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Development and Characterization of Liposomal Disodium Ascorbyl Phytostanyl Phosphates (FM-VP4)

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ABSTRACT

The specific objectives of this project were (1) to develop liposomal disodium ascorbyl phytostanyl phosphate (FM-VP4) formulations, (2) to develop a liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS) assay for quantification of FM-VP4 in liposomal formulations and plasma sample, and (3) to characterize liposomal FM-VP4 formulations by finding optimal drug-to-lipid ratios and determining the degradation of FM-VP4 in liposomes. Section 2 describes an LC/MS/MS assay developed for the identification and quantification of FM-VP4 in liposomal formulations to provide estimates of drug concentrations and encapsulation efficiency. The extra step of removing plasma proteins prior to LC/MS/MS assay yields an analysis of FM-VP4 in plasma samples. Section 3 describes experiments designed to find the optimal drug-to-lipid ratio for liposomal FM-VP4 formulations by comparing encapsulation efficiencies and varying the lipid compositions. Additionally, this section details our degradation studies to determine if liposomes have any protective effects on FM-VP4; these studies tested various lipid compositions at 37°C in rabbit plasma. The mechanism of how FM-VP4 lowers low-density lipoprotein (LDL) cholesterol and total cholesterol levels in various animal models is presently unknown. However, before the mechanism of action could be studied, FM-VP4 first had to be delivered efficiently into plasma or cultured cell. The low systemic bioavailability and cellular uptake of FM-VP4 further suggested the importance of finding an efficient delivery vehicle for this drug. This project proposed a framework

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for such delivery and paves the way for further investigation into how FM-VP4 works in vivo and in vitro.

Key Words: FM-VP4; LC/MS/MS; Liposomes; Phytostanols.

INTRODUCTION

Heart disease caused by atherosclerosis is one of the leading causes of death in North America. Although smoking and high blood pressure are considered major risk factors for this fatal disease, elevated concentrations of plasma cholesterol have been widely accepted as playing an equally important role in the development of cardiovascular disease. The prevalence of elevated levels of low-density lipoprotein (LDL) cholesterol to a large extent accounts for the high attendant risk for developing coronary heart disease over a lifetime, 49% in men and 32% in women. The benefits of reducing LDL cholesterol are well established.[1,2]

Recently, a novel, water-soluble plant stanol derivative, FM-VP4, has been developed. Although the lipid-lowering property of FM-VP4 has been demonstrated in dogs, gerbils, and rats,[3–6] the overall contribution of FM-VP4 to the inhibited cholesterol accumulation in Caco-2 cells was shown to be independent of pancreatic lipase activity, *p*-glycoprotein activity, or cholesterol incorporation in micelles.[7] Further insight into the role of FM-VP4 in the possible extracellular effects and intracellular effects will likely be obtained from animal or cell culture studies.

However, a limitation in the study of the mechanism of FM-VP4 is the low proportion of the drug that is absorbed after oral administration to animals. As little as 6.5% of ^{3}H-FM-VP4 at a dose of 20 mg/kg was shown to distribute in plasma 8 hours after oral administration to rats.[8] Even after 1 hour of incubation, only about 25% of ^{3}H-FM-VP4 at a concentration of 50 μM was shown to associate with intestinal epithelial (IEC)-6 cells.[9] Therefore, it is logical to enhance and optimize the delivery of FM-VP4 to animals or cultured cells prior to the investigation of its mechanism of action.

Liposomes have been used as carriers for various therapeutic agents. When phospholipids are hydrated with water, they form sheets of lipid bilayers that self-close to form spherical structures, called ''multilamellar liposomes,'' when the mixture is agitated. Once formed, multilamellar liposomes can be reduced in size by energy input either in the form of sonic energy (sonication) or mechanical energy (extrusion), resulting in the formation of uniformly sized large unilamellar vesicles (LUVs). Such vesicles have been shown to efficiently deliver biological macromolecules to animals and cultured cells.[10–13]

In order to characterize the liposomal FM-VP4 formulations, an assay that identifies and quantifies FM-VP4 in both liposomes and plasma was warranted. We used a liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS) assay for this quantification.

QUANTIFICATION OF FM-VP4 BY LC/MS/MS

Chemistry of FM-VP4

The two major components of FM-VP4 are disodium ascorbyl campestanyl phosphate and disodium ascorbyl sitostanyl phosphate. FM-VP4 is a semisynthetic esterified phytostanol derivative produced by the catalytic hydrogenation of campesterol and sitosterol to form campestanol and sitostanol, each of which is covalently linked to ascorbic acid by a phosphodiester bond (Fig. 1). A number of minor components are also present in FM-VP4, including the parent phytostanols, campestanol, and sitostanol. For purposes of molarity calculation in the following parts of this report, such minor components are assumed to be negligible and the adjusted material potency comprises only of two major components, disodium ascorbyl campestanyl phosphate and disodium ascorbyl sitostanyl phosphate in a 1 to 2 ratio.

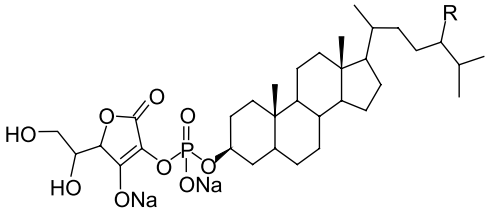

Figure 1. Structures of FM-VP4. Disodium ascorbyl campestanyl phosphate (R=methyl) and disodium ascorbyl sitostanyl phosphate (R=ethyl) are considered the major compounds of FM-VP4 and they are derived from phytostanols by the attachment of an ascorbic acid through a phosphate group. FM-VP4 is generally obtained in a mixture of disodium ascorbyl campestanyl phosphate and disodium ascorbyl sitostanyl phosphate in a ratio around 1:2.

MATERIALS AND METHODS

Materials

1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine (DMPC) was purchased from Lipoid GmbH (Ludwigshafen, Germany). Cholesterol, ammonium acetate, HEPES, ammonium hydroxide, sodium hydroxide, and sodium chloride were obtained from Sigma (Mississauga, Ontario, Canada), while FM-VP4 was manufactured by Forbes Medi-Tech, Inc. (Vancouver, British Columbia, Canada). An extruder and its accessories were purchased from Northern Lipids (Vancouver, BC). Tetrahydrofuran (THF) and acetonitrile (ACN) were obtained from Fisher (Vancouver, BC). Rabbit plasma was produced by Bioreclamation, Inc. (Hicksville, NY). Spectra/Por 2® membrane tubing (12,000 to 14,000 Dalton MWCO) was purchased from Fisher (Vancouver, BC).

Preparation of Buffers and Mobile Phases

Ammonium acetate solution at a concentration of 5 mM and a pH of 8.4 was prepared by dissolving 770.8 mg of ammonium acetate crystal in 2 L of distilled water. The final pH of this solution was adjusted to 8.4 by the addition of a few drops of 20% ammonium hydroxide. A THF/ACN 50/50 solution was prepared by mixing equal amounts of THF and ACN. A fourfold HEPES-buffered saline (HBS) solution was prepared by dissolving 38.128 g of HEPES and 70.128 g of sodium chloride in 2 L of distilled water, and the final pH of the solution was adjusted to 7.4 by the addition of sodium hydroxide. Before use, this fourfold solution was reconstituted with distilled water at a 1:3 ratio to yield HBS (1X), which has a final concentration of 15 mM of HEPES and 150 mM of NaCl.

Instrumentation

Analyses were performed on a Micromass Quattro (I) LC/MS/MS connected to a Hewlett Packard 1090 Series II Liquid Chromatograph, which is comprised of an autosampler, a column compartment, and a binary pump. The data acquisition program used was MassLynx software Version 3.5.

Chromatography and Mass Spectrometric Condition

The compounds of interest were separated on a Phenomenex C_{18}, 30×2.0 mm (Torrance, CA) 3-μL column at a flow rate of 0.3 mL/min. The mobile phases were 5 mM ammonium acetate, pH 8.4 (A), and THF/ACN 50/50 (B). Mobile phase B was set at 5% initially, increased to 90% over 2 min, and maintained at that gradient for 2 min. The final gradient of 5% was held for 5 minutes to give a run time of 9 min; the injection volume was 10 μL.

Under negative electrospray ionization, the mass spectrometer was operated in the multiple reaction monitoring (MRM) mode for quantitative analysis and synchronization with the liquid chromatography.[14,15] The nebulizing gas, nitrogen, was set at 100 V, while the collision gas, argon, at 38 eV.

Preparation of Standard Solution and Calibration Curve for Liposomal Samples

Initially, the reference standards (FM-VP4) were dissolved in HBS to produce stock solutions that in turn were diluted in 5 mM ammonium acetate (pH 8.4) and a solution of THF/ACN 50/50 containing 0.2 mg/mL of liposomal DMPC/cholesterol mixtures, so as to obtain solutions of various standards (range: 2.50 to 20.0 μg/mL). Calibration curves with five points were used for routine sample analysis; calibration curves with nine points were used for quality assurance. All standard solutions were freshly prepared for every assay.

Limit of Detection (LOD) Determination

The usual limit of detection (LOD) for an analyte is three standard deviations (SD) above the mean of the blank. The blank in the assay for FM-VP4 in liposomal samples contained 0.2% lipids (DMPC/cholesterol) in the ACN/THF 50/50 solution. The blank for the assay of FM-VP4 in plasma samples contained 50% plasma diluted 10-fold and 50% ACN/THF 50/50 solution; the plasma protein in the blank was then removed by ultracentrifugation. The LOD was determined by liquid chromatography (LC) system analysis of three prepared sets of each of the two different blanks. The magnitudes of the signal produced by the blank were determined at the retention times corresponding to ascorbyl campestanyl phosphate and to ascorbyl sitostanyl phosphate. The minimum detectable signal can be expressed by the following equation:

$$S_m = S_{blank} + 3\ SD_{blank}$$

where S_m, S_{blank}, and SD_{blank} stand for the minimum detectable signal, the mean of the blank signal, and the standard deviation of the blank signal, repectively. This calculation of the minimum detectable signal applied to

a standard curve yields the minimum detectable concentration of FM-VP4 in either medium.

Limit of Quantification (LOQ) Determination

The limit of quantification (LOQ) is often set at the point where the coefficient of variance (CV) is larger than one is prepared to accept, because as the concentration of the analyte falls, the measurement becomes less precise. On a given day, intraday CVs were determined through analysis of data obtained from three sets of standards with known concentrations at the following levels: 0.00, 0.156, 0.313, 0.625, 1.25, 2.50, 5.00, 10.0, and 20.0 μg/mL. After these sets were prepared, they were analyzed in the LC/MS/MS system. The magnitude of signals yielded from the analytical system could in turn be used on a calibration curve of the same range to calculate concentrations of the above standards. Calculated concentrations of the above standards were determined by a calibration curve of the same range. Then the average and SD of the calculated concentration could be found, so that, finally, the intraday CVs could be determined. Interday CVs were calculated through the same process of analysis, except that a single set of standards of the same concentrations was prepared and then analyzed each day, for several days. Their calculated concentrations were determined by a calibration curve derived from another set of the same concentrations prepared on the same day that each analysis took place. Thus, the interday average, SD, and CV of the calculated concentrations could be found. Both the intraday and interday CV values allow one to determine the precision of the analytical machine.

Preparation of Liposomal FM-VP4

The DMPC/cholesterol (55:45 molar ratio) liposomes were prepared by extrusion. (DMPC/cholesterol at molar ratios of 70/30, 85/15, and 100/0 will be discussed in ''Preparation of Large Unilamillar Vesicles'' under ''Development and Characterization of Liposomal FM-VP4'') Lipids were dissolved in chloroform and then dried down to a lipid film under a stream of nitrogen gas. The lipid film was dried further under vacuum for 2 hours to remove any residual chloroform. The lipid film was then hydrated with 15 mM HBS (pH 7.4) to achieve a drug-to-lipid molar ratio of 0.1. The resulting multilamellar vesicle mixture was frozen and thawed five times and extruded 10 times through two stacked 100-nm polycarbonate filters with an extrusion device. The size of the large unilamellar vesicle was analyzed by a Zetasizer 3000HS (Malvern Instruments, Southborough, MA), and drug-loaded liposomes were collected after 30 hours of dialysis.

Extraction of Plasma Samples

Plasma samples obtained from normoalipidemic rabbits were used for the extraction studies. One hundred microliters of plasma sample was added to clean 2-mL eppendorf® tubes. Nine hundred microliters of 5 mM ammonium acetate was then added to achieve a 10-fold dilution of the samples. Protein was precipitated with an equal volume of ACN/THF by vortexing the solution for 30 seconds and sonicating it for 10 minutes. After centrifugation for 5 minutes at $8000 \times g$, the supernatant layer was transferred to an autosampler vial; 10 μL was injected into the liquid chromatograph.

Preparation of Standard Solutions and Calibration Curves for Plasma Samples

Blank plasma was diluted by 10 times in 5 mM ammonium acetate prior to preparation of the calibration curve. Plasma samples were then spiked with different amounts of FM-VP4. Standards of lower concentrations were subsequently prepared by serial dilution in the same medium. Protein was removed from plasma as described above. Calibration curves with five points (2.50, 5.00, 10.0, 15.0, and 20.0 μg/mL) prepared in diluted plasma were used for routine sample analysis, while calibration curves with nine points (0.156 to 20.0 μg/mL) were used for quality assurance.

Extraction Efficiency Studies

Specific concentrations of FM-VP4 were added to diluted plasma samples, and the protein of the samples was removed as described previously. The concentrations of FM-VP4 recovered by the extraction procedure were determined using a calibration curve constructed with standard FM-VP4 in 5 mM ammonium acetate and ACN/THF solution.

Sample Collection of FM-VP4

Plasma samples obtained from experiments involving incubation of FM-VP4 at 37°C were used to test the application of this assay to the study of the

degradation of FM-VP4. FM-VP4 was incubated in rabbit plasma at 37°C for 7 days. Aliquots were obtained for determining the stability of FM-VP4.

DEVELOPMENT AND CHARACTERIZATION OF LIPOSOMAL FM-VP4

Materials and Methods

Materials

The DMPC we used was purchased from Lipoid GmbH (Ludwigshafen, Germany); cholesterol, HEPES, INFINITY cholesterol diagnostic reagent, and sodium chloride from Sigma (Mississauga, Ontario, Canada). The FM-VP4 was manufactured by Forbes Medi-Tech Inc. (Vancouver, BC). The extruder and accessories were purchased from Northern Lipids (Vancouver, BC), and the phospholipid assay kits from Roche Diagnostics (Mannheim, Germany). The EDTA anticoagulated red blood cells and rabbit plasma were produced by Bioreclamation, Inc. (Hicksville, NY). Spectra/Por 2® membrane tubing (12,000 to 14,000 Dalton MWCO) was purchased from Fisher (Vancouver, BC).

Preparation of Buffers

HEPES-buffered saline 15 mM was prepared by dissolving 4.766 g of HEPES powder and 8.766 g of NaCl in 1 L of distilled water; the final pH of this buffer was adjusted by the addition of sodium hydroxide.

Preparation of Large Unilamillar Vesicles (LUVs)

The DMPC/cholesterol liposomes at molar ratios of 55/45, 70/30, 85/15, and 100/0 were prepared by extrusion as previously described.[16,17] Lipid mixtures of DMPC and cholesterol were dissolved in chloroform and dried under a stream of nitrogen gas to produce a lipid film, which was dried further under vacuum for more than 2 hours to remove residual organic solvent. To achieve drug-to-lipid ratio, the lipid film was then hydrated in a 55°C water bath in a 15 mM HEPES-buffered saline (pH 7.4) containing FM-VP4. This suspension was frozen and thawed five times with liquid nitrogen and a 55°C water bath. The hydrated lipid mixture was extruded 10 times through a two-stacked 100-nm polycarbonate filter at 55°C with an extruder. The phospholipid and cholesterol contents of the LUVs were determined using the phospholipids assay kit and cholesterol diagnostic reagent. The quantification of FM-VP4 was measured by our LC/MS/MS assay.

FM-VP4 Encapsulation Experiments

We dissolved FM-VP4 in HBS prior to the hydration of the lipid film to incorporate it into the LUV. The concentration of FM-VP4 employed in each experiment varied, depending on the desired drug-to-lipid ratios. After the addition of FM-VP4, the multilamellar lipid concentration of about 5 mM was extruded 10 times. Drug-loaded liposomes were collected after 30 hours of dialysis. The drug-to-lipid ratios were determined at three steps in the process: before extrusion, after extrusion, and after dialysis. The encapsulation efficiency of FM-VP4 was defined as the drug-to-lipid ratio after dialysis divided by the original drug-to-lipid ratio multiplied by 100%.

Preparation of FM-VP4 Micelles

The FM-VP4 was dissolved in HBS, producing a 10 mM stock solution, and FM-VP4 micelles formed spontaneously due to its structural characteristics. Solutions of lower concentrations were subsequently prepared by further diluting the stock solution in HBS. Each concentration was subjected to size analysis by a Zetasizer.

Quantification of FM-VP4

Briefly, the assay was performed employing a Micromass Quattro (I) LC/MS/MS connected to a Hewlett Packard 1090 series II Liquid Chromatograph. FM-VP4 was separated on a Phenomenex C_{18}, 30 × 2.0 mm, 5 μ Column (Torrance, CA). The reference standards were dissolved initially in HBS and then diluted in appropriate media to produce standards of various concentrations (range: 2.5–20.0 μg/mL or 3.6–28.8 μmol/L). Samples were diluted by 5 mM ammonium acetate and their protein contents were removed by ACN/THF solution when necessary.

Particle Size Determination

The sizes of liposomes and FM-VP4 micelles were determined by photon correlation spectrometry (PCS),

also known as dynamic light scattering (DLS) or quasi-elastic light scattering (QELS), using a Zetasizer 3000HS (Malvern Instruments, Southborough, MA).

Phospholipid Assay

Phospholipid concentrations were determined by phospholipid enzymatic colorimetric test kits produced by Roche Diagnostics (Mannheim, Germany). Testing reagents were prepared according to the manufacturer's guidelines just before the assay: briefly, 20 μL of either a sample or a standard were added to 3 mL of the premixed testing reagents, which contain phospholipase D, choline oxidase, phenol, 4-aminophenzone, and peroxidase. Each mixture was incubated for 10 minutes at 37°C. Then its concentration of phospholipids was analyzed by UV-vis spectroscopy at 500 nm, which allowed a standard curve with a linear range of 0.188 mg/mL to 3.00 mg/mL to be constructed

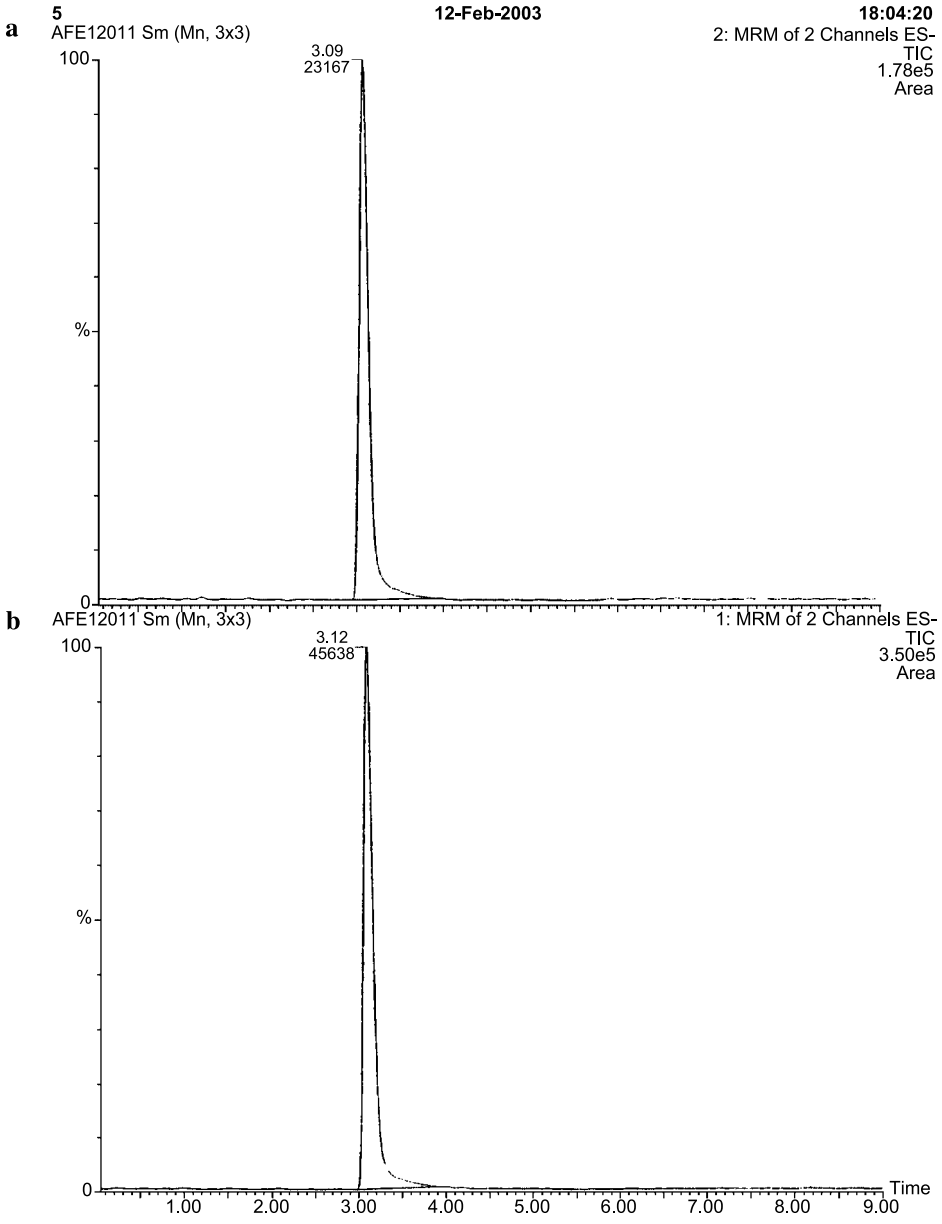

Figure 2. Total ion chromatogram obtained from LC/MS/MS assay of FM-VP4. a) Ascorbyl campestanyl phosphate. b) Ascorbyl sitostanyl phosphate.

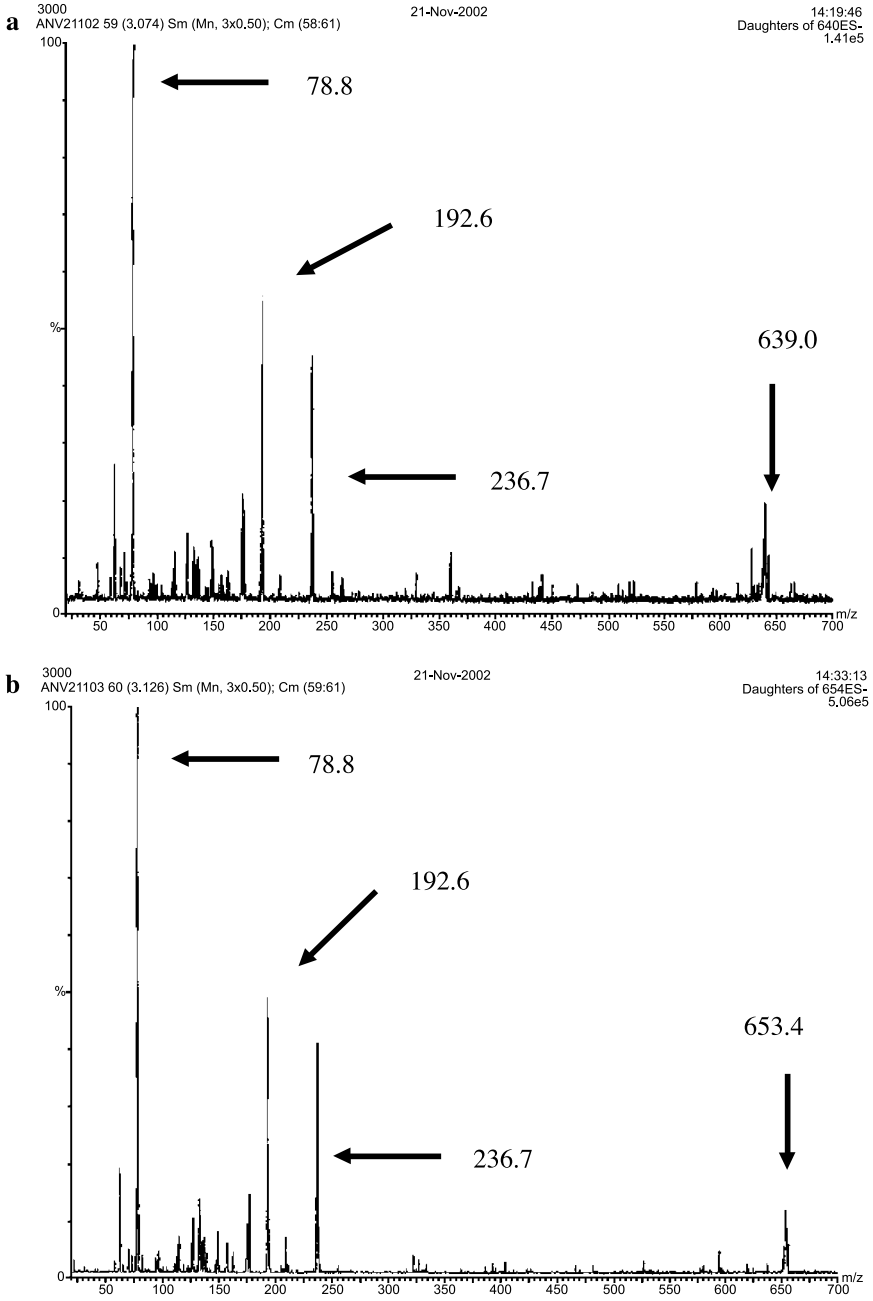

Figure 3. Mass spectrum obtained from LC/MS/MS assay of FM-VP4 reference standard. a) Ascorbyl campestanyl phosphate. b) Ascorbyl sitostanyl phosphate.

for every assay. The testing principle of this phospholipid assay is described below:

$$\text{Phospholipid} + H_2O \xrightarrow{\text{phospholipase D}} \text{choline} + \text{phosphatidic acid}$$

$$\text{Choline} + 2\ O_2 + H_2O \xrightarrow{\text{choline oxidase}} \text{betaine} + 2\ H_2O_2$$

$$2\ H_2O_2 + \text{4-aminophenazone} + \text{phenol} \xrightarrow{\text{phospholipase D}} \text{4-(}p\text{-benzoquinone-mono-imino)-phenazone} + 4\ H_2O$$

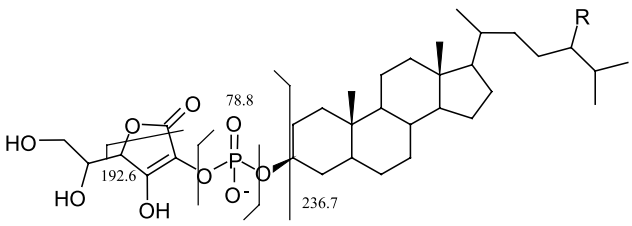

Figure 4. Possible *m/z* assignment of FM-VP4 for the mass spectrum.

Cholesterol Assay

The cholesterol concentrations in the liposomal samples were measured with a Sigma INFINITY Cholesterol Reagent. This testing reagent was reconstituted with distilled water, according to the manufacturer's instructions. Briefly, 10 μL of either a sample or a standard were mixed thoroughly into 1 mL of testing reagent. After incubation at 37°C for 5 minutes, each solution was analyzed by UV-vis spectrometry at 500 nm. The calibration curve was within a range of 0.125 mg/mL to 4.00 mg/mL. The series of reactions of the assay is as follows:

$$\text{Cholesterol ester} \xrightarrow{\text{cholesterol esterase}} \text{Cholesterol} + \text{Fatty acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{cholesterol oxidase}} \text{Cholest-4-en-3-one} + H_2O_2$$

$$2H_2O_2 + \text{hydroxybenzoic acid} + \text{4-aminoantipyrine} \xrightarrow{\text{peroxidase}} \text{Quinoneimine Dye} + 4\ H_2O$$

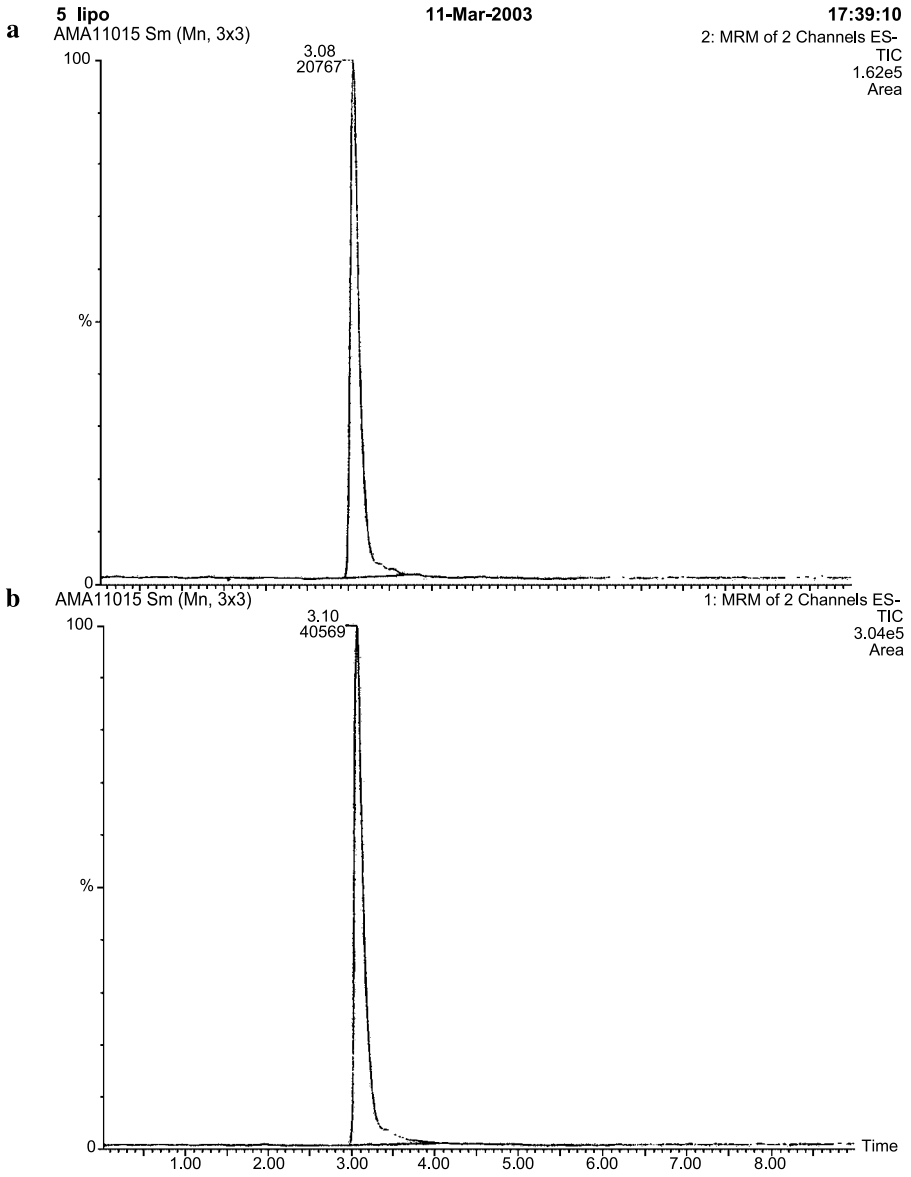

Figure 5. Total ion chromatogram of FM-VP4 standard obtained from DMPC/cholesterol lipid mixture. a) Ascorbyl campestanyl phosphate. b) Ascorbyl sitostanyl phosphate.

Degradation Studies

A liposomal FM-VP4 formulation loaded at a final drug-to-lipid ratio of 0.5 was used in the degradation studies. Each sample was incubated in rabbit plasma at 37°C for 7 days and the final concentration of FM-VP4 adjusted to about 100 μM. Aliquots were obtained at specific time points for determining the stability of FM-VP4.

Statistical Analysis

A one-way analysis of variance (ANOVA, JumpIn, SAS Institute, Inc.) was used for determining the statistical significance of results for all the liposomal formulation studies. The critical difference was set at $p < 0.05$ and the data were expressed as mean ± standard deviation. FM-VP4 LC/MS/MS chromatographs are presented as representative figures of numerous determinations.

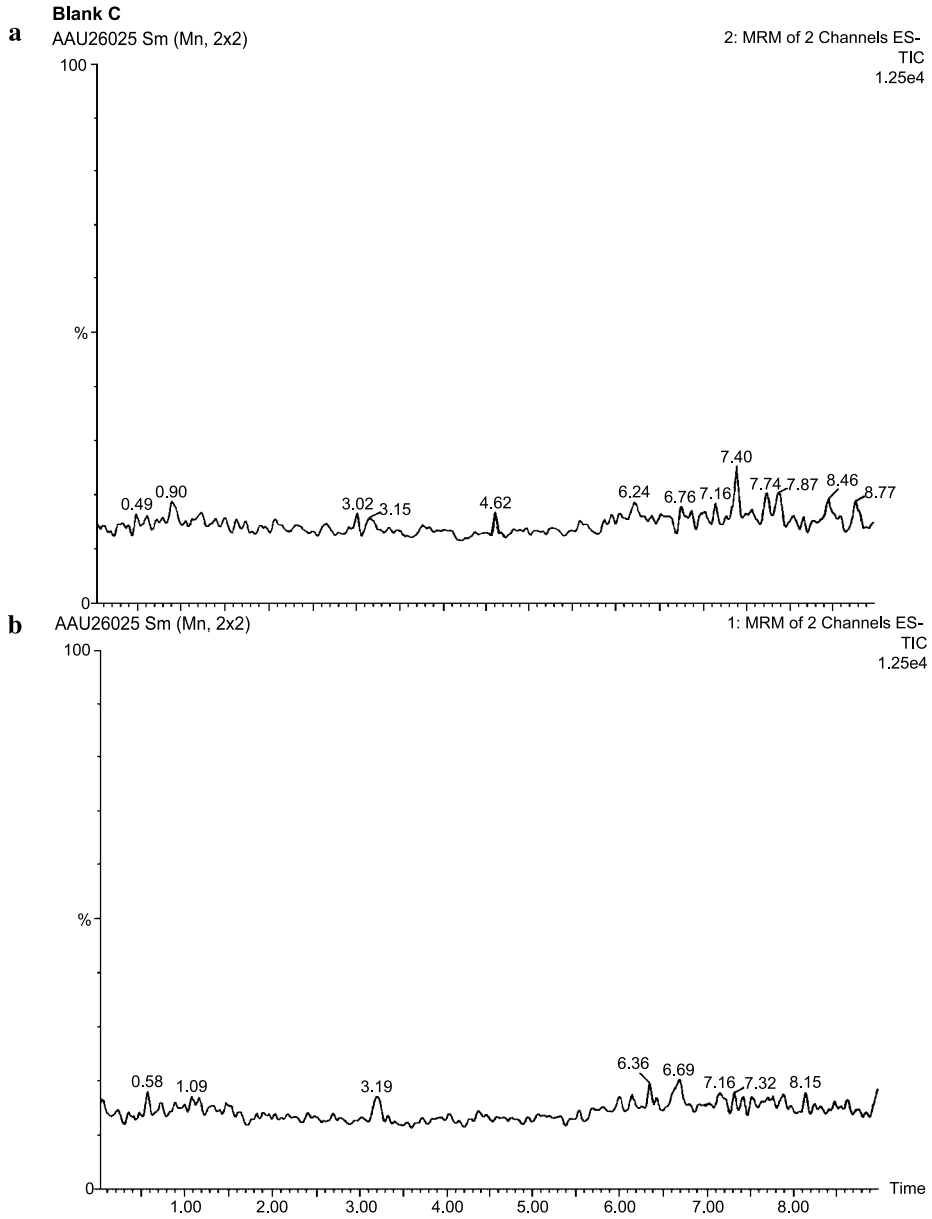

Figure 6. Total ion chromatogram of a blank for the assay of FM-VP4 in liposomal samples. a) Ascorbyl campestanyl phosphate. b) Ascorbyl sitostanyl phosphate.

RESULTS

Quantification of FM-VP4 by LC/MS/MS

Chromatography and Mass Spectra

Figure 2 shows the total ion chromatograms obtained from FM-VP4 (ascorbyl sitostanyl phosphate and ascorbyl campestanyl phosphate) dissolved in HBS. Ascorbyl campestanyl phosphate was eluted out from the separating column a little earlier than ascorbyl sitostanyl phosphate was. Figure 3 shows the mass spectra of FM-VP4. The suggested *m/z* assignments of some of the fragments are shown in Fig. 4. Figure 5 shows the total ion chromatograms obtained from FM-VP4 standard solution prepared with a mixture of DMPC/cholesterol lipid. Figure 6 represents the blank chromatograms obtained from a mixture of DMPC/cholesterol lipid. Figure 7 shows the total ion chromatograms obtained from FM-VP4 standard solution prepared with rabbit plasma diluted 10-fold. Figure 8

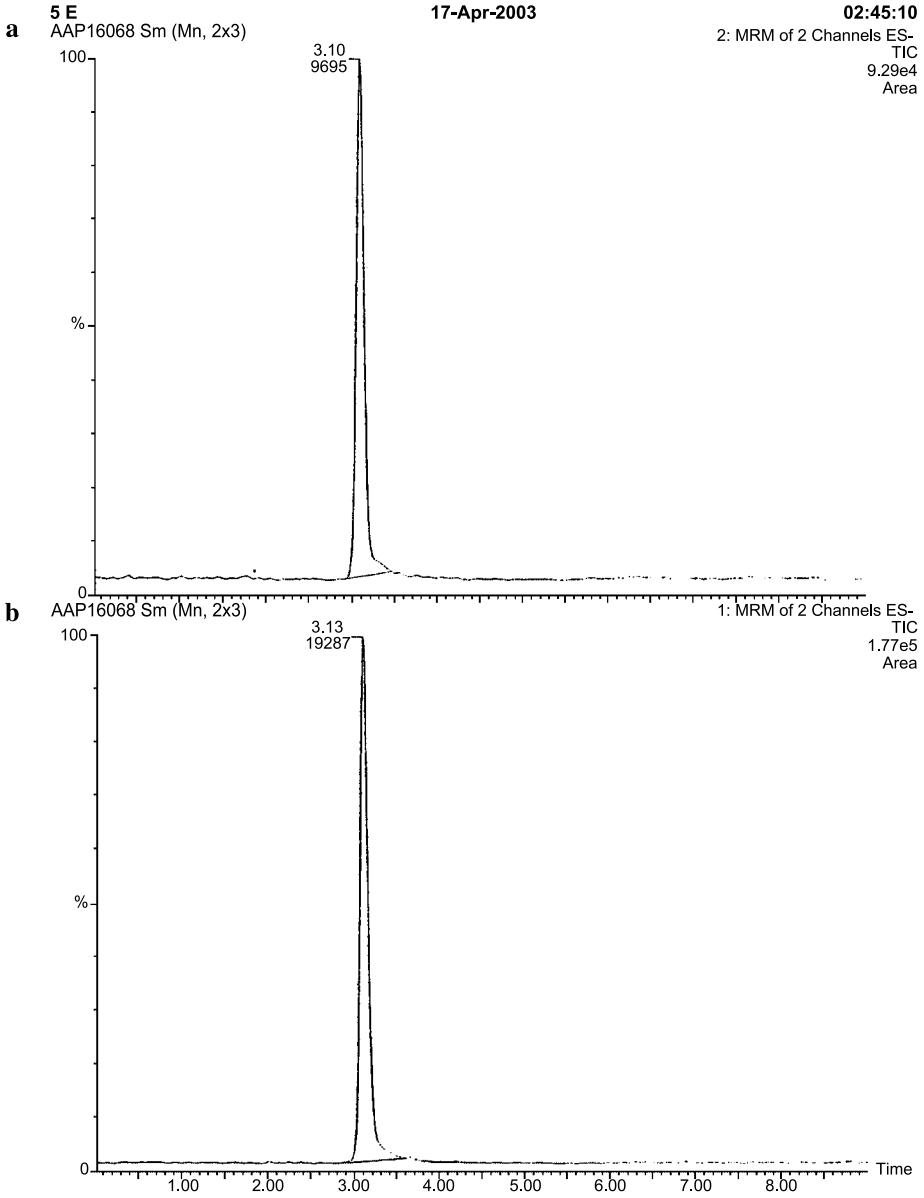

Figure 7. Total ion chromatogram of FM-VP4 standard obtained from ten-fold diluted rabbit plasma. a) Ascorbyl campestanyl phosphate. b) Ascorbyl sitostanyl phosphate.

represents the blank chromatograms obtained from rabbit plasma diluted 10-fold.

Quantification of FM-VP4 in Liposomes

Quantification was based on the sum of peak areas plotted against the corresponding concentrations of FM-VP4. Figure 9, showing a typical calibration curve obtained following dilution of the reference standards, indicates that quantification was linear in the examined range of 2.50–20.0 μg/mL of FM-VP4 ($0.99 \leq r^2 \leq 1.00$). Aliquots of liposome samples were used to estimate the concentration of FM-VP4 in liposomal formulation. The FM-VP4 concentrations in these samples were well above the minimum quantification limit of 1.25 μg/mL. A typical total ion chromatogram obtained from FM-VP4 liposomal formulation is shown in Fig. 10. Here, the peaks show baseline resolution; there is no interference from other substances in liposomes. The encapsulation efficiency (drug-to-lipid ratio after dialysis/original drug-to-lipid

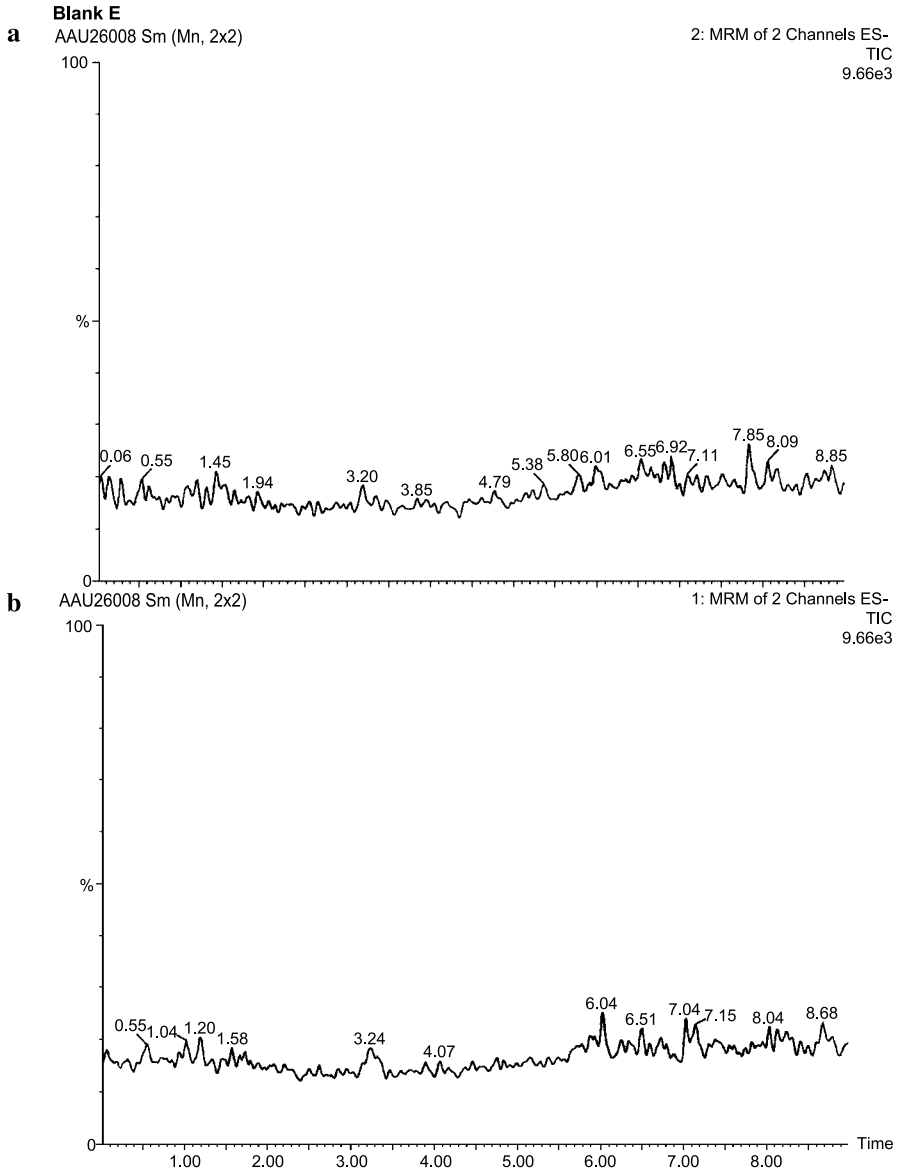

Figure 8. Total ion chromatogram of a blank for the assay of FM-VP4 in plasma samples. a) Ascorbyl campestanyl phosphate. b) Ascorbyl sitostanyl phosphate.

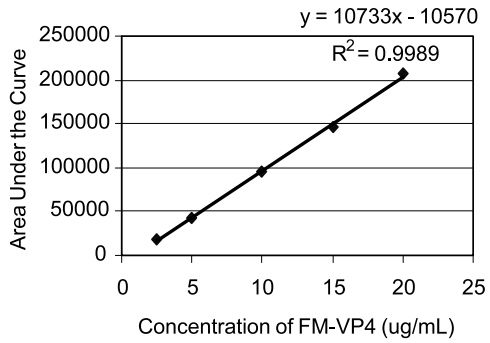

Figure 9. A typical calibration curve of FM-VP4 for liposomal samples (range: 2.50–20.0 μg/mL).

ratio multiplied by 100%) at a drug-to-lipid ratio of 0.1 was about 91%.

Validation of Assay Procedure

Figure 11 shows a representative calibration curve obtained following extraction of spiked rabbit plasma samples. Quantification was found to be linear in the range of 2.50–20.0 μg/mL FM-VP4 ($0.99 \leq r^2 \leq 1.00$). The interday coefficients of variation were found to be less than 5% in the examined concentrations of 2.50, 5.00, 10.0, 15.0, and 20.0 μg/mL. When the efficiency of the extraction procedure was studied at the above concentration, the mean extraction recovery was found to be 112–7%.

Identification of FM-VP4

The concentration of FM-VP4 in the plasma samples obtained from 37°C incubation was used to estimate the degradation of FM-VP4 after 7 days. A typical total ion chromatogram obtained from rabbit plasma containing FM-VP4 was shown in Fig. 12. The FM-VP4 concentration in these samples was well above the limit of quantification of 1.25 μg/mL. The concentration of FM-VP4 remaining in the 37°C rabbit plasma after 7 days was found to be 58%.

Development and Characterization of Liposomal FM-VP4

Drug-to-Lipid Ratio

Initially, one drug-to-lipid ratio, 0.1, was utilized to study the encapsulation efficiency of FM-VP4 in liposomes. The drug-to-lipid ratios were traced throughout the manufacturing process. Figure 13 shows the drug-to-lipid ratios of various formulations before extrusion, after extrusion, and after dialysis. Apparently, the steps of preparation led to a slight deviation from the original drug-to-lipid ratios; both drug and lipids could have been lost during the processes. The original drug-to-lipid ratios and the drug-to-lipid after dialysis were used to calculate the encapsulation efficiency.

Figure 14 shows that all formulations demonstrated similar encapsulation efficiencies greater than 90%. The same procedures were repeated for three additional drug-to-lipid ratios, 0.3, 0.6, and 1.0. Further details were given in ''Preparation of Large Unilamillar Vesicles'' under ''Development and Characterization of Liposomal FM-VP4.''

Lipid Content

Our lipid composition analysis of the DMPC and the cholesterol contents of liposomes using the phospholipid assay kits and cholesterol diagnostic reagents before extrusion and after dialysis indicates that the manufacturing processes had no effect on the lipid contents of various formulations. After extruding the liposomal mixture for 10 cycles and dialyzing it for 30 hours, the ratios of DMPC/cholesterol were almost the same as in the original preparations. For example, in the presence of FM-VP4 at a drug-to-lipid ratio of 0.1, the reestablished DMPC/cholesterol ratios of the 55/45, 70/30, 85/15, and 100/0 formulations after the procedures were 57/43, 71/29, 85/15, and 100/0, respectively (Fig. 15). The comparable lipid compositions identified following extrusion and dialysis of liposomes with drug-to-lipid ratios of 0.3, 0.6, and 1.0 are shown in Fig. 15. The DMPC/cholesterol ratios were not changed after the incorporation of FM-VP4. The fact that the lipid content ratios were maintained suggests that the lipid compositions of liposomes are not affected by FM-VP4.

Encapsulation Efficiency

In our assay, FM-VP4 was loaded in the liposomes at various drug-to-lipid ratios: 0.1, 0.3, 0.6, and 1.0. Up to a drug-to-lipid ratio of 0.6, an encapsulation efficiency of approximately 80% was achieved (Fig. 16). Formulations at different lipid compositions under 0.6 demonstrated a similar loading efficiency; however, the loading efficiency of FM-VP4 decreased to about 60% for most formulations when the drug-to-lipid ratio

was 1.0. Therefore, formulations prepared at a drug-to-lipid ratio of 0.6 were used to further characterize liposomal FM-VP4.

Degradation of FM-VP4

To assess the stability of FM-VP4 in liposomal formulations, we conducted in vitro degradation studies in rabbit plasma. The FM-VP4 was loaded at a final drug-to-lipid ratio of 0.5. The liposomes thus loaded with FM-VP4 were diluted by rabbit plasma and incubated at 37°C for 7 days, creating a final FM-VP4 concentration in rabbit plasma of 100 μM. Over the first 2 days of this incubation period, the concentration of the free FM-VP4 decreased to 79% and 58% by the end of the 7-day period (Fig. 17). This initial drop in the percentage of FM-VP4 remaining indicated that it degraded promptly in plasma.

Encapsulation of FM-VP4 increased its stability. The 55/45 formulation apparently virtually did not degrade in the first 4 days, and its concentration remained at greater than 95%. The free FM-VP4, in

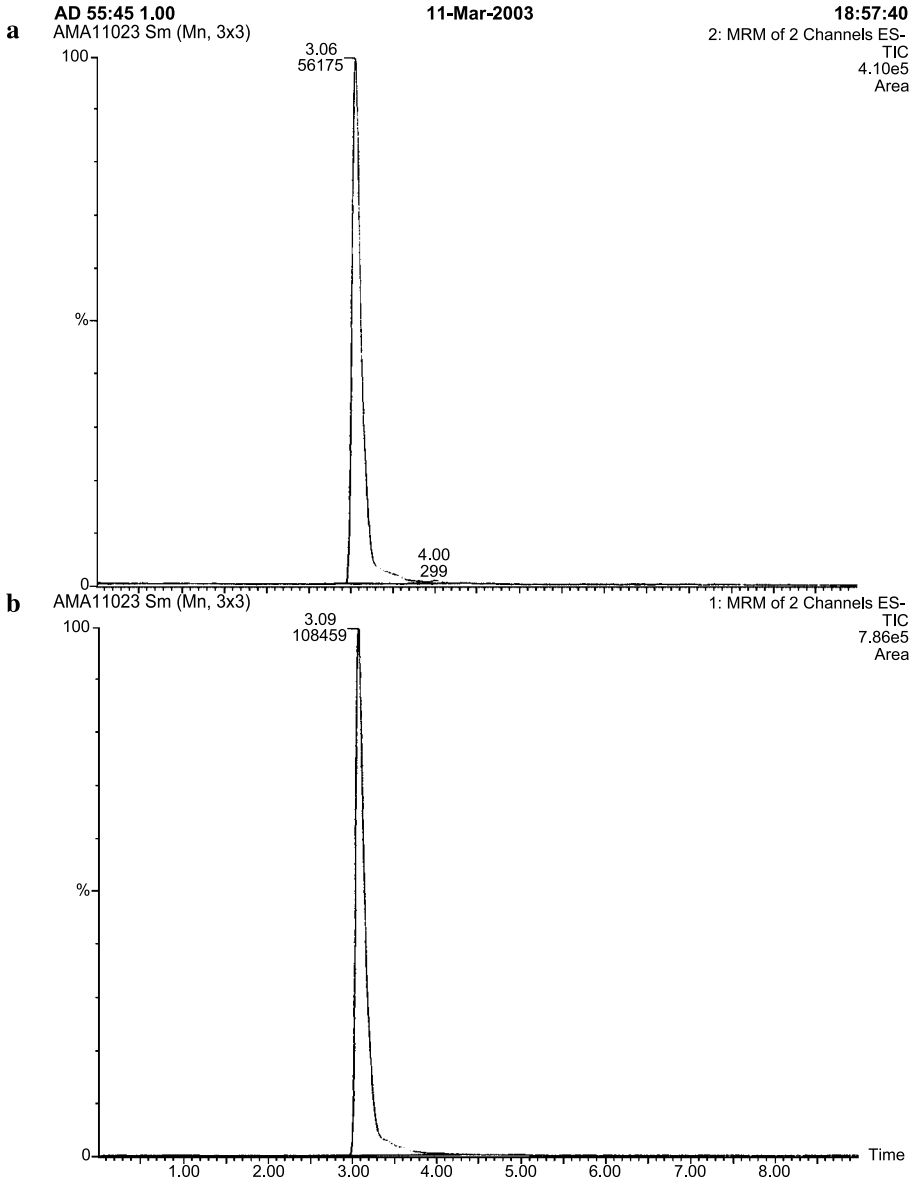

Figure 10. Total ion chromatogram of FM-VP4 sample obtained from DMPC/cholesterol liposomal formulation. a) Ascorbyl campestanyl phosphate. b) Ascorbyl sitostanyl phosphate.

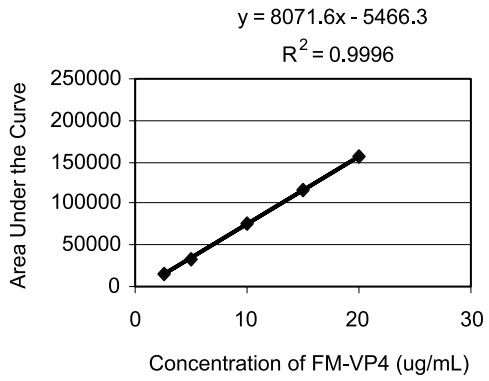

Figure 11. A representative calibration curve obtained following extraction of spiked rabbit plasma samples (range: 2.50–20.0 μg/mL).

contrast, degraded to 69% during this period. Because of the reduced cholesterol content in the 70/30, 85/15, and 100/0 lipid compositions, FM-VP4 in these formulations degraded slightly faster than in the 55/45 formulation, varying from 79–90% for the first 2 days, and then decreasing to 67–78% after 4 days.

DISCUSSION

One objective of these studies was to develop an assay for the identification and quantification of FM-VP4 in liposomal formulation to estimate the drug's concentration and encapsulation efficiency, following extrusion of the lipid mixtures to reduce the size of liposomes, and dialysis to remove free drug from the drug-loaded liposomes.

Figure 10 shows the presence of FM-VP4 in the liposomal formulation. Identification of FM-VP4 in samples suggests the incorporation of FM-VP4 in liposomes. A preliminary estimation of the encapsulation efficiency of FM-VP4 in liposomes made up of DMPC/cholesterol is about 100%. Our results also suggest that at least some of the FM-VP4 remained stable throughout the manufacturing process.

The mass spectra of ascorbyl campestanyl phosphate and ascorbyl sitostanyl phosphate show that the *m/z* 78.8–78.9 fragment is very intense, but this intensity may be due to the presence of a phosphate group in each molecule. Therefore, despite the intensity of the *m/z* 78.8–78.9 fragment in the mass spectra, two larger ions (192.6 and 236.7 *m/z*) representing ascorbyl phosphate groups in each of the two compounds were chosen for quantification instead of the more abundant phosphate ions because these larger ions would improve the selectivity of the assay. The parent ions of FM-VP4 were not included in the quantification of the drug, because the signal produced by these parent ions in the second mass spectrometer was observed to be very small and because the 192.6 and 236.7 *m/z* fragments are highly representative of the molecules of FM-VP4.

Negative ions were obtained through the use of negative electrospray ionization and through the use of 5 mM ammonium acetate (pH 8.4) as the mobile phase and as part of the diluent for FM-VP4. The limit of detection of an assay may be defined as the minimum concentration of an analyte that, by the magnitude of its signal, can be clearly distinguished from background noise. To reach this minimum, the magnitude of the sample's signal should be three standard deviations above the mean of the blank. The minimum concentration can then be identified from the slope of the standard curve.[18] The limit of detection for liposomal samples in this assay was 0.17 μg/mL. The limit of quantification, the point beyond which variation is unacceptable, depends on the precision of measurements.[18] The limit of quantification of this assay was 1.25 μg/mL, because below this concentration the interday coefficient of variance (CV) rose above 15%.

Not much is know about FM-VP4's mechanism, but its lipid-lowering effect has been well demonstrated. It is interesting to note that since the absorbability of FM-VP4 is about 6.5%, liposomal FM-VP4 may, at least in part, mediate the intravenous administration of FM-VP4. Also, liposomes may increase the cellular uptake of FM-VP4, so that the intracellular mode of action of FM-VP4 can then be studied. Further experiments to elucidate the mechanism of FM-VP4 are underway.

Another objective of this study was to develop an assay to identify and quantify FM-VP4 in plasma.

Figure 12 shows the presence of FM-VP4 after incubating at 37°C in rabbit plasma. The decreased concentration of FM-VP4 suggests its degradation in rabbit plasma: as FM-VP4 degraded in rabbit plasma, about 79% of the FM-VP4 dose remained unchanged after 2 days and the concentration of FM-VP4 further decreased to 58% after 7 days. However, while FM-VP4 thus clearly undergoes degradation in plasma, its degradation pathways are not fully understood. The limit of detection of this assay for plasma samples was 0.51 μg/mL. The limit of quantification was 1.25 μg/mL; below this concentration, the interday coefficient of variance (CV) was found to be larger than 15%.

Even though the intact compounds of FM-VP4 were detected in the plasma samples, it is possible that

phytostanol, the parent compound of FM-VP4, mediates the observed lipid-lowering effects in different animal models. Interestingly, phytostanol, the parent compound of FM-VP4, was not detected in the FM-VP4-injected plasma by this assay method. Our laboratory is undertaking further experiments to explore how FM-VP4 and phytostanol act.

FM-VP4 was derived from plant sterols and stanols, compounds which naturally occur in vegetable oils, seeds, and nuts. Plant sterols and stanols, called ''phytosterols'' and ''phytostanols,'' have been shown to decrease LDL cholesterol levels in animals and humans.

Chemically, FM-VP4 was synthesized from campestanol and sitostanol, which are covalently linked to an ascorbic acid group by a phosphodiester bond. FM-VP4 has been found to be effective in lowering lipid levels in different animals; it has also been shown to delay the progression of atherosclerotic lesions in ApoE deficient mice. However, the mechanisms of FM-VP4's actions remain unknown at present.

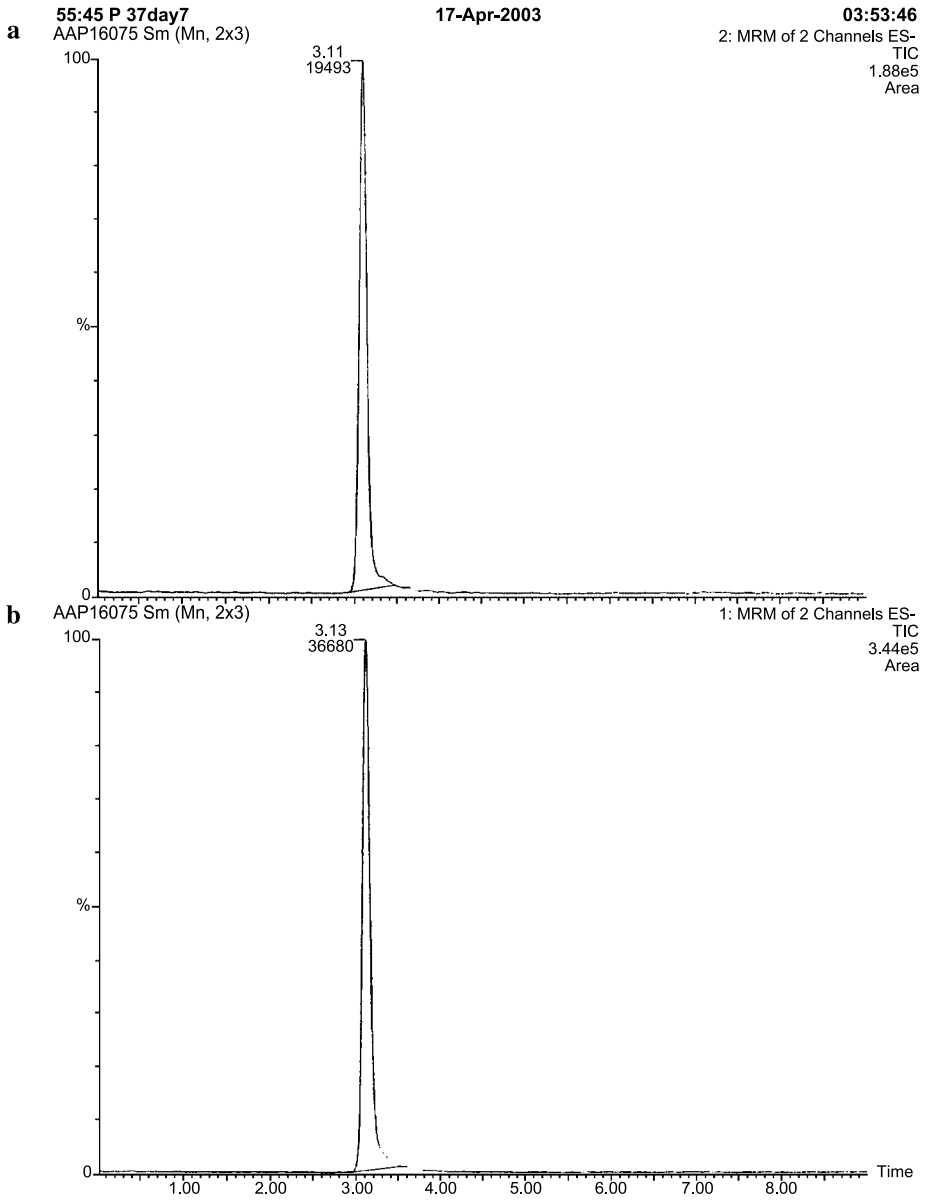

Figure 12. Total ion chromatogram of FM-VP4 sample obtained from rabbit plasma samples. a) Ascorbyl campestanyl phosphate. b) Ascorbyl sitostanyl phosphate.

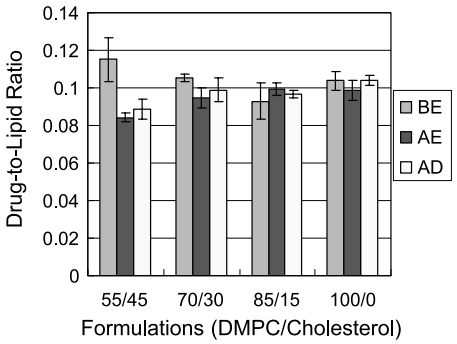

Figure 13. Drug-to-lipid ratio established before extrusion, after extrusion, and after dialysis for formulations at different DMPC/cholesterol ratios. The original drug-to-lipid ratio was 0.1. Data presented as mean ± standard deviation (n=6).

We discovered that a high concentration of FM-VP4 in plasma may be difficult to achieve: only 6.5% of an orally administered dose was absorbed. In addition, when FM-VP4 is incubated in rabbit plasma at 37°C, it is metabolized and its concentration decreases to 80% in 2 days. As a consequence, we have recently investigated whether the encapsulation of FM-VP4 in liposomes would prevent FM-VP4 from degrading in plasma.

This study recorded that the ability of liposomes to protect FM-VP4 in plasma varies according to the lipid compositions of the liposomes. Further, degradation studies with liposomal FM-VP4 incubated in rabbit plasma showed that about 95% of the FM-VP4 concentration remained, even after 4 days of incubation, when FM-VP4 was in a 55/45 formulation. When the cholesterol content of the lipid compositions was decreased,

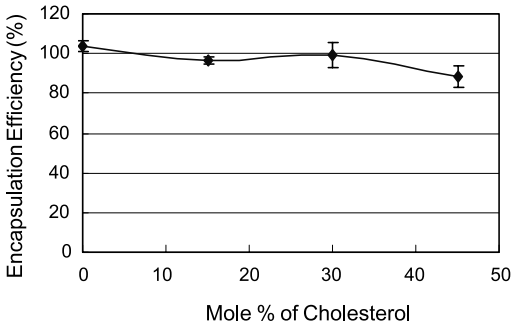

Figure 14. Encapsulation efficiency after manufacturing processes for formulation at different DMPC/cholesterol ratios. The original drug-to-lipid ratio was 0.1. Efficiency was calculated by drug-to-lipid ratio after dialysis divided by the original drug-to-lipid ratio multiplied by 100%. Data presented as mean ± standard deviation (n=6).

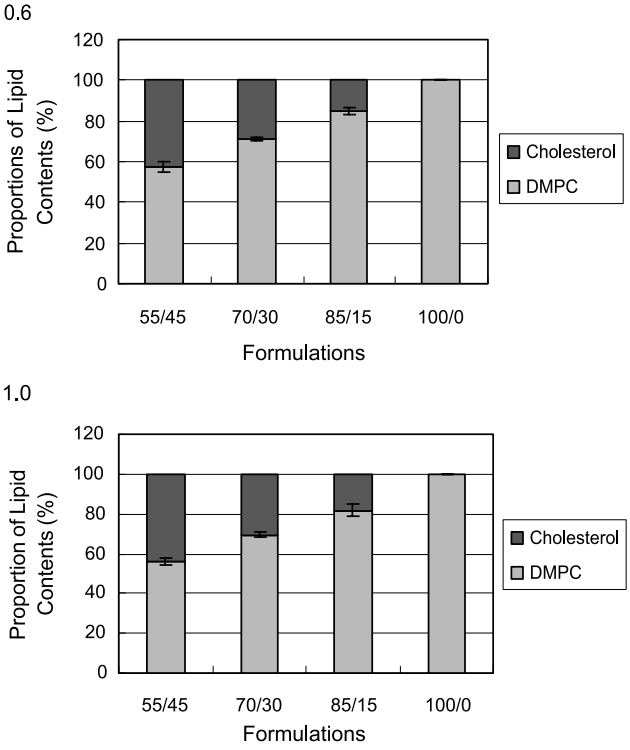

Figure 15. DMPC/cholesterol ratios of different formulations after steps of preparations. Liposomes were freeze-thawed five times, extruded 10 times, and dialyzed for 30 hours.

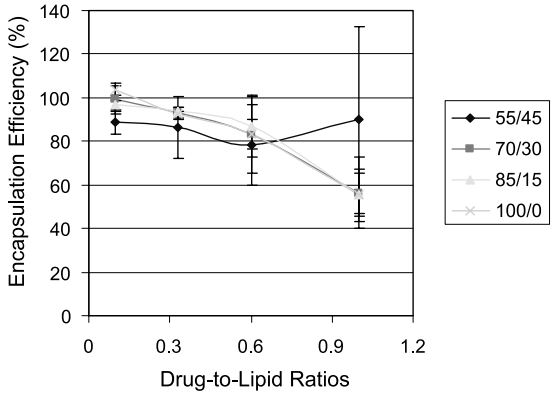

Figure 16. Encapsulation efficiency after preparation steps (before extrusion, after extrusion, and after dialysis) for a series of formulations. Original drug-to-lipid ratios were 0.1, 0.3, 0.6, and 1.0. Efficiency was calculated by drug-to-lipid ratio after dialysis divided by the original drug-to-lipid ratio multiplied by 100%. Data presented as mean ± standard deviation (n=6).

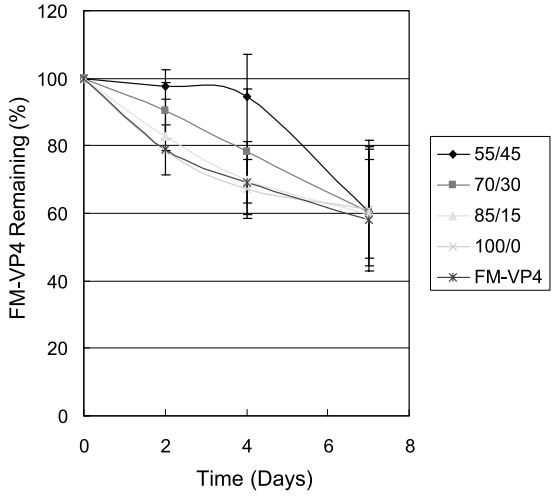

Figure 17. Percentage of FM-VP4 remaining after incubation in rabbit plasma at 37°C for a 7-day period. The FM-VP4 was either encapsulated in various liposomal formulations at a drug-to-lipid ratio of 0.5 or dissolved in HBS prior to the studies. Data presented as mean ± standard deviation (n=6).

however, degradation of FM-VP4 started earlier; for example, FM-VP4 in the 85/15 formulation decreased to 83% in 2 days.

The stability factors of the liposomal FM-VP4 formulation will be explained, and the advantages and potential uses of the formulation will be discussed in the following section. Each lipid has a phase transition. Energy is required to convert the lipid from the gel to the liquid crystalline state; a transition occurs within a narrow temperature range. The midpoint of the transition is often known as the ''melting'' temperature.[19] When phospholipids are hydrated in water, stacks of liquid sheets form large multilamellar vesicles. Sonic energy and mechanical energy are often used to produce unilamellar liposomes by reducing the size of multilamellar liposomes. When the phase transition between the gel and liquid crystalline phases occurs in unilamellar liposomes, the high-density, tightly packed molecules, and more ordered fatty acyl chains, characteristics at low temperatures give way to the more disordered and more permeable lipid bilayer of the liquid crystalline phase.[20]

The incorporation of cholesterol in liposome membranes has shown to play an important role in the development of a drug delivery system. Cholesterol is found in mammalian membranes. Investigations by differential scanning calorimetry of phospholipids/cholesterol liposomes have shown a decrease in the heat of transition as cholesterol content increases. As the concentration of cholesterol rose to between 33% and 50% mol, the lipid phase transition was essentially eliminated. In fact, when cholesterol was present in high enough concentrations, it promoted conformational order in the liquid crystalline phase and destroyed the crystalline structure in the gel phase. Clearly, cholesterol possesses a modulating effect, condensing lipids in the liquid crystalline phase and liquefying them in the gel phase. At temperatures higher than the phase transition of the phospholipids, a relatively low permeability of the phospholipids bilayer is likely to

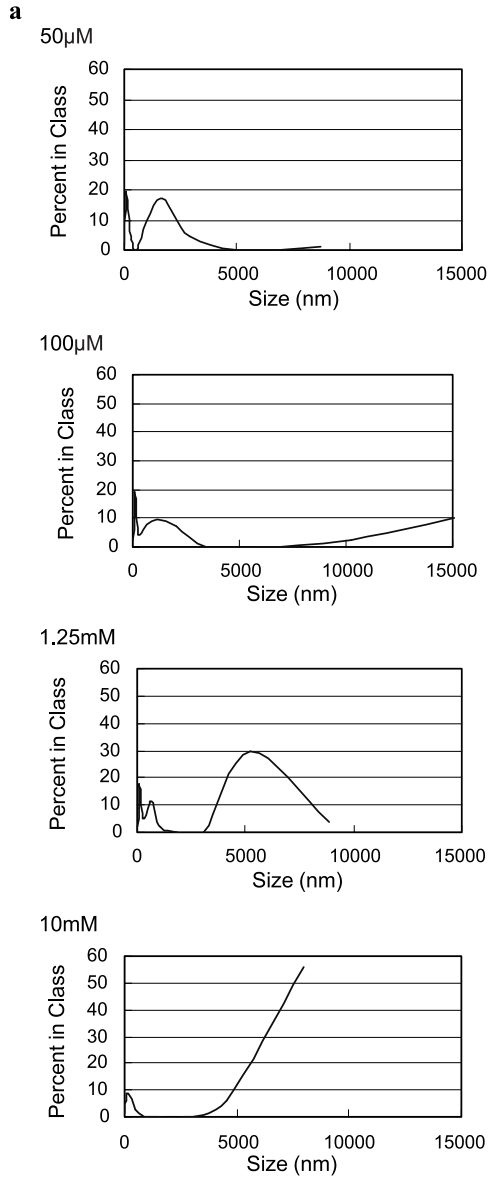

Figure 18. Size distribution of FM-VP4 micelles. a) Representative FM-VP4 micelles size distribution at selected concentrations. b) Characteristics of FM-VP4 micelles varying from 50 μM to 10 mM.

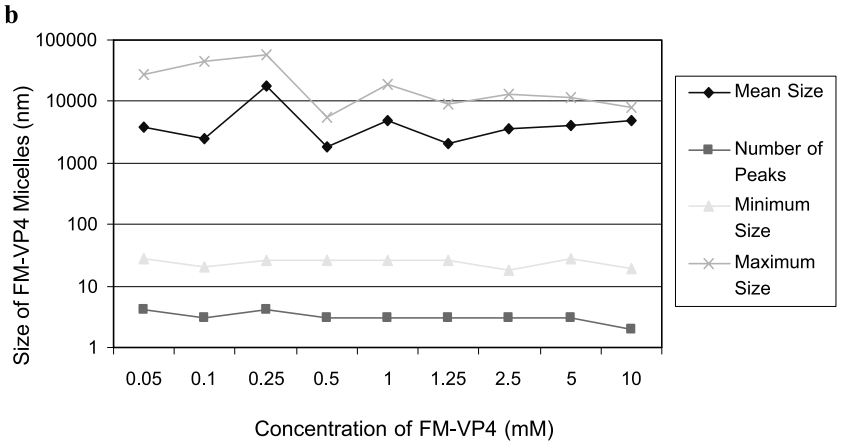

Figure 18. Continued.

occur.[20–24] This finding supports previous reports, suggesting that the cholesterol-rich lipid bilayer was more resistant than the cholesterol-poor lipid bilayer to destabilization by blood components.[25]

FM-VP4 is an amphipathic molecule with a polar head group (ascorbyl phosphate) and a nonpolar hydrocarbon body. When FM-VP4 is mixed with water, its polar region interacts favorably with the water molecules and tends to dissolve, but its nonpolar, hydrophobic region has the opposite tendency, avoiding contact with water. Therefore, the polar regions are at the surface in contact with water, and the nonpolar regions cluster together to present the smallest possible hydrophobic area to solvent. These stable structures found in water are called micelles.[26] At higher concentrations, however, a gradual change in micellar shape occurs, elongating to form cylindrical or lamellar structures. This phenomenon may be observed in many ionic molecules.[27] The formation of FM-VP4 micelles with a critical micelle concentration of 14 μM has been previously determined by 1,6-diphenyl-1,3,5-hexatriene (DPH), which is a hydrophobic fluorescent probe in water.[28] The size of FM-VP4 micelles in HBS varies widely (Fig. 18a), and apparently randomly sized distribution being represented by more than one peak in each concentration. Figure 18b summarizes the size characteristics of FM-VP4 micelles when dissolved in HBS at various concentrations: the mean size of micelles ranged from 1866 nm to 17970 nm at 5 μM to 10 mM. These micelles reached a maximum size of over 10000 nm, but they could also occur at a size of just 30 nm.

We have studied the effects on the size distribution of the drug particles when FM-VP4 was incorporated into various formulations of liposomes on size distribution. Specifically, we used the Zetasizer to analyze the size of liposomal FM-VP4 after we reduced the size of liposomal FM-VP4 to about 100 nm, the maximum and minimum sizes of the liposomes ranging only between 231 nm and 37 nm, respectively. Only one sharp peak was observed in these formulations, indicating that the liposomes were homogenously distributed by size (Fig. 19).

Studies have shown that liposomes can be used as carrier vehicles to facilitate the delivery of a variety of materials into cells in vitro. The processes of stable

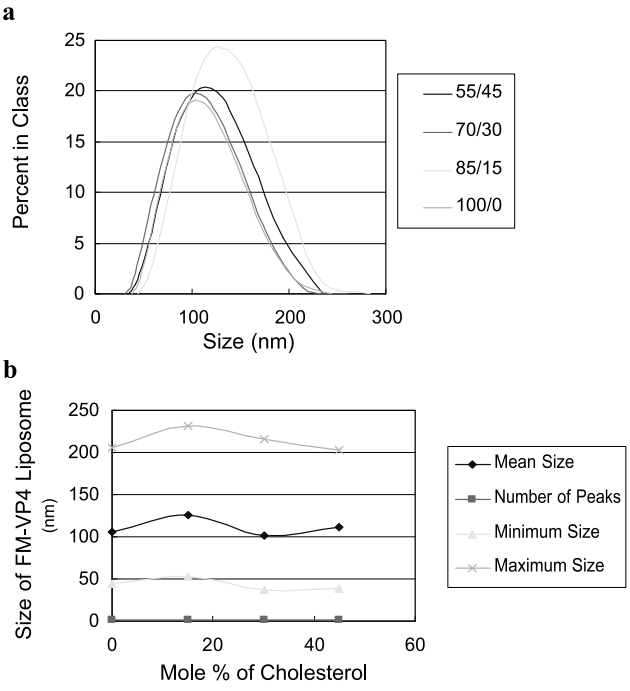

Figure 19. Size distribution of liposomal FM-VP4. a) Size distribution of liposomal FM-VP4 in different formulations. b) Size characteristics of liposomal FM-VP4 formulations at different DMPC/cholesterol ratios.

adsorption, endocytosis, lipid exchange, and fusion have been proposed to explain how liposomes interact with cells.[13,29] Liposomes containing DMPC exhibit a long circulation half-life of 74 minutes in mice.[25] Because we found that animals do not absorb FM-VP4 efficiently, we suggest that the extracellular activity of FM-VP4 needs to be examined through trials involving the administration of the drug by injection. Therefore, vehicles that promote long circulation times in plasma are desirable. Moreover, our in vitro degradation studies reveal that FM-VP4 demonstrates excellent stability for at least 4 days at 37°C in liposomes consisting of DMPC/cholesterol at a ratio of 55/45. Finally, we observed that the formation of aggregates of FM-VP4 was minimized when FM-VP4 was incorporated in liposomes that are uniform in size (about 100 nm). These properties suggest that FM-VP4 in cholesterol-rich liposomes might have benefits in the investigation of the extracellular effects of FM-VP4 in animals and the intracellular mechanisms in cells.[30,31]

CONCLUSIONS AND FUTURE DIRECTIONS

At present, the mechanism by which FM-VP4 lowers LDL cholesterol levels and total cholesterol levels in various animal models remains unknown. However, before the mechanism of action could be studied, ways of efficiently delivering FM-VP4 into plasma or cultured cells had to be developed. FM-VP4's low systemic bioavailability and cellular uptake make the development of such delivery particularly important. Our work provides a framework that suggests ways to efficiently deliver FM-VP4 so that its mechanism of action can be studied in vitro and in vivo.

Liposomal FM-VP4 can be used to increase the accumulation of FM-VP4 in cultured cells; to determine if FM-VP4 and liposomal FM-VP4 stimulate cholesterol efflux through an ATP-binding cassette cholesterol transporter, ABC1A; to ascertain whether FM-VP4 and liposomal FM-VP4 inhibit cholesterol ester transfer protein (CETP) activity; and to evaluate if FM-VP4 and liposomal FM-VP4 lower lipid levels.

LIST OF ABBREVIATIONS

ABC	ATP-binding cassette cholesterol transporter
ACN	Acetonitrile
ADME	Absorption, distribution, metabolism, and excretion
ANOVA	One-way analysis of variance
AUC	Area under the curve
CETP	Cholesteryl ester transfer protein
CRP	c-reactive protein
CV	Coefficient of variance
DAPC	Diarachidoylphosphatidylcholine
DC	Direct current
DMPC	1,2-Dimyristoyl-*sn*-glycero-3-phosphatidylcholine
DOTAP	Dioleoyloxy-3-(trimethylammonio) propane
DPH	1,6-Diphenyl-1,3,5-hexatriene
DPPC	1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine
DSPC	1,2-Distearoyl-*sn*-glycero-3-phosphatidylcholine
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FER	Fractional esterification rate
HBS	HEPES-buffered saline
HDL	High-density lipoprotein
HMG-CoA	β-hydroxy-β-methyl-glutaryl-CoA
L	Liter
LC	Liquid chromatography
LCAT	Lecithin: cholesterol acyltransferease
LC/MS/MS	Liquid chromatography/mass spectrometry/mass spectrometry
LDL	Low-density lipoprotein
LOD	Limit of detection
LOQ	Limit of quantification
LUV	Large unilamellar vesicle
MDR1	Multiple drug resistance protein
MER	Molar esterification rate
MPS	Mononuclear phagocyte system
MRM	Multiple reaction monitoring
MS	Mass spectrometry
PEG	Polyethylene glycol
RES	Reticuloendothelial system
Rf	Radio frequency
SD	Standard deviation
THF	Tetrahydrofuran

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