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Research paper

Direct lipid quantitation of cationic liposomes by reversed-phase HPLC in lipoplex preparation process

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Abstract

The proposed reversed-phase high-performance liquid chromatography method with ultraviolet detection provides a simple and rapid procedure to separate and quantitate lipids from cationic liposomes used in gene transfer. We describe experimental conditions which do not require lipid extraction from liposomes prior to sample analysis. Evaluation of the method reported here showed suitable lipid separation capacity and quantitation accuracy from cationic liposomes composed of either the pentammonio lipid pcTG90 and dioleoyl phosphatidylethanolamine, or 1,2-dioleoyl-3-trimethylamonium propane and cholesterol. Detection limits were in the range of 0.5–1 μg depending on the lipid. This quantitative method has proven useful in lipoplex formulation processing development and its application may be extended to a wide range of lipid-based gene and drug delivery systems. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Lipid quantitation; Reversed-phase HPLC; Liposomes; Cationic lipid; Colipid; Lipoplex; Gene transfer

1. Introduction

Cationic liposome-DNA complexes (lipoplexes) can mediate gene expression in vivo [1]. Evaluation of lipoplexes in preclinical studies and in human clinical trials requires high reproducible batch-to-batch lipoplex characteristics. Among numerous parameters, inclusion of colipids, cationic lipid/colipid ratio, and charge ratio were reported to affect in vivo lipoplex activity [2,3]. It is therefore crucial to accurately determine the concentration of each liposomal component before mixing cationic liposomes with plasmid DNA in order to ensure high reproducibility of lipoplex preparations.

High-performance liquid chromatography (HPLC) is a technique of choice in lipid analysis [4]. A recent study describes a normal phase HPLC method for quantitation of cationic liposome components [5]. However, the reported procedure requires evaporative light-scattering detector and the error prone and time-consuming solvent lipid extraction from preformed liposomes prior to sample analysis. Moreover, quantitation of the commonly used colipid cholesterol was not investigated.

We describe here a simple and fast reversed-phase high-performance liquid chromatography (rp-HPLC) procedure using ultraviolet (UV) detection that provides efficient separation and quantitation of cationic (pcTG90, 1,2-dioleoyl-3-trimethylamonium propane (DOTAP)) and neutral (dioleoyl phosphatidylethanolamine (DOPE), cholesterol (Chol)) lipids from liposomes. The lipid extraction step could be avoided by choosing an appropriate single mobile phase composed of isopropanol in which liposomal lipids were completely dissolved. This quantitative method is used systematically in our laboratory as a control technique in the process of lipoplex preparation for in vivo use.

2. Materials and methods

2.1. Chemicals and reagents

The dioleoyl pentammonio lipid pcTG90 was synthesized as reported previously [6]. DOTAP (cat. # 890890) and DOPE (cat. # 850725) were purchased from Avanti Polar Lipids (Alabaster, AL) and Cholesterol (Chol) from Sigma (cat. # C7402). Chloroform was from Aldrich (Steinheim, Germany, cat. # 31,998-8), HPLC analytical grade isopropanol and ethanol were from Carlo Erba (Milano, Italy, cat. # 412421 and 414607, respectively). Deionized Milli-Q water (Millipore) was used. Methanol and anhydrous D(+) glucose were purchased from Merck (cat. # 1.06009 and 1.08337,

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respectively). Trifluoroacetic acid (TFA) was from Pierce (cat. # 25904).

2.2. Column and HPLC conditions

A reversed-phase C_{18} LC ABZplus + column (25 × 0.46 cm, 5 μm particle; Supelcosil, Supelco, Bellefonte, PA) was used on a HP1090 HPLC system (Hewlett–Packard Co., Camas). A guard column (Supelguard ABZ + plus, 2 cm, Supelco, cat. # 59535-U) was mounted between the injector and the analytical column. Ten microliters of each liposome preparation were diluted with 50 μl of solution A (0.15% TFA in water) and 60 μl of solution B (0.05% TFA in isopropanol). One hundred microliter of these diluted sample mixtures were injected. Lipids were eluted using linear gradients starting from a mixture of 50% solution A and 50% solution B to 100% solution B in 10 min. This gradient was followed by a 10 min plateau at 100% solution B before going back to the initial solvent mixture in 2 min. An interval of 10 min was allowed between two subsequent runs. All runs were performed at room temperature at a flow rate of 0.5 ml/min. UV detection was done at 205 nm using a diode array detector. Standard curves were obtained from lipid powder weighed and dissolved in isopropanol for DOPE and DOTAP, methanol for Chol and ethanol/water (90/10) for pcTG90.

2.3. Liposome preparation

Lipids were mixed in chloroform and solvent removal was performed overnight at 45°C using a Rapidvap vortex evaporator (Labconco, Uniequip, Martinsried, Germany). The resulting lipid films were hydrated with a 5% glucose (w/v) solution (5–15 mg/ml cationic lipids) and sonicated (Bransonic 221 ultrasonic water bath from Branson Ultrasonics Corp., Danbury, CT) until lipids were entirely resuspended. Small liposomes were formed by sequential extrusion through 400 and 200 nm pore diameter polycarbonate membranes (Nuclepore, Costar, Cambridge, MA) using a Lipex Biomembranes extruder (Vancouver, Canada). Preformed liposomes were stored at 4°C under inert atmosphere (argon) until use.

3. Results and discussion

Each lipid starting material has been individually analyzed by rp-HPLC. Superimposed chromatograms of tested lipids are presented in Fig. 1. Lipids emerged as single major peaks in the order of increasing hydrophobicity with retention times of 13.2, 14.8, 16.1 and 18.8 min for pcTG90, DOTAP, DOPE, and Chol, respectively. For all lipid tested, standard curves fitted well with linear relationships between elution peak areas and absolute amounts of injected lipids up to 120 μg of pcTG90, DOTAP, or DOPE and up to 40 μg of Chol (Fig. 2). The r^2 for a linear curve fitting varied between 0.991 and 0.997.

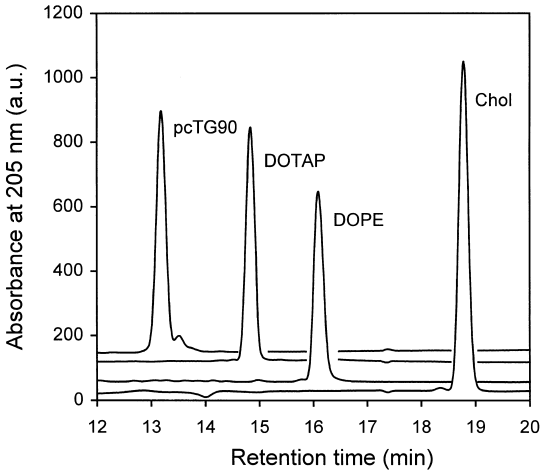

Fig. 1. Superimposed chromatograms of cationic lipids pcTG90 and DOTAP and neutral colipids DOPE and Chol used as standards for liposomal lipid quantitation. Quantity of injected material was 60 μg in the case of pcTG90, DOTAP, and DOPE, and 20 μg in the case of Chol. Details of sample preparation and elution conditions are described in Section 2.2.

Liposome components were efficiently baseline separated, both in formulations of pcTG90/DOPE (1:2, mol/mol) (Fig. 3A) and DOTAP/Chol (1:1, mol/mol) (Fig. 3B). Importantly, there was no need for organic solvent lipid extraction from preformed liposomes prior to sample injection. This is advantageous compared to most methods requiring the error prone and time-consuming multiple lipid extractions from liposomes. Moreover, solvents and/or solvent mixtures used for extraction such as chloroform and acetone are not suitable when UV detection is used because of their high extinction coefficients. On the contrary, short-chain alcohols such as isopropanol constitute good solvents for lipids and are poor UV absorbers. By choosing a 50% mixture of isopropanol in water, liposomal lipids were efficiently dissolved in a homogenous phase that could be injected directly onto the HPLC column. Although the so-called 'disintegration-by-dilution' method had been successfully applied to the quantitative determination of cholesterol in various liposome drug formulations [7], this

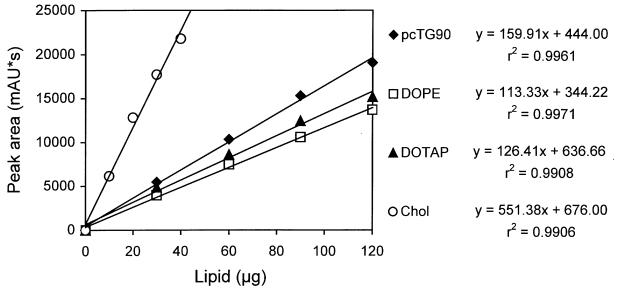

Fig. 2. Standard curves of lipids analyzed by rp-HPLC and UV detection at $\lambda = 205$ nm. The peak area of each lipid class is plotted against the absolute amount of injected lipid. Slopes, intercepts and correlation coefficients were determined from linear regression analysis. Each point is the mean of three determinations. Details of sample preparation and elution conditions are described in Section 2.2.

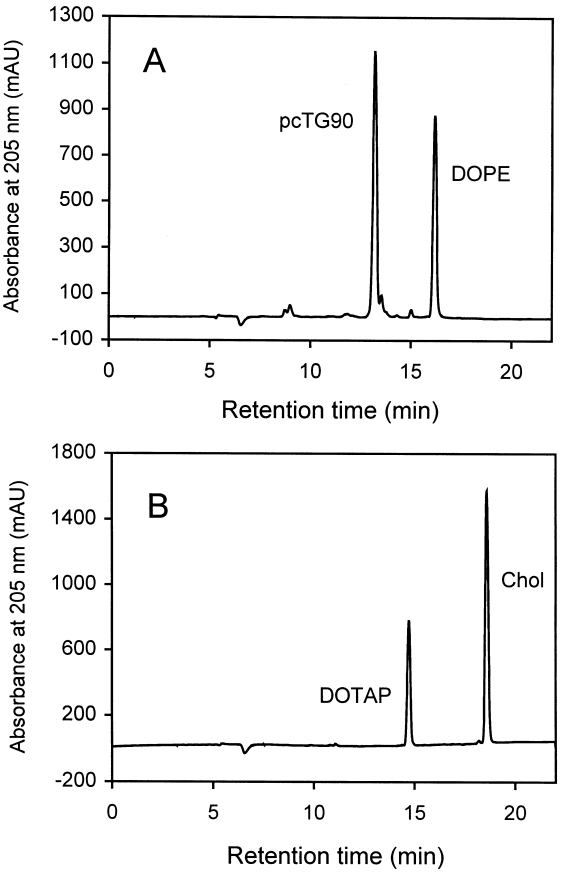

Fig. 3. Chromatograms of cationic liposomes. (A) pcTG90/DOPE (1:2, mol/mol) and (B) DOTAP/Chol (1:1, mol/mol) liposome formulations. Liposomes were diluted in isopropanol and directly injected as such without prior lipid extraction. Injected liposomal lipids correspond to (A) 83 μg of pcTG90 and 84 μg of DOPE, and (B) 70 μg of DOTAP and 39 μg of Chol. Details of sample preparation and elution conditions are described in Section 2.2.

strategy has never been described in combination to rp-HPLC for direct quantitation of cationic liposome content.

In addition to general experimental errors related to accuracy of volumes of volatile solvents and weights of small quantities of materials, errors in lipid quantities may also result from lipid loss occurring during extrusion of liposomes through small pore diameter membranes. Therefore, a very accurate lipid quantitation of all final cationic liposome preparations must be performed before using them to form lipoplexes, especially since the lipid/DNA ratio can affect both lipoplex stability and activity [3]. Table 1 compares injected quantities of liposome components, corresponding concentrations and lipid ratios estimated from raw material weights used to form liposomes with those calculated from rp-HPLC analysis of injected liposomes. Estimated and HPLC-calculated values were in good accordance suggesting that our multi-step liposome preparation procedure does not lead to significant lipid loss.

Reversed-phase HPLC was previously used to separate and analyse isomers of spermine and spermidine derivatized cholesterol [8]. This is a method of choice in the case of polyvalent positively-charged lipids such as pcTG90 that are prone to stick too avidly to normal polar matrix stationary phase. Importantly, the method we have developed efficiently separated lipids from two different cationic liposome formulations composed of cationic, zwitterionic and neutral lipids highly different by their structure and physicochemical characteristics. Although the two cationic lipids utilized in this study contain identical hydrophobic moieties (dioleoyl), they strongly differ by the nature of their polar head. pcTG90 comprises one primary and four secondary amino groups whereas DOTAP contains a single quaternary amine. Furthermore, it was important to validate our method with formulations containing the two most commonly used colipids described in the literature, i.e. DOPE and Chol.

In order to determine lipid concentration from stock solutions and to validate lipid quantitation by rp-HPLC for colipids, colorimetric assays were performed for quantitation of DOPE [9] and Chol [10]. The ratios $R_{H/C}$ of absolute amounts of these colipids in liposomes determined by rp-

Table 1
Determination of liposomal lipid concentrations and cationic lipid/colipid molar ratios by rp-HPLC[a]

	pcTG90/DOPE liposomes		DOTAP/Chol liposomes	
	pcTG90	DOPE	DOTAP	Chol
Estimated injected lipid (μg)	83.3	84	70	38.9
[b]HPLC-calculated injected lipid (μg)	87.69	83.68	69.6	39.84
Estimated concentration (mM)	6.78	13.56	10.02	10.06
[b]HPLC-calculated concentration (mM)	7.14	13.52	9.96	10.37
Estimated lipid molar ratio	1/2		1/1	
[b]HPLC-calculated lipid molar ratio	1/1.89		1/1.04	

[a] pcTG90/DOPE and DOTAP/Chol liposomes diluted in isopropanol (50% final) were injected in the rp-HPLC system and quantitation of each liposome component was deduced from the corresponding surface peak area of the chromatogram and corresponding standard curves (see Section 2 for details).
[b] Means of two determinations.

HPLC over those determined by colorimetric assays were $R_{H/C} = 1 \pm 0.4$ for all samples tested.

The detection limit of evaporative light-scattering was reported to be near 0.05 μg for the cationic lipid 1,2-dimyristoyl-oxypropyl-3-dimethyl ammonium bromide (DMRIE) and DOPE that showed twice the peak height of the mobile phase background [5]. Such high sensitivity may only be required when high cost and difficult to produce and/or purify materials are used. In our case, the detection limits with signal to noise ratio of at least ten were near 1 μg for pcTG90, DOTAP and DOPE and 0.5 μg for Chol, which provided acceptable sensitivity for quantitative determination. Contrary to evaporative light-scattering, UV detection is not restricted to non-volatile materials and volatile mobile phase but its use can be limited in the case of poor UV absorbers such as saturated aliphatic chain lipids.

In summary, the HPLC method reported here is simple, fast, and allows efficient direct separation and quantitation of cationic liposome components without requirements for prior organic solvent lipid extraction. This method can be used routinely as a control technique in lipoplex preparation process and may be applied to various liposomal components used in gene transfer.

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