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Short communication

A high-performance liquid chromatographic method for the analysis of lipids from lyophilized formulations

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Abstract

A simple, sensitive and rapid, high-performance liquid chromatography (HPLC) method has been developed for the simultaneous determination of cardiolipin, cholesterol and egg phosphatidylcholine from lyophilized lipid formulations. The proposed HPLC method utilizes a μPorosil column (300×3.9 mm I.D., 10 μm) and UV detection at 205 nm. A good resolution was observed with a mobile phase of water–methanol–acetonitrile (5:70:25) at a flow-rate of 2 ml/min. This method is capable of measuring levels of about 0.2–0.8 mg/ml of cardiolipin, 0.25–1.0 mg/ml of cholesterol and 0.8–3.2 mg/ml of egg phosphatidylcholine (EPC). The proposed analytical method has been validated according to USP compliance standards for accuracy, precision and reproducibility and thus may be employed for routine analysis of the lipid components from a typical placebo liposomal formulation.

Keywords: Lipids; Cardiolipin; Cholesterol; Phosphatidylcholine

1. Introduction

HPLC has limited applications in lipid analysis because of non-specific absorption maxima observed with many lipids. However, due to the unsaturated centers of the fatty acid moieties and presence of a polar end group, direct UV detection at the 200-nm region could be applied for lipid analysis [1,2]. This complex absorption behavior makes quantitation difficult because, depending on the average unsaturation degree, the molar extinction coefficients of the individual phospholipids vary for each lipid source. Similar difficulties are found in gas chromatographic estimation where flame ionization detection is used for lipid quantitation [3,4]. GC analysis also requires conversion of lipids to their methyl ester derivatives [5–7].

Several other derivatization methods have been developed for the detection and quantitation of lipids [8]. Ethanolamine- and serine-containing phospholipids can be easily derivatized whereas suitable derivatives of choline-containing lipids are not easily derivatized. Fluorometric methods [9] and phosphorus microassays based on the methods of Fiske and Subbarow [10] could be applied although they are laborious and time consuming for routine use. Phosphorus-containing compounds can also be quantitated with an automated phosphorous analyzer [11–13]. Barnes [14] has reported an HPLC method for the analysis of phospholipids using UV detection at 205 nm.

The objective of this study was to develop a

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simple, sensitive and rapid HPLC method with UV detection for the simultaneous determination of cardiolipin, cholesterol and egg phosphatidylcholine in lyophilized lipid formulations and to validate the method to establish its suitability for routine analysis.

2. Experimental

2.1. Chemicals and reagents

Cardiolipin (or diphosphatidylglycerol), cholesterol and egg phosphatidylcholine (EPC) were obtained from Avanti Polar Lipids. Acetonitrile and methanol (HPLC grade) were purchased from Fisher Scientific. De-ionized water was used throughout.

2.2. Preparation of stock solution

About 25 mg of cardiolipin, 50 mg of cholesterol and 175 mg of egg phosphatidylcholine were accurately weighed in a 50-ml volumetric flask. About 30 ml of methanol was added to the flask and sonicated for 5 min. The volume was made up to the mark with methanol. This stock solution was appropriately diluted for the preparation of calibration curve and method validation.

2.3. Sample preparation

About 300 mg of the lyophilized lipid cake was accurately weighed in a 10-ml volumetric flask. The material was dissolved and the volume made up to the mark using methanol. The standard as well as sample solutions were filtered through a 0.45-μm syringe filter (Acrodisc, 13CR PTFE, Gelman Sciences) prior to filling in autosampler vials.

2.4. Calibration samples

Calibration samples were prepared by diluting the stock solution appropriately, giving a concentration range of 206.25–825.00 μg/ml for cardiolipin, 267.25–1017.0 μg/ml for cholesterol and 814.37–3257.5 μg/ml for EPC.

2.5. HPLC conditions

HPLC was performed using a SpectraSeries P2000 solvent delivery system (Spectra Physics) fitted with an UV detector (SpectraSeries UV150) and an automatic injector (SP8780). A DS650 Chromatography Data System (Kratos Analytical) was used for integration. Chromatographic separation was achieved at ambient temperature with a normal-phase μPorosil column (30 cm×3.9 mm I.D., 10 μm; Waters, Millipore, Milford, MA, USA). The mobile phase was water–methanol–acetonitrile (5:70:25) at a flow-rate of 2 ml/min. It was filtered through a 0.45-μm membrane filter (FP-450, Varicel Gelman) and degassed using helium purge. The wavelength of spectrophotometric detection was 205 nm. The injection volume was 20 μl.

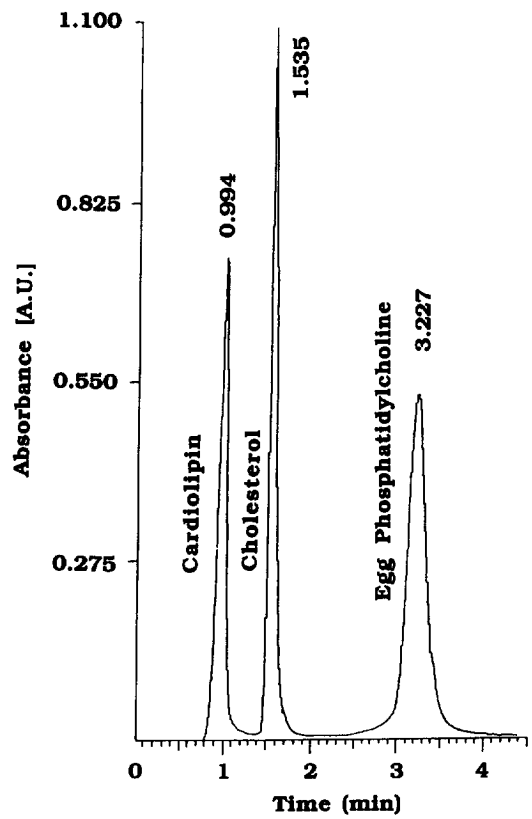

Fig. 1. Detector response for cardiolipin (412.5 μg/ml), cholesterol (535.5 μg/ml) and egg phosphatidylcholine (1628.3 μg/ml) peaks, detected simultaneously from lyophilized liposomal formulation.

Table 1
HPLC method validation for accuracy and reproducibility

Assay	Standard level of lipid (μg/ml)														
	Cardiolipin					Cholesterol					Egg phosphatidylcholine				
	206.25	412.50	550.00	660.00	825.00	267.25	535.50	714.00	856.80	1071.00	814.37	1628.25	2171.60	2606.00	3257.50
1	207.21	412.19	551.32	655.97	829.66	267.75	543.09	701.74	862.38	1059.90	824.64	1627.06	2173.96	2586.49	3264.38
	207.20	411.58	551.72	654.86	829.90	266.75	544.08	705.00	859.90	1063.05	816.14	1625.53	2171.72	2586.12	3274.66
2	207.35	413.21	551.68	656.74	830.16	266.49	541.47	703.53	857.43	1060.10	820.84	1626.14	2173.15	2594.43	3266.07
	207.39	415.01	551.64	656.74	830.16	268.02	542.12	702.56	858.14	1059.94	820.31	1627.14	2168.08	2596.02	3271.98
Mean	207.29	412.99	551.64	656.17	829.27	267.25	542.69	703.21	859.46	1060.75	820.48	1626.47	2171.72	2590.77	3269.27
S.D.	0.097	1.500	0.221	0.999	1.617	0.747	1.141	1.401	2.203	1.552	3.478	0.774	2.602	5.192	4.849
n	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
C.V.	0.047	0.363	0.040	0.152	0.195	0.280	0.210	0.199	0.256	0.146	0.424	0.047	0.120	0.200	0.148
A.R.	100.50	100.12	100.30	99.42	100.52	100.00	101.34	98.49	100.31	99.04	100.75	99.89	100.00	99.42	100.36

C.V. (coefficient of variation) (%)=S.D.×100/mean; A.R. (analytical recovery) (%)=mean×100/std. pool level.

2.6. Validation parameters

The proposed analytical method was validated according to USP XXII compendial standards [15].

Accuracy and precision of the method were established by assessing the inter-assay analytical recovery of the samples at five levels. Precision was evaluated from the reproducibility of six injections for each of the lipids.

3. Results and discussion

The presence of unsaturated centers and polar end groups on the fatty acid moieties satisfactorily enabled direct UV detection in the 200-nm region. Though the wavelength of spectrophotometric detection is not a selective one, it gave significant absorption with a high signal-to-noise ratio rendering good quantitation of all the peaks under investigation. A good resolution was observed with a mobile phase of water–methanol–acetonitrile (5:70:25). A representative HPLC chromatogram for the analysis of cardiolipin, cholesterol and EPC is shown in Fig. 1.

The validation protocol established for all other HPLC methods developed and used in this laboratory are in compliance with US compendial standards. The standard curve for lipids was linear for cardiolipin (0.206–0.825 mg/ml), cholesterol (0.267–1.017 mg/ml) and EPC (0.814–3.257 mg/ml) with squared linear regression coefficients of 0.995, 0.993 and 0.999, respectively. Statistical evaluation of observed data was done using Student's *t*-test.

The accuracy of the analytical procedure is confirmed by the results of two independent inter-assays as shown in Table 1. Good analytical recovery was observed over the range of 98–102%. The average inter-assay coefficient of variation was found to be 0.16% for cardiolipin, 0.22% for cholesterol and 0.19% for EPC.

The precision of the method was calculated by assessing the coefficient of variation of six injections. The average intra-assay coefficient of variation was found to be 0.15% for cardiolipin, 1.12% for cholesterol and 0.29% for EPC.

The statistical parameters, such as the coefficient of variation and standard deviation, show the suitability of this method for the routine analytical testing of lipids from liposomal formulations. Analysis carried out on six additional batches of liposomal formulations yielded consistent results. However, the method is not very stable and the effect of degradation products due to peroxidation and the presence of impurities are still under investigation. In spite of this drawback, the proposed analytical method can be used to quantitate the lipid content of liposomal formulations, stored under refrigerated conditions over a period of one year without interference from impurities and products of normal and forced degradation.

In conclusion, a simple, sensitive and rapid HPLC method (normal-phase) was successfully developed for the simultaneous analysis of cardiolipin, cholesterol and egg phosphatidylcholine. The method showed good linearity, accuracy and precision over the concentration range usually found in liposomal formulations. The method could be readily employed for routine quality control analysis of the lipid components of the formulations.

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References

[1] F.B. Jungalwala, J.E. Evans and R.H. McCluer, Biochem. J., 155 (1976) 155.
[2] W.S.M. Van Kessel, W.M. Hax, R.A. Demel and J. De Gier, Biochim. Biophys. Acta, 486 (1977) 524.
[3] K. Kluchi, T. Ohta and H. Ebine, J. Chromatogr., 133 (1977) 226.
[4] J.K. Kaitaranta and N. Nicolaides, J. Chromatogr., 205 (1981) 339.
[5] M. Masatoshi and U. Nobuo, Comp. Biochem. Physiol., 92B (1989) 319.
[6] G.G. Orgambide, Z. Huang, A.A. Gage and F.B. Dazzo, Lipids, 28 (1993) 975.
[7] N.L. Pruitt, Am. J. Physiol., 254 (1988) R870.
[8] M.J. Cooper and M.W. Anders, J. Chromatogr. Sci., 13 (1975) 407.

[9] H.G. Sciefer and V. Neuhoff, Z. Hoppe-Seyler's Physiol. Chem., 352 (1971) 913.
[10] C.H. Fiske and Y. Subbarow, J. Biol. Chem., 66 (1925) 375.
[11] S.P. Bessman, Anal. Biochem., 59 (1974) 524.
[12] J.K. Kaitaranta, P.J. Geiger and S.P. Bessman, J. Chromatogr., 206 (1981) 327.
[13] J.K. Kaitaranta and S.P. Bessman, Anal. Chem., 53 (1981) 1232.
[14] J.A. Barnes, D.J. Pehowich and T.M. Allen, J. Lipid Res., 28 (1987) 130.
[15] The United States Pharmacopeia XXII, 1990, United States Pharmacopeial Convention, Rockville, MD, Mack Printing Company, Easton, PA, pp. 1710–1712.