Prolongation of the Circulation Time of Doxorubicin Encapsulated in Liposomes Containing a Polyethylene Glycol-Derivatized Phospholipid: Pharmacokinetic Studies in Rodents and Dogs

Alberto A. Gabizon,[1,4] Yechezkel Barenholz,[2] and Meir Bialer[3]

Received August 10, 1992; accepted October 16, 1992

The pharmacokinetics of doxorubicin (DOX) encapsulated in liposomes containing polyethylene glycol-derivatized distearoylphosphatidylethanolamine (PEG/DSPE) were investigated in rodents and dogs. The plasma levels of DOX obtained with PEG/DSPE-containing liposomes were consistently higher than those without PEG/DSPE or when PEG/DSPE was replaced with hydrogenated phosphatidylinositol (HPI). Despite the inclusion of PEG/DSPE in liposomes, there was a significant drop in the plasma levels of DOX when the main phospholipid component, hydrogenated phosphatidylcholine, was replaced with lipids of lower phase transition temperature (dipalmitoylphosphatidylcholine, egg phosphatidylcholine), indicating that phase transition temperature affects the pharmacokinetics of liposome-encapsulated DOX. In beagle dogs, clearance was significantly slower for DOX encapsulated in PEG/DSPE-containing liposomes than in HPI-containing liposomes, with distribution half-lives of 29 and 13 hr, respectively. In both instances, almost 100% of the drug measured in plasma was liposome-associated. The apparent volume of distribution was only slightly above the estimated plasma volume of the dogs, indicating that drug leakage from circulating liposomes is insignificant and that the distribution of liposomal drug is limited mostly to the intravascular compartment in healthy animals.

KEY WORDS: liposome; doxorubicin; pharmacokinetics; polyethylene glycol.

INTRODUCTION

Polyethylene glycol-derivatized phospholipids and certain glycolipids have been shown to prolong the residence time of liposomes in the circulation of experimental animals (1–9). Further, there is a direct correlation between circulation time of liposomes in mice and their accumulation in transplanted tumors (2,7,10,11). Thus, long-circulating liposomes may enhance the delivery of anticancer drugs to tumors. The therapeutic index of anthracyclines was found to be increased by encapsulation in polyethylene glycol- or hydrogenated phosphatidylinositol-containing liposomes (7,11). Particularly, a small fraction of negatively charged polyethylene glycol-distearoyl phosphatidylethanolamine (PEG/DSPE), a semisynthetic phospholipid, appears to be useful in the development of an anticancer drug carrier.

In the present study, we examined the pharmacokinetics of the chemotherapeutic agent, doxorubicin, encapsulated in PEG/DSPE-containing liposomes in rodents and dogs. Several long-circulating liposome formulations were compared and the role played by the gel-to-liquid crystalline phase transition temperature (T_m) of the matrix liposome component was addressed.

MATERIALS AND METHODS

Liposome Preparation. Egg phosphatidylcholine (EPC), fully hydrogenated soybean phosphatidylcholine (HPC), and fully hydrogenated soybean phosphatidylinositol (HPI) were from Avanti Polar Lipids (Birmingham, AL). HPI was also obtained from Karlshamns LipidTeknic (Sweden). PEG/DSPE, molecular weight ca. 2600, was provided by Liposome Technology Inc. (Menlo Park, CA). Cholesterol (Ch), dipalmitoylphosphatidylcholine (DPPC), and α-tocopherol were from Sigma (St. Louis, MO). DOX (doxorubicin hydrochloride RDF) was a gift from Farmitalia-Carlo Erba (Milano, Italy). For dialysis, dilutions, and intravenous injections, sterile and pyrogen-free fluids from the Hadassah University Hospital Pharmacy were used. Liposomes were prepared by thin lipid film hydration and downsized by extrusion as previously described (11). The molar ratio of the lipid components was as follows: HPC:Ch, 10:8; HPI:HPC:Ch, 1:9:8; PEG/DSPE:HPC:Ch, 0.75:9.25:8; PEG/DSPE:DPPC:Ch, 0.75:9.25:8; and PEG/DSPE:EPC:Ch, 0.75:9.25:8. α-Tocopherol was present in all liposomes formulations at a ratio of 1:100 of total phospholipid. Doxorubicin encapsulation into the liposome water phase was achieved through a proton gradient generated by ammonium sulfate, following a technique previously reported (12). Unencapsulated DOX was removed by passage trough a Dowex resin column (13). The liposomes obtained had the following characteristics: a mean vesicle size between 65 and 95 nm with a monodisperse distribution as determined by dynamic laser scattering; less than 5% free drug in the stored preparation; a final drug concentration in the range of 1.5 to 2.5 mg/mL; and a drug-to-phospholipid ratio in the range of 40 to 60 μg/umol. DOX and phosphate determinations were done as previously reported (11).

Animals. The rodents used in this study were outbred Sabra (25–30 g) female mice and Sprague–Dawley (150–200 g) female rats from the Hebrew University Animal Breeding Center. Beagle dogs (11–19 kg, 6 females and 4 males) were obtained from the Research Animal Unit of Hadassah University Hospital.

Pharmacokinetic Studies. Drug preparations were diluted using 5% dextrose to 1 mg/mL for rodent injections and 1.8 mg/mL for dog injections. In rodents, the drug was injected by IV bolus through the tail vein. Blood (0.5–1 mL) was collected from the retroorbital sinuses under ether anesthesia. Mice were sacrificed immediately after bleeding. Rats were allowed to recover, to enable daily blood sampling

[1] Sharett Institute of Oncology, Hadassah University Hospital, P.O.B. 12000, Jerusalem 91120, Israel.
[2] Department of Membrane Biochemistry, Hebrew University–Hadassah School of Medicine, Jerusalem, Israel.
[3] Department of Pharmacy, School of Pharmacy, The Hebrew University of Jerusalem, P.O.B. 12065, Jerusalem 91120, Israel.
[4] To whom correspondence should be addressed.

0724-8741/93/0500-0703$07.00/0

over 3 consecutive days. Dogs were randomly assigned to three groups (free DOX, two females and one male; DOX encapsulated in HPI:HPC:Ch liposomes—hereafter referred to as HPI-DOX, two females and two males; DOX encapsulated in PEG/DSPE:HPC:Ch liposomes—hereafter referred to as PEG-DOX, two females and one male). Each treatment group was started on a different day to cope with the logistic difficulties resulting from the crowded sampling in the first couple of hours. Twenty-four hours before injection, the animals were weighed and their upper and lower limbs shaved. An 18-G Venflon IV catheter was inserted in a superficial limb vein. The drug was injected by quick bolus (approx. 15 sec). After drug injection, the catheter was rinsed with physiologic saline and then with 1 mL diluted heparin (250 U/mL) to prevent clotting. Four-milliliter blood samples were obtained before drug injection and at 5, 10, 15, 30, and 45 min, 1, 2, 4, 6, 8, 10, 12, 24, 48, and 72 hr following drug injection. In the liposome groups, blood was also obtained after 96, 120, 144, and 168 hr. Before each sampling, 2 mL of blood was withdrawn and discarded as dead space volume. After each sampling, the catheter was flushed with diluted heparin. After the 12-hr time point, the catheter was removed and further sampling was done by vein puncture. Blood was collected in Vacutainer tubes with K_3-EDTA anticoagulant and kept at 5°C. Plasma was separated by centrifugation within less than 24 hr after blood collection. To determine the total amount of drug (liposome-bound, protein-bound, and free drug) and its metabolites in dog plasma, HPLC analysis was used (see next section). To determine liposome-associated drug in plasma, a simpler total fluorescence assay was used (see next paragraph).

A fraction of plasma (0.1 to 0.2 mL in rodents, 0.5 mL in dogs) from liposome-injected animals was passed through a Dowex resin column to separate the liposome-associated fraction from free and protein-bound drug which are bound by the resin (12,14). The Dowex resin also binds and removes from plasma the daunosamine-containing metabolites, such as doxorubicinol, and a fraction of the deoxyaglycones (14). The plasma samples were stored at −20°C. To determine DOX and DOX equivalents by a fluorescence assay, the samples were treated with 0.075 *N* HCl in 90% isopropanol as described previously (11). Measurement was made by determining the intensity of fluorescence emission at 590 nm, with an excitation wavelength of 470 nm. The fluorimetric reading was transformed to μg/mL by interpolation with the readings of a standard curve of DOX in the linear range.

Plasma Determination of DOX and Its Metabolites in Dogs by HPLC. DOX and its metabolites were extracted by a procedure modified from Andrews *et al.* (15). For extraction 400 μL plasma was pipetted in an Ependorff test tube, to which 400 μL isopropanol, 400 μL chloroform, and 0.5 g ammonium sulfate were added. After 15 sec of vigorous vortexing, the test tube was centrifuged for 15 min at 10,000 rpm. Two phases and an interphase were formed: the clear chloroformic enriched upper phase was transferred to another Ependorff test tube. The solvents were evaporated by a stream of nitrogen at 30°C. The samples were stored dry at −20°C. The desired volume of isopropanol (100–200 μL) was added before injection to the HPLC system. HPLC analysis of DOX and metabolites was done following the procedure of Beijnen *et al.* (16) with modifications as previously reported (17). The system includes Kontron 420 HPLC pump, Kontron HPLC 460 autosampler, and Kontron 450 data system (Kontron, Switzerland).

Detection and quantification are based on the intensity of fluorescence emission of doxorubicin and were determined using a Jasco FR-210 spectrofluorometer (excitation, 470 nm; emission, 580 nm) equipped with 75-W xenon ozoneless lamp. A reverse-phase column (RP-C8, Alltech, Deerfields, IL) measuring 150 × 4.6 mm was used. The column was eluted with a solvent system of acetonitrile–water (4:6, v/v) containing 10 mg/L desipramine HCl to reduce adsorption of DOX to glassware and column. The pH of the mobile phase was adjusted to 2.60 with perchloric acid. The following metabolites were prepared as described elsewhere (18): doxorubicinol [retention time (RT), 3.16 min], DOX aglycone (RT, 1.95 min), and 7-deoxydoxorubicin aglycone (RT, 2.83 min). The RT of DOX was 4.46 min. All standard metabolites were stored in HPLC-grade ethanol at −20°C in the dark. In all cases, recovery of doxorubicin after extraction was better than 80%, and in most cases better than 90% as determined by data obtained from measurements of total plasma fluorescence emission intensity at 590 nm (excitation, 470 nm) after solubilization of plasma samples with 9 vol of 0.075 *N* HCl in 90% isopropanol (11). To correct for losses due to the extraction procedure a known amount of daunorubicin (RT, 8.00 min) was added to plasma samples as internal standard before extraction.

The above HPLC extraction procedure has been previously validated for human pharmacokinetic studies with a formulation of liposome-associated DOX containing low phase transition-temperature phospholipids (19). To ensure that this extraction is also applicable to liposomes composed of high phase transition-temperature phospholipids such as those used in this study, dog and human plasma were spiked with known amounts of PEG-DOX. Daunorubicin was added as internal standard. We then compared the DOX concentration obtained by HPLC analysis after either of the following extraction procedures:

(i) isopropanol/chloroform/$(NH_4)_2$ SO_4 extraction, as described above; and

(ii) solubilization by 0.075 *N* HCl in 90% isopropanol.

The agreement between the two methods was excellent. The relationship between the measured and the spiked doxorubicin level, in the range of 0.05–15 μg/mL, was described by a straight line in both cases. For i, [DOX] measured = 0.07 + 1.0585 * [DOX] spiked (r^2 = 0.996). For ii, [DOX] measured = 0.04 + 1.08 * [DOX] spiked (r^2 = 0.998).

Pharmacokinetic Analysis. The terminal slope of log*C* (DOX plasma concentration) versus time (*t*) was calculated by the method of least squares. The terminal half-life ($t_{1/2}$) was calculated from the quotient ln2/slope. The area under the *C* versus *t* curve (AUC) was calculated by the trapezoidal rule with extrapolation to infinity (20). Clearance (CL) was calculated by dividing dose over AUC. Volume of distribution (V_β) was calculated by dividing CL over the terminal slope. Volume of distribution at steady state (V_{ss}) and mean residence time (MRT) were calculated using Eqs. (1) and (2), respectively (21,22):

$$V_{ss} = \text{Dose} * \text{AUMC}/(\text{AUC}^2) \quad (1)$$

$$MRT = AUMC/AUC \quad (2)$$

AUMC is the area under the product of $C * t$ versus t, from time 0 to infinity, and was calculated by the trapezoidal rule with extrapolation to infinity. All pharmacokinetic parameters were calculated in a noncompartmental manner based on the statistical moment theory (20,23).

RESULTS

Effect of Liposome Composition on the Plasma Levels of Liposome-Associated DOX in Rodents

Table I shows the plasma levels of liposome-associated DOX, 24 hr after the iv administration in mice of DOX encapsulated in a variety of liposome formulations. The PEG/DSPE:HPC:Ch formulation produced the highest plasma levels. When PEG/DSPE was replaced with HPI or deleted from the formulation, as in the case of HPC:Ch, plasma levels were significantly lower. When HPC was replaced with phospholipids of lower T_m (DPPC, EPC), while retaining PEG/DSPE in the liposome composition, lower plasma levels were observed in the order of decreasing T_m: HPC > DPPC > EPC. In addition, in *in vitro* stability tests with DOX-containing, PEG/DSPE liposomes in human plasma, the percentage of DOX retained in liposomes after 1 hr of incubation was 85, 60, and 38%, for PEG/DSPE:HPC:Ch, PEG/DSPE:DPPC:Ch, and PEG/DSPE:EPC:Ch, respectively. Thus, the trend observed is superior drug retention in the following order, HPC > DPPC > EPC.

When three of these formulations were injected into Sprague–Dawley rats, the plasma levels of liposome-associated DOX examined at 4, 24, and 48 hr (Fig. 1) agree with the mouse data, with the order of length of circulation time: PEG/DSPE:HPC:Ch > HPI:HPC:Ch > HPC:Ch.

Pharmacokinetics of DOX Encapsulated in HPI- and PEG/DSPE-Containing Liposomes in Dogs

The next step was to assess the pharmacokinetic behavior of DOX-encapsulated in long-circulating liposomes in a large mammal species, such as dogs, for eventual scale-up to humans. In view of our results in rodents, we chose for the dog studies the two best-rating liposome preparations: HPI:HPC:Ch and PEG/DSPE:HPC:Ch. A third group of dogs was injected with free DOX. The dose given was relatively subtoxic (0.5 mg/kg) to avoid any complications (vomiting, dehydration) which could distort the results.

Table I. Effect of Liposome Composition on Plasma Levels of Liposome-Associated DOX in Mice[a]

Liposome composition	μg DOX/mL plasma (SD)	%ID in plasma
Experiment 1		
HPC-Ch	3.6 (0.9)	1.8
HPI-HPC-Ch	12.5 (0.3)	5.6
PEG/DSPE-HPC-Ch	34.8 (9.1)	17.4
Experiment 2		
PEG/DSPE-HPC-Ch	37.4 (2.4)	16.1
PEG/DSPE-DPPC-Ch	16.4 (0.7)	7.4
PEG/DSPE-EPC-Ch	0.7 (0.0)	0.3

[a] Sabra female mice injected iv with 10 mg/kg DOX and bled 24 hr after dosing. Each experimental group consisted of four mice.

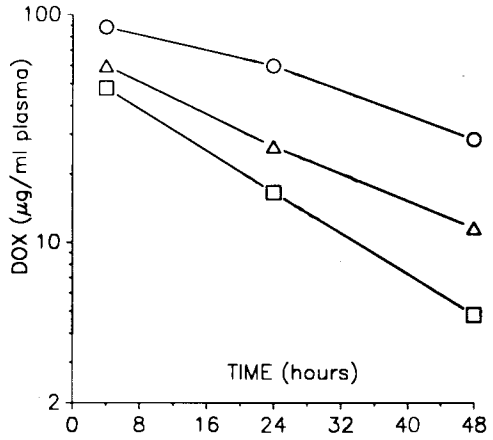

Fig. 1. Effect of liposome composition on plasma clearance of liposome-encapsulated DOX in rats. DOX dose, 10 mg/kg iv. Each point represents the mean value of two rats. Liposome composition: ○, PEG/DSPE:HPC:Ch; △, HPI:HPC:Ch; □, HPC:Ch.

Figure 2 and Table II show, respectively, the plasma concentration/time curves and mean pharmacokinetic parameters for the two liposome preparations tested based on the total DOX levels in plasma measured by HPLC. The plasma concentration/time curve of free DOX is also depicted in Fig. 2, but the pharmacokinetic parameters were not calculated since plasma levels during the terminal clearance phase were undetectable with the dose used in this study (0.5 mg/kg). However, it is well-known that the pharmacokinetics of free DOX follows a biphasic pattern with a rapid distribution phase ($t_{1/2}$, 5–10 min) and a slow terminal-elimination phase ($t_{1/2}$, 25–30 hr), clearance in the order of 15 mL/min/kg, and a large volume of distribution (25 L/kg) (for example, Ref. 24). Significant differences between the two DOX liposome preparations were found. The mean clearance value of HPI-DOX was four times greater than that of PEG-DOX, although no significant difference was observed in the volume of distribution. It appears, therefore, that the relatively longer half-life and MRT of PEG-DOX result from a reduced clearance rate. Interestingly, the volume of distribution for liposomal drug is only slightly greater than the plasma volume pointing at the liposome restriction to the central compartment. The distribution phase of free DOX was extremely rapid, in the order of minutes (Fig. 2). The peak plasma concentration of free DOX was significantly below that observed for liposomal DOX. Clearly, the pharmacokinetics of DOX is drastically changed by liposome encapsulation. No detectable amounts of DOX metabolites were seen by HPLC in any of the experimental groups.

The plasma concentration/time curves of liposome-associated DOX and total DOX as determined by fluorometric measurements on acidified isopropanol extracts were almost identical for both liposome preparations, HPI-DOX and PEG-DOX (Fig. 3). Therefore, at least more than 90% circulates in liposome-associated form. Since liposome-associated drug is not exposed to metabolic processes, this observation may account for the absence of detectable DOX

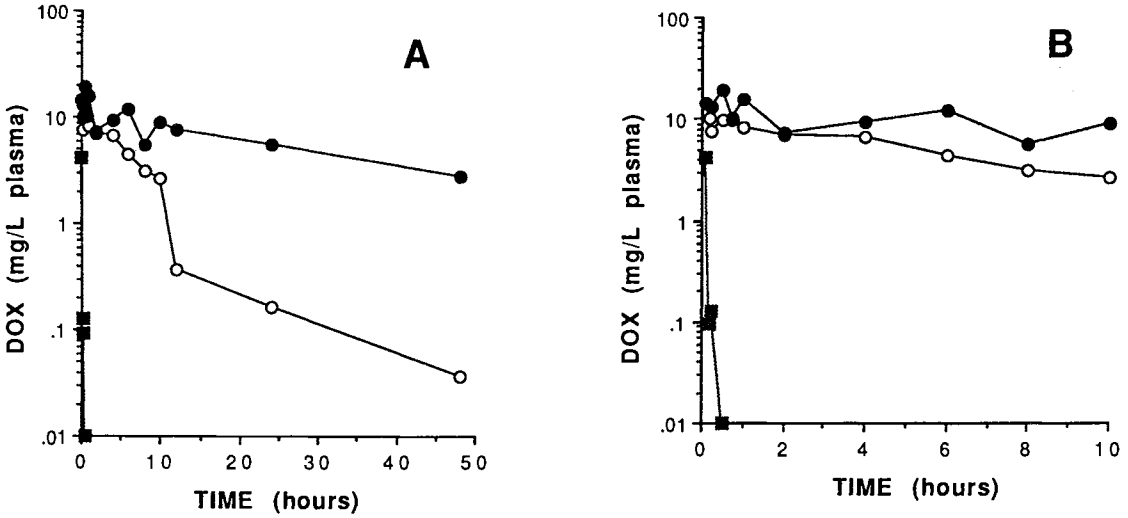

Fig. 2. Comparison of the pharmacokinetics of DOX in three representative beagle dogs receiving either free DOX, HPI-DOX, or PEG-DOX. DOX dose, 0.5 mg/kg iv. DOX levels in plasma determined by HPLC. ■, free DOX; ○, HPI-DOX; ●, PEG-DOX. A, 0- to 50-hr view; B, 0- to 10-hr view.

metabolites. Table III shows the pharmacokinetic parameters for plasma liposome-associated drug after administration of PEG-DOX or HPI-DOX. In both groups, the clearance of liposome-associated drug was accounted for by a slow monoexponential distribution phase ($t_{1/2}$; 13 and 29 hr for HPI-DOX and PEG-DOX, respectively). The measured peak plasma concentrations of liposome-associated drug in HPI-DOX- and PEG-DOX-treated dogs were similar (7.5 ± 0.9 and 7.4 ± 0.5 mg/L, respectively). In agreement with the observations on total plasma doxorubicin (Table II), the clearance of liposome-associated DOX was significantly faster in the animals receiving HPI-DOX liposomes compared to those receiving PEG-DOX, although no significant differences in volume of distribution were noticed (Table III). There was a remarkable similarity between the pharmacokinetic parameters obtained for plasma total DOX (Table II) and those for plasma liposome-associated DOX (Table III) within each experimental group.

Table II. Pharmacokinetic Parameters of DOX Following iv Administration (0.5 mg/kg) of PEG-DOX and HPI-DOX Liposomal Preparations to Beagle Dogs

	Mean ± SD	
	PEG-DOX	HPI-DOX
$t_{1/2}$ (hr)*	27 ± 5	10 ± 5
AUC^{0-168} (mg × hr/L)	281 ± 108	57 ± 26
$AUC^{0-\infty}$ (mg × hr/L)	304 ± 118	70 ± 22
CL (mL/min)	0.42 ± 0.12	1.5 ± 0.3
CL (mL/min/kg)	0.031 ± 0.014	0.13 ± 0.04
V_β (L)	1.0 ± 0.1	1.2 ± 0.4
V_β (L/kg)	0.07 ± 0.02	0.11 ± 0.05
V_{ss} (L)	0.9 ± 0.1	0.9 ± 0.3
V_{ss} (L/kg)	0.07 ± 0.02	0.07 ± 0.02
MRT (hr)	37 ± 7	11 ± 7

* The differences between the terminal slope and the half-life mean values of PEG-DOX and HPI-DOX were statistically significant ($P < 0.01$, Student's t test).

DISCUSSION

The pharmacokinetics of DOX can be drastically modified by encapsulation in long-circulating liposomes (12). Our results are the first to document these pharmacokinetic changes in a large mammalian species, such as dogs, and to compare various long-circulating liposomes as carriers of DOX.

The *in vivo* clearance rate of liposome-encapsulated DOX after iv injection is determined by two main factors: (i) clearance of the intact liposomes; (ii) leakage of drug from circulating liposomes. Liposome clearance is determined to a large extent by the liposome uptake activity of the reticuloendothelial system (RES) of liver, spleen, and bone marrow (25). Liposome localization in non-RES tissues, as in the case of tumors, is a slow process requiring prolonged liposome stay in circulation (2,11) and apparently related to increased microvascular permeability and neovascularization (26). Stable retention of the liposome contents for prolonged circulation times is another prerequisite to achieve optimal drug delivery to extra-RES tissues. In fact, as liposome formulations with longer circulation times are designed, the stability requirements become more stringent since the vesicles remain longer in the blood but must retain their contents. One important factor controlling vesicle bilayer permeability is the T_m of the matrix phospholipid (25). As seen in Table I, the use of phospholipids with lower T_m resulted in lower plasma levels of liposome-associated DOX. This is likely to be the result of reduced stability rather than shortened vesicle circulation time. The *in vitro* stability data in plasma (see Results) and the fact that no significant differences in circulation time between radiolabeled PEG/DSPE-HPC-Ch and PEG/DSPE-EPC-Ch liposomes have been reported (27) strongly support this point. Thus, despite the presence of a PEG-derivatized phospholipid, the T_m of the bulk component of the liposome bilayer is an independent factor con-

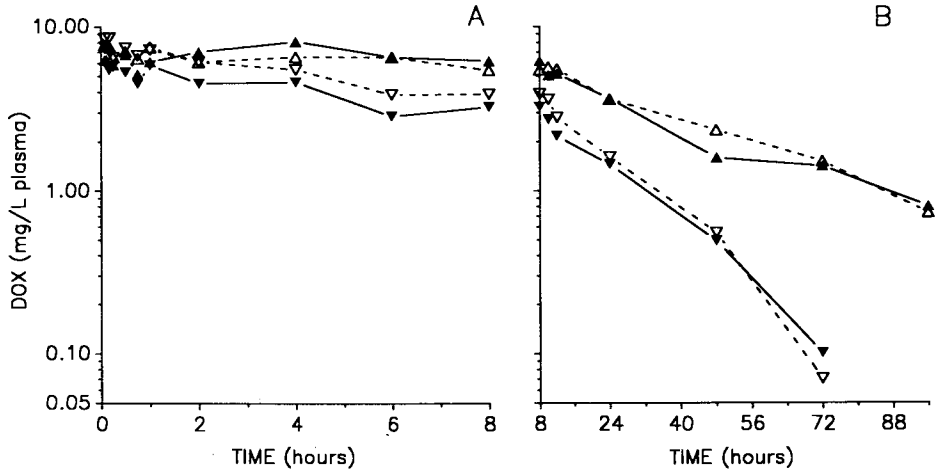

Fig. 3. Comparison of the pharmacokinetics of liposome-associated DOX in receiving either HPI-DOX or PEG-DOX. DOX dose, 0.5 mg/kg iv. DOX levels in plasma determined by a fluorescence assay. Each point represents the mean value of three (PEG-DOX) or four (HPI-DOX) dogs. Filled symbols and solid lines stand for total DOX concentration in plasma. Open symbols and dashed lines stand for liposome-associated DOX concentration on plasma. (▼, ▽), HPI-DOX; (▲, △) PEG-DOX. (A) 0- to 8-hr view; (B) 8- to 96-hr view.

trolling vesicle stability. An increased permeability to protons or ammonium ions in liposomes with low T_m components would lead to a gradual loss of the ion gradient holding DOX in ionized form in the vesicle aqueous phase (G. Haran and Y. Barenholz, unpublished results).

Small (<100-nm), neutral vesicles made with high-T_m (>37°C) phospholipids and Ch are known to be stable in plasma and have long half-lives in circulation (reviewed in Ref. 25). Further prolongation in blood residence time can be obtained by addition of a small molar fraction of certain negatively charged glycolipids or PEG-derivatized phospholipids (2–9). The mechanism accounting for this extended vesicle survival in the circulation appears to be related to a phenomenon of steric stabilization (28). In agreement with results obtained with radiolabeled liposomes, DOX encapsulated in PEG/DSPE liposomes stay longer in blood than DOX encapsulated in other stable formulations (HPI: HPC:Ch and HPC:Ch). Since the accumulation of liposome-associated drug in tumors depends on its circulation residence time (11), this study indicates that PEG/DSPE liposomes are more efficient DOX carriers than any other formulations tested thus far across rodents and dogs.

Table III. Pharmacokinetic Parameters of Liposome-Associated DOX Following iv Administration (0.5 mg/kg) of PEG-DOX and HPI-DOX Liposomal Preparations to Beagle Dogs

	Mean ± SD	
	PEG-DOX	HPI-DOX
$t_{1/2}$ (hr)*	29 ± 8	13 ± 1
AUC^{0-168} (mg × hr/L)	241 ± 58	102 ± 29
$AUC^{0-\infty}$ (mg × hr/L)	276 ± 88	106 ± 28
CL (mL/min)	0.48 ± 0.15	1.05 ± 0.30
CL (mL/min/kg)	0.032 ± 0.009	0.086 ± 0.017
V_β (L)	1.1 ± 0.2	1.1 ± 0.4
V_β (L/kg)	0.07 ± 0.01	0.09 ± 0.03
V_{ss} (L)	1.1 ± 0.3	1.0 ± 0.3
V_{ss} (L/kg)	0.07 ± 0.02	0.08 ± 0.02
MRT (hr)	40 ± 10	18 ± 2

* The differences between the terminal slope and half-life mean values of PEG-DOX and HPI-DOX were statistically significant ($P < 0.01$, Student's t test).

When the pharmacokinetic parameters in dogs after HPI-DOX injection are compared to those after PEG-DOX injection (Tables II and III), the latter group shows a longer half-life and slower clearance. These differences must be accounted for by reduced RES uptake and/or reduced leakage rate. The respective contribution of each of these processes cannot be directly assessed from these experiments. Nevertheless, the fact that we could not detect any significant amount of nonliposome-associated drug in plasma in either treatment group (Fig. 3) suggests that the rate of RES uptake is the main factor accounting for the differences between PEG-DOX and HPI-DOX. Since there were no differences in the small volume of distribution between HPI-DOX and PEG-DOX, both preparations appear to be restricted to the central compartment to a similar degree.

In a preliminary report on the pharmacokinetics of PEG-DOX in humans (29), a half-life of 42 hr was found for liposome-associated DOX. This value is consistent with the $t_{1/2}$ of PEG-DOX in dogs (29 hr) (Table III), indicating that the present study can predict the pharmacokinetic behavior of DOX liposomes in humans.

The finding most relevant to clinical applications is the drastic change of the pharmacokinetics of DOX achieved through encapsulation in liposomes of specific compositions (Fig. 2). The differences in half-life, clearance, and volume of distribution between free and liposomal drug are striking in magnitude, emphasizing the complete control of drug pharmacokinetics with the liposome carrier. It is thus conceivable that significant changes in the tissue distribution

and pharmacodynamics of DOX will be achieved using optimized liposome formulations.

ACKNOWLEDGMENTS

This work was supported by Liposome Technology Inc. (Menlo Park, CA). Alberto A. Gabizon is a recipient of a Research Career Development Award from the Israel Cancer Research Fund. We are grateful to S. Samuel, R. Cohen, M. Chemla, and T. Barenholz for their technical help.

REFERENCES

1. T. M. Allen and A. Chonn. Large unilamellar liposomes with low uptake by the reticuloendothelial system. *FEBS Lett.* 223:42–46 (1987).
2. A. Gabizon and D. Papahadjopoulos. Liposome formulations with prolonged circulation time in blood and enhanced uptake by tumors. *Proc. Natl. Acad. Sci. USA* 85:6949–6953 (1988).
3. T. M. Allen, C. Hansen, and J. Rutledge. Liposome with prolonged circulation times: Factors affecting uptake by reticuloendothelial system and other tissues. *Biochim. Biophys. Acta* 981:27–35 (1989).
4. A. L. Klibanov, K. Maruyama, V. P. Torchilin, and L. Huang. Amphipathic polyethyleneglycols effectively prolong the circulation time of liposomes. *FEBS Lett.* 268:235–237 (1990).
5. G. Blume and G. Cevc. Liposomes for sustained drug release in vivo. *Biochim. Biophys. Acta* 1029:91–97 (1990).
6. J. Senior, C. Delgado, D. Fisher, C. Tilock, and G. Gregoriadis. Influence of surface hydrophilicity of liposomes on their interaction with plasma protein and clearance from the circulation: Studies with polyethyleneglycol-coated vesicles. *Biochim. Biophys. Acta* 1062:77–82 (1991).
7. D. Papahadjopoulos, T. Allen, A. Gabizon, E. Mayhew, K. Matthay, S. K. Huang, K.-D. Lee, M. C. Woodle, D. D. Lasic, C. Redemann, and F. J. Martin. Sterically stabilized liposomes: Improvements in pharmacokinetics and anti-tumor therapeutic efficacy. *Proc. Natl. Acad. Sci. USA* 88:11460–11464 (1991).
8. T. M. Allen, C. Hansen, F. Martin, C. Redemann, and A. Yau-Young. Liposomes containing synthetic lipid derivatives of polyethylene glycol show prolonged circulation half-lives in vivo. *Biochim. Biophys. Acta* 1066:29–36 (1991).
9. A. Gabizon and D. Papahadjopoulos. The role of surface charge and hydrophilic groups on liposome clearance *in vivo*. *Biochim. Biophys. Acta* 1103:94–100 (1992).
10. A. Gabizon, D. C. Price, J. Huberty, R. S. Bresalier, and D. Papahadjopoulos. Effect of liposome composition and other factors on the targeting of liposomes to experimental tumors: Biodistribution and imaging studies. *Cancer Res.* 50:6371–6378 (1990).
11. A. Gabizon. Selective tumor localization and improved therapeutic index of anthracyclines encapsulated in long-circulating liposomes. *Cancer Res.* 52:891–896 (1992).
12. A. Gabizon, R. Shiota, and D. Papahadjopoulos. Pharmacokinetics and tissue distribution of doxorubicin encapsulated in stable liposomes with long-circulation times. *J. Natl. Cancer Inst.* 81:1484–1488 (1989).
13. G. Storm, L. Van Bloois, M. Brouwer, and D. J. Crommelin. The interaction of cytostatic drugs with adsorbents in aqueous media: The potential implications for liposome preparations. *Biochim. Biophys. Acta* 818:343–351 (1992).
14. S. Druckmann, A. Gabizon, and Y. Barenholz. Separation of liposome-associated doxorubicin from non-liposome-associated doxorubicin in human plasma: Implications for pharmacokinetic studies. *Biochim. Biophys. Acta* 980:381–384 (1989).
15. P. A. Andrews, D. E. Brenner, F. E. Chou, H. Kubo, and N. R. Bachur. Facile and definitive determination of human adriamycin and daunorubicin metabolites by high-pressure liquid chromatography. *Drug Metab. Disp.* 8:152–156 (1980).
16. J. H. Beijnen, H. Rosing, P. A. De Vries, and W. J. Underberg. Stability of anthracycline antitumor agents in infusion fluids. *J. Parent. Sci. Technol.* 39:220–223 (1985).
17. S. Amselem, R. Cohen, S. Druckmann. A. Gabizon, D. Goren, R. M. Abra, A. Huang, R. New, and Y. Barenholz. Preparation and characterization of liposomal doxorubicin for human use. *J. Liposome Res.* 2:93–123 (1992).
18. S. Takanashi and N. R. Bachur. Adriamycin metabolism in man: evidence from urinary metabolites. *Drug Metab. Disp.* 4:79–87 (1976).
19. A. Gabizon, R. Chisin, S. Amselem, S. Druckmann, R. Cohen, D. Goren, I. Fromer, T. Peretz, A. Sulkes, and Y. Barenholz. Pharmacokinetic and imaging studies in patients receiving a formulation of liposome-associated adriamycin. *Br. J. Cancer* 64:1125–1132 (1991).
20. M. Gibaldi and D. Perrier. *Pharmacokinetics,* Marcel Dekker, New York, 1982, pp. 445–449.
21. L. Z. Benet and R. L. Galazzi. Non-compartmental determination of steady state volume of distribution. *J. Pharm. Sci.* 68:1071–1074 (1979).
22. K. Yamaoka, T. Nakagawa, and T. Uno. Statistical moments in pharmacokinetics. *J. Pharmacokinet. Biopharm.* 6:547–558 (1978).
23. K. Yamaoka. *Methods for Pharmacokinetic Analysis for Personal Computers,* Nanko-D Ltd., Tokyo, 1986, pp. 145–175.
24. R. F. Greene, J. M. Collins, J. F. Jenkins, J. L. Speyer, and C. E. Myers. Plasma pharmacokinetics of adriamycin and adriamycinol: Implications for the design of *in vitro* experiments and treatment protocols. *Cancer Res.* 43:3417–3421 (1983).
25. J. H. Senior. Fate and behavior of liposomes in vivo: A review of controlling fators. *CRC Crit. Rev. Ther. Drug Carrier Syst.* 3:123–193 (1987).
26. R. K. Jain. Vascular and interstitial barriers to delivery of therapeutic agents in tumors. *Cancer Metastas. Rev.* 9:253–266 (1990).
27. M. C. Woodle and D. D. Lasic. Sterically stabilized liposomes. *Biochim. Biophys. Acta* 1113:171–199 (1992).
28. D. D. Lasic, F. J. Martin, A. Gabizon, S. K. Huang, and D. Papahadjopoulos. Sterically stabilized liposomes: A hypothesis on the molecular origin of the extended circulation times. *Biochim. Biophys. Acta* 1070:187–192 (1991).
29. A. Gabizon, R. Catane, B. Uziely, B. Kaufman, T. Safra, Y. Barenholz, and A. Huang. A pilot study of doxorubicin encapsulated in long-circulating (Stealth®) liposomes in cancer patients. *Proc. Am. Soc. Clin. Oncol.* 11:124 (1992).