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Method for Rapid Separation of Liposome-Associated Doxorubicin from Free Doxorubicin in Plasma

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To understand and predict the efficacy and/or toxicity of liposomal drugs *in vivo*, it is essential to have rapid, reliable methods of separating and quantitating both the free and the liposomal forms of the drug. A method using solid-phase extraction chromatography columns was developed to separate and quantitate unencapsulated doxorubicin and liposome-associated doxorubicin in plasma following the intravenous injection of liposomal doxorubicin. The method facilitated the recovery and quantitation of free and liposomal drug. The separation and recovery of doxorubicin were linear across the entire range of possible mixtures (0 to 100%) of the two forms of the drug in plasma. Free drug and liposomal drug were readily separated for liposomal doxorubicin systems varying in size (0.1–1.0 μm) and lipid composition (egg yolk phosphatidylcholine/cholesterol and distearylphosphatidylcholine/cholesterol). The method is rapid and allows for multiple samples to be processed simultaneously.

The use of liposomes as a drug delivery system has been an area of increasing interest in pharmaceutics [see reviews (1,2)]. The use of antineoplastic agents encapsulated in liposomes has proven useful in attenuating toxicity while maintaining or increasing efficacy of certain compounds, thus enhancing the therapeutic index (3–6). The mechanism for the increase in the therapeutic activity is, however, not known. Alterations in drug pharmacokinetics resulting from liposome encapsulation may provide an insight into this enhanced therapeutic effect. Pharmacokinetic studies have been conducted in animal models for several liposome encapsulated antineoplastics (7,8). However, the lack of methodology which accurately separates and recovers liposomal drug and free drug (not entrapped in liposomes) in plasma has significantly limited these studies. In order to fully understand liposomal drug therapy, the development of techniques which are able to quantitate both free and encapsulated forms of liposomal drugs is essential. This is particularly important in the case of the antineoplastic drug doxorubicin, which is currently undergoing extensive evaluation in clinical trials using three different liposomal formulations.

A satisfactory method for separating free and liposomal drug from biological fluids should (i) be fast and simple in order to accommodate large numbers of samples, (ii) provide immediate separation of free from liposomal drug, (iii) result in efficient recovery of each fraction, (iv) avoid excessive sample dilution, and (v) allow for collection of free and liposomal fractions which are suitable for subsequent analysis such as HPLC. We describe here a method that separates free from liposome encapsulated doxorubicin in a manner which satisfies these criteria.

MATERIALS AND METHODS

Egg yolk phosphatidylcholine (EPC)[2] and distearylphosphatidylcholine (DSPC) were purchased from Avanti Polar Lipids. Cholesterol (Chol) and Amberlite XAD-2 were purchased from Sigma Chemical Co. Carboxylic acid-2 (CBA-2), ethyl (C-2), octyl (C-8), octadecyl (C-18) solid-phase extraction columns (100 mg) and vacuum manifold were purchased from World Wide Monitoring (Horsham, PA). [1,2-^{3}H(N)]Cholesteryl

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[2] Abbreviations used: EPC, egg yolk phosphatidylcholine; DSPC, distearylphosphatidylcholine; Chol, cholesterol; MLV, multilaminar large vesicle; QELS, quasi-elastic light scattering; HBS, Hepes-buffered saline; PBS, phosphate-buffered saline; BSA, bovine serum albumin.

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hexadecyl ether was acquired from New England Nuclear. Doxorubicin was purchased from Adria Laboratories and [14-^{14}C]doxorubicin purchased from Amersham Laboratories.

Liposomal preparation. EPC/Chol (55/45, mol/mol) vesicles were prepared by hydrating a lipid film in the presence of 300 mM citrate buffer (pH 4.0). The MLVs were then frozen and thawed 5 times as described previously (9) and extruded 10 times through two (stacked) polycarbonate filters of indicated pore size by employing a liposome extruder obtained from Lipex Biomembranes (Vancouver, BC). For DSPC/Chol (55/45, mol/mol) vesicles extrusion was carried out at 65°C. Vesicle size distributions were determined by quasi-elastic light scattering (QELS) employing a Nicomp Model 270 particle sizer.

Doxorubicin encapsulation. Doxorubicin was encapsulated into liposomes using a pH gradient as previously described (10). Briefly, for preparing small volumes of liposomal doxorubicin used for *in vitro* testing, the liposomal preparations were made as follows: A pH gradient (acidic inside) across the vesicles was created by passing the extruded vesicles down a G-50 desalting column to exchange the untrapped citrate buffer (pH 4.0) for 20 mM Hepes-buffered saline (HBS) (pH 7.8) or alternatively adding 0.5 M Na_2CO_3 to increase the extravesicular pH to 7.8 (11). The phospholipid content of the vesicle solution was determined by phosphorous analysis (12). The vesicles were then mixed with a solution of doxorubicin (with or without [^{14}C]doxorubicin) to achieve a final drug to lipid ratio of 0.2:1 (w/w) and then heated at 60°C for 5 min with intermittent vortex mixing. Doxorubicin-trapping efficiencies determined as previously described were routinely greater than 99% (6). The doxorubicin content of liposomal doxorubicin used for animal injection or standards was determined by taking an aliquot of the vesicles, disrupting them with 0.5% Triton X-100, and measuring the absorbance of released doxorubicin at 480 nm.

CBA-2 column separation of free and liposomal doxorubicin. Free doxorubicin was separated from liposomal doxorubicin in plasma using CBA-2 solid-phase extraction columns attached to a vacuum manifold apparatus. The separation procedure is summarized in Table 1. The columns were conditioned with 2 × 1 ml of methanol followed by 2 × 1 ml of phosphate-buffered saline (PBS), pH 7.4. All solutions were drawn through the columns with a manifold vacuum pressure of 3–5 mm Hg unless otherwise noted. Column sorbent was maintained in a wet state at all times (no air was pulled through unless noted). Following the column conditioning, the valves connecting the columns to the manifold were closed. Plasma (0.1 ml minimum) containing the liposomal and/or free drug was applied to the top of the columns. With little or no vacuum being applied, the valves were opened to allow the plasma to settle into the columns. After the plasma had settled into the column sorbent, 100 μl PBS was added to facilitate the movement of the liposome containing plasma into the sorbent. Plasma passing through the column (containing the liposomal drug fraction) was collected in 15-ml culture tubes. The remaining liposomal drug fraction was eluted with 2 × 1 ml of PBS and collected in the same culture tube. Doxorubicin in the liposomal fraction was then extracted with chloroform:2-propanol (1:1). The columns were further washed with 3 × 1 ml of PBS followed by 5 × 1 ml of 10 mM HCl. Air was pulled through the columns for 1 min at maximum vacuum, followed by a wash with 2 × 1 ml of hexane (vacuum 5 mm Hg) and air-dried for 1 min at maximum vacuum. The free drug was eluted with 1 ml of methanol and collected in tubes for analysis by direct fluorescence measurement or by HPLC coupled with fluorescence detection. In the case of HPLC analysis the collection tubes for both free and liposomal doxorubicin fractions contained daunorubicin as an internal standard. For experiments using [14-^{14}C]-doxorubicin and [1,2-^{3}H(N)]cholesteryl hexadecyl ether as markers for drug and lipid, respectively, 1-ml fractions were collected from CBA-2 columns and counted in a Beckman LS 3801 (Beckman Instruments, Fullerton, CA) scintillation counter using standard scintillation counting techniques.

TABLE 1

Protocol for Separating Liposomal Doxorubicin from Free Doxorubicin in Plasma, Using a CBA-2 Column

(1) Column preparation
  (a) Methanol wash (2 × 1 ml)
  (b) Buffer wash (2 × 1 ml)
(2) Separation and recovery of liposomal drug
  (a) Layer plasma sample (0.1 to 1.0 ml) onto column
  (b) While collecting the eluate, draw sample through column, followed by buffer (2 × 1 ml)
(3) Column wash
  (a) Buffer wash (3 × 1 ml)
  (b) Acid wash (3 × 1 ml) (pull maximum vacuum at the end, for 30 s)
  (c) Hexane wash (2 × 1 ml) (Pull maximum vacuum at the end, for 1 min)
(4) Free drug recovery
  (a) Add 1 ml methanol and collect the eluate

Fluorescence assay for doxorubicin. The liposomal doxorubicin which passes freely through the CBA-2 column was determined by direct fluorescence (13). Briefly, the initial 2 ml of buffer eluted from the CBA-2 columns containing the liposomal doxorubicin fraction was extracted with 4 ml of chloroform:2-propanol (1:1). The quantity of doxorubicin in the free drug fraction (methanol fraction) and that in the liposomal drug fraction

(chloroform:2-propanol extract) were determined by measuring the relative fluorescence intensity of the respective solutions using a Shimadzu RF-540 spectrofluorophotometer (excitation, 500 nm; emmission, 560 nm). Samples were quantitated by comparison with standard solutions containing known amounts of free or liposomal doxorubicin prepared in the same manner.

HPLC analysis for doxorubicin. The free and liposomal doxorubicin samples eluted from the CBA-2 column were prepared for HPLC analysis as follows. The liposomal doxorubicin fraction (2 ml) was extracted with 4 ml of chloroform:2-propanol (1:1) and the organic phase was collected and evaporated under nitrogen. The free doxorubicin fraction (in methanol) from the CBA-2 columns was evaporated under nitrogen. All samples were stored at −20°C until HPLC analysis. The samples were reconstituted in 150 μl of methanol and 25–50 μl was injected into the HPLC. Any precipitate present in the free doxorubicin samples following reconstitution with methanol was removed by either centrifugation or filtration with a 0.2-μm filter. The HPLC system was a 5-μm C-18 Nova-Pak radial compression column with a C-18 guard column, Waters M510 pump, and U6K injector (Waters Associates, Milford MA). The HPLC chromatographic conditions were similar to those described by Roland (14). The mobile phase consisted of an isocratic mixture of methanol (67%) and aqueous solution (23%) of 10 mM ammonium acetate:acetic acid (30:1, v/v) at a flow rate of 2.4 ml/min. Doxorubicin, metabolites, and internal standard (daunorubicin) were detected using a Shimadzu RF-540 spectrofluorophotometer fitted with an HPLC microflow cell. Excitation and emission wavelengths were 500 and 560 nm, respectively. Peak areas were integrated using a Waters 740 data module. The quantity of doxorubicin was calculated by comparing the peak area ratios of doxorubicin/internal standard with peak area ratios of known standards of free or liposomal doxorubicin prepared via the same sample preparation procedure.

Animals studies. Female CD-1 mice weighing 20 to 25 g were obtained from Charles River Breeding Laboratories. Liposomal and free doxorubicin were administered (20 mg/kg) in a volume of 0.2 ml via tail-vein injection. At the indicated time after injection, three mice were anesthetized with ether and blood was collected by cardiac puncture. Immediately following, the plasma was separated and placed on ice and an aliquot was processed through the CBA-2 column within 3 h of blood collection.

RESULTS

Initial studies were conducted to characterize the binding and recovery of free doxorubicin from plasma using CBA-2 solid extraction columns. Aliquots (0.1 ml) of plasma containing varying amounts of doxorubicin with [14-^{14}C]doxorubicin as a tracer were applied to the columns. Each fraction that eluted from the column was counted for radioactivity and compared to the total amount applied to the column. Column retention and recovery of free doxorubicin were good, averaging 87% over the range (0.004–20 μg) of doxorubicin which was applied (data not shown). Approximately 8% of the free doxorubicin in plasma passed through the column without binding.

To characterize the liposome interaction with the CBA-2 column, vesicle preparations containing [1,2-^{3}H(N)]cholesteryl hexadecyl ether as a liposome marker were used. When plasma (0.1 ml) containing liposomes was applied to the column, greater than 99% of the liposomal-associated radioactivity passed through the column and was collected in the aqueous fraction (buffer and HCl washes) (Table 2). As shown in Table 2, the free passage of the liposomes through the column was not affected by liposome composition (EPC/Chol vs DSPC/Chol) or size (ranging from 0.1 to 1 μm). Free passage of the liposomes through the column was, however, dependent upon the presence of plasma as a carrier. This was evident by the fact that less than 10% of the liposomes mixed with HBS and put through the column passed through with the aqueous fraction (Table 2). This retention of liposomes was prevented by the addition of bovine serum albumin (BSA) (minimum of 1%) to the HBS as a carrier (data not shown). Liposomes in plasma taken from mice previously injected with EPC/Chol vesicles (with and without a pH gradient) also passed freely through the column.

Because of the limited volume of plasma available from small laboratory animals, it was necessary to determine the minimum volume of plasma to apply on the column to achieve effective separation and recovery of free and liposomal doxorubicin. As shown in Fig. 1, a minimum sample volume of 0.1 ml plasma was required to achieve consistent separation and recovery of the two forms of doxorubicin. Applications less than 0.1 ml resulted in an increased percentage of the total doxorubicin being recovered as free doxorubicin with a concurrent decrease in the percentage recovered as liposomal doxorubicin. Volumes of 1.0 ml were separated equally as well as 0.1-ml samples (data not shown).

The linearity of the separation and recovery of free doxorubicin and liposomal doxorubicin from plasma were determined across the entire range of possible mixtures of the two forms of doxorubicin. Free doxorubicin and liposomal doxorubicin were mixed together at ratios of 4:0, 3:1, 2:2, 1:3, and 0:4 on a weight/weight basis for doxorubicin, to achieve total doxorubicin concentrations of 20 and 200 μg/ml. A volume of 0.1 ml of each sample was applied to the columns. Doxorubicin in the liposomal fraction (aqueous buffer) was extracted with

TABLE 2

Liposomal Lipid Recovery from Plasma and Saline: Effect of Liposome Type and Size and the Presence of pH Gradient Across the Membrane

Liposomal[a] formulation	Vesicle size, (μm)	Carrier	Total dpm applied	% of total radioactivity eluted with: Aqueous wash: Buffer	Aqueous wash: HCl	Organic wash: Hexane	Organic wash: Methanol	Organic wash: Chloroform	Total % recovered
EPC/Chol	0.2	Plasma	5,538	93.6	6.1	0.1	0.1	0.2	100.1
EPC/Chol	0.2	Saline	4,733	0.7	0.0	88.3	9.2	4.2	102.4
DSPC/Chol	0.2	Plasma	13,026	100.4	0.6	0.3	0.2	0.0	101.5
DSPC/Chol	0.2	Saline	13,105	9.4	0.4	77.2	7.8	3.0	97.8
DSPC/Chol	1.0	Plasma	43,709	97.5	1.7	0.0	0.4	0.1	99.7
EPC/Chol[b]	0.1	Plasma	59,284	96.7	3.2	Not collected	0.5	Not collected	100.5
EPC/Chol[c]	0.1	Plasma	66,089	104.0	0.6	Not collected	0.2	Not collected	104.8

[a] [1,2-$^3H_2$(N)]Cholesteryl hexadecyl ether was incorporated into the liposomes as a lipid marker. The mole ratio of EPC:Chol and DSPC:Chol in the liposome formulations was 55:45.

[b] Plasma collected from mice 4 h following iv injection of EPC/Chol liposomes with a pH gradient.

[c] Plasma collected from mice 4 h following iv injection of EPC/Chol liposomes without a pH gradient.

chloroform:2-propanol (1:1) and measured by fluorescence, while the free doxorubicin was eluted from the columns with methanol and measured directly by fluorescence, as described under Materials and Methods. As shown in Table 3, the measured amounts of free and liposomal doxorubicin were in agreement with the theoretical quantities which were mixed together in the plasma. The average total recovery of doxorubicin was 97 and 103%, respectively, for the 2 and 20 μg samples applied to the columns (Table 3). The linearity of the separation and recovery is shown by plots of the measured ratios versus the theoretical ratios of free doxorubicin/total doxorubicin (Fig. 2A) and liposomal doxorubicin/total doxorubicin (Fig. 2B). The slope of the line for measured free doxorubicin/total doxorubicin versus theoretical free doxorubicin/total doxorubicin was $y = 1.088x - (-0.01)$ with an $r^2 = 0.9939$ (Fig. 2A). The slope for the line for measured versus theoretical liposomal doxorubicin/total doxorubicin was $y = 0.931x - (0.03)$ with an $r^2 = 0.9917$ (Fig. 2B).

In order to determine the levels of free doxorubicin and liposomal doxorubicin *in vivo,* mice were injected iv with liposomal doxorubicin (EPC/Chol, 0.2 μm, 0.2:1 drug to lipid weight ratio) at a dose of 20 mg/kg of doxorubicin. At 2 min, 30 min, and 4 h after injection, plasma was collected, placed on ice, and immediately passed through the CBA-2 column to separate free and liposo-

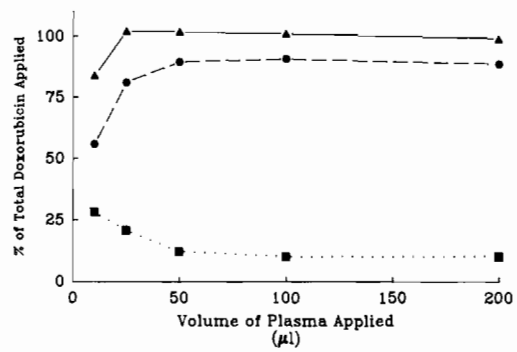

FIG. 1. The effect of plasma volume applied to the CBA-2 column on recovery of free doxorubicin, liposomal doxorubicin, and total doxorubicin. Plasma containing added [14-^{14}C]doxorubicin-loaded EPC/Chol vesicles was applied to CBA-2 columns in various volumes and processed as described under Materials and Methods. The data are expressed as percentage of the total doxorubicin applied which is recovered as free doxorubicin (■), liposomal doxorubicin (●), and total recovered doxorubicin (▲).

TABLE 3

Recovery of Free and Liposomal Doxorubicin from a Mixture in Plasma

Total amount doxorubicin applied (μg)	Lipo:free (μg:μg): Theoretical	Lipo:free (μg:μg): Measured[a]	Total % recovered
2	2.0:0.0	1.76:0.07	91.5
	1.5:0.5	1.37:0.53	95.0
	1.0:1.0	0.92:1.07	99.5
	0.5:1.5	0.48:1.52	100.0
	0.0:2.0	0.03:1.99	101.0
20	20.0:0.0	18.39:0.24	93.2
	15.0:5.0	15.30:5.10	102.0
	10.0:10.0	10.36:9.86	101.1
	5.0:15.0	5.41:16.40	109.1
	0.0:20.0	0.07:21.79	109.3

[a] Measured by fluorescence, as described under Materials and Methods. Values represent an average of duplicate samples.

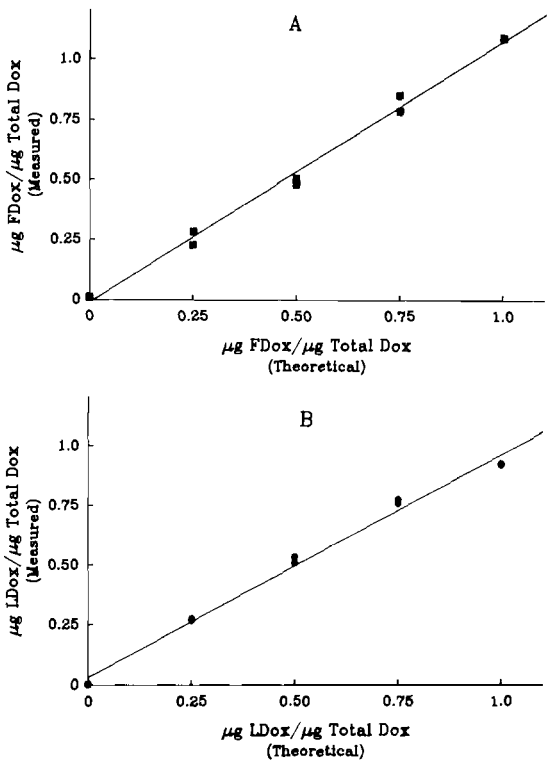

FIG. 2. The measured ratios versus the theoretical ratios of free doxorubicin/total doxorubicin (A) and liposomal doxorubicin/total doxorubicin (B) in plasma. Free doxorubicin and liposomal doxorubicin were added to plasma at various ratios to achieve a total doxorubicin concentration of 20 μg/ml for each particular sample. Doxorubicin content in each fraction was determined by fluorescence as described under Materials and Methods. Each sample was done in duplicate.

mal doxorubicin. Doxorubicin was quantitated by HPLC analysis. As shown in Table 4, the initial plasma concentration of liposomal doxorubicin (2 min after injection) was 162 μg/ml. After 30 min and 4 h, the liposomal doxorubicin levels had decreased to 157 and 49 μg/ml, respectively. The free doxorubicin concentrations at these respective times were 10.0, 3.76, and 0.83 μg/ml, representing 5.9, 2.3, and 1.7% of the total doxorubicin concentration in the plasma, respectively.

Although the percentage of free doxorubicin in the plasma following an iv injection of liposomal drug is low, the actual amount of free drug may be significant, especially when the level of total circulating drug is high, as often occurs with liposomal doxorubicin. It is possible that some of the free drug which is measured is a result of crossover of liposomal drug into the free drug fraction during processing on the column. Therefore, an attempt was made to define the limit of liposomal and free drug separation. The CBA-2 column is a solid-phase extraction column consisting of a carboxylic acid moiety combined with a hydrophobic phase bonded to silica. Compounds are retained primarily by ionic and/or hydrophobic interactions. The possibility existed that the neutral liposomes used in this study were interacting with the hydrophobic phase, resulting in the crossover of a small percentage of drug into the free drug fraction. In order to test whether column hydrophobicity was affecting liposome retention, plasma spiked with either EPC/Chol or DSPC/Chol vesicles with encapsulated [14-^{14}C]doxorubicin was passed through CBA-2, ethyl (C-2), octyl (C-8), and octadecyl (C-18) solid-phase extraction columns. No difference was observed in the percentage of free doxorubicin recovered from these various types of columns. The percentage of free doxorubicin found in plasma containing EPC/Chol liposomal doxorubicin vesicles ranged from 4.0 to 4.3% and in plasma spiked with DSPC/Chol vesicles, the level of free doxorubicin ranged from 0.8 to 0.9% (data not shown). The percentage of free drug found in these samples could be a result of incomplete encapsulation of the drug and/or release of free drug from the liposome during the mixing of the vesicles with the plasma during the sample preparation.

Another approach that was taken to determine the limit of free and liposomal drug separation was to first remove free drug which was released as a result of mixing of liposomal doxorubicin with plasma during the sample preparation. The subsequent determination of free drug would represent the amount of liposomal drug which crosses over into the free drug fraction during the processing on the column. First, the percentage of free doxo-

TABLE 4

Free Doxorubicin and Liposomal Doxorubicin Plasma Levels in Mice Injected with Liposomal Doxorubicin[a]

Time after injection	Total doxorubicin (μg/ml)	Liposomal doxorubicin		Free doxorubicin	
		μg/ml	% of total	μg/ml	% of total
2 min	171.61 ± 7.44	161.57 ± 8.13	94.1	10.04 ± 0.78	5.9
30 min	160.87 ± 6.95	157.11 ± 6.82	97.7	3.76 ± 0.13	2.3
4 hour	49.66 ± 5.92	48.83 ± 5.81	98.3	0.83 ± 0.12	1.7

[a] Values represent the mean ± SD doxorubicin plasma levels (in 0.1-ml samples) of three mice at each of the indicated times after iv injection of liposomal doxorubicin (EPC/Chol) (20 mg doxorubicin/kg). Doxorubicin was quantitated by HPLC as described under Materials and Methods.

TABLE 5

Limit of Liposomal Doxorubicin Separation in Plasma Containing Liposomal Doxorubicin

Liposome formulation	Plasma[a] source	Time of plasma sampling, (h)	Column A[b] Doxorubicin applied (μg)	Column A[b] % Eluted with methanol (% FDox)	Column C[c] Doxorubicin applied (μg)	Column C[c] % Eluted with methanol (% FDox)
EPC/Chol[d]	Spiked plasma	NA	5.99	3.40	2.38	1.66
DSPC/Chol[d]	Spiked plasma	NA	5.63	0.93	2.67	0.06
EPC/Chol[e]	Mouse plasma	0.5	7.49	2.49	2.50	0.56
EPC/Chol[e]	Mouse plasma	4.0	4.46	0.74	2.01	0.52

[a] The separation procedure (0.1-ml sample size) was carried out on human plasma containing added liposomal doxorubicin or pooled (n = 3) plasma samples collected from mice 0.5 and 4 h after injection with liposomal doxorubicin (0.2 μm EPC/chol, 20 mg/kg).

[b] The initial free doxorubicin (FDox) level was determined using column A.

[c] The remaining plasma sample was put through a separate CBA-2 column (column B) to remove free doxorubicin (FDox) from the original sample, followed by a second determination of free doxorubicin on a third column (column C).

[d] Liposomal doxorubicin preparations consisted of standard 0.2 μm EPC/Chol and DSPC/Chol vesicles previously described under Materials and Methods, containing [14-^{14}C]doxorubicin (sp act, 0.0134 μCi/μg). Doxorubicin was quantitated using standard scintillation counting techniques.

[e] Doxorubicin was quantitated using HPLC analysis as described under Materials and Methods.

rubicin found in plasma containing added liposomal doxorubicin was determined using a CBA-2 column (column A). The remainder of the spiked plasma sample was passed through a second, separate CBA-2 column (column B) (prepared and wetted with blank plasma to avoid changing the plasma protein concentration in the collected sample) to remove the free doxorubicin. Following this preparative step, an aliquot of the processed plasma sample was put through a third CBA-2 column (column C) and the amount of free doxorubicin determined again. This preparative procedure decreased the percentage of free doxorubicin for EPC/Chol liposomal doxorubicin vesicles from 3.40 to 1.66% and for DSPC/Chol vesicles it decreased from 0.93 to 0.06% (Table 5). In a separate experiment, plasma pooled from mice 30 min and 4 h after receiving intravenous liposomal doxorubicin was put through the same preparative column procedure to remove the free drug. As shown in Table 5, the preparative column step decreased the percentage of free doxorubicin found in the mouse plasma 30 min (2.49%) and 4 h (0.74%) after injection to equivalent levels of 0.56 and 0.52%, respectively.

DISCUSSION

We have described here a rapid method which accurately and reproducibly separates free doxorubicin from liposomal doxorubicin in plasma. This method utilizes a solid-phase extraction column consisting of a weak cationic exchanger combined with a hydrophobic phase, which takes advantage of the weak cationic and hydrophobic nature doxorubicin to bind and remove it from plasma. Fortuitously, liposomes in plasma or in the presence of plasma protein such as BSA pass directly through the columns with the aqueous washes while the free drug is retained. The free drug is subsequently eluted with methanol. The separation of free and liposomal drug is linear across the entire range of possible mixtures of the two drug forms.

Separation of liposomes from free drug can be accomplished by centrifugation, dialysis, size-exclusion chromatography, ultrafiltration, and ion-exchange chromatography (15,16). The method described in this paper has some unique advantages over these other methods. First, the actual separation of free and liposomal drug occurs within 30 s and each of the two fractions of the drug can be quantitatively recovered within approximately 10 min. This contrasts with separation procedures which employ centrifugation or dialysis. Second, the ability to process plasma samples as small as 0.1 ml makes the method suitable for studies using small laboratory animals such as mice and rats. Third, the 12-sample vacuum manifold allows the convenient processing of multiple plasma samples. Fourth, the method can be used for liposomal preparations which vary in vesicle size and composition. However, since liposome preparations can vary widely, each liposomal formulation should be tested for compatibility with the method. This may be especially true for liposomal systems made of charged phospholipids. Fifth, both the liposomal and the free drug fractions are easily recovered and quantitated directly. This contrasts with previous methods that do not recover free drug (17,18). Finally, this method is likely to be applicable to liposomal formulations of a wide variety of drugs, particularly those that are weak bases.

Because plasma protein binding of doxorubicin is extensive, ranging from 50 to 90% (19,20), the high recov-

ery of free doxorubicin from plasma suggests that the column readily removes the drug from these protein binding sites. Therefore, it must be noted that the free drug, which is measured in the plasma using this method alone, does not discriminate between protein bound drug and actual free drug.

The separation of liposomal doxorubicin and free doxorubicin in plasma utilizing this procedure appears to be extremely efficient. This is especially true for the DSPC/Chol formulation used in this study, which demonstrated little (0.06%, Table 5) crossover of liposomal drug into the free drug fraction. The crossover for the EPC/Chol preparation, however, was higher and somewhat variable as might be expected with a "leakier" unsaturated vesicle formulation. The lowest crossover found with the EPC/Chol formulation was 0.52 and 0.56% of the total doxorubicin plasma concentration (Table 5). Since these values were equivalent and found in plasma taken from mice at different times following an injection with liposomal doxorubicin, it is likely that this represents the limit of separation for this formulation. Whether this small percentage of free drug is a result of liposomal release on the column as the vesicles pass through or free drug which leaks during the dilution and preparation of the sample prior to application to the column is difficult to determine. If it is being released on the column, column hydrophobicity does not appear to play a great role, since there was no difference in the percentage recovered from a C-2 and a C-18 column. Interactions with lipoproteins may also be involved with the release/leakage of free drug in this formulation. Regardless, it appears that the baseline separation (crossover of liposomal drug into the free drug fraction) of this EPC/Chol preparation is approximately 0.5–0.6% of the total doxorubicin plasma concentration.

The importance of employing a sensitive method for determining free and liposomal drug such as the one described here is illustrated in the strikingly low levels of free doxorubicin observed in the plasma of mice administered liposomal doxorubicin (Table 5). Clearly, predictions of toxicity and/or efficacy on the basis of pharmacokinetic behavior of liposomal drug formulations would be quite different for analysis based on total drug concentrations (traditional methods) or free drug levels (present study). The use of this separation method in pharmacokinetic studies may therefore greatly enhance the level of understanding of the therapeutic activity of liposomal doxorubicin and directly address issues concerning its mechanism of action.

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REFERENCES

1. Ostro, M. J. (1987) Liposomes: From Biophysics to Therapeutics, Dekker, New York.
2. Ostro, M. J., and Cullis, P. R. *Amer. J. Hosp. Pharm.*, in press.
3. Rahman, A., Kessler, A., More, N., Sikic, B., Rowden, G., Woolley, P., and Schein, P. S. (1980) *Cancer Res.* **40,** 1532–1537.
4. Gabizon, A., Dagan, A. Goren, D., Branholz, Y., and Fuks, Z. (1982) *Cancer Res.* **42,** 4734–4739.
5. Rahman, A., White, G., More, N., and Schein, P. S. (1985) *Cancer Res.* **45,** 5427–5432.
6. Mayer, L. D., Tai, L. C. L., Ko, D. S. C., Masin, D., Ginsberg, R. S., Cullis, P. R., and Bally, M. B. (1989) *Cancer Res.* **49,** 5922–5930.
7. Rahman, A., Charmichael, D., Harris, M., and Roh, J. K. (1986) *Cancer Res.* **46,** 2295–2299.
8. Abraham, I., Hilchie, J. C., and Mezei, M. (1983) *J. Pharm. Sci.* **72,** 1412–1415.
9. Mayer, L. D., Hope, M. J., Cullis, P. R., and Janoff, A. S. (1986) *Biochim. Biophys. Acta* **817,** 193–196.
10. Mayer, L. D., Bally, M. B., and Cullis, P. R. (1986) *Biochim. Biophys. Acta* **857,** 123–126.
11. Hope, M. J., Bally, M. B., Webb, G., and Cullis, P. R. (1985) *Biochim. Biophys. Acta* **812,** 55–65.
12. Fiske, C. H., and Subbarow, Y. (1925) *J. Biol. Chem.* **66,** 375–400.
13. Bachur, N. R., Moore, A. L., Bernstein, J. G., and Lio, A. (1970) *Cancer Chemother. Rep.* **54,** 89–94.
14. Rolland, A. (1988) *Int. J. Pharmaceut.* **42,** 145–154.
15. Magin, R. L., and Chan, H. C. (1987) *Biotechnol. Tech.* **1,** 185–188.
16. Druckmann, S., Gabizon, A., and Barenholz, Y. (1989) *Biochim. Biophys. Acta* **980,** 381–384.
17. Harris, P. A., and Gross, J. F. (1975) *Cancer Chemother. Rep.* **59,** 819–825.
18. Reich, S. D. (1983) *in* Pharmacokinetics of Anticancer Agents in Humans (Ames, M. M., Powis, G., and Kovich, J. S., Eds.), pp. 29–48, Elsevier, New York.