Pergamon

Journal of Pharmaceutical & Biomedical Analysis, Vol. 12, No. 8, pp. 961–968, 1994

0731–7085/94 $7.00 + 0.00

0731–7085(94)00044–1

A rapid method for the separation and analysis of leaked and liposomal entrapped phosphoramide mustard in plasma*

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Abstract: Pharmacokinetic studies of liposomal drugs should include simultaneous determination of leaked and entrapped drug in biological specimens. Due to the limited stability of many liposomal preparations in biological samples, a rapid analytical procedure is often necessary. Phosphoramide mustard (PM), a key cytotoxic metabolite of a widely used alkylating drug cyclophosphamide, has recently been entrapped into a liposomal formulation and the preparation has been found to be rather unstable in plasma. We have, therefore, developed a rapid method for the separation of liposome-associated PM from the unassociated drug and a method for their quantitation in plasma. This method involves the use of size exclusion mini-gel column and requires minutes to process. Due to the use of internal standards, this method tolerates low recovery and requires the collection of a single fraction of each of liposome-associated PM and the unassociated drug. The recovery of liposomal PM from the first fraction of the gel column was found to be 82.4 ± 7.9% (SD, $n = 8$), whereas that of liposome-unassociated PM from the major fraction was 16.8 ± 2.8% (SD, $n = 8$). However, the low recovery problem of liposome-unassociated PM was circumvented by adding the internal standard [α,β-$^2H_8$] PM prior to separation, thus compensating for the loss of liposome-unassociated PM due to incomplete collection. Two types of standard curve were constructed for quantitation of liposome-associated PM and unassociated PM and the linearity for both was excellent. Assay validation indicated that within-run RSD values at 213 ng, 426 ng and 1065 ng for liposomal PM were 4.2, 4.3 and 3.0%, respectively. For liposome-unassociated PM at 100 ng, 200 ng and 500 ng levels, within-run RSD values of 9.7, 3.6 and 2.1% respectively, were found. Between-run RSD values was 2.9% for liposome-associated PM and 6.3% for unassociated PM. For total PM in plasma, the within- and between-run RSD values were 10.3 and 11.3%, respectively, at 100 ng ml^{-1} level. This method was applied to separate liposome-associated PM from unassociated PM in the plasma obtained from rats following intravenous administration of liposomal PM at 5 mg kg^{-1}. Surprisingly, liposomal PM was found to be quite stable *in vivo* since about 70–90% of total plasma PM levels was accounted for by PM associated with the liposomal form at least up to a 1-h period of observation.

Keywords: *Separation method; liposome-associated and unassociated drugs; phosphoramide mustard; stable isotope; gas chromatographic-mass spectrometry.*

Introduction

Following the characterization of liposomes by Bangham in 1964 as a model for biological membrane studies [1], liposomes have emerged as a drug delivery system for anticancer drugs, antibiotics, enzymes, hormones, and immunomodulators [2, 3]. The major aim for utilizing liposomes for drug delivery is to improve the therapeutic index of entrapped drugs by either prolonging the circulating half-lives or attenuating the toxicities. Liposomes have a unique capability to trap water soluble molecules in the aqueous compartment and incorporate lipid soluble molecules in the membrane bilayers [1, 4]. Liposomes are widely used as drug carriers due to their nontoxic and biodegradable nature and ease of preparation. Lipid composition, size and charge or surface properties can be manipulated to meet specific needs, e.g. for targeting drug to specific sites or as a controlled release drug carrier system [2, 5–7]. For several anticancer drugs, pharmacokinetic and tissue distribution studies on their liposomal formulations have been conducted in animals [8–11]. Due to the instability of most conventional liposomes in plasma, circulating drugs usually exist in three forms: liposome-associated drug, free leaked drug, and protein-bound leaked

*Presented in part at the Analysis and Pharmaceutical Quality Section of the Eighth Annual American Association of Pharmaceutical Scientists Meeting, November 1993, Orlando, Florida, USA.

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drug, and interpretation of pharmacokinetic and tissue distribution data of liposomal formulated drug based on total drug levels may be misleading. Unfortunately, most of the studies to date only report total drug levels in plasma following liposomal drug administration. Thus, the lack of a rapid and reliable method to separate and determine liposome-associated and unassociated drug from total drug levels has hampered proper pharmacokinetic studies of these liposomal formulated drugs. Only two methods for the separation of liposomal drug from the unentrapped entity have been reported to date for liposome-encapsulated doxorubin (DXN) [12, 13]. These methods are limited to negatively charged liposomes and drugs that exist in a positively charged form at physiological pH. The underlying separation mechanism was solely based on the repulsion of negatively charged liposomes and the affinity of positively charged DXN by cationic exchange resin. Since these assays did not employ either external or internal standard for quantitation, full recovery of liposome-associated and unassociated drug from the resin and precision of the assay were required. We have recently entrapped a reactive key cytotoxic metabolite, phosphoramide mustard (PM), of a widely used anticancer drug cyclophosphamide in a liposomal formulation and the preparation has been found to be rather unstable in plasma. We described here a simple, rapid, and reliable method using Sephadex G-50 minicolumn for separation and quantitation of liposome-associated PM (Lip–PM) and unassociated PM (ULip-PM) in plasma. This method is suitable for pharmacokinetic study and does not require full recovery of ULip-PM from the gel column.

Materials and Methods

Chemicals

[β-$^2H_4$] PM (PM-d_4) and [α,β-$^2H_8$] PM (PM-d_8), synthesized according to the literature procedures [14] with appropriate modifications, were provided in Dr K. Chan's Laboratory. Unlabelled PM as its cyclohexylamine salt was supplied by Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD, USA). Sphingomyelin (SM) was purchased from Avanti Polar Lipids, Inc. (Birmingham, AL, USA). Cholesterol (CH) was obtained from The Sigma Chemical Co. (St Louis, MO, USA). SM and CH were used without further purification. Methanol, chloroform and methylene chloride are of HPLC grade (Fisher Scientific, Pittsburgh, PA, USA). Methylsilyltrifluoroacetamide (MSTFA) and Triton® X-100 were obtained from Pierce (Rockford, IL, USA). Sephadex G-50 was obtained from Pharmacia Fine Chemicals (Piscataway, NJ, USA). Bath-type of sonicator and Liposofast® assembly were purchased from Laboratory Supplies Co. (Hickville, NY, USA) and from Avestin (Ottawa, Canada), respectively. Centrifugation was accomplished on a Accuspin FR® (Beckman, Inc., Palo Alto, CA, USA). Disposable syringes were obtained from Western Medical Supplies (Arcadia, CA, USA). Microcentrifuge tubes were obtained from PGC Scientific (Gaitherburg, MD, USA).

GC/MS analysis

A Finnigan MAT ITS40 ion trap mass spectrometer (Finnigan MAT, San Jose, CA, USA) coupled to a Varian 3400 gas chromatograph (Varian, Walnut Creek, CA, USA) was used for the analysis of PM. The temperatures of transfer line and ion source were maintained at 280°C and 220°C, respectively. Chemical ionization mode with ammonia as the reagent gas was used and the ionization current was set at 10 μA. Helium was used as the carrier gas with a constant head pressure at 15 psi. Trimethylsilylated and dehydrochlorinated PM was chromatographed on a 30 m DB-5 capillary column (0.25 mm i.d. and 0.25 μm film thickness) (J&W Scienfific, Folsom, CA, USA). The oven temperature was programmed from 150°C to 230°C at the rate of 10°C per min and the injection port temperature set at 220°C. Under this condition the retention time of derivatized PM was 8.3 min. Quantitation was performed using ions at *m/z* 329 and 337 for derivatized PM and PM-d_8, respectively [15].

Preparation of liposomal PM

The dried lipid thin film composed of SM and CH at 1:1 mole ratio was first prepared. SM, 88 mg and CH, 46.4 mg, were dissolved in chloroform methanol (2:1, v/v) in a 13 × 100 mm pyrex test tube. The organic solvent was evaporated to dryness under a stream of nitrogen leaving a lipid thin film around the tube. The tube was placed in a vacuum

chamber to remove the trace amount of chloroform. Unlabelled PM as the cyclohexylamine salt, 88 mg, was dissolved in 1 ml of PBS. The resultant solution was added to the tube containing the dried lipid thin film. Multilamellar vesicles (MLV) were first prepared by vigorously shaking the tube followed by sonication in an ice-cold water bath for 20 min using a bath-typed sonicator. The MLV suspension was extruded back and forth for 11 passes through two polycarbonate membranes of 100 nm pore size, employing a 'Liposofast®' assembly. At the end of the extrusion process the liposomal preparation turned from an opaque to a clear solution. Unentrapped PM was separated from this crude liposomal PM by means of Sephadex G-50 minicolumn centrifugation method. Briefly, a 100 μl aliquot of the crude liposomal PM preparation was loaded dropwise onto the top of each of 10 Sephadex G-50 minicolumns, resting in a rack of 16 × 100 mm tubes. The columns were prepared as described below under 'Separation of liposome-associated PM and unassociated PM'. To each of the columns was added 400 μl of PBS and the tube centrifuged at 180*g* and at 0°C for 1 min. Most of the liposomal PM was found to be eluted from the column at this fraction. Purified liposomal PM was collected and kept in an ice bath until use. Examination of liposomal PM vesicles prepared by this method under the negative stained electron microscopy revealed homogeneous vesicles with the average diameter of 33 ± 2.4 nm (SD, $n = 256$) [15]. To quantitate the amount of entrapped PM, a 50 μl aliquot of liposomal PM was diluted to 1 ml with PBS. To 25 μl of the diluted liposomes was added a proper amount of PM-d_8 as the internal standard and 0.1% (v/v) Triton X-100 to disrupt the liposomes. The resulting mixture was processed by solid-phase extraction, derivatization, and quantitation as described under 'Quantitation of total PM, liposome-associated PM and unassociated PM'.

Separation of liposome-associated PM and unassociated PM

Lip-PM and ULip-PM were separated by Sephadex column chromatography. Sephadex G-50 was first swollen overnight in pH 7.4, 0.067 M sodium phosphate buffer in 0.9% sodium chloride (PBS). The gel was degassed and about 4 g was packed into each of several 3 cm^3 disposable syringes (1 cm × 6 cm) plugged with filters. The excess buffer was removed from the gel by centrifugation at 180*g* for 1 min and at 0°C.

To 100–200 μl PBS solution or plasma sample containing a mixture of Lip-PM and ULip-PM was added 2000 ng of PM-d_8 in 200 μl normal saline as the internal standard for ULip-PM, and the content was mixed and loaded onto the Sephadex G-50 minicolumn. The column was eluted with 400 μl of PBS and centrifuged at 180*g* and at 0°C for 1 min. Liposomal PM was collected in the first fraction which was kept frozen at −70°C until analysis. The minicolumn was then washed twice with 400 μl each of PBS and eluents were discarded. The final elution was accomplished with an additional 500 μl of PBS and the column centrifuged at 730*g* and at 0°C for 1 min. The eluent was kept frozen at −70°C until analysis for ULip-PM.

Quantitation of total PM, liposome-associated PM and unassociated PM

Total PM. The total PM was analysed by the procedure below. To a sample containing PM was added a suitable amount of PM-d_8 as the internal standard. The content was mixed and passed onto a Poly-prep column (BioRad, Richmond, CA, USA) packed with 400 mg of C-18 reversed-phase resin (Analytichem International, Harbor City, CA, USA) which has been prewashed with methanol and water. After centrifugation at 800*g* and at 4°C for 3 min the resin was washed with 0.5 ml of ice-cold normal saline. The water was removed from the resin by centrifugation at 2000*g* and at 4°C for 10 min. Then, the resin was eluted with 1 ml of methanol to a 12 × 75 mm culture tube and the methanol evaporated to dryness under a stream of nitrogen. The residue was derivatized with 30 μl MSTFA at 120°C for 30 min and an aliquot of it was analysed by GC/MS.

Lip-PM. To quantitate Lip-PM, samples collected through the first fraction of minicolumn were thawed and kept cold in an ice bath. Then, a proper amount of PM-d_4 was added as the internal standard. Liposomes were disrupted by addition of 0.1% (v/v) Triton X-100 as before. The resultant solution was extracted for PM using solid-phase method and residue derivatized as described under 'Total PM analysis'. The derivatized residue was analysed for PM by the GC/MS procedure. A standard curve was constructed by spiking

known amounts of PM, e.g. 100, 200, 500, 1000 and 2000 ng in diluted blank liposomes containing an equivalent lipid concentration to that in the sample. The calibration standards were processed at the same time as the samples using identical procedures. The plot of PM to PM-d_4 ratio versus amount of PM constituted the standard curve for Lip-PM.

ULip-PM. For quantitation of ULip-PM, samples collected through a minicolumn at Fraction No. 4 were thawed and kept cold in an ice bath. For the standard curve for ULip-PM, pure PM ranged between 100 and 2000 ng in diluted blank liposomes plus 2000 ng each of PM-d_8 as the internal standard was first passed onto a mini-gel column and fractionated accordingly to collect free PM at Fraction No. 4. The samples and calibration standards were then processed through extraction, derivatization, and GC/MS analysis similar to the procedures described for both total PM and Lip-PM. The plot of PM to PM-d_8 ratio vs amount of PM constituted the standard curve for ULip-PM.

Recovery study of liposome-associated PM and unassociated PM from mini-gel column. Prepared liposomal PM was diluted by PBS to an approximate concentration of 100 μg ml^{-1}. Eight replicates of 20 μl each of the diluted liposomal PM were processed through mini-gel column chromatography as before. The Lip-PM fractions were kept at −70°C until analysis. Eight replicates of 20 μl each of the diluted liposomal PM were also stored at −70°C for analysis for total PM. The recovery of Lip-PM from mini-gel column was calculated as the percentage of average amount of PM obtained from the first fraction of the column as compared with the average amount of total PM prior to column processing.

For the recovery of ULip-PM, a solution of free PM at 100 μg ml^{-1} was first prepared in normal saline. Then eight replicates of 30 μl each of the PM solution were processed through mini-gel column chromatography as before. The fraction (Fraction 4) which contained free PM was collected and kept at −70°C until analysis. Eight replicates of 30 μl each of free PM were also stored at −70°C until analysis. After addition of appropriate amounts of the internal standard PM-d_8, the column eluant and calibration standards were then processed through extraction, derivatization, and GC/MS analysis for PM as described for total PM as before. The recovery of ULip-PM from mini-gel column was calculated as the percentage of the average amount of free PM obtained from Fraction No. 4 column eluant as compared to that of the unprocessed free PM.

Within-run assay of liposome-associated PM and unassociated PM. Purified liposomal PM was first prepared and diluted to an appropriate concentration of 20 μg ml^{-1}. The solution of free PM-d_4 at 10 μg ml^{-1} was also prepared in normal saline. Eight replicates of a sample containing a mixture of approximately 200 ng of liposomal PM and 100 ng of free PM-d_4 were prepared. Separation of Lip-PM and ULip-PM-d_4 was accomplished as outlined previously. Following extraction and GC-MS analysis, amounts of Lip-PM and ULip-PM-d_4 were calculated based on the appropriate standard curves. The above steps were repeated for two more sets of replicate of samples each containing a mixture of approximately 430 ng of liposomal PM and 200 ng of free PM-d_4, and 1065 ng of liposomal PM and 500 ng of free PM-d_4, respectively. Precision of the assay at each level of liposomal PM and free PM was determined as RSD values and accuracy of the assay as % of the observed values from the theoretical values.

Between-run assay of liposome-associated PM and unassociated PM. Six sets of each standard curve of liposomal PM and free PM were constructed at PM levels ranging between 100 ng and 2000 ng. Details of the construction of standard curves were described previously. Each standard curve was performed separately on different days. The precision of this assay was estimated from RSD of the slope.

Between- and within-run assays of total PM in plasma. Six replicates of human plasma spiked with PM at 100 ng ml^{-1} were analysed for PM at the same day through the extraction, derivatization, and GC/MS procedures as described above, along with a calibration curve. The RSD value was calculated and provided the within-run assay variation. Similarly, six plasma samples spiked with PM at 100 ng ml^{-1} were analysed on 6 separate days, each along with a separate calibration curve. The RSD value was computed which indicated the between-run variation.

Application of the analytical method in biological samples. This rapid separation method of Lip-PM and ULip-PM was applied to a preliminary pharmacokinetic study of liposomal PM following its intravenous (iv) administration to the rat. The right jugular vein of four male Sprague–Dawley rats (Harlan, Indianapolis, IA, USA) weighing 360–390 g, was each cannulated under ether anaesthesia according to the published procedures [16]. Following the cannulation and recovery, each rat was given an iv injection of liposomal PM at 5 mg kg^{-1} via the jugular vein cannula. The cannula was rinsed with 0.5 ml of normal saline once following drug administration. Blood samples at 0.2–0.5 ml each were collected at 5, 15, 30 and 60 min into microcentrifuge tubes containing 20 μl of 1000 IU ml^{-1} heparin and kept in an ice bath. The tubes were centrifuged at 2000*g* and at 4°C for 10 min to separate plasma from red blood cells. An aliquot of plasma at each time point was processed immediately to separate Lip-PM from ULip-PM using mini-gel column as outlined previously. The remaining plasma samples were used for the quantitation of total PM. Samples were kept at −70°C until analysis. Total PM, Lip-PM and ULip-PM were analysed by the procedures as previously described.

Results

The recovery of spiked free PM and liposomal PM from mini-gel column is shown in Table 1. Only 16.8% of free PM or ULip-PM was recovered from the column as the major fraction (elution volume 1.7 ml, Fraction No. 4). In contrast, recovery of Lip-PM from the first fraction of the column was high, averaging 82.4% of the amount applied (Table 1). The reproducibility of the assay method as determined by RSD values for ULip-PM and Lip-PM in the mixtures is shown in Table 2. As shown, RSD values were found to be 9.7, 3.6, and 2.1% for ULip-PM at levels of 100, 200

Table 1
Recovery of spiked free PM and liposomal PM from Sephadex G-50 minicolumn

Sample no.	Total free PM (μg)	Free PM from Fraction No. 4 of mini-gel column (μg)	% Recovery	Total liposomal PM (μg)	Liposomal PM from Fraction No. 1 of mini-gel column (μg)	% Recovery
1	1.56	0.33	21.1	2.86	2.46	86.0
2	1.78	0.36	20.2	3.09	2.35	76.1
3	1.78	0.33	18.5	2.99	2.37	79.3
4	1.89	0.28	14.8	3.01	2.47	82.1
5	1.88	0.30	16.0	3.21	2.52	78.5
6	1.92	0.28	14.6	3.32	2.43	73.2
7	1.88	0.29	15.4	2.50	2.47	98.8
8	1.86	0.25	13.4	2.95	2.52	85.4
Average			16.8			82.4
SD			2.8			7.9

Table 2
Within-run variation of liposome-associated PM and liposome-unassociated PM at three different mixtures of liposomal PM and free PM-d_4

	Mixture 1 Free PM-d_4 100 ng plus liposomal PM 213 ng		Mixture 2 Free PM-d_4 200 ng plus liposomal PM 426 ng		Mixture 3 Free PM-d_4 500 ng plus liposomal PM 1065 ng	
No.	ULip-PM	Lip-PM	ULip-PM	Lip-PM	ULip-PM	Lip-PM
1	92.7	202.9	178.9	337.7	489.2	976.5
2	103.2	192.8	189.3	364.0	477.5	1037.7
3	91.2	195.9	176.4	341.8	485.0	1038.5
4	101.5	182.1	175.3	345.5	466.7	982.3
5	100.0	193.5	174.5	358.8	466.3	981.1
6	84.4	178.1	172.4	329.0	482.3	1003.2
7	86.0	186.2	174.1	373.1	494.5	1016.5
8	109.7	188.2	188.1	362.3	483.9	1055.0
Average	91.6	190.0	178.6	352.0	480.7	1011.0
RSD (%)	9.7	4.2	3.6	4.3	2.1	3.0

and 500 ng, respectively. RSD values of 4.2, 4.3 and 3.0% were observed for liposomal PM at levels of 213, 426 and 1065 ng, respectively. The quantitation of Lip-PM utilized calibration curve constructed by free PM plus blank liposomes. This was justified, since for the quantitation of PM, liposomes were disrupted by Triton X-100 prior to the analysis. In Table 3, the observed values of ULip-PM were in close agreement with the theoretical values between 100 ng and 500 ng giving the average accuracy of 92.3%. As shown in Table 3, the observed values of Lip-PM after corrected for the recovery were in proximity with the theoretical values giving the average accuracy of 108%. Table 4 showed the result of crossover of Lip-PM into the ULip-PM fraction at three different levels of their mixtures. As shown, at liposomal PM levels of 213 ng and 426 ng, no detectable crossover was found. However, at the liposomal PM level of 1065 ng, only 0.8% crossover was detected in the liposome-unassociated drug fraction. The assay was linear from 100 ng to 2000 ng monitored for both species and RSD values of the between-run assay for Lip-PM and ULip-PM were found to be 2.9 and 6.3%, respectively. The within- and between-run RSD values for total

Table 3
Accuracy of the assay for liposome-unassociated PM and liposome-associated PM

	ULip-PM				Lip-PM			
Sample level	Theoretical values (ng)	*n*	Mean observed values (ng)	Accuracy*	Theoretical values (ng)	*n*	Mean observed value (ng)	Accuracy*
Low	100	8	91.6	91.6	213	8	213	108.5
Medium	200	8	178.6	89.3	426	8	427	100.2
High	500	8	480.7	96.1	1065	8	1227	115.2
Average				92.3				108.0

*Determined as % of theoretical values.

Table 4
Percentage crossover of liposome-associated PM into liposome-unassociated PM fraction as obtained from the within-run experiment

Level no.	% Crossover
(1) Liposomal PM 213 ng and free PM-d_4 100 ng	Not detectable
(2) Liposomal PM 426 ng and free PM-d_4 200 ng	Not detectable
(3) Liposomal PM 1065 ng and free PM-d_4 500 ng	0.8

Following the separation of liposome-associated PM and liposome-unassociated PM in the mixtures, liposome-associated PM in the unassociated drug fraction was quantitated by GC/MS using the ion at *m/z* 329 while liposome-unassociated PM in the same fraction was analysed using the ion at *m/z* 333. Data are expressed as percentage of liposome-associated PM in the first fraction which is spilled over to the liposome-unassociated PM fraction.

Table 5
Liposome-associated PM and liposome-unassociated PM plasma levels in rats ($n = 4$) given liposomal PM intravenously at 5 mg kg^{-1}

		Lip-PM		ULip-PM	
Time (min)	Total PM ($\mu g\ ml^{-1}$)	($\mu g\ ml^{-1}$)*	% of total	($\mu g\ ml^{-1}$)*	% of total
5	62.1 (8.8)	47.5 (3.0)	76.5	9.2 (3.9)	14.8
15	39.5 (7.7)	27.3 (4.0)	69.1	5.8 (3.2)	14.7
30	22.4 (2.6)	17.3 (3.0)	77.2	3.8 (3.0)	17.0
60	7.3 (1.2)	6.6 (1.1)	90.4	0.96 (0.40)	13.2

*Values represent the mean (SD) of four rats at each of the indicated times following the iv injection of liposomal PM.

PM at 100 ng ml^{-1} in human plasma were 10.3 and 11.3%, respectively. The application of this separation method was shown in the *in vivo* experiment where rats were given liposomal PM intravenously at 5 mg kg^{-1}. Table 5 showed Lip-PM and ULip-PM plasma levels at 5, 15, 30 and 60 min post-injection. Surprisingly, 70–90% of the circulating total PM levels were associated with liposomes at least up to 60 min following the administration, indicating that the formulation was rather stable *in vivo*.

Discussion

Due to the limited stability of PM in buffer and biological media, evaluation of the *in vivo* pharmacokinetics of a new formulation of PM, liposomal PM, requires a rapid procedure to separate the entrapped drug from its free form. A rapid and efficient separation method was developed using size exclusion mini-gel column which required only minutes to process. This method can quantitate the liposome-unassociated drug without the need for its full recovery from the gel column (16.8%, Table 1) while most of liposome-associated drug can be recovered within the first elution (82.4%, Table 1). This procedure involves the use of the stable isotopically labelled internal standard, PM-d_8, which was added to samples prior to separation. Thus, any liposome-unassociated drug fraction eluted from the gel column would have the fixed PM-d_0 to PM-d_8 ratio. This alleviates the need of full recovery of ULip-PM. A major and specific fraction, e.g. Fraction No. 4, which contained the most amount of drug eluted from the column, was collected for quantitation of ULip-PM. Lip-PM was quantitated by the use of the external standard PM-d_4. This method differs from previous methods reported for liposomal DXN [12, 13] which determined the total drug and liposome-associated drug levels, and their difference provided the free drug levels. However, this subtraction method would often impart significant errors. The main advantage of this method is the ability to quantitate the free drug directly.

The use of different labelled PM as markers for free drug (PM-d_4) in a mixture of liposomal PM and free PM was found to be very powerful in examining the possible cross-contamination between two forms of PM during the course of separation. This cross-contamination may arise from leakage and diffusion of PM between these two sources. Using stable isotope technology, the extent of crossover of PM between these two forms could be accurately determined and the results indicated no crossover of free PM to liposomal PM. The crossover of liposomal PM into the free PM fraction was not detectable until above 1 μg in the mixture and the value was still less than 1%. Therefore, the negligible crossover observed here also attested to a rather stable formulation of liposomal PM during the separation process. The separation method described here was also found to be very efficient. This was evident from the good accuracy and reproducibility (Table 2 and Table 3) in a wide range of free drug (100–500 ng) and in the presence of liposomal PM (200–1000 ng).

Since we employed Sephadex G-50 resin rather than ion exchange resin to separate liposome-associated drug from the unassociated drug, this method could be applied to liposomal system and leaked drug of any charge state. Liposomal system of any size should be equally separated. The only disadvantage of this method is its inability to separate protein bound drug from liposomal drug and therefore, may be limited to drugs that do not bind to plasma protein extensively. When this method was employed to separate Lip-PM from ULip-PM in rat plasma given liposomal PM intravenously at 5 mg kg^{-1}, liposomal PM was found to be quite stable in circulation as evidenced from the low levels of ULip-PM (Table 5). The observed prolonged levels of Lip-PM were in contrast to the fast disappearance of free PM with the elimination $t_{½}$ of less than 10 min [15]. Clearly, therapeutic activity of liposomal PM will depend on plasma levels of intact liposomes which serve as a controlled release system. Finally, the separation method developed here may be useful for future pharmacokinetic studies.

References

[1] A.D. Bangham and R W. Horne, *J. Mol. Biol.* **8**, 660 (1964).
[2] R. Perez-Soler, *Cancer Treat. Rep.* **16**, 67–82 (1989).
[3] J.N. Weinstein and L.D. Leserman, *Pharmacol. Ther.* **24**, 207–233 (1984).
[4] R.R.C. New, in *Liposomes: a Practical Approach* (R.R.C. New, Ed.), pp. 1–32. Oxford University Press, New York (1990).
[5] K.J. Hwang, in *Liposomes: From Biophysics to Therapeutics* (M.J. Ostro, Ed.), pp. 109–156. Marcel Dekker Inc., New York (1987).

[6] V.V. Ranade, *J. Clin. Pharmacol.* **29**, 685–694 (1989).
[7] D.E. Brenner, *J. Natl. Cancer Inst.* **81**, 1436–1438 (1989).
[8] J. Lautersztain, R. Perez-Soler, A.R. Khokhar, R.A. Newman and G. Lopez-Berestein, *Cancer Chemother. Pharmacol.* **18**, 93–97 (1986).
[9] C.E. Swenson, K.A. Stewart, J.L. Hammett, W.E. Fitzsimmons and R.S. Ginsberg, *Antimicrob. Agents Chemother.* **34**, 235–240 (1990).
[10] J. Freise, W.H. Mueller, P. Magerstedt and H.J. Schmoll, *Arch. Int. Pharmacodyn.* **258**, 180–192 (1982).
[11] A. Gabizon, R. Shiota and D. Papahadjopoulos, *J. Natl. Cancer Inst.* **81**, 1484–1488 (1989).
[12] R.L. Thies, D.W. Cowens, P.R. Cullis, M.B. Bally and L.D. Mayer, *Anal. Biochem.* **188**, 65–71 (1990).
[13] S. Druckmann, A. Gabizon and Y. Barenholz, *Biochim. Biophys. Acta* **980**, 381–384 (1989).
[14] A. Takamizawa, S. Matsumoto, T. Iwata, Y. Tochino, K. Katagiri, K. Yamaguchi and K. Shiratori, *J. Med. Chem.* **18**, 376–382 (1975).
[15] A. Srigritsanapol, Ph.D. Dissertation, University of Southern California at Los Angeles (1993).
[16] S.K. Baker and S. Niazi, *J. Pharm. Sci.* **72**, 1027–1029 (1983).

[Received for review 21 December 1993; revised manuscript received 4 March 1994]