Journal of Chromatography, 348 (1985) 221–227
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 110

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF LIPIDS IN VESICLES

M. S. McCRACKEN* and N. J. HOLT
Procter and Gamble Company, Miami Valley Laboratories, P.O. Box 39175, Cincinnati, OH 45247 (U.S.A.)
(Received August 14th, 1985)

SUMMARY

Accurate quantitation of the four lipid components in a vesicle formulation has been accomplished by selectively changing the high-performance liquid chromatographic mobile phase composition to arrive at an optimal separation requiring less than 10 min. A systematic approach to selection of the mobile phase conditions has minimized the number of experiments required to achieve the necessary separation. Relative standard deviations between one and four percent are obtained for the four lipid components. Accuracy, as measured by recovery experiments for spiked vesicle formulations, varies between one and two percent of target levels at the nominal lipid concentrations investigated.

INTRODUCTION

Vesicles have been investigated for almost twenty years as model membrane systems and more recently as drug delivery systems[1]. As a drug carrier, the vesicle bilayer composition can effect the *in vitro* and *in vivo* stability of the vesicle[1–4]. Quantitation of the vesicle components is necessary to determine the bilayer composition for formulations produced with different lipid components and by different vesicle making processes. In addition, stability testing of the vesicle requires accurate and precise methods to determine the bilayer components and the occurrence of any degradation products.

A recent review article[5] discusses a number of different approaches to analysis of lipids in different biological membranes. For vesicle formulations, one approach used to measure the lipids in the bilayer is an assay based on phosphorous analysis of extracted samples[6]. Results are corrected for the presence of non-phosphorus-containing lipids. This procedure is time consuming and not specific for the different bilayer components. Time-consuming, two-dimensional thin-layer chromatography has been used on lipid extracts[7] to provide lipid levels of individual vesicle components.

In cases where large numbers of vesicle solutions need to be analyzed, a high-performance liquid chromatography (HPLC) method that is rapid and provides

0021-9673/85/$03.30

quantitative information on the individual bilayer components would be desirable. The time required for the development of the chromatographic conditions can be minimized by using a systematic approach rather than a trial-and-error approach. One such approach reported in the literature is the four-solvent optimization method[8]. This paper describes the application of the four-solvent triangle technique to identify a suitable separation of the lipid components in a vesicle formulation. The approach used to achieve the final separation conditions is described. Data supporting the accuracy and precision of the method are presented.

EXPERIMENTAL

Chemicals

Cholesterol was obtained from Byron Chemical (Long Island City, NY, U.S.A.). Dipalmitoyl phosphatidyl choline (DPPC) was purchased from Avanti Biochemicals (Birmingham, AL, U.S.A.). The glycolipid used in the vesicle bilayer (digalactosyl diglyceride) was isolated from wheat flour. The isolated material was primarily a mixture of two digalactosyl diglycerides (DGDG) differing in the aliphatic chain length composition. Twenty-five percent of the material has a chain length composition of C_{16} and C_{18} (DGDG1) with the remainder differing only in that the chain length composition was C_{18} (DGDG2). Identification of the composition was performed using mass spectrometry on the two fractions isolated from the chromatographic procedure described below.

Dipalmitoyl phosphatidyl glycerol (DPPG) was obtained from Sigma (St. Louis, MO, U.S.A.). All solvents used were of HPLC grade. Solvents were thoroughly degassed individually before use and were mixed with Nanopure-treated water (Barnstead, Boston, MA, U.S.A.). Tromethamine hydrochloride (Tris) was obtained from American Research Products (Euclid, OH, U.S.A.) and edetate disodium (EDTA) from Sigma. The buffer solution for the vesicles contained 17.5 mg/ml of Tris and 1.0 mg/ml of EDTA at a pH of 7.4. Standard solutions of the lipid components for use in the working curves were prepared in a 50:50 mixture of the Tris–EDTA buffer and tetrahydrofuran. Porcine insulin that was encapsulated in the vesicles was obtained from E. R. Squibb & Sons (Atlanta, GA, U.S.A.).

Apparatus

The HPLC method was developed using a Spectra-Physics (Arlington Heights, IL, U.S.A.) Model SP8700 pump with a Valco (Houston, TX, U.S.A.) injector and a 200-μl sample loop. Mobile phase flow-rate was maintained at 1.5 ml/min. An on-line degasser ERC-3000 from Erma Optical Works (Tokyo, Japan) was used to remove any gas dissolved in the mobile phase.

The detector was a Spectra-Physics Model 8440 variable wavelength detector set at 214 nm. Detector response was recorded with a Spectra-Physics Model 4100 computing-integrator. An Ultrasphere-Octyl (15 cm × 4.6 mm) column from Altex (Berkeley, CA, U.S.A.) was used. The column temperature was maintained at 50°C with a Sys-tec (Minneapolis, MN, U.S.A.) Model CH-1448 dual zone temperature controller. Earlier work had shown increased resolution at this temperature.

Preparation of vesicles

A lipid solution containing cholesterol, dipalmitoyl phosphatidyl choline, digalactosyl diglycerides and dipalmitoyl phosphatidyl glycerol in a molar ratio of 40:40:15:5 was dissolved in chloroform. The chloroform was then evaporated under vacuum leaving a lipid crust. An aqueous solution containing porcine insulin was added to the crust and sonicated for 10 min at 50 W and 55°C using a cup-horn sonicator from heat Systems-Ultrasonic (Pittsburg, PA, U.S.A.). The resulting mixture was centrifuged at 20000 *g* to remove any solid components which were not converted to vesicles and any oversized vesicles. Unencapsulated insulin was removed by ion exchange. The vesicle concentration was adjusted by dilution in Tris–EDTA buffer to produce a vesicle level equivalent to about 1 mg/ml cholesterol.

Vesicles produced by this manner were typically unilamellar with a mean diameter of about 800 Å and a final cholesterol concentration of 1.0 mg/ml. Vesicle diameters were examined by electron microscopy following negative staining. The vesicles were disrupted prior to injection onto the HPLC column by mixing one-to-one with tetrahydrofuran (THF).

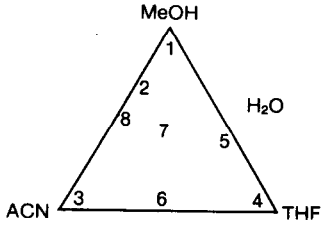
RESULTS AND DISCUSSION

Mobile phase optimization

A systematic approach was taken to achieve the separation using the four-solvent optimization strategy[8,9]. This approach allows rapid identification of the best

TABLE I

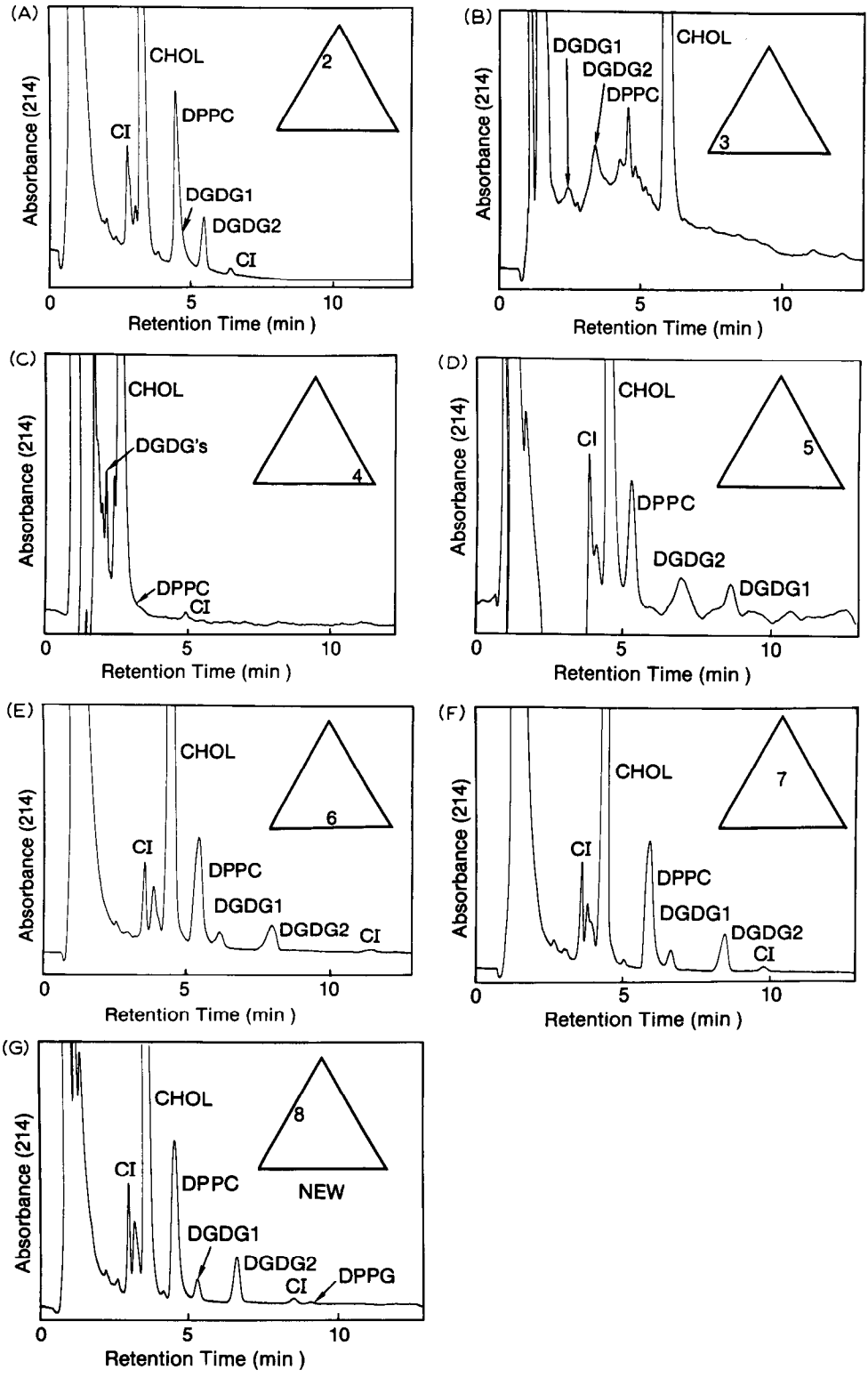
MOBILE PHASE COMPOSITION FROM SOLVENT SELECTIVITY TRIANGLE



	Mobile phase composition			
	MeOH (%)	*ACN (%)*	*THF (%)*	*H_2O (%)*
1*	97	0	0	3
2	60	35	0	5
3	0	94	0	6
4	0	0	66	34
5	25	0	48	27
6	0	50	30	20
7	32	31	22	15
8**	25	25	32	18

* Sample not soluble in mobile phase.

** Best mobile phase.


(A)
Absorbance (214)
CHOL
CI
DPPC
DGDG1
DGDG2
CI
2
0
5
10
Retention Time (min)
(B)
Absorbance (214)
DGDG1
DGDG2
DPPC
CHOL
3
0
5
10
Retention Time (min)
(C)
Absorbance (214)
CHOL
DGDG's
4
DPPC
CI
0
5
10
Retention Time (min)
(D)
Absorbance (214)
CHOL
CI
DPPC
DGDG2
DGDG1
5
0
5
10
Retention Time (min)
(E)
Absorbance (214)
CHOL
CI
DPPC
DGDG1
DGDG2
CI
6
0
5
10
Retention Time (min)
(F)
Absorbance (214)
CHOL
CI
DPPC
DGDG1
DGDG2
CI
7
0
5
10
Retention Time (min)
(G)
Absorbance (214)
CHOL
CI
DPPC
DGDG1
DGDG2
CI
DPPG
8
NEW
0
5
10
Retention Time (min)

separation that can be achieved using a given stationary phase packing. A C_8 stationary-phase was chosen because of our earlier success in separation of similar types of compounds. The solvent selectivity triangle organizes solvents in groups according to their contribution factors (proton acceptor, proton donor or dipole interaction). Experiments are conducted using solvents from groups nearest the apexes of the triangle to obtain the widest selectivity difference. Intermediate selectivities are obtained by blending solvents.

Each solvent is assigned a strength weighing factor. Mixtures of solvents with equal solvent strengths will provide an approximately equivalent range of capacity factors, k'. Initial strength of the mobile phase was chosen to be about 2.9 so as to provide k' values for each of the lipid components between 1 and 10. The three solvents used for the reversed-phase experiments were methanol, acetonitrile and THF with water being used as a strength adjusting solvent. Table I shows the mobile phase compositions 1–7 designed to identify the necessary separation and the final optimal composition 8. The chromatograms obtained from the different mobile phase mixtures are shown in Fig. 1A–G corresponding to composition 2–8 in Table I. The elution times for the lipid components of the vesicles are labeled. Other peaks are due to the impurities present in the cholesterol used to make the vesicles.

Evaluation of the chromatograms focussed primarily on the separation of DPPC and DGDG1. Because of the low levels of DGDG1 (*ca.* 0.15 mg/ml) and the low molar absorptivity of this glycolipid at 214 nm, routine quantitation of DGDG1 is difficult unless adequate separation is achieved. This aspect of the separation had proven difficult in earlier trial-and-error approaches to mobile phase optimization.

Fine-tuning of the mobile phase composition focused on Separations 2, 6 and 7. Separation 7 provides the necessary separation of the components of the four lipid components of interest (cholesterol, DGDG1, DGDG2 and DPPC) and the cholesterol impurities. Separation 2 provides a shorter analysis time, but does not provide adequate resolution of DPPC and DGDG1. Separation 6 provided similar separation as seen in Separation 7. The compromise between the decreased analysis time and necessary separation was achieved in Separation 8. Separation 8 does not necessarily represent the optimal separation, but a suitable separation that could be quickly identified by the systematic approach described in this work.

Impurities in the cholesterol are not baseline resolved from cholesterol in Separation 8; however, any contribution of the impurities to the cholesterol area have little effect on quantitation of cholesterol. The low levels of DPPG in the samples (*ca.* 0.1 mg/ml) and its low molar absorptivity at 214 nm made it difficult to quantitate. In each case it eluted after the other components and only in Separation 8 was DPPG distinguishable from the baseline for routine samples.

Linearity, precision and accuracy

Working curves for cholesterol, DPPC, DGDG1 and DGDG2 were run using Separation 8. The concentration of cholesterol standards was from 0.1 to 1.0 mg/ml with DPPC standards being from 0.2 to 2.0 mg/ml. DGDG1 standards were from 0.05 to 0.25 mg/ml while DGDG2 standards were from 0.15 to 0.75 mg/ml. Corre-

Fig. 1. Chromatographic separation of lipid components. (A) No. 2 (see Table I); (B) No. 3; (C) No. 4; (D) No. 5; (E) No. 6; (F) No. 7; (G) No. 8. Abbreviations: CI = cholesterol impurity found in starting material. See the chemicals section for other abbrevations.

TABLE II

PRECISION DATA FOR A VESICLE FORMULATION

		Cholesterol (mg/ml)	*DPPC (mg/ml)*	*DGDG (1) (mg/ml)*	*DGDG (2) (mg/ml)*
Within-day precision*	Mean	0.486	0.988	0.144	0.376
	Std. dev.	0.003	0.012	0.004	0.011
	R.S.D. (%)	0.7	1.3	3.1	3.0
Day-to-day precision**	Mean	0.508	0.986	0.144	0.374
	Std. dev.	0.016	0.018	0.004	0.015
	R.S.D. (%)	3.1	1.8	3.1	3.9

* Data based on analysis of one sample seven times.

** Data based on results for eight days for a vesicle sample with the data for each day an average of triplicate analysis.

lation coefficients for the working curves for the four lipid components generally exceed 0.999 and values of 0.9999 have been achieved. Relative *Y*-intercepts are less than 1% of the middle standards.

The precision obtained using Separation 8 was evaluated on a representative

TABLE III

SPIKING ACCURACY DATA FOR A VESICLE FORMULATION

	Spiking level (mg/ml)	*Analytical result* (mg/ml)*	*Recovery (%)*
Cholesterol	0.120	0.120	100.0
	0.330	0.327	99.1
	0.620	0.619	99.8
	0.820	0.822	100.2
	1.014	1.013	99.9
DPPC	0.240	0.231	96.3
	0.650	0.642	98.8
	1.200	1.209	100.8
	1.600	1.590	99.4
	2.030	1.990	98.0
DGDG (peak 1)	0.034	0.048	141.2
	0.091	0.103	113.2
	0.170	0.170	100.0
	0.226	0.222	98.2
	0.283	0.277	97.9
DGDG (peak 2)	0.086	0.079	91.9
	0.229	0.235	102.6
	0.430	0.440	102.3
	0.574	0.568	99.1
	0.717	0.722	100.7

* Data based on duplicate results.

vesicle preparation on a single day and over a period of eight days. These data are summarized in Table II. The higher relative standard deviation for the DGDG components reflects the difficulty in routinely quantitating the glycolipids at the low levels present in the vesicle formulation.

The accuracy was evaluated by spiking mixtures of lipids at different concentrations into a Tris–EDTA–THF matrix equivalent to that found after the sample preparation step. The spiked solutions as shown in Table III represent levels between 24 and 200% of normal samples (0.5 mg/ml cholesterol). The results in Table III indicate good recovery. In most cases the difference at the target level of lipids in the vesicles was less than 1%. Only at the low levels of the DGDG's was there large deviations in the recoveries from the spiking levels. The deviations at those points again reflects the difficulty in quantitating the glycolipids at the low levels.

CONCLUSIONS

This study has shown that isocratic method development for major lipids in a vesicle formulation can be achieved quickly by an organized route. By using the solvents around the solvent selectivity triangle, a suitable mobile phase was identified by qualitatively comparing the seven chromatograms. Fine-tuning of the method provided optimal peak resolution in an additional step. The resulting chromatographic conditions enabled accurate and precise quantitation of the lipids in a vesicle formulation in less than ten minutes.

Although this method was developed for a particular vesicle formulation, a similar approach may be useful to establish mobile phase conditions for separation of lipids in other formulations.

REFERENCES

1 H. M. Patel and B. E. Ryman, in C. G. Knight (Editor), *Liposomes: From Physical Structure to Therapeutic Applications*, Elsevier/North-Holland Biomedical Press, New York, 1981, p. 411.
2 R. N. Rowland and J. F. Woodley, *Biochim. Biophys. Acta*, 620 (1980) 400.
3 P. S. Wu, G. W. Tin, J. D. Baldeschwieler, T. Y. Shen and M. M. Ponipom, *Proc. Nat. Acad. Sci. U.S.A.*, 78 (1981) 6211.
4 C. M. Gupta, A. Bali and S. Dhawan, *Biochim. Biophys. Acta*, 648 (1981) 192.
5 A. R. N. Eberendu, B. J. Vernables and K. E. Daugherty, *LC, Liq. Chromatogr. HPLC Mag.*, 3 (1985) 424.
6 R. M. Abra, C. A. Hunt and D. T. Lau, *J. Pharm. Sci.*, 73 (1984) 203.
7 A. T. M. van der Steen, B. de Kruijff and J. de Gier, *Biochim. Biophys. Acta*, 691 (1982) 13.
8 J. L. Glajch, J. J. Kirkland, K. M. Squire and J. M. Minor, *J. Chromatogr.*, 199 (1980) 57.
9 L. R. Snyder, *J. Chromatogr. Sci.*, 16 (1978) 223.