Separation and Quantitation of Cationic Liposome Components by High Performance Liquid Chromatography with Evaporative Light-Scattering Detection

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INTRODUCTION

Cationic lipids have been widely used to deliver genes and enhance their expression in vitro (1–3). Plasmid DNA and other negatively charged molecules can be complexed with cationic liposomes and delivered into cells possibly by mechanism involving fusion and endocytosis (2–5). Cationic liposomes used for in vitro transfection are commonly composed of a cationic lipid (e.g. DOTMA, DDAB, DOGS, DOTAP, DOSPA, or DMRIE), and a fusogenic neutral lipid (e.g. DOPE). In vivo application of cationic liposomes is broadly investigated as well. Human clinical trials have been initiated with HLA-B7 or IL2 plasmid DNA and DMRIE/DOPE cationic liposomes (6–8). For these studies it became necessary for an analytical assay to be developed for determining the purity and the stability of the cationic liposome components. TLC was initially used to check the degradation profile of the lipid components. However, this assay was not adequate for accurate quantitation.

Normal phase HPLC is a common technique for the analytical separation of lipid components based on their relative hydrophobicity, such as alkyl/acyl chain lengths and head group polarity (9–10). The separation depends on differences in the partition coefficients of the various components between the stationary phase (polar matrix) and mobile phase (relatively non-polar solvent). A Diol column (bonded normal phase) was evaluated in conjunction with an evaporative light scattering detector (11–12) for the separation and detection of DMRIE and DOPE in the clinical formulation (13).

The light scattering detector has been proven to be effective in detecting poor UV-absorbers, such as most lipids (14–17). Effluent from the column is nebulized to form a homogeneous mist. This aerosol then enters a heated tube where the mobile phase evaporates to leave non-volatile analytes. The light scattering caused by the non-volatile analytes is detected by a photomultiplier and converted to an analog signal which is output to a signal recording device. The amount of scattered light is a function of the mass of the analyte.

Several mobile phases with different volatile pH modifiers, ammonium hydroxide, acetic acid and trifluoroacetic acid were studied to improve the separation of lipid components of cationic liposomes. 0.1% trifluoroacetic acid (TFA) in mobile phase composed of chloroform, methanol and water was able to produce effective separation between cationic lipid and other neutral lipid.

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MATERIALS AND METHODS

Chemicals and Reagents

1,2-dimyristyloxypropyl-3-dimethyl-hydroxyethyl ammonium bromide (DMRIE) was synthesized by the Chemistry Department of Vical Inc. Dioleoylphosphatidylethanolamine (DOPE) was purchased from Avanti (catalog# 850725). Chloroform and methanol were obtained from Burdik & Jackson (catalog# 048-4*DK) and Fisher (catalog# A454-4) respectively. Trifluoroacetic acid (TFA) was obtained from Pierce (catalog# 28901). Distilled water and 0.9% sodium chloride were from Gibco BRL (catalog# 670-5235AG) and Baxter (catalog# 2B-13-24) respectively.

Chromatographic Conditions

A Perkin-Elmer liquid chromatographic system consisting of a Model 410 B10 pump and a model of ISS-100 autosampler (maximum volume of injection: 150 μl) were used. A HP3394 integrator was used for recording and integration with attenuation range set between 7 and 10. The detection was done by an evaporative light-scattering detector (ELSD) from SEDERE model Sedex 55. Nebulization occurred at 40 ~ 42°C and at a pressure of 2.2 bar using a nitrogen source. The setting for the gain on the detector was between 8 and 9.

The column was a prepacked DIOL cartridge column from Advanced Separation Technologies Inc. (ASTEC) (catalog# 51087 for cartridge column and catalog# 50101 for fittings; 5 μm spherical, 25 cm × 4.6 mm).

Elution System

The mobile phase contained 670 parts of chloroform, 297.5 parts of methanol, 32.5 parts of water, and 1 part of TFA. The water was deionized water and passed through a Millipore system (Milli-Q). The mixed mobile phase was equilibrated overnight and then degassed by sonicating for 5 to 10 minutes before use.

Liposome Preparation

The cationic liposome consisted of equal molar amount of DMRIE and DOPE. Both lipids were dissolved in chloroform and the solvent was evaporated to produce a dry lipid film. The dry lipid film was hydrated with aqueous vehicle and vortexed at the top speed for 1 to 2 minutes to form DMRIE/DOPE cationic liposomes.

Lipid/DNA Complex Preparation

pCMV-int-CAT plasmid DNA was used for complexation with DMRIE/DOPE liposomes in this study. The DNA was purified from transformed E. coli culture by alkaline lysis and double CsCl gradient ultracentrifugation (18). The DNA was

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first diluted to the desired concentration with water or aqueous vehicle and then mixed with cationic liposomes to form complexes.

Modified Bligh-Dyer Extraction (19)

0.8 volume of aqueous buffered liposome or DNA/liposome complex was sequentially mixed by brief vortexing with 2 volumes of methanol, 1 volume of chloroform, another 1 volume of chloroform, and 1 volume of 0.9% NaCl. The final mixture was spun at 1000 rpm for about 3 minutes and the bottom organic layer was dried by speed-vac before dissolving in the mobile phase for subsequent HPLC analysis.

Sample Preparation

For cationic lipid/DOPE present as a dry lipid film, the lipid was dissolved in chloroform first and then diluted with mobile phase prior HPLC analysis. For liposomes present in sterile water for injection, the liposomes were first dried by vacuum or speed-vac and then dissolved in chloroform. The lipid solution in chloroform was then further diluted in mobile phase prior to injection. For liposomes or lipid/DNA complexes present in aqueous buffered solution, the preparations were extracted by modified Bligh-Dyer method. Known volume of organic phase was collected and evaporated by speed-vac, the non-volatile lipid extracts was then dissolved in mobile phase prior to injection.

RESULTS

An acidic organic mobile phase (containing volatile 0.1% TFA as a pH modifier, pH 2 ~ 3) was found effective for separating DMRIE and DOPE with only 10 minutes elution using an ASTEC Diol column. A typical chromatogram of the cationic liposome containing equal moles of DMRIE and DOPE is shown in Figure 1 (4.8 ug DMRIE and 5.6 ug DOPE were injected to the column with the detector gain set at 8 and integrator attenuation set at 10). Neutral DOPE was eluted approximately 1 to 2 minutes earlier than cationic lipid DMRIE in this HPLC system. The numbers over the elution peaks were the retention times in Figure 1. Retention times varied slightly, probably due to minor variation of chloroform, methanol and water composition in the mobile phase, since they were measured out by a graduated cylinder. However, the elution profile was reproducible for a mobile phase prepared in a given day.

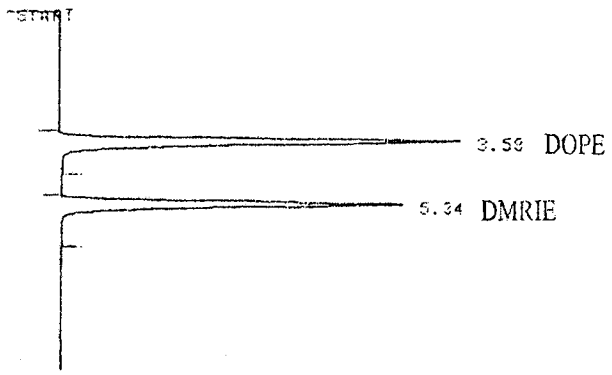

Fig. 1. Typical chromatograms of DMRIE and DOPE on ASTEC Diol column with an ELS detector. 4.8 μg DMRIE and 5.6 μg DOPE (7.5 nmole each) were injected to the column with detector gain at 8 and integrator attenuation at 10.

An evaporative light scattering detector was used to quantitate the eluted product from the HPLC column. Lipid standard curves (showing the relationship between lipid mass and scattered light) of DMRIE and DOPE are shown in Figure 2. The plot indicates a linear response between Log (scattered light signal or HPLC peak area) versus Log (lipid mass). Thus the diffused light or scattered light signal, A, is a function of the lipid mass, m, which apparently obeys an exponential relationship:

$$A = am^b$$

Both a and b are constants dependent upon the mobile phase and the temperature of evaporation. Curves from both lipids overlap closely which indicates similar scattered light signals generated by both lipid species. These observations were reproducible from day to day operation.

The method has been validated with DMRIE and DOPE lipids for the precision and accuracy. Coefficients of variation (CV) of the assay from five replicates of samples ranged from 0.7% to 3.1% for DMRIE and 1.1% to 2.8% for DOPE from three independent sets of experiments. The accuracy of the assay was also determined by comparing the unknown sample to the reference standard, the results were 103.8% for DMRIE and 100.2% for DOPE from five replicates analysis.

Sensitivity of the detection system for DMRIE and DOPE was also studied. A signal-to-noise ratio of 2:1, i.e. twice the noise level, is generally accepted as the detection limit (15,20). The detection limit was approximately 0.05 μg for each lipid which showed twice the peak height of the mobile phase background.

For liposomes present in aqueous buffered solution or in lipid/DNA complex form, lipids were extracted with chloroform and methanol using a modified Bligh-Dyer method as described in the method section. Lipid recovery of DMRIE and DOPE from 0.9% NaCl were determined to be 99% and 91% respectively by HPLC analysis. The modified Bligh-Dyer extraction was effective to extract DNA from our current clinical DNA lipid complex formulation completely in this study. Nearly 100% of total DNA was recovered in the upper aqueous phase after the extraction and measured by UV spectrophotometry as compared to the DNA control.

DISCUSSION

Over the years, it has been difficult to perform quantitative HPLC analysis of lipids due to the detection limitation. Most lipids are lack of a chromophore and fail to be detected by the most widely used ultraviolet spectrophotometric detector. The detection wavelength often lies at an extreme UV region (190 to 210 nm) and requires highly purified transparent solvents. Furthermore, the quantitation is not reliable as various lipids exhibit a different extinction coefficient according to the degree of unsaturation of the fatty acid chains. Refractive index detectors, another commonly used detection method, are not highly sensitive and are very susceptible to solvent and temperature fluctuations. In this work, the evaporative light scattering detector was studied to analyze the cationic liposome components in clinical formulations. The method described here successfully

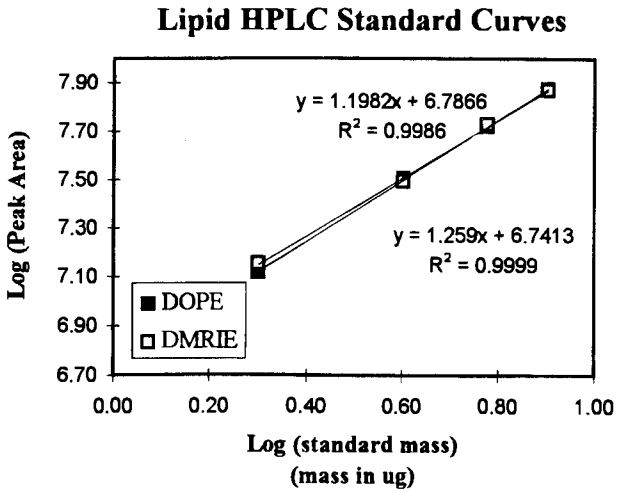

Fig. 2. Standard Curves of DMRIE and DOPE. 2, 4, 6, and 8 μg of each lipid were injected to the column. Log (mass) was plotted against log (peak area) for each lipid, both curves obeyed linear response empirically.

separated DMRIE (a cationic lipid) and DOPE (a neutrally charged phospholipid), and accurately quantitated their contents in the dry lipid film or aqueous DMRIE/DOPE cationic liposome formulations.

The pH of the mobile phase was determined to be important parameter leading to the success of this HPLC method. A high pH mobile phase which has been used successfully for separating either neutral or negatively charged phospholipids (10,14) was not effective for eluting the cationic lipids. This is likely due to the deprotonated silanol groups on the column matrix at high pH which interact with the positively charged head group of the cationic lipids, and interfere with the desired normal phase separation and elution. Therefore, acidic mobile phase conditions were found to be critical for this method, TFA provided sharper resolution as compared to acetic acid.

Results from the work also demonstrated that the method provided excellent quantitation and sensitivity (less than 0.05 μg for both lipids). The detection for both of the DMRIE and DOPE lipids which were examined showed similar signal response. Thus, the detection of the method was not dependent on the degree of fatty acid saturation or net charge of the molecule. A linear response with excellent correlation coefficient was observed between log(scattered light signal) and lot(-mass) for both lipids. The scattered light signal was therefore obeying an exponential relationship with the lipid mass (Figure 2). This empirical mathematical relationship was useful and allowed accurate quantitation.

The method was shown acceptable for the evaluation of the manufacturing processes, and for determining the stability of cationic liposome formulations. Furthermore, this procedure could be applied to the separation of other types of phospholipids or positively charged lipids. The method was found to be effective in separating other positive charged lipid, GAP-DLRIE, by modifying to a slightly more polar mobile phase (600 chloroform/350 methanol/50 water/1 TFA). GAP-DLRIE, (±)-N-(3-aminopropyl)-N,N-dimethyl-2,3-bis(dodecycloxy)-1-propanaminium bromide), is another type of cationic lipid with two positive charged groups.

However, the evaporative light scattering detection system is limited to non-volatile materials. For the same reason the mobile phase or modifying agents need to be volatile. Any non-volatile buffers, salts, or other excipients present in the liposome or DNA/lipid complex formulations require a separation procedure (e.g. modified Bligh-Dyer extraction or solid phase extraction) prior to subsequent HPLC analysis. In sum, the HPLC method described here using an evaporative light scattering detection, an acidic pH mobile phase and a diol normal phase column offers an important improvement in the area of cationic lipid analysis.

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