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Short Communication

Determination of dimyristoylphosphatidylglycerol in human serum by liquid–liquid extraction and reversed-phase liquid chromatography

KISHOR M. WASAN,† ALAN C. HAYMAN‡ and GABRIEL LOPEZ-BERESTEIN*†

†*Department of Clinical Investigations, The University of Texas M.D. Anderson Cancer Center, Houston, TX 77030, USA*
‡*Argus Pharmaceuticals, The Woodlands, TX 77381, USA*

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Introduction

Dimyristoylphosphatidylglycerol (DMPG) (Fig. 1), an anionic phospholipid not found in human serum, is commonly used in the preparation of liposomes [1–5]. The determination of phosphatidylglycerol in rat alveolar macrophages [6] using cyanopropyl columns and bronchoalveolar lavage fluid in humans [7] has been previously demonstrated. Furthermore, Bonanno *et al.* have determined phosphatidylglycerol from a pulmonary surfactant using an on-line coupled silica/reversed-phase high-performance liquid chromatography system [8]. However, to date, a technique to determine DMPG in human serum has not been developed.

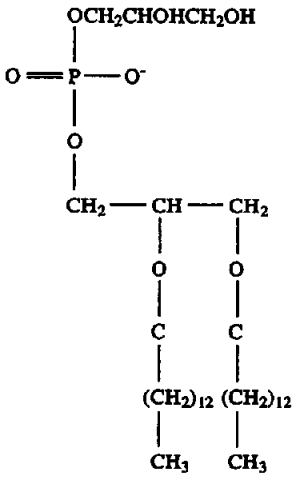

Figure 1
Chemical structure of dimyristoylphosphatidylglycerol.

A number of clinical studies are ongoing with drugs incorporated into liposomes including liposomal amphotericin (L-AmpB) [9], all *trans* retionic acid [10], nystatin [11], and *cis-bis*-neodecanoato-*trans*-R,R-1,2 diaminocyclohexane platinum (II) [12] where DMPG is used in their formulation. A technique to further understand the fate of these liposomal compounds within the bloodstream would be useful. This paper reports a simple and rapid method for measuring DMPG in human serum by utilizing liquid–liquid extraction followed by reversed-phase isocratic high-performance liquid chromatography.

Materials and Methods

Apparatus

The chromatographic system employed consisted of a constametric II G pump (LDC/Milton Roy, Riviera, FL), a Rheodyne 7126 injector (Rainin Instruments), and a variable-wavelength detector (Spectromonitor D; LDC/Milton Roy). Results were recorded on a Hewlett–Packard HP 3396 integrator. For chromatographic separation, a Zorbax C_{18}

* Author to whom correspondence should be addressed.

column (5 μm particle size, 250 mm × 4.6 mm i.d.; Mac-Mod) was used.

Chemicals

Chromatographically pure (96% purity) DMPG was obtained from Nippon Fine (Tokyo, Japan). Methyl-*t*-butyl ether (MTBE), and other solvents were purchased from J.T. Baker (Phillipsburg, NJ, USA). Phosphoric acid was purchased from Sigma (St Louis, MO, USA) and pooled human serum was obtained from the M.D. Anderson Blood Bank.

Liquid chromatography

All experiments were carried out at ambient room temperature (approximately 23°C). The column was equilibrated with the mobile phase for at least 30 min prior to analysis of samples. The mobile phase consisted of MTBE and water containing 10 mM of phosphoric acid (70:30, v/v; pH 4.6) at a flow rate of 2 ml min^{-1}. DMPG was detected by UV absorbance at 213 nm (AUFS 0.005).

Sample preparation

Different concentrations of DMPG (125, 62.5, 31.3, 15.6 and 7.8 μg of DMPG ml^{-1} of serum) were extracted from human serum with MTBE and water containing 10 mM of phosphoric acid (90:10, v/v; pH 5.9; 2:1, v/v extractant:serum). This mixture was vortexed for 10 s, extracted by shaking at 37°C in an incubator for 30 min and the organic extractant (50 μl) was injected onto the HPLC column. The within-day and between-day precision was established by assaying the different concentrations of DMPG (n = 6) at four different

Table 1
Mean (±SD) extraction efficiency of DMPG from human serum

Initial DMPG conc. (μg ml^{-1})	DMPG conc. in extractant* (μg ml^{-1})	Percent extracted* (%)
125	113 ± 2.4	90.4 ± 2.0
62.5	61.0 ± 1.2	97.5 ± 1.7
31.25	29.3 ± 1.5	93.6 ± 4.7
15.6	14.8 ± 0.5	94.4 ± 3.2
7.8	7.7 ± 0.2	98.7 ± 2.8

*(n = 6).

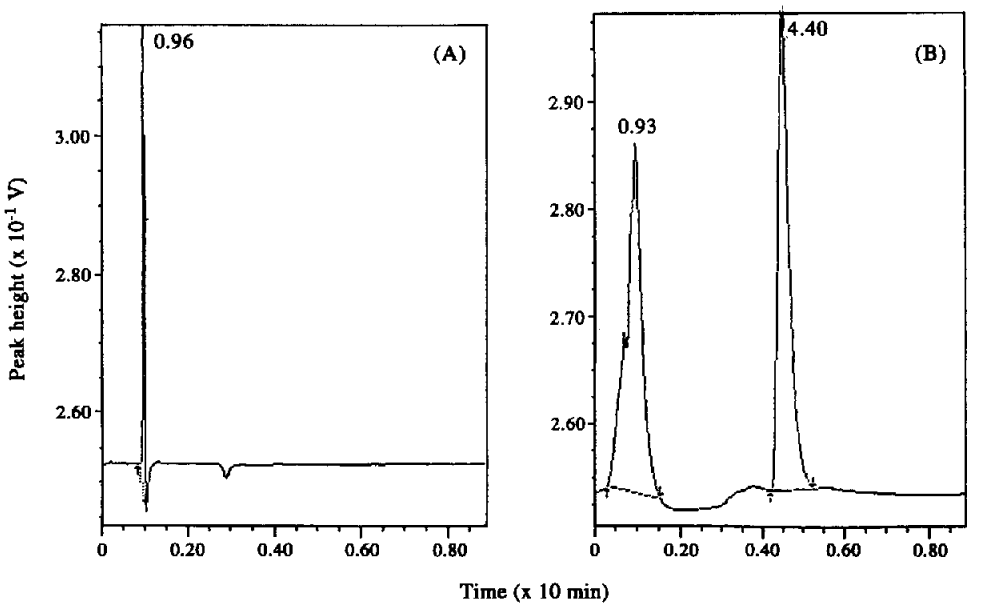

Figure 2
Elution pattern for (a) blank human serum (solvent front retention time = 0.93 min) and (b) dimyristoylphosphatidylglycerol (DMPG 125 μg ml^{-1}) extracted from human serum on a Zorbax C_{18} column [DMPG retention time = 4.4 min; (5 μM particle size, 250 mm × 4.6 mm i.d.)]. Flow rate 2.0 ml min^{-1}; wavelength = 213 nm. Eluent–methyl *t*-butyl ether and 10 mM phosphoric acid in water (70:30, v/v; pH 4.6).

times during the day and on four different days.

The assay was evaluated by incubating L-AmpB (which contains 60 μg ml^{-1} of DMPG for every 20 μg ml^{-1} of amphotericin B) in non-treated human serum for 60 min at 37°C. Following the incubation, serum samples were separated into their high-density (HDL) and low-density (LDL) lipoprotein fractions by size-exclusion and affinity chromatography as previously described [13]. Concentrations of DMPG in each lipoprotein fraction were evaluated by determining the peak area of DMPG in each lipoprotein fraction and then comparing it with the standard curves (in both serum and the separated lipoprotein fractions) obtained after regression analysis of the calibration samples.

Results and Discussion

The mean recovery for DMPG was in excess of 90% (relative standard deviation of less than 5%) over the range of the calibration curves for DMPG (7.8–125 μg ml^{-1}) (Table 1) when compared to the direct injection of the standard dissolved in MTBE and water containing phosphoric acid. Figure 2 shows typical elution profiles for blank serum and a sample containing DMPG. The standard curve in serum was linear for both within-day and between-day DMPG concentrations over a range of 7.8–125 μg ml^{-1} and the correlation coefficient was greater than 0.99 for each regression line (Table 2). The relative standard deviations for DMPG concentrations ranged from 2.3 to 5.5% for within-day and 2.7 to 5.5% for between-day determinations (Table 3). The retention time of DMPG following liquid–liquid extraction from serum was 4.4 min. The limit of quantification for DMPG was 7.8 μg ml^{-1} (signal-to-noise ratio = 8). When L-AmpB was incubated in non-treated human serum for 60 min at 37°C over 80% of the initial DMPG concentration was found in the HDL fraction and 12% in the LDL fraction.

This assay may be used to determine the pharmacokinetics and serum distribution of the DMPG component of liposomes [6] and to further explain the behaviour of liposomes within the bloodstream.

Conclusions

An LC assay for the determination of DMPG in serum has been developed involving liquid–liquid extraction followed by isocratic HPLC analysis. The DMPG peak was eluted within 5 min, following sample injection, without interference from other endogenous compounds. This simple and rapid assay for DMPG can be utilized to further follow the distribution and behaviour of liposomes within the bloodstream.

Table 2
Mean (±SD) linear calibration curve for DMPG carried out at four different times during the day and on four different days

Calibration curve	Equation	Correlation coefficient
Within-day DMPG conc.	$y = 2371\ (123)\ x + 1095.9\ (54)$	0.997 (0.006)
Between-day DMPG conc.	$y = 2308.5\ (167)\ x + 721.49\ (76)$	0.996 (0.023)

y = Peak area (μV s^{-1}).
x = Concentration of DMPG.

Table 3
Precision date for DMPG in human serum

Intial DMPG conc. (μg ml^{-1})	Within-day ($n = 6$)* DMPG conc. (μg ml^{-1})	RSD (%)	Between-day ($n = 6$)* DMPG conc. (μg ml^{-1})	RSD (%)
125	114.0 ± 3.5	3.1	117.1 ± 3.9	3.3
62.5	60.0 ± 1.9	3.2	61.7 ± 3.0	4.8
31.25	30.0 ± 0.9	3.1	30.6 ± 1.7	5.5
15.6	15.0 ± 0.4	2.7	15.1 ± 0.3	2.3
7.8	7.7 ± 0.4	5.5	7.4 ± 0.3	3.7

*Mean ± standard deviation (SD).

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