy between the HPLC-calculated and targeted lipid molar ratios. A close examination of the sample chromatogram revealed the presence of DSPC hydrolysis products of 1- and 2-acyl lyso-forms and stearic acid eluted before DOTAP. Therefore, the hydrolysis of DSPC accounted for the HPLC-calculated lipid molar ratio deviated from the targeted one.

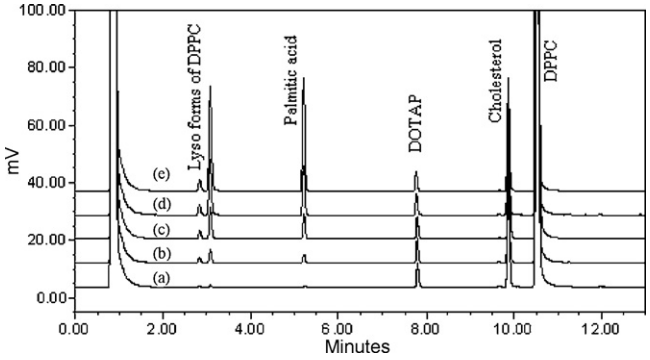

Fig. 5. (a–e) Stability evaluation of cationic liposome under accelerated thermal condition of 50 °C. The liposome formulation was composed of DOTAP/Cholesterol/DPPC with targeted lipid molar ratio of 5.0/20.0/75.0.

The developed HPLC-ELSD system was also used to assess liposome sample stability under accelerated thermal conditions. The obtained results indicated that the hydrolysis of DOTAP, DPPC and DSPC followed pseudo-first-order kinetics, which was consistent with other studies on phosphatidylcholine [13,15]. Fig. 5 shows the evolution of lipid hydrolysis of a liposome sample composed of DOTAP/Cholesterol/DPPC under thermal condition of 50 °C. Samples were taken at appropriate intervals and analyzed by HPLC. Cholesterol has been demonstrated to be chemically stable in forced degradation studies and there was no change of choles-

Table 2
Quantitative analysis of liposome components.

Liposomes and components	HPLC-calculated concentration (mg/mL)	HPLC-calculated lipid molar ratio (%)	Targeted lipid molar ratio (%)
Liposome sample a			
DOTAP/DSPC	4.49 27.54	15.56/84.44	15.00/85.00
Liposome sample b			
DOTAP/Cholesterol/DPPC	2.26 2.40 38.23	5.25/10.10/84.65	5.00/10.00/85.00
Liposome sample c			
DOTAP/Cholesterol/DSPC	1.69 4.24 27.02	5.07/23.04/71.89	5.00/20.00/75.00

terol concentration observed during the storage period (data not shown). The hydrolysis of DOTAP and DPPC was assessed by plotting the concentration remaining against the storage time on a semi-logarithmic scale. The data were well fitted with a pseudo-first-order kinetic model. The hydrolysis of DPPC seemed to be about 3 times faster than that of DOTAP as illustrated by their first-order rate constants of 3.7×10^{-3} and $1.3 \times 10^{-3}\ h^{-1}$, respectively. The rising concentrations of hydrolysis products of 1- and 2-acyl lyso-forms and palmitic acid from DPPC were also chromatographically detected during the storage. It was reported that the hydrolysis of phospholipids to lyso-forms and fatty acids to some extent could have a deteriorative effect on physical stability as well as the efficacy of liposomes as a delivery system [13,14], and their formation during shelf life should be limited. Although chemical hydrolysis of aqueous liposome dispersions is nevertheless unavoidable, a fine-tuning of formulation parameters, e.g. pH modification, can slow down this process and lead to optimal physical and chemical stability and prolonged shelf life [15]. The HPLC-ELSD method described herein provides a useful tool for formulation optimization.

4. Conclusions

A simple and rapid reversed-phase HPLC-ELSD method was developed for quantitative analysis of the components of cationic liposomes without any cumbersome lipid extraction from liposomes prior to sample injection. The method was validated to be linear, precise, accurate, specific, sensitive and well suited to the simultaneous determination of DOTAP, cholesterol, DPPC, DSPC, as well as their degradation products in liposome-based drug formulations. The developed HPLC method using ELSD is compatible with on-line mass spectrometry, which allows for further peak identification. The baseline separation of lipid hydrolysis products, 1- and 2-acyl lyso-forms and free fatty acids, from the liposomal components makes this method ideal for stability testing of liposome-based formulations.

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