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Quantitative determination of cholesterol in liposome drug products and raw materials by high-performance liquid chromatography

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SUMMARY

Most liposomes used as drug delivery systems contain cholesterol as a major structural component. Cholesterol has profound effects on the chemical, physical and metabolic stability of liposomes and lipsome drug products and must be accurately monitored during formulation and processing development, stability testing and manufacturing. Before analyzing their components, the liposomes must be disintegrated and solubilized by dilution with methanol or 2-propanol. This high-performance liquid chromatographic assay is applicable to the resulting lipid-rich matrices and allows a direct quantitative analysis of cholesterol.

Cholesterol separates well from common ingredients of liposome-based drug products and cholesterol oxidation products. Calibration curves are linear over two orders of magnitude and the cholesterol detection limit is 1.5 μl/ml. Method precision for an anticancer liposome drug formulation was 0.9% relative standard deviation. The assay is also useful for measuring cholesterol in phospholipid and cholesterol raw materials.

INTRODUCTION

Liposomes are vesicles composed of single or multiple phospholipid bilayers that form spontaneously when these lipids are exposed to an aqueous environment[1]. They have shown great promise as biocompatible pharmaceutical drug carriers[2]. Liposomes can carry drugs of various compositions. Water-soluble drugs can be carried in the enclosed aqueous space and hydrophobic molecules can be incorporated in the lipid bilayer. The bilayer-forming phospholipids are commonly composed of phosphatidylcholine (PC) and phosphatidylglycerol (PG); cholesterol is also frequently a component in the bilayer structure. Although cholesterol is not essential for the formation and stability of liposomes, it has several beneficial effects, such as increased retention of water-soluble drugs[3,4], prevention of lipid phase transitions[5] and increased resistance to *in vivo* liposome degradation[4]. The phospholipid bilayer can accommodate up to 50 mol% of cholesterol.

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The development of pharmaceutical liposome formulations requires careful monitoring of the key liposomal ingredients during process development and stability testing. Among the various techniques described for the quantitative determination of cholesterol, only chromatographic assays offer the selectivity required for the complex matrices of liposome formulations. Thin-layer chromatography (TLC)[6–8] is labor intensive and of limited reproducibility. Gas chromatography[9,10] generally requires prior derivatization to more volatile compounds. For determining the cholesterol content in liposome formulations, high-performance liquid chromatography (HPLC) is the assay technique of choice, as it is compatible with liquid matrices, requires no derivatization and can be easily automated. While several HPLC methods for the quantitative analysis of cholesterol have been described[11–13], they cannot tolerate the high levels of lipids typically present in liposome drug formulations.

This assay system was developed for the precise quantitative determination of cholesterol in lipid-rich matrices, such as liposome formulations, cholesterol and phospholipid raw materials.

EXPERIMENTAL

Reagents

All solvents were of HPLC grade. Cholesterol external standard was from the National Bureau of Standards (NBS, Gaithersburg, MD, U.S.A.); 7-ketocholesterol, 25-hydroxycholesterol, 7-α-hydroxycholesterol, 7-β-hydroxycholesterol, 3,5-cholestadien-7-one, desmosterol and lanosterol were from Steraloids (Wilton, NH, U.S.A.), 4-cholesten-3-one was from Fluka (Buchs, Switzerland), 5-cholesten-3-one from Sigma (St. Louis, MO, U.S.A.), bulk cholesterol [United States Pharmacopeia (U.S.P.) grade] from Croda (Edison, NJ, U.S.A.), partially hydrogenated egg phospholipids were from Asahi Chemical (Ibarakiken, Japan) obtained through Austin Chemical (Rosemont, IL, U.S.A.).

Chromatography system

The HPLC apparatus was a Hewlett-packard 1090 with a diode-array detector and column heater. A non-endcapped 25 × 0.46 cm I.D. Spherisorb S-5 ODS-1 column was operated at 40°C with 100% HPLC-grade methanol at a flow-rate of 1.0 ml/min. In some instances an Alltech (Deerfield, IL, U.S.A.) ODS guard column was mounted between the injector and the analytical column. Cholesterol was detected with maximum sensitivity at 207 nm. The injection volume was 20 μl.

RESULTS

Sample preparation and the "disintegration-by-dilution" concept

In general, liposome drug formulations are composed of zwitterionic PC and anionic PG, with varying amounts of cholesterol (5–50%) in an aqueous buffer solution. In addition, they may contain antioxidants, such as α-tocopherol, salts, sugars and other excipients. Encapsulated drugs cover a wide spectrum in size and polarity, from small organic molecules to polypeptides and proteins. Liposomes may be viewed as a two-phase system, consisting of the hydrophobic bilayer and the aqueous medium, in which lipids, drugs, and excipients exist in a dynamic equilibrium. Unlike

monomolecular solutions of drugs, the liposome drug formulations require distintegration of the liposomes to form a solution before individual components can be analyzed by HPLC. Frequently, the liposomes are "dissolved" by extracting the liposomal lipids from the aqueous liposome suspension with an organic solvent, *e.g.*, chloroform–methanol mixtures. Usually, multiple extractions are necessary to recover the hydrophilic and/or lipophilic components completely. Extraction procedures are time consuming, error prone, difficult to automate and often produce solvent mixtures that are incompatible with subsequent HPLC analysis. For these reasons, sample preparation procedures which result in a single phase are preferable. Many liposome drug formulations can be "dissolved" to form a single phase by dilution with short-chain alcohols like methanol or 2-propanol. The resulting solvent mixtures (methanol, 2-propanol–water) constitute good solvents for phospholipids, cholesterol and lipid excipients and are sufficiently polar to keep moderate amounts of buffer salts and hydrophilic excipients in solution. By judicious choice of solvents and dilution volumes, the heterogeneous liposome suspension can be diluted with methanol and/or 2-propanol to form a homogeneous solution that can be readily injected into the HPLC system. However, the success of this fast and simple approach requires HPLC conditions in which all sample components are soluble in the mobile phase and do not interfere with the detection of the compound of interest.

Cholesterol in liposome drug formulations

The "disintegration-by-dilution" concept has been successfully applied to the determination of cholesterol in various liposome drug formulations. Fig. 1 shows chromatograms of liposome drug formulations of an anticancer drug (doxorubicin,

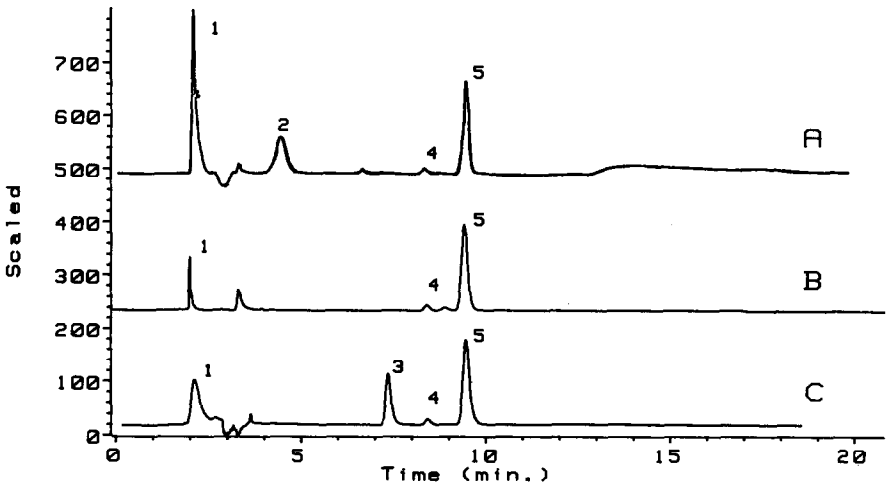

Fig. 1. Chromatograms of liposome drug formulations of doxorubicin, albuterol and an oligopeptide. (A) Doxorubicin liposome formulation was diluted with four volumes of distilled water and solubilized by adding five volumes of 2-propanol; 20 μl of the solution, equivalent to 2 μl of liposome formulation, were injected. (B) Albuterol liposome formulation: 60 mg was dissolved in 10 ml methanol; 20 μl, equivalent to 0.12 mg of liposome formulation, were injected. (C) Liposome formulation of an oligopeptide diluted with an equal volume of 2-propanol; 20 μl, equivalent to 10 μl of liposome formulation, were injected. Peaks: 1 = solvent front and PG; 2 = unidentified; 3 = α-tocopherol; 4 = bulk cholesterol contaminant; 5 = cholesterol.

A), a bronchodilator (albuterol, B) and an oligopeptide (C). The liposome formulations were prepared for cholesterol HPLC analysis by dilution with methanol and/or 2-propanol and were injected into the HPLC system without further treatment. All formulations were composed of PC, PG and cholesterol as major lipophilic liposome-forming components. The polypeptide formulation also contained α-tocopherol, detectable in the cholesterol assay system as a distinct peak between 7 and 8 min. In every case, the cholesterol peak is narrow, symmetrical, and baseline separated. Comparison of diode-array spectra which had been obtained at the leading edge, apex and trailing edge of the cholesterol peak were identical. This peak purity check, made possible by diode-array technology, is a good indication for the absence of interfering peaks. Under the conditions of the cholesterol assay, PG is eluted with or close to the solvent front. PC is eluted with longer retention times, well separated from cholesterol. Depending on its origin, PC may be eluted as a broad, ill-defined peak, (Fig. 1A) as its molecular species are partially separated by chain length and unsaturation. Hydrogenated or saturated synthetic PC may not be visible due to its lower UV absorptivity (Fig. 1B,C). A small peak preceding the cholesterol originates most likely from the cholesterol raw material (Fig. 4).

A partial validation of the cholesterol determination in a doxorubicin liposome formulation showed excellent linearity of the cholesterol response. Calibration curves were established with dilutions of NBS cholesterol from 0.01 to 3.0 m*M* and based on peak areas. Slope and *y*-intercept [± standard error (S.E.)] and R^2 were calculated by unweighted linear regression analysis as 2144 ± 38, 16.7 ± 49.7 and 0.9998, respectively. Method precision for a typical doxorubicin liposome sample was 0.9% relative standard deviation ($n = 6$); the detection limit (defined as signal-to-noise ratio 2) was 1.5 μg cholesterol/ml. There was no measurable interference of the sample matrix with the cholesterol response, as determined by adding a known amount of cholesterol standard to a doxorubicin liposome sample.

Cholesterol oxidation in liposomes

The assay has also proven useful for the analysis of cholesterol and its major oxidation products in experimental liposome formulations, incubated at elevated temperature (50°C) in the absence of antioxidants (Fig. 2). All oxidation products are well separated from the main cholesterol peak (Table I, Fig. 2). Major oxidation products, such as isomeric 7-hydroxycholesterols, 7-ketocholesterol and 3,5-cholestadien-7-one, are well resolved and can be quantitated by external standards, with sensitivities around 1 μg/ml. 3,5-Cholestadien-7-one, 4-cholesten-3-one and 5-cholesten-3-one are eluted with very similar retention times but can be distinguished by their UV spectra. Some oxidation products, like 7-ketocholesterol and 3,5-cholestadien-7-one, can be determined with higher sensitivities by additional monitoring at 240 nm (Fig. 2).

Cholesterol in phospholipid raw materials

Most bulk phospholipids used in the manufacture of liposome drug products are isolated from egg yolk and are contaminated by varying amounts of cholesterol. The sensitivity of the cholesterol determination in phospholipid raw materials is determined by the solubility of the phospholipid in methanol, which, in turn depends on the phospholipid type (PC, PG) as well as the chain length and unsaturation of its

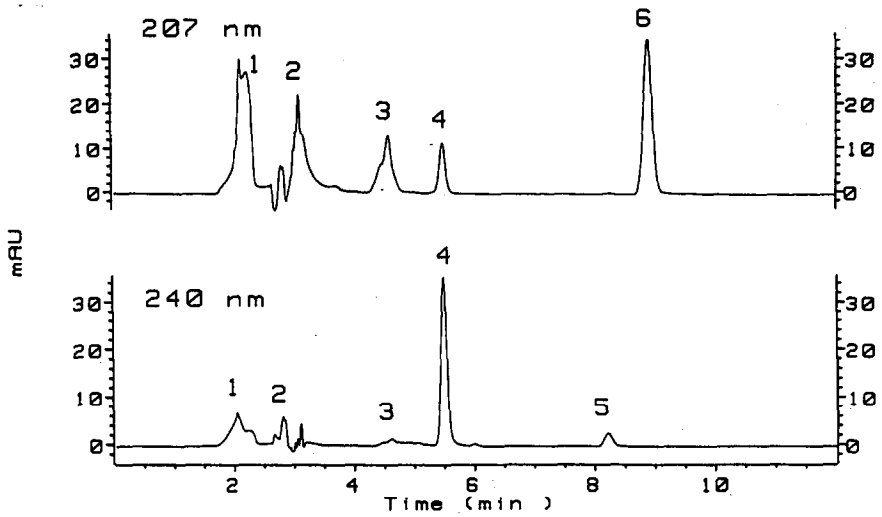

Fig. 2. Chromatogram of a thermally stressed experimental liposome formulation, recorded at 207 and 240 nm. A PC–PG–cholesterol formulation after 2 months incubation at 50°C. Liposomes were dissolved by adding nine volumes of 2-propanol; 20 μl, equivalent to 2 μl of formulation, were injected. Peaks: 1 = solvent front; 2 = unidentified; 3 = 7-α- and 7-β-hydroxycholesterol; 4 = 7-ketocholesterol; 5 = 3,5-cholestadiene-7-one; 6 = cholesterol.

fatty acids. Detection limits for cholesterol are in the best case (native egg PC) 0.1 mg/g, in the worst case (fully hydrogenated PG) *ca.* 2 mg/g. PG and PC are well separated from the cholesterol peak, PG is eluted with or close to the void peak, PC is eluted with longer retention times as a large cluster of convoluted peaks, due to the partial separation of the molecular species varying in chain length and unsaturation of the fatty acids. The phospholipid peak response increases with unsaturation of the phospholipid, because the double bonds of unsaturated fatty acids contribute to the UV absorbance. Fig. 3 shows a typical chromatogram of egg-derived PC raw material.

TABLE I

RETENTION TIMES OF CHOLESTEROL AND CHOLESTEROL AUTOXIDATION PRODUCTS

Compound	*Retention time (min)*
7-α-Hydroxycholesterol	4.55
7-β-Hydroxycholesterol	4.69
25-Hydroxycholesterol	4.76
7-Ketocholesterol	5.45
5-Cholesten-3-one	7.95
3,5-Cholestadien-7-one	8.12
4-Cholesten-3-one	8.16
Cholesterol	8.80

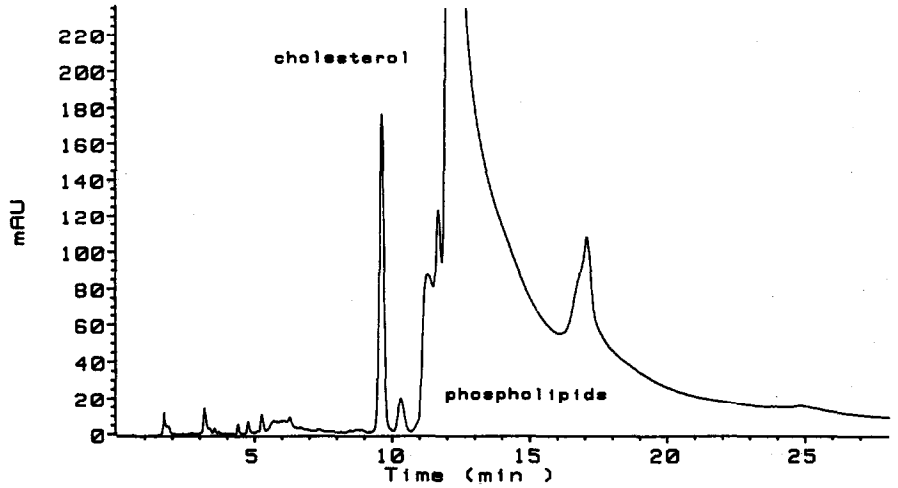

Fig. 3. Chromatogram of an egg-derived PC raw material. A 560-mg amount of egg-derived, partially hydrogenated PC raw material was dissolved in 10 ml of methanol and 20 μl, equivalent to 1.12 mg PC, were injected.

Bulk cholesterol raw material

Bulk cholesterol is frequently contaminated with precursors of its biosynthesis, such as desmosterol or lanosterol[14] and may also contain cholesterol autoxidation products[15]. Cholesterol, desmosterol, lanosterol and cholesterol autoxidation products are well separated from the cholesterol peak (Table I, Fig. 4). The cholesterol content (purity) of the raw material can be determined by external standardization with NBS cholesterol. As cholesterol is quite soluble in methanol, large quantities of

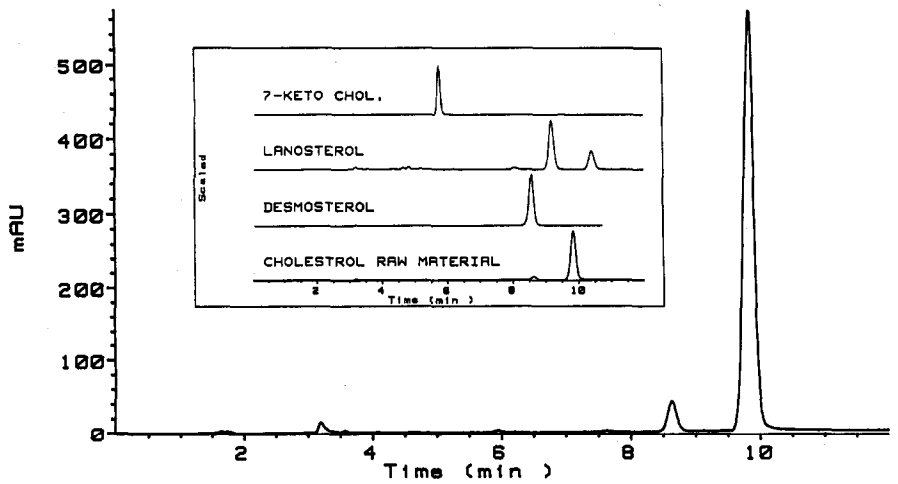

Fig. 4. Chromatogram of a cholesterol raw material. A 116-mg amount of U.S.P.-grade cholesterol raw material was dissolved in 100 ml methanol; 20 μl of the solution, equivalent to 0.023 mg cholesterol, were injected.

the bulk material can be injected to achieve excellent sensitivities for the contaminating compounds.

CONCLUSIONS

The major advantages of this assay system are its versatility, ruggedness, good linearity over a wide concentration range and its tolerance of lipid-rich matrices, common in liposome formulations. Retention time shifts, as often seen with assays operating at ambient temperature, are avoided by controlling the column temperature. The assay does not require mobile phase preparation. The 'disintegration-by-dilution' approach to sample preparation is faster and more reproducible than conventional lipid extraction procedures. This is reflected by the precision of the cholesterol determination in doxorubicin liposomes. The system efficiently separates cholesterol from phospholipids, common liposome ingredients (*e.g.*, α-tocopherol), raw material contaminants, and oxidation products of cholesterol. This allows an accurate quantitative measurement of cholesterol in a wide variety of liposome drug products and raw materials.

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