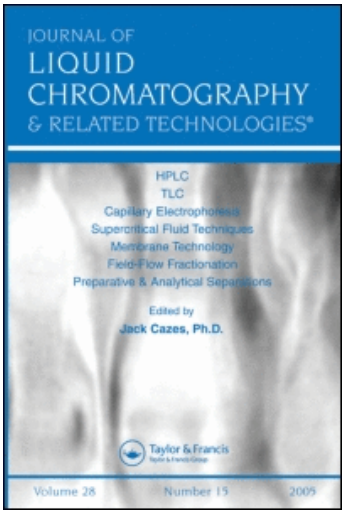

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REVERSED-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF CATIONIC LIPID-BASED GENE TRANSFER AGENTS

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ABSTRACT

Cationic lipid-mediated gene transfer represents a promising approach for the treatment of a number of diseases. Since the successful introduction of DOTMA:DOPE (Lipofectin), a variety of cationic lipids have been developed for use in gene transfer. Some of the more active cationic lipid formulations, including GL-67:DOPE, DC-chol:DOPE, DMRIE:DOPE and DOTAP, have been used in human clinical trials. It is of critical importance to develop robust analytical methods for the determination of the chemical purity of these formulations. We report here efficient, sensitive, and reproducible reversed-phase HPLC methods for use in determining the chemical purity of cationic lipid formulations. GL-53:DOPE, GL-67:DOPE, DMRIE:DOPE, DC-chol:DOPE, GAP-DLRIE:DOPE, DOTMA: DOPE (Lipofectin), DDAB:DOPE (Lipofectace), DOSPA:DOPE (Lipofectamine), DOGS (Transfectam), and DOTAP were analyzed by HPLC on C8 or C18 bonded phase columns with

aqueous/mixed organic mobile phases containing trifluoroacetic acid and with ELSD detection in the gradient elution mode. Baseline resolution of the components of the GL-53:DOPE formulation was achieved by optimization of the solvent system and gradient profile. Capacity factors (k') of the cationic lipids were greatly affected by the end-capping chemistry of the C18 bonded phases. The calibration curves for GL-53, DC-chol, DMRIE, and DOPE were determined in the range of 1.6-200.0 μg. The detection limits for these compounds were determined to be 0.4-1.6 μg.

INTRODUCTION

Gene therapy has the potential to become a breakthrough technology for the treatment of inherited and acquired diseases including cystic fibrosis, arteriosclerosis, cancer, and AIDS.[1-3] One of the fundamental challenges that must be met for gene therapy to become a widely used treatment strategy is the development of safe and effective delivery vehicles. Cationic lipid-mediated gene transfer is one of the most promising non-viral gene delivery systems developed to date.[4-5] A number of early stage clinical trials have been reported utilizing cationic lipid-mediated gene transfer. A list of the cationic lipid-based gene transfer agents examined and the disease indications investigated in these trials is given in Table 1.[5] Note that the majority of the cationic lipids thus far developed are found to be most effective when formulated with a neutral co-lipid usually dioleoylphosphatidylethanolamine (DOPE).

Cationic lipids consist of a lipophilic group, capable of interacting with membrane bilayers, an amine containing moiety, capable of holding a positive charge and interacting with the negatively charged plasmid DNA, and a linking group to hold the two together. The structures of a number of cationic lipids are depicted in Figure 1. We have developed a diverse set of cationic lipids and have found a number of these compounds, especially N^4-Sperminecarbamic acid cholesteryl ester (GL-67) and N^4-Spermidinecarbamic acid cholesteryl ester (GL-53), to be particularly effective gene transfer agents *in vivo*.[6]

Thin-layer chromatography (TLC) historically has been the primary method used for the determination of the chemical purity of cationic lipid-based gene transfer agents. Unfortunately, TLC suffers from a number of limitations including the inadequate resolution of closely eluting solutes, poor sensitivity and poor reproducibility when compared with high performance liquid chromatography (HPLC). The analysis of a variety of lipids (e.g., cholesterol

Table 1

Clinical Investigations of Cationic-Lipid Mediated Gene Transfer (5)

Cationic Lipd-Based Agents[a]	Diseases
DOTMA:DOPE	Cancer (glioma and melanoma)
DDAB:DOPE	Cancer (breast)
DOTAP	Cystic Fibrosis
DMRIE:DOPE	Cancer (colon, renal and melanoma) and Cystic Fibrosis
DC-chol:DOPE	Cancer (melanoma), Cystic Fibrosis and AAT deficiency
GL-67:DOPE	Cystic Fibrosis

[a] Lipid names and structures are shown in Figure 1.

and phospholipids) by HPLC with an evaporative light scattering detector (ELSD) has been reported with satisfactory levels of resolution, sensitivity and analysis time achieved by gradient elution in the normal-phase mode.[7-16] However, the development of bare silica based analyses of cationic lipid-based gene transfer agents has proved to be much more challenging. Recently, a cationic lipid-based gene transfer agent, (DDAB:DOPE, 8:15 mole/mole), has been resolved and quantified using a silica-based aminopropyl bonded phase column with ELSD detection in the isocratic mode.[17-18] This method has three areas in which improvement is warranted. The cationic lipid, DDAB, was insufficiently retained ($k'<1$) and inadequately resolved from other early eluted, non-volatile solutes (i.e. inorganic ions). Secondly, the method has a significant peak-tailing problem arising from the secondary interaction of the amine functionality of cationic lipid and the silanol groups of the stationary phase.[17] Finally, the column lifetime of aminopropyl bonded phases tends to be short and over time problems with reproducibility are common.

In this work, we describe efficient and sensitive reversed-phase HPLC (RP-HPLC) methods with ELSD in the gradient mode for the analysis of cationic lipid-based gene transfer agents. These methods have a C8 or C18 bonded stationary phase and a mobile phase consisting of water/mixed organic modifiers and trifluoroacetic acid. Utilizing these methods, a number of cationic lipid-based gene transfer agents were analyzed with significant improvements in resolution, peak shape, quantification, reproducibility and column stability over methods previously reported.

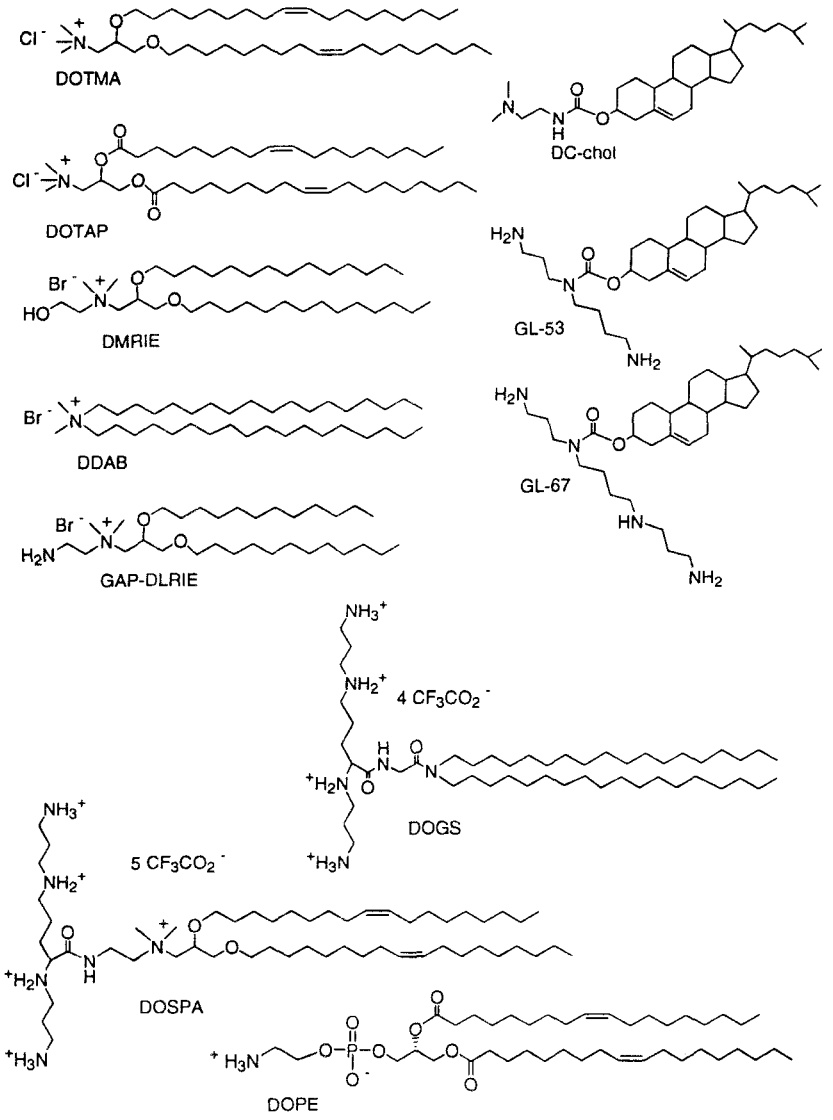


Figure 1. Chemical Structures of the cationic lipids and the co-lipid DOPE discussed in this work.

MATERIALS AND METHODS

The silica-based C18 bonded phase columns (4.6 x 250 mm, 5 μm) were obtained from various manufacturers. The ODS-AQ, Symmetry C18, Kromasil C18 and Zorbax RX-C18 columns were obtained from YMC (Wilmington, NC), Waters (Milford, MA), Eicon Scientific (Osterville, MA), and MAC-MOD Analytical (Chadds Ford, PA) respectively. N^4-Spermidinecarbamic acid cholesteryl ester (GL-53) and N^4-Sperminecarbamic acid cholesteryl ester (GL-67) were obtained from Genzyme (Cambridge, MA). 1,2-Dimyristyloxypropyl-3-dimethylhydroxyethylammonium bromide (DMRIE) and N-(3-Aminopropyl)-N,N-dimethyl-2,3-bis(dodecyloxy)-1-propanaminium bromide (GAP-DLRIE): DOPE (1:1 mole/mole) were obtained from Dr. Philip Felgner (Vical Inc.). 3(-(N-(N',N'-Dimethylaminoethane)carbamoyl) cholesterol (DC-chol) was obtained from Professor Leaf Huang (University of Pittsburgh). Dimethyldioctadecyl-ammonium bromide (DDAB) was purchased from Sigma (St. Louis, MO). Transfectam agent, Dioctadecylamidoglycyl spermine (DOGS), was purchased from Promega (Madison, WI). Lipofectin, Lipofectace, and Lipofectamine agents were obtained from Gibco/BRL (Grand Island, NY). Lipofectin agent is a 1:1 (w/w) liposomal formulation of the cationic lipid 2,3-dioctadecenyl-oxypropyl-N,N,N,-trimethylammonium chloride (DOTMA) and DOPE in membrane filtered water. Lipofectace agent is a 1:2.5 (w/w) liposomal formulation of the cationic lipid DDAB and DOPE in membrane filtered water. Lipofectamine agent is a 3:1 (w/w) liposomal formulation of the cationic lipid 2,3-Dioleyloxy-N-[2-(spermine-carboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate (DOSPA) and DOPE in membrane filtered water. 1,2-Dioleoyl-3-trimethylammonium methylpropane (DOTAP) and DOPE were purchased from Avanti Polar Lipids (Pelham, AL). The deionized (DIUF) water was purchased from Fisher (Fair Lawn, NJ). HPLC grade methanol, acetonitrile, 2-propanol, chloroform, and water were purchased from Burdick & Jackson (Muskegon, MI). Trifluoroacetic acid (TFA) and triethylamine (TEA) were obtained from Aldrich (Milwaukee, WI) and Sigma (St. Louis, MO) respectively.

The HPLC system consists of a Waters 600E gradient pump, 712 WISP autosampler, TCM, Maxima chromatography workstation (Milford, MA), and ACS model 750/14 evaporative light scattering detector (Polymer Laboratories, Amherst, MA). The stationary phase consisted of a silica-based C8 or C18 column maintained at 40°C. A 15-200 μg sample of the cationic liposomal dispersion was directly injected into the HPLC system. Lipid sample weights of 1.6, 8.0, 40.0, and 200.0 μg were evaluated in the calibration curve study. Typically, a binary solvent system, consisting of aqueous/mixed organic mobile phases with the addition of trifluoroacetic acid, was used in the gradient mode (see Table 2 for the gradient programs).

Table 2

Reversed-Phase High Performance Liquid Chromatographic Analysis of Cationic Lipid-Based Gene Transfer Agents

Cationic Lipid Agent[a]	Mobile Phase	Gradient[b]	Column	k'$_1$[c]	k'$_2$[d]
DMRIE:DOPE	A. CH_3CN:CH_3OH:H_2O =70:25:5 (0.1%TFA) B. CH_3CN:CH_3OH:$CHCl_3$ =70:10:20 (0.1%TFA)	I	C18[e]	2.50	3.89
DC-chol:DOPE	A. CH_3CN:CH_3OH:H_2O =70:15:15 (0.1% TFA, 0.05% TEA) B. CH_3CN:CH_3OH:$CHCl_3$ =70:10:20 (0.1%TFA, 0.05% TEA)	I	C8[f]	2.84	3.73
GL-53:DOPE	A. CH_3OH:IPA:H_2O =60:20:20 (0.1% TFA) B. CH_3OH:IPA=70:30 (0.1% TFA) C. CH_3OH:IPA:$CHCl_3$ =60:20:20 (0.1 TFA)	II	C18[e]	4.10	5.58
	A. CH_3OH:IPA:H_2O =55:15:30 (0.1% TFA) B. CH_3OH:IPA=70:30 (0.1% TFA)	III	C18[e]	3.83	7.21
GL-67:DOPE	A. CH_3OH:IPA:H_2O =55:15:30 (0.1% TFA) B. CH_3OH:IPA=70:30 (0.1% TFA)	IV	C18[e]	4.16	6.05
GAP-DLRIE:DOPE	A. CH_3OH:IPA:H_2O =55:15:30 (0.1% TFA) B. CH_3OH:IPA=70:30 (0.1% TFA)	IV	C18[e]	4.19	6.05
Lipofectin	A. CH_3OH:IPA:H_2O =55:15:30 (0.1% TFA) B. CH_3OH:IPA=70:30 (0.1% TFA)	III	C18[e]	5.69	7.21
Lipofectace	A. CH_3OH:IPA:H_2O =55:15:30 (0.1% TFA) B. CH_3OH:IPA=70:30 (0.1% TFA)	III	C18[e]	5.36	7.21
Lipofectamine	A. CH_3OH:IPA:H_2O =55:15:30 (0.1% TFA) B. CH_3OH:IPA=70:30 (0.1% TFA)	III	C18[e]	4.55	7.21

Table 2 (continued)

Reversed-Phase High Performance Liquid Chromatographic Analysis of Cationic Lipid-Based Gene Transfer Agents

Cationic Lipid Agent[a]	Mobile Phase	Gradient[b]	Column	k'$_1$[c]	k'$_2$[d]
Transfectam	A. CH_3OH:IPA:H_2O =55:15:30 (0.1% TFA) B. CH_3OH:IPA=70:30 (0.1% TFA)	III	C18[e]	4.89	
DOTAP	A. CH_3OH:IPA:H_2O =55:15:30 (0.1% TFA) B. CH_3OH:IPA=70:30 (0.1% TFA)	III	C18[e]	4.95	

[a] Lipid names and structures are shown in Figure 1. Lipofectin, Lipofectace, Lipofectamine and Trasfectam are DOTMA:DOPE, DDAB:DOPE, DOSPA:DOPE and DOGS respectively (see Materials and Methods section).

[b] Gradient I: (1) 100%A hold for 1.5 minutes, (2) Linear gradient to 100%B for 6 minutes, (3) 100%B hold for 15 minutes, (4) Linear gradient to 100% A for 2 minutes, (5) 100%A hold for 5.5 minutes. Gradient II: (1) 100%A hold for 7.5 minutes, (2) Linear gradient to 100%B for 3 minutes, (3) 100%B hold for 2 minutes, (4) Linear gradient to 100%C for 2.5 minutes, (5) 100%C hold for 5.5 minutes, (6) Linear gradient to 100%B for 2 minutes, (7) 100%B hold for 5 minutes, (8) Linear gradient to 100%A for 2.5 minutes, (9) 100%A hold for 10 minutes. Gradient III: (1) Linear gradient from 0%B to 88%B for 10 minutes, (2) 88%B hold for 14 minutes, (3) Linear gradient from 12%A to 100%A for 4 minutes, (4) 100%A hold for 12 minutes. Gradient IV: (1) 100%A hold for 5 minutes, (2) Linear gradient from 0%B to 95%B for 5 minutes, (3) 95%B hold for 14 minutes, (4) Linear gradient from 5%A to 100% A for 4 minutes, (5) 100%A hold for 12 minutes.

[c] Capacity factor (k_1') of the cationic lipid, $k'=(t_r-t_0)/t_0$.

[d] Capacity factor (k_2') of DOPE, $k'=(t_r-t_0)/t_0$.

[e] 250 x 4.6 mm 5μm YMC ODS-AQ analytical column.

[f] 250 x 4.6 mm 5μm Zorbax RX-C8 analytical column.

A 1.0 mL/min flow rate was used for all the analyses. The optimum conditions for the evaporative light scattering detector were found to be an evaporative temperature of 55°C and an air pressure of 20 psi. The cationic lipid-based gene transfer agents were prepared by mixing chloroform solutions of the cationic lipids with the chloroform solution of the co-lipid DOPE in appropriate ratios. The dried lipid films were produced by evaporating the chloroform under a stream of nitrogen.

The cationic liposomal dispersions were prepared by hydration of the dried lipid films[19] with DIUF water followed by vortex. These aqueous liposomal dispersions were readily injected into the HPLC system directly upon completion of the hydration procedure.

RESULTS AND DISCUSSION

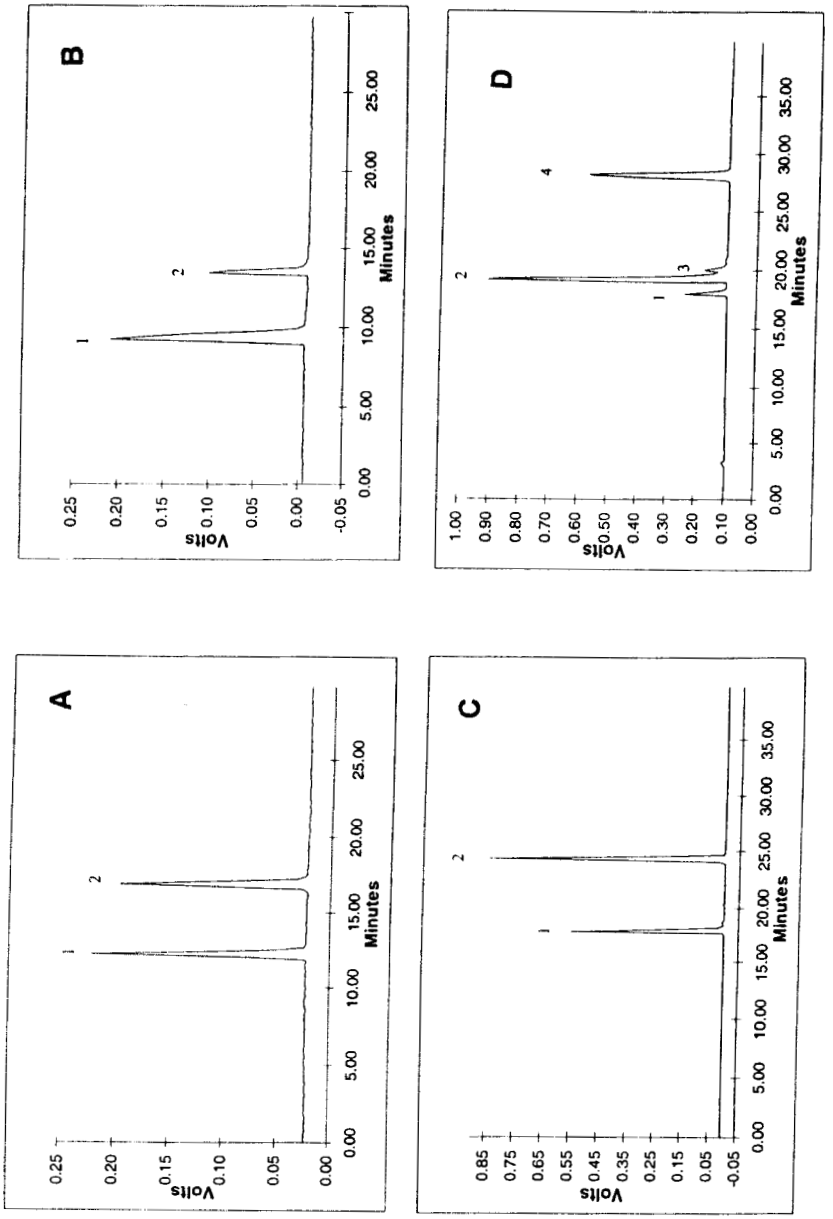
The HPLC methods described herein were useful for the analysis of a wide range of cationic lipid structure types (see Figure 1). The cationic lipids examined in this study contain a variety of amine groups, linker functionalities, and lipid moieties. We analyzed cationic lipids containing quaternary (e.g., DOTMA), tertiary (e.g., DC-chol), secondary (e.g., GL-67), and primary (e.g., GAP-DLRIE) amine functionalities. Five cationic lipids (GAP-DLRIE, GL-53, GL-67, DOGS, and DOSPA) possess multiple amine moieties, whereas the others (DOTMA, DOTAP, DMRIE, DDAB, and DC-chol) contain a single amine moiety. Many cationic lipids are used and were analyzed as amine salts (e.g., DOGS), while some others are used and were analyzed as the free base (e.g., GL-53).

The cationic lipids in this study have cholesterol or fatty acid derived alkyl chains as the lipophilic groups. Three of the cationic lipids (DC-chol, GL-53 and GL-67) contain the cholesterol group as the lipid moiety. The remaining cationic lipids have lipophilic groups comprised of alkyl chains of various length that are either saturated or have a single double bond. A number of these cationic lipids have a lipid component comprised of two 18 carbon chains, each with a cis double bond, attached to a dihydroxypropyl group via ester moieties (DOTAP) or ether moieties (DOTMA and DOSPA). Two cationic lipids (DDAB and DOGS) contain 18 carbon saturated chains bonded to a single nitrogen atom. Two others (DMRIE and GAP-DLRIE) contain 14 and 12 carbon saturated chains, respectively, attached to a dihydroxypropyl group via ether moieties. The amine containing groups are attached to the lipophilic groups by amide linkages (e.g., DOGS), carbamate linkages (e.g., GL-67 and DC-chol) or simply by amine linkages (e.g., DMRIE and DDAB). All the cationic lipids hold positive charges ranging from +1 (e.g., DOTMA) to +5 (e.g., DOSPA). DOPE, by far the most widely used co-lipid, is zwitterionic.

Table 2 describes the optimum RP-HPLC conditions for ten cationic lipid formulations. DOPE is a component of most of these formulations. The mobile phases used are either binary or ternary aqueous/organic solvent systems. Up to 30 % H_2O is added to the solvent systems for generating appropriate retention (capacity factor k'=1-10) by RP-HPLC.

The organic modifiers used in these methods were CH_3OH, CH_3CN, 2-propanol and $CHCl_3$, with CH_3OH being the most widely used solvent. Although 2-propanol is viscous and increases the back pressure, its judicious use as a co-solvent was found to increase sample loading significantly. For example the change to CH_3OH:2- propanol, 55:15 increased the sample loading from 15 μg to 200 μg (see Figure 2 (C-D)). As a result, this provides greater sensitivity for the detection of minimal amounts of degradation products or other impurities (Peak 1 and 3 in Figure 2 (D)). TFA was used as a buffer in every method listed in Table 2. This was done for several reasons. TFA is a volatile acid, and thus, is compatible with ELSD detection. The addition of TFA to the eluent mix resulted in a significant improvement in the retention of the cationic lipids due to ion pairing. These cationic lipid ion pairs have enhanced hydrophobic interactions with the C8 or C18 bonded phases. Furthermore, the acidity of the mobile phase (apparent pH $\leq$ 2) leads to the protonation of the residual silanol groups of the silica-based C8 or C18 bonded phases. This minimizes the ionic interactions of the cationic lipids with the silanol groups. As a result, peak-tailing problems are reduced or eliminated and quantitative measurements of the cationic lipids are improved. If TFA was substituted with formic acid or acetic acid the improvements in retention time and peak tailing vanished. As is demonstrated in Figure 2 (A-D), the utilization of gradient elution for RP-HPLC of the cationic lipid-based agents (e.g., DC-chol:DOPE) gave appropriate retention times for the cationic lipids (9.14 min for DC-chol) and the co-lipids (13.42 min for DOPE). Higher efficiency (as compared to the isocratic elution) is also achieved with the use of gradient elution. Irrespective of whether a cationic lipid was analyzed as the free base or as a salt form, it always gave a constant k'. For example, the k' of GL-53 remained constant at a k' of 4.10 for the free base form, as well as, for the hydrochloride and trifluoroacetate salt forms (data not shown). In this study, the cationic lipids were always found to be the first components of the formulation to be eluted (see Table 2 and Figure 2 (A-D)).

It is well known that optimization of the capacity factor can significantly improve the separation or resolution of solutes in HPLC.[20] This can be achieved through the correct choice of the solvent systems and gradient programs. The effect that capacity factor optimization can have on the resolution of cationic lipids is illustrated by a comparison of the chromatograms of the GL-53:DOPE (1/1 mole/mole) formulation shown in Figure 3.[21] As can be seen in Figure 3 (A), three major peaks were eluted at retention times of 17.62 (peak 2), 20.21 (peak 4), and 22.75 (peak 5) minutes using gradient program II and a ternary solvent system (Table 2). In contrast, this sample can be resolved to six corresponding peaks at 16.13 (peak 3), 16.86 (peak 4), 17.24 (peak 5), 22.29 (peak 6), 22.69 (peak 7), and 28.63 (peak 8) minutes using



gradient program III and a binary solvent system (Table 2) as shown in Figure 3 (B). This dramatic increase in resolution, to nearly baseline separation, was achieved by the use of a weaker solvent system as the first eluent of the gradient profile (CH_3OH:IPA:H_2O=55:15:30 (0.1% TFA) verses CH_3OH:IPA: H_2O= 60:20:20 (0.1% TFA)) and by the use of a linear gradient with a more shallow slope. Both of these changes resulted in the increase in the capacity factors for all the components of the GL-53:DOPE formulation.

The peak assignments for Figure 3 (A) are as follows: peak 1, N^4-spermidine 7-dehydro-cholesteryl carbamate; peak 2, GL-53; peak 3, unknown component; peak 4, oleic amide derivatives of GL-53; and peak 5, DOPE. The peak assignments for Figure 3 (B) are as follows: peak 1, unknown component; peak 2, N^4-spermidine 7-dehydro-cholesteryl carbamate; peak 3, GL-53; peaks 4 and 5, 2- and 1-oleoylphosphatidylethanolamine; peaks 6 and 7, regioisomers of the oleic amide derivative of GL-53, and peak 8, DOPE. A number of the peaks in these chromatograms are due to a transacylation reaction that can occur between GL-53 and DOPE in an aqueous environment. This phenomenon will be discussed in detail elsewhere.[22]

A comparison of the capacity factors of three cationic lipids on silica-based C18 bonded phase columns made by four different manufacturers is shown in Figure 4. The characteristics of the silica supports and bonding chemistries contribute to the variations of these C18 bonded phases. According to the specifications of the manufacturers, the Zorbax RX-C18 has the smallest surface area (180 m^2/g), lowest carbon load percentage (12%), and a relatively low bonding density (2.98 $\mu mole/m^2$). It is also the only C18 bonded phase without end capping used in this study.

The Kromasil C18 bonded phase, on the other hand, has the largest surface area (340 m^2/g), highest carbon load percentage (19%), and a relatively high bonded phase coverage (3.10 $\mu mole/m^2$).

Figure 2 (left). RP-HPLC chromatograms of: (A) DMRIE:DOPE (1/1, w/w, 15 μg sample) peak 1 (DMRIE, 12.09 min) and peak 2 (DOPE, 16.87 min) on a 4.6x250 mm YMC-ODSAQ column at 40°C; (B) DC-chol:DOPE (2/1, w/w, 15 μg sample) peak 1 (DC-chol, 9.14 min) and peak 2 (DOPE, 13.42 min) on a 4.6x250 mm Zorbax RX-C8 column at 40°C; (C) GL-67:DOPE (1/2 mole/mole, 200 μg sample) peak 1 (GL-67, 17.86 min) and peak 2 (DOPE, 24.38 min) on a 4.6x250 mm YMC-ODSAQ column at 50°C; (D) Lipofectamine (200 μg sample) peak 2 (DOSPA, 19.19 min), peak 4 (DOPE, 28.22 min), and peak 1 and 3 (two unknown components) on a 4.6x250 mm YMC-ODSAQ column at 40°C. The gradient conditions can be found in Table 2.

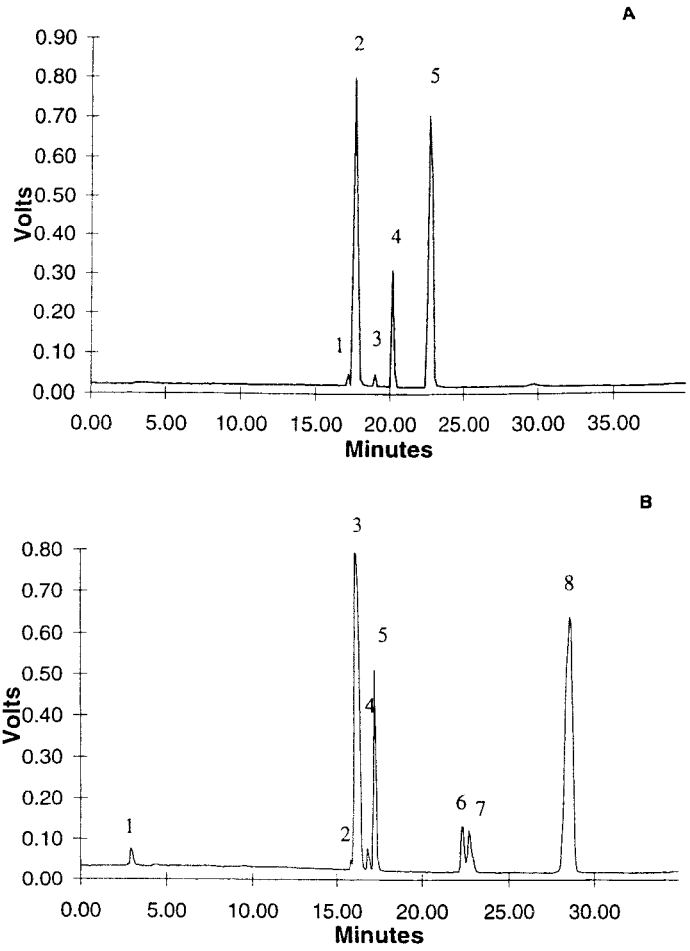


Figure 3. RP-HPLC chromatograms of GL-53:DOPE (1/1 mole/mole, 200 μg sample) on a YMC-ODSAQ column at 40°C: (A) Peak 2 and 5 are GL-53 and DOPE respectively; (B) Peak 3 and 8 are GL-53 and DOPE respectively. See text for the other peak assignments. Separation conditions can be found in Table 2.

The major difference between the YMC-ODSAQ bonded phase and the Symmetry C18 and Kromasil C18 bonded phases is the presence of hydrophilic end capping groups on the former compared with the shorter chain silane end capping groups (e.g., chlorotrimethylsilane) on the latter. The differences in the C18 bonded phases of the various manufacturers have had significant effects on the capacity factors of the cationic lipids. Interestingly, the Zorbax

RX-C18 bonded phase was determined to be the most retentive C18 phase (see Figure 4) in spite of having the lowest carbon load percentage (12%) and a relatively low bonding density (2.98 μmole/m^2). The Zorbax RX-C18 bonded phase column is the one column that we examined that lacks end capping groups and thus has relatively more residual silanol sites. The enlarged *k'* values and longer retention times observed with this column may be due to hydrogen bonding interactions of these silanol groups with the cationic lipids. On the other hand, the YMC-ODSAQ bonded phase gives the least retention apparently due to the effect of the hydrophilic end capping groups. The presence of end capping groups and their hydophilicity has a profound effect on the capacity factors of cationic lipids. As can be seen in Table 2, the YMC-ODSAQ bonded phase was chosen for the analysis of most of the cationic lipid-based agents. This column provided good peak resolution along with a short enough retention time to allow for a reasonable rate of sample throughput. The improved resolution achieved with the YMC-ODSAQ bonded phase may be due to secondary interactions of the end capping groups with components of the cationic lipid formulations. Such interactions have been reported previously in the RP-HPLC of other compounds.[23] In spite of differences in the C18 bonded phases from the different manufacturers, the elution order of the cationic lipids remained constant: GL-53 followed by DC-chol and then DMRIE.

A correlation was observed between the cationic lipid structural features and the capacity factors obtained for GL-67 (3.63), GL-53 (3.83), DC-chol (4.62), DOTAP (4.95), and DOTMA (5.69) on the YMC-ODSAQ column (gradient III, see Table 2). As would be expected in RP-HPLC, we found the most polar molecule in a similar structural series to have the smallest *k'*. DOTAP and DOTMA differ only in the presence of two ester groups in the former as opposed to two ether groups in the latter. DOTAP with its more polar ester groups has the smaller *k'*. In the other structurally related series GL-67 has three amine groups, GL-53 has two amine groups, and DC-chol has a single amine group. The retention order of this series shows the same trend with the most polar compound (GL-67) having the smallest *k'* and the least polar compound (DC-chol) having the largest *k'*.

The use of ELSD provides greater sensitivity as compared with conventional UV and RI detectors for the detection of nonchromogenic compounds such as lipids, carbohydrates, steroids, and inorganic ions.[11,24-27] A major benefit of ELSD detection, and a critical one for this work, is its compatibility with gradient elution. Unfortunately, many mobile phase additives that greatly improve the selectivity of RP-HPLC separations, including crown-ethers, cyclodextrins, and macrocyclic antibiotics, are not compatible with ELSD detection.

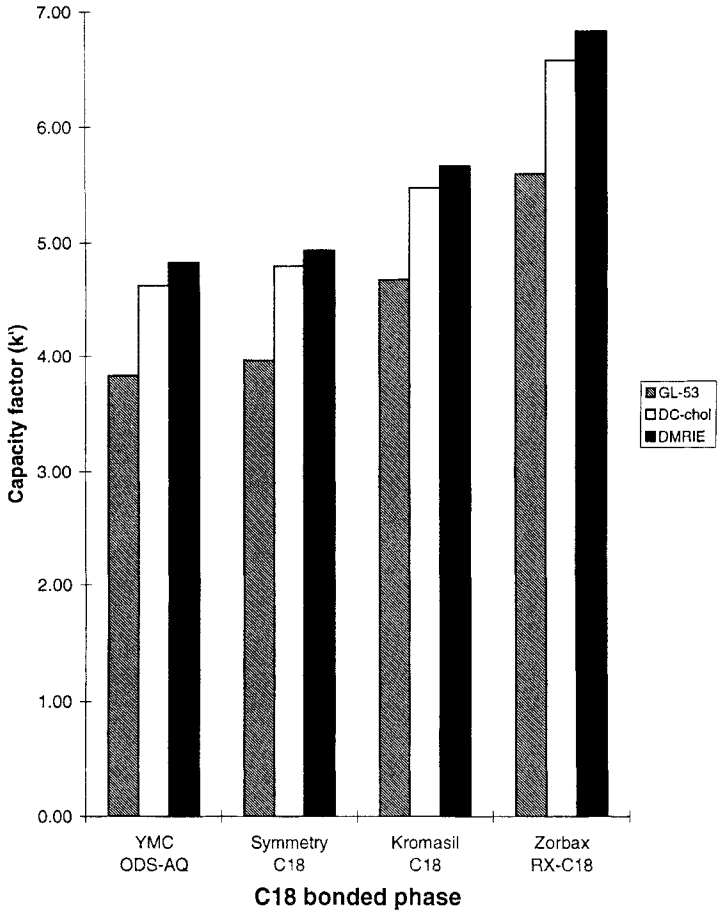


Figure 4. Effect of the C18 bonded phase from various manufactures on the capacity factor (k') of the cationic lipids GL-53, DC-chol, and DMRIE. See Materials and Methods section for RP-HPLC and ELSD conditions.

The sensitivity of ELSD can be maximized by optimization of the mobile phase system, flow rate, evaporative temperature, and nitrogen (or air) pressure.[28] After considerable experimentation, the optimum evaporative temperature was found to be 55°C and the optimum air pressure was found to be 20 psi in order to obtain the maximum sensitivity for this ACS model. The calibration curves of GL-53, DC-chol, DMRIE, and DOPE generated by RP-HPLC with ELSD detection are shown in Figure 5. The relationship of detector response with respect to sample weight for the cationic lipids is non-

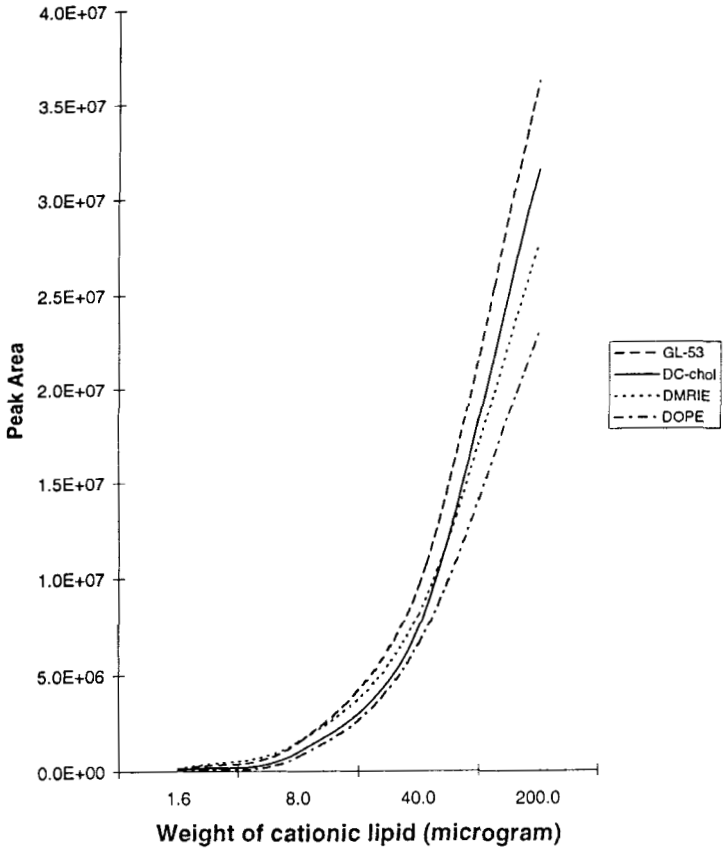


Figure 5. Calibration curves of ELSD response with respect to sample weight of GL-53, DC-chol, DMRIE, and DOPE. Lipid weights ranging from 1.6, 8.0, 40.0, and 200.0 μg are evaluated. See Materials and Methods section for RP-HPLC and ELSD conditions.

linear below 40.0 μg and approximately linear in the range of 40.0 to 200.0 μg for GL-53, DC-chol, DMRIE, and DOPE. ELSD detector non-linearity over broad weight ranges has been reported previously.[28] In the present study, the limits of detection were determined to be 0.4 μg for DMRIE, 0.4 μg for DDAB, 0.6 μg for GL-53, 0.7 μg for GL-67, 0.8 μg for DC-chol, and 1.6 μg for DOPE.

It is likely that a variety of other cationic amphiphile-based agents or formulations can be separated and characterized with these RP-HPLC methods. These methods have already proven to be useful in the identification of degradation products and in the analyses of purity and stability of the various cationic lipids and cationic lipid formulations prepared in our laboratories for research and for the clinic. The C8 and C18 bonded stationary phases, that we have examined, have exhibited long-term stability in the presence of mobile phases with high organic modifier concentrations and can be readily scaled-up for preparative separations. These methods should be readily adaptable for the LC-MS and for use in metabolism and pharmacokinetic studies of cationic lipid-based agents. Currently, we are applying this method to the analysis of a number of other classes of amphiphile-based agents.

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