Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1073 (2005) 347–353
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

A rapid isocratic high-performance liquid chromatography method for determination of cholesterol and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine in liposome-based drug formulations

Ramsharan Singh, Monsurat Ajagbe, Shastri Bhamidipati, Zafeer Ahmad, Imran Ahmad*

Process Development and Manufacturing Department, Research and Development, NeoPharm Inc., 1850 Lakeside Drive, Waukegan, IL 60085, USA

Available online 19 January 2005

Abstract

A high-performance liquid chromatography (HPLC) method for the determination of cholesterol and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) in liposome-based drug formulations has been developed. Liposome formulations of anticancer agents (viz., paclitaxel, docetaxel, 7-ethyl-10-hydroxycamptothecin (SN38), doxorubicin, mitoxantrone and an antisense oligodeoxyribonucleotide, etc.) were prepared. These formulations contain DOPC, cholesterol and other lipids, such as tetramyristoyl cardiolipin or 1,3-bis(1,2-bis-tetradecyloxy-propyl-3-dimethylethoxyammonium bromide)propan-2-ol [(*R*)-PCL-2] in product-specific ratios. A simple HPLC method that uses isocratic elution and UV detection has been developed for simultaneous quantification of cholesterol and DOPC components of the liposome formulations. The chromatographic separation of these components is achieved using a C_8 analytical column with 50 mM ammonium phosphate buffer (pH 2.7)–methanol (15:85, v/v) as mobile phase. Both cholesterol and DOPC peaks are well resolved and free of interference from other excipients or degraded impurities in the formulation. The method has been found to be linear ($r > 0.999$) over a wide concentration range of both analytes. This method offers the advantage of simultaneous quantitation of cholesterol and DOPC in various liposome-based formulations without any preprocessing of the sample, and has quantitation limits of 0.5 and 10 μg/mL for cholesterol and DOPC, respectively.

Keywords: Lipids; Cholesterol; 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine; Tocopherols; Antioxidants; Phospholipids; Liposomes; Anticancer drugs

1. Introduction

Liposomes are lipid vesicles prepared by dispersing various lipids in an aqueous phase. Liposomes have been used as a carrier of drugs and antigens [1]. One of the most significant advantages of using liposomes as a drug delivery carrier is the reduced toxicity and improved therapeutic efficacy. While several different lipids could be used for formulating drugs in liposomes, invariably most formulations contain cholesterol and a phospholipid with some degree of unsaturation. Most commonly used phospholipids are egg phosphatidylcholine, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC). We developed several liposome-based drug formulations containing different anticancer agents, e.g., paclitaxel, docetaxel, 7-ethyl-10-hydroxycamptothecin (SN38), doxorubicin, mitoxantrone and an antisense oligodeoxyribonucleotide [2–5]. These formulations contain DOPC, cholesterol, (+)-α-tocopherol acid succinate (TAS), and tetramyristoyl cardiolipin or 1,3-bis(1,2-bis-tetradecyloxy-propyl-3-dimethylethoxyammonium bromide)propan-2-ol [(*R*)-PCL-2], in product-specific ratios.

There are several reported high-performance liquid chromatography (HPLC) methods in the literature that use silica columns with UV detection and wide and varied mobile phase compositions of organic solvents (viz., acetonitrile, hexane, methanol and isopropanol) and water for detection and quantitation of phospholipids [1,6–11]. Separation of several classes of phospholipids was achieved using silica column by gradient elution with hexane–isopropanol–water mobile phase [6]. While quantitation of cholesterol and other

* Corresponding author. Tel.: +1 847 8870800; fax: +1 847 8879281.
E-mail address: imran@neophrm.com (I. Ahmad).

0021-9673/$ – see front matter © 2004 Published by Elsevier B.V.
doi:10.1016/j.chroma.2004.12.036

phospholipids in liposome formulation has previously been reported [12–15], some methods require lipid extraction from samples prior to injecting in to HPLC system [14,15], whereas others use complicated gradient elution for separation [12]. The detection limit achieved for cholesterol in a method that uses isocratic elution does not permit its quantitation in our formulations [13]. More recently, evaporative light scattering detection (ELSD) has been used for quantitation of cholesterol, and other phospholipids in liposome-based formulations [16]. While the methods that use ELSD are considered universal, they are cumbersome to reproduce, calibration of standards require non-linear regression anlayses due to varying sensitivity of the detector and often give results with high variations compared to UV detection methods.

In this report, we describe a rapid HPLC method for separation and simultaneous quantitation of cholesterol and DOPC components of liposome formulations. This method uses a C_8 column, isocratic mobile phase and UV detection at 205 nm. This method was tested for several liposome-based formulations described above and results were found to be similar. The results generated show that this method can also be used to quantitate (+)-α-tocopherol acid succinate which was included in the formulations as an antioxidant. In addition, we have also used this method to determine other phospholipids similar to DOPC such as 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) in our liposome-based formulations of NeoPhectin family of products.

2. Experimental

2.1. Materials

Cholesterol used for calibration standard was prepared by JBL Scientific (San Luis Obispo, CA, USA) and was purchased from National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA) as standard reference material (SRM). Cholesterol for formulation use, DOPC, tetramyristoyl cardiolipin, and 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (lyso-PC) were purchased from Avanti Polar Lipids (Alabaster, AL, USA) and were of either research or cGMP grade. Myristic acid, oleic acid and (+)-α-tocopherol acid succinate were purchased from Aldrich (Milwaukee, WI, USA) and were of research grade. HPLC grade methanol, monobasic ammonium phosphate and 85% phosphoric acid (H_3PO_4) were purchased from Fisher Scientific (Pittsburgh, PA, USA). High-purity water was obtained from a Milli-Q purification system (Millipore, Bedford, MA, USA).

2.2. High-performance liquid chromatography

The HPLC system was equipped with Agilent 1100 Series systems (Agilent Technology, Palo Alto, CA, USA) consisting of vacuum degasser, temperature controlled well-plate autosampler, column thermostat, quaternary pump and photo diode array detector. Chromatographic analysis was performed using a Hypersil BDS C_8 (15 cm × 4.6 mm i.d., 5 μm particle size, 80 Å pore size) column from Alltech (Deerfield, IL, USA) or from Thermo (Bellefonte, PA, USA) at column temperature of 60 °C and sample temperature of 20 °C. The analytical column was protected with a disposable KrudKatcher pre-column 0.5 μm filter from Phenomenex (Torrance, CA, USA). Mobile phase flow rate was set to 2.0 mL/min with a run time of 20 min. The diode array detector was operated at 205 nm with 4 nm of bandwidth and slit setting, off reference mode and 2 s of response time setting. Injection volume was set at 50 μL. An isocratic mobile phase containing 50 mM solution of ammonium phosphate buffer (pH 2.7)–methanol (15:85, v/v) was used.

2.3. Preparation of reagents

Ammonium phosphate buffer (50 mM, pH 2.7) was prepared by dissolving ammonium phosphate in water and adjusting pH to 2.7 by adding 85% H_3PO_4. The clear solution was filtered through a 0.45 μm nylon membrane filter and stored at room temperature and used for 1–2 weeks. Mobile phase was prepared by mixing the buffer solution and methanol in 15:85 (v/v) ratio.

Standard stock solutions of cholesterol (1.0 mg/mL) and DOPC (2.0 mg/mL) were prepared using methanol. Intermediate stock solutions were prepared by mixing the stock solutions of cholesterol and DOPC in the product-specific ratio to bracket the target concentration for analysis. Calibration standards were prepared by further diluting the intermediate solution. Five levels of calibration standards were prepared by diluting this intermediate solution. Mobile phase was used as diluent for intermediate and calibration standard solutions. The calibration standards used for liposome-based SN38 (LE-SN38) analysis were prepared in the range of 8–200 and 40–1000 μg/mL for cholesterol and DOPC, respectively. For other drug formulations, similar preparations were made based on the lipid ratio in the particular formulation.

2.4. Preparation of liposome-based formulations for HPLC analysis

Liposome-based formulations were prepared as described previously [2–5]. The samples for HPLC injections were prepared by diluting the liposome formulations with mobile phase. To bring the concentration of the samples into the calibration range, the required dilution depends on the expected concentration of cholesterol and DOPC in samples. Concentration and ratio of cholesterol and DOPC in the samples depend on the specific product. For example, an LE-SN38 sample containing about 15 mg/mL cholesterol and 40 mg/mL of DOPC can be diluted to 100-fold for HPLC injection.

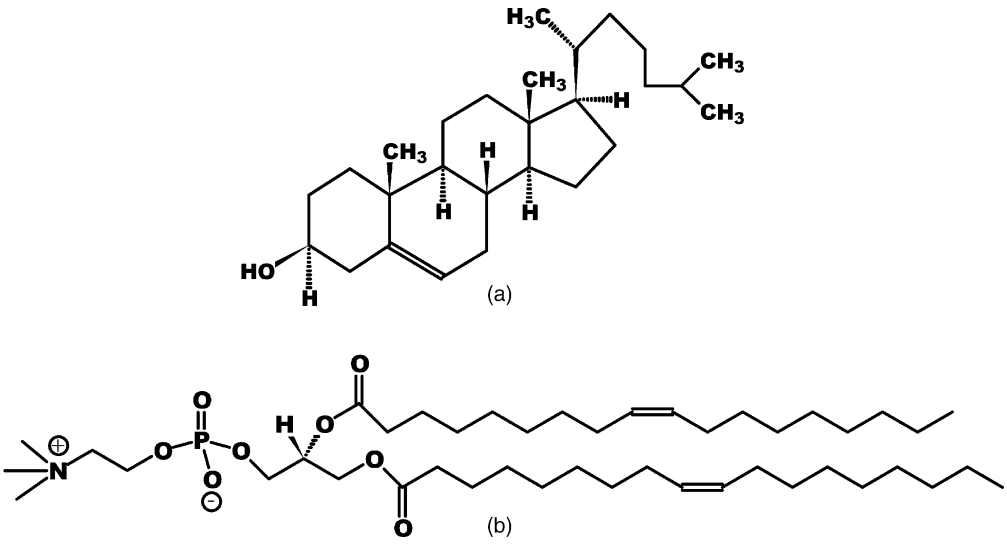

Fig. 1. Structures of (a) cholesterol and (b) 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC).

Table 1
Chromatographic performance data of the method

Analyte	Retention time (min)	Tailing[a]	Retention factor[b]	Plate count[c]	Resolution between critical band pair
Cholesterol	5.145	1.033	4.562	6870	3.467
DOPC	14.517	1.180	14.694	5827	–

[a] Tailing is defined as $W_{0.05}/2t_w$, where $W_{0.05}$ is peak width at 5% of peak height (min) and t_w is distance between peak front and peak retention measured at 5% of the peak height (min).
[b] Retention factor is defined as $(t_R - t_0)/t_0$, where t_R is retention time of peak (min) and t_0 is void time (min). Void time = 0.925 min for the method.
[c] Plate count is defined as $16(t_R/W_B)^2$, where W_B is the tangent peak width (min).

3. Results and discussion

The HPLC method described here was developed for quantitation of cholesterol and DOPC (Fig. 1) following the FDA and ICH guidelines [17–20]. Linearity, accuracy, precision, specificity, quantitation limit and robustness of the method were tested to ensure that the method is suitable for identification and quantitation of the cholesterol and DOPC components of different liposome-based drug formulations. Chromatographic performance data for a typical run are presented in Table 1. Resolution of 2.0 or greater is desired for critical band pair. Critical resolution of 3.467 was observed between cholesterol and TAS peaks. Tailing factors for both cholesterol and DOPC are <1.20. Retention factor in the range of $0.5 < k' < 20.0$ is desired to clearly separate the first peak from void time and to avoid higher retention time for the last band. Retention factors of 4.362 and 14.694 were found for cholesterol and DOPC, respectively. Retention factors of the cholesterol and DOPC peaks were optimized by varying mobile phase composition. However, it was necessary to increase column temperature to 60 °C to achieve acceptable resolution between critical band pair of cholesterol and TAS.

3.1. Linearity and range

Linearity of the calibration standards was tested in the concentration rage of 8.0–200.0 and 40.0–1000.0 μg/mL for cholesterol and DOPC, respectively. Five levels of calibration standards were prepared at various concentration levels. Correlation coefficients (r) were found to be >0.999 for both cholesterol and DOPC (Table 2). It is clear that the curves are linear in this range of concentration and the correlations are suitable for quantitation. Typical chromatogram of a standard solution is shown in Fig. 2. Representative chromatograms of four liposome-based formulations are presented in Fig. 3.

3.2. Accuracy

The accuracy study was performed to determine the closeness between the true concentration value and the experimental results. Cholesterol and DOPC were spiked in to the excipients that include other lipids, antioxidant, sucrose and the active drug components expected to be present in

Table 2
Summary of linear regression data for calibration standards

Analyte	Linear regression parameters			
	Intercept	Slope	Correlation coefficient (r)	Coefficient of determination (r^2)
Cholesterol	8.478	13.935	0.99997	0.99994
DOPC	2.431	4.116	0.99996	0.99992

Table 3
Summary of method accuracy results

Analyte	Recovery solution at target level (%)	Theoretical concentration (μg/mL)	Recovered average concentration[a] (μg/mL)	RSD[a] (%)	Analytical recovery[a] (%)
Cholesterol	60	85.179	85.240 (0.171)	0.200	100.071
	100	141.965	141.918 (0.167)	0.118	99.967
	140	198.751	197.663 (0.273)	0.138	99.452
DOPC	60	218.052	218.159 (3.342)	1.532	100.049
	100	363.420	363.117 (4.379)	1.206	99.917
	140	508.788	504.486 (2.514)	0.498	99.154

[a] Based on six data points. Standard deviation is given in parentheses.

the formulations. Spike recovery was performed at three levels (60, 100 and 140%) of the target concentration. For each level, three preparations were performed. Table 3 summarizes the results from accuracy experiments. Average recovery of 99.83 and 99.71% were observed for cholesterol and DOPC, respectively, which is within ±2.0% of normally accepted value.

3.3. Precision

Instrument precision was performed as part of each sequence run at the beginning of the sequence. Six injections of the target level of calibration standard were performed and the data were evaluated. Table 4 shows the mean retention and area response for both cholesterol and DOPC. Relative standard deviations (RSDs) for both cholesterol and DOPC are <1.000%, indicating sufficient instrument reproducibility with this method.

For sample precision measurements, six replicate samples were prepared and analyzed on the first day. For each sample preparation, three injections were performed. On the second day, the same analyst prepared a second set of six samples from the same sample vial and independently analyzed on the same system. Results are summarized in Table 5. The RSD values are found to be <1.4% for both cholesterol and DOPC on each day and <2.0% between days 1 and 2. These results clearly indicate sufficient sample repeatability with this method.

Intermediate precision was evaluated by comparing the results between two analysts on two systems. Results of intermediate precision are presented in Table 6. Overall RSD between analysts 1 and 2 are 0.467 and 2.141% for cholesterol

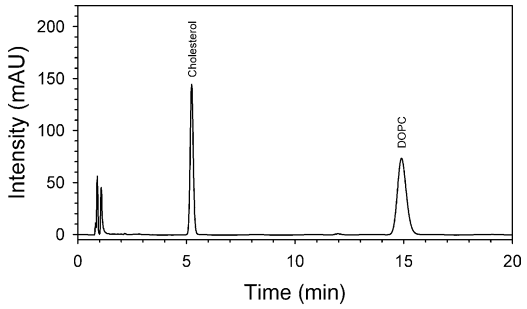

Fig. 2. Chromatogram of a calibration standard containing 100 and 500 μg/mL of cholesterol and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), respectively.

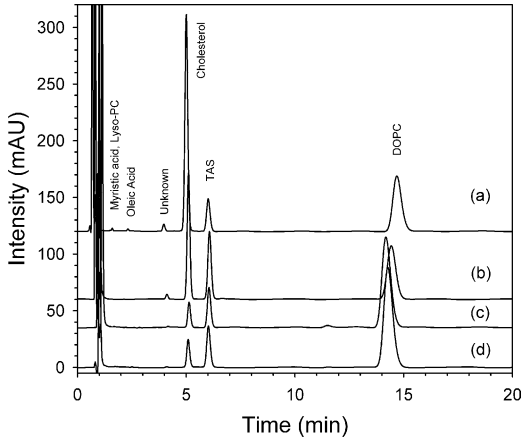

Fig. 3. Typical chromatograms of liposome-based SN-38 (a), antisense oligodeoxyribonucleotide (b), docetaxel (c) and paclitaxel (d).

Table 4
Reproducibility of retention times and peak areas of standard compounds

Analyte	Retention time[a]		Peak area[a]	
	Mean (min)	RSD (%)	Mean (mAU s)	RSD (%)
Cholesterol	5.591(0.006)	0.099	2009.883 (2.949)	0.147
DOPC	16.642(0.026)	0.905	1515.582 (13.721)	0.905

[a] Results are based on six injections. Standard deviation is given in parentheses.

Table 5
Summary of method precision results (analyst 1)

	Analyte	Average concentration[a] (mg/mL)	RSD[a] (%)
Day 1	Cholesterol	15.337 (0.165)	1.075
	DOPC	33.083 (0.279)	0.844
Day 2	Cholesterol	14.946 (0.125)	0.835
	DOPC	32.537 (0.433)	1.332
Between days 1 and 2	Cholesterol	15.142 (0.245)	1.619
	DOPC	32.810 (0.454)	1.383

[a] Based on 18 data points. Standard deviation is given in parentheses.

Table 6
Summary of intermediate precision measurement studies

Analyte	Analyst 1		Analyst 2		Results between two analysts	
	Average concentration[a] (μg/mL)	RSD[a] (%)	Average concentration[a] (μg/mL)	RSD[a] (%)	Average concentration[a] (μg/mL)	RSD[a] (%)
Cholesterol	13.357 (0.077)	0.579	13.330 (0.040)	0.302	13.343 (0.062)	0.467
DOPC	37.483 (0.390)	1.041	36.618 (0.865)	2.361	37.050 (0.793)	2.141

[a] Based on 18 data points. Standard deviation is given in parentheses.

and DOPC, respectively, indicating sufficient reproducibility with this method.

3.4. Quantitation limit

There are at least four different ways to determine quantitation limits of analytes [21] and signal-to-noise ratio (S/N) is one of the most commonly used procedures. We have used this procedure for determining quantitation limits of cholesterol and DOPC. Stock solutions of individual lipid components, namely, cholesterol and DOPC, were progressively diluted and signal-to-noise ratios for cholesterol and DOPC were determined until a minimum S/N ratio of 10 was achieved. Using this method, quantitation limits of 0.5 and 10.0 μg/mL for cholesterol and DOPC, respectively, were observed. Detection limit of 1.5 μg/mL (defined as signal-to-noise ratio of 2) for cholesterol in liposome-based drug formulation samples has been previously reported [9].

3.5. Specificity

The specificity of the method was evaluated by spiking expected degradants of the lipids on excipients and by performing forced degradation of liposome-based samples under different stress conditions. Forced degradation was conducted using 0.05 M NaOH, 0.05 M HCl, 3% H_2O_2 and thermal treatment at 60 °C. The extent of degradation under each condition was evaluated based on the recovery in the sample after being stressed. Methanol and mobile phase were injected as blanks and no detectable peaks were observed. A typical chromatogram containing expected degradants is shown in Fig. 4. Peak identification was achieved using retention time by injecting individual components. Myristic acid, oleic acid, lyso-PC and TAS were individually injected to determine the retention time in order to identify the degradant and TAS peaks in the formulations. Oleic acid and lyso-PC are the expected degradants from DOPC where as myristic acid is an expected degradant from cardiolipin. TAS is added in the formulation as an antioxidant. For all the liposome formulations, the drug compound eluted at the front with void volume. Identification was performed by collecting the UV–vis spectrum using diode array detection. UV–vis spectrum of the strong peak observed at the void time was found to be the same as for authentic drug compound. Both cholesterol and DOPC peaks were resolved from any expected degradants as well as from TAS antioxidant peak. Fig. 5 represents a chromatogram where LE-SN38 sample was stressed for degradation under basic condition. Peaks for unidentified degradants are found in the chromatogram at retention time of approximately 4 min. However, these peaks were clearly well separated from the adjacent cholesterol peak. Specificity results clearly indicate that the method is specific for resolving cholesterol and DOPC peaks from any of the degradant peaks and are free of interference.

3.6. Robustness

Robustness is the capacity of a method to remain unaffected by small, deliberate variations in method parameters and measures reliability of the method. To test the robustness of the method, we deliberately varied four parameters, pH of the buffer, column temperature, mobile phase composition and flow rate.

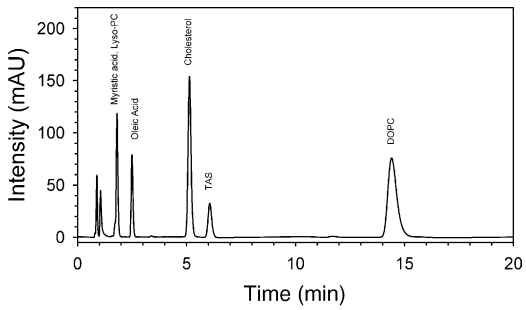

Fig. 4. Chromatogram of various lipids and expected degradants in presence of other excipients.

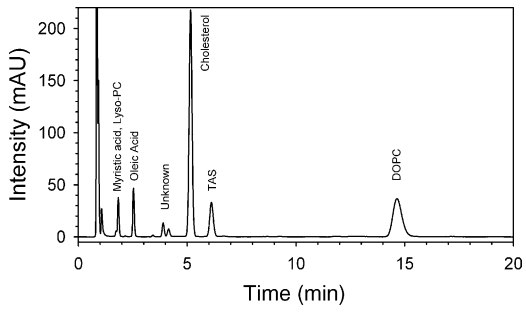

Fig. 5. Chromatogram of a 100-fold diluted LE-SN38 sample with ca. 28% degradation of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC).

Table 7
Summary of the robustness study results

Parameter		Critical resolution	Tailing factor		Plate count		Overall RSD (%)[a]		Correlation coefficient (r)		Retention time	
			Cholesterol	DOPC	Cholesterol	DOPC	Cholesterol	DOPC	Cholesterol	DOPC	Cholesterol	DOPC
Column temperature (°C)	58	4.74	1.06	1.12	7505	6432	1.62	1.54	0.99991	0.99991	5.52	16.13
	60	4.64	1.06	1.16	7281	6286	0.73	1.15	0.99986	0.99975	5.28	14.93
	62	4.46	1.07	1.17	7135	5881	0.99	1.15	0.99987	0.99987	5.02	13.90
Flow rate (mL/min)	1.8	4.76	1.06	1.15	7659	6444	0.43	0.74	0.99983	0.99962	5.89	16.83
	2.0	4.64	1.06	1.16	7281	6286	0.73	1.15	0.99986	0.99975	5.26	14.93
	2.2	4.44	1.08	1.17	6670	5496	0.77	1.01	0.99982	0.99978	4.86	13.96
Mobile phase (buffer–methanol, v/v)	13:87	3.87	1.06	1.17	6688	5622	0.52	0.48	0.99977	0.99977	3.98	9.45
	15:85	4.64	1.06	1.16	7281	6286	0.73	1.15	0.99986	0.99975	5.26	14.93
	17:83	5.15	1.06	1.17	6940	5270	0.24	0.74	0.99988	0.99979	7.08	24.47
Buffer pH	2.5	4.45	1.06	1.18	6713	5250	0.18	0.46	0.99985	0.99981	5.28	15.19
	2.7	4.64	1.06	1.16	7281	6286	0.73	1.15	0.99986	0.99975	5.26	14.93
	2.9	4.42	1.06	1.17	6775	5324	0.14	0.37	0.99976	0.99969	5.19	14.78

[a] For each sequence run, a calibration standard solution (100% of target level) was injected at the beginning of the sequence (six injections), after the five levels of calibration standards (two injections) and at the end of the sequence (two injections) to verify the system suitability of the overall run sequence. The percent RSD data are based on the response (area counts) of these 10 system suitability injections for each sequence run.

The buffer solutions with pH values of 2.5, 2.7 and 2.9 were used to study the effect of pH variation on the performance of the method. With each variation of buffer pH, analysis of a sample solution was performed and the chromatographic results were evaluated. Data were collected at three column temperature settings at 58, 60 and 62 °C to study the effect of column temperature variation on the performance of the method. With each variation of column temperature, analysis of a sample solution was performed and the chromatographic results were evaluated. The mobile phase flow rates were set at 1.8, 2.0 and 2.2 mL/min to study the effect of mobile phase flow rate variation on the performance of the method. Three different mobile phase compositions (13:87, 15:85 and 17:83 (v/v) buffer–methanol) were used to study the effect of varying the mobile phase composition on the performance of the method.

Robustness data from the above experiments are summarized in Table 7. The retention times for both cholesterol and DOPC decreased with increasing column temperature, mobile phase flow rate and pH of the buffer, which is expected. The chromatographic performance of the method does not change significantly. The retention times for cholesterol and DOPC increased with increasing polarity of the mobile phase composition. However, it is important to note that the overall performance of the method remained unaffected.

4. Conclusions

A rapid isocratic HPLC method for simultaneous quantitation of cholesterol and DOPC in liposome-based formulations has been developed. The method is linear, precise, accurate, sensitive, robust and specific for quantitation of cholesterol and DOPC in liposome-based formulations. In addition, this method is applicable to several of the liposome-based formulations for quantitation of cholesterol and DOPC and does not require preprocessing of the samples. This method has improved quantitation limits compared to other methods for analyzing cholesterol in liposome-based formulations.

References

[1] F.B. Jungawala, J.E. Evans, R.H. McCluer, Biochem. J. 145 (1975) 517.
[2] J.L. Hohnson, A. Ahmad, S. Khan, Y.-F. Wang, A.W. Abu-Qare, J.E. Ayoub, A. Zhang, I. Ahmad, J. Chromatogr. B 799 (2004) 149.
[3] P.C. Gokhale, J. Pei, C. Zhang, I. Ahmad, A. Rahaman, U. Kasid, Anticancer Res. 21 (2001) 3313.
[4] P.C. Gokhale, C. Zhang, J.T. Newsome, J. Pei, I. Ahmad, A. Rahman, A. Dritschilo, U.N. Kasid, Clin. Cancer Res. 8 (2002) 3611.
[5] J.A. Zhang, T. Xuan, M. Parmar, L. Ma, S. Ugwu, S. Ali, I. Ahmad, Int. J. Pharm. 270 (2004) 93.
[6] W.M.A. Hox, W.S.M. Geurts van Kessel, J. Chromatogr. 142 (1977) 735.
[7] R.M. Carrol, L.L. Rudel, J. Lipid Res. 22 (1981) 359.
[8] G.M. Patton, J.M. Fasulo, S.J. Robins, J. Lipid Res. 23 (1983) 190.
[9] J.G. Hamilton, K. Comai, J. Lipid Res. 25 (1984) 1142.
[10] T.L. Kaduce, K.C. Norton, A.A. Spector, J. Lipid Res. 24 (1983) 1398.
[11] J.R. Yandrasitz, G. Berry, S. Segal, J. Chromatogr. 225 (1981) 319.
[12] O. Meyer, O. Roch, D. Elmlinger, H.J.J. Kolbe, Eur. J. Pharm. Biopharm. 50 (2000) 353.
[13] J.K. Lang, J. Chromatogr. 507 (1990) 157.
[14] A.M. Samuni, Y. Barenholz, D.J.A. Crommelin, N.J. Zuidam, Free Radic. Biol. Med. 23 (1997) 972.
[15] C.-Y. Chow, T.D. Heath, Biochim. Biophys. Acta 1239 (1995) 168.
[16] Y. Shimizu, M. Nakata, J. Matsunuma, T. Mizuochi, J. Chromatogr. B 754 (2001) 127.
[17] Draft Guidance for Industry: Analytical Procedures and Methods Validation, US Department of Health and Human Services, US Food and Drug Administration, Center for Drug Evaluation and Research,

Center for Biologics Division of Research, Rockville, MD, August 2000.
[18] ICH Q2B Validation of Analytical Procedures: Methodology, International Conference on Harmonization of Technical Requirements for Registration of Pharmaceutical for Human Use, Geneva, May 1997.
[19] ICH Q2A Text on Validation of Analytical Procedures, International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use, Geneva, March 1995.
[20] Reviewer Guidance: Validation of Chromatographic Methods, US Department of Health and Human Services, US Food and Drug Administration, Center for Drug Evaluation and Research, Rockville, MD, November 1994.
[21] T.C. Paino, A.D. Moore, Pharm. Technol. (1999) 86.