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Research Article

In Vitro and *In Vivo* Studies Evaluating a Liposome System for Drug Solubilization

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A liposome system was developed which demonstrates suitability as an intravenous drug carrier for a lipophilic drug compound (RS-93522, a dihydropyridine CA^{2+} channel blocker). An aqueous phospholipid suspension was employed as a nontoxic solubilizing vehicle for this drug. The liposome formulation, composed of a 3% mixture of dioleoylphosphatidylcholine and dioleoylphosphatidylglycerol, produced a physically and chemically stable preparation which solubilized the lipophilic drug compound at a concentration 500 times above its intrinsic aqueous solubility. Characterizing the liposome–drug system by gel filtration chromatography showed that the drug comigrated with the lipid constituents of the liposome. Further *in vitro* studies established that the liposome–RS-93522 formulation allowed for rapid and complete transfer of the drug from the liposome to bind with albumin when added to human serum. *In vivo* studies with rats were performed in which the pharmacokinetics of the liposomal–RS-93522 system were compared to those of a cosolvent-solubilized RS-93522 solution. This study showed that the pharmacokinetic profiles of the two solutions were identical. All the evidence indicates that a liposome formulation of this type does not alter the distribution of the drug in serum and is, therefore, not likely to affect the intrinsic pharmacological or toxicological parameters of the drug relative to the conventional solvent/excipient-containing formulation. This liposome system demonstrates utility as a biocompatible, nontoxic drug delivery vehicle.

KEY WORDS: liposome; drug solubilization; drug delivery; lipophilic drug.

INTRODUCTION

Many reports have demonstrated specific therapeutic advantages for the incorporation of lipophilic drugs into liposomes (1–8). These reports tend to emphasize that specifically optimized liposome formulations can favorably alter pharmacokinetic parameters or tissue distribution. The formulations can be designed either to target the drug to disease sites (4,9,10), to increase its duration of action (5,6,11,12), or to divert the drug from organs that are particularly sensitive to its toxic effects (1,2,5). To produce these effects, attention has generally focused on developing lipid compositions which give good stability in serum (12). In general, the most stable liposomes are comprised of phospholipid with a high melting temperature, cholesterol, and negatively charged glycolipids (13).

The compound, RS-93522 (Fig. 1), is a dihydropyridine structure (molecular weight, 526.54 g/mol) with demonstrated activity as a calcium channel blocker and antihypertensive agent. Characterization of its solubility shows it to be sparingly water soluble at 0.012 mg/ml (Leo Gu, Syntex Research, personal communication). In order to develop an iv formulation and achieve the requisite concentration of this compound, a large amount of cosolvent or cosolvent combination (at a concentration greater than 40%) was required. Various cosolvents have demonstrated detrimental toxicological effects (14,15). Hence, solvents and excipients exhibiting a low toxicity are sought in order to circumvent this pervasive drug formulation problem (16). Phospholipids are conceptually attractive as solubilizing agents because they can solubilize a broad range of water-insoluble drugs, and compared to other solvents, surfactants, or excipients, they are relatively nontoxic (1,2).

In this report, we demonstrate the rationale for developing nontoxic liposome-solubilized drug compositions that are physically stable in the formulation but are intrinsically *unstable* in serum. We show *in vitro* and *in vivo* results with a phospholipid-solubilized cardiovascular drug formulation in order to demonstrate two general concepts: (1) that phospholipids are effective as nontoxic solubilizing agents for lipophilic compounds and (2) that selected liposome formulations rapidly deliver lipophilic drugs into the same serum pool reached when conventionally formulated.

MATERIALS AND METHODS

Chemicals

Both dioleoylphosphatidylcholine (DOPC) and dioleoylphosphatidylglycerol (DOPG) were obtained from

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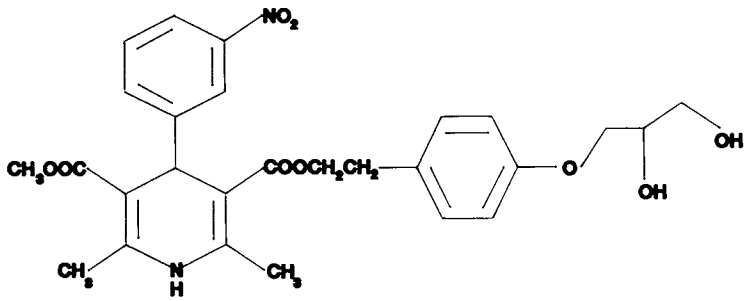

Fig. 1. RS-93522. 2-[4-(2,3-Dihydroxypropoxy)phenyl]ethyl methyl-1,4-dihydro-2,6-dimethyl-4-(3-nitro phenyl)-3,5-pyridinedicarboxylate.

Avanti Polar Lipid as 20 mg/ml solutions in chloroform. RS-93522 was synthesized by, and received from, the Institute of Organic Chemistry, Syntex Research. ^{14}C-DOPC was supplied by New England Nuclear (NEN) with a specific activity of 114 mCi/mmol and a radiochemical purity of 98%. Sepharose 6B gel filtration medium was received from Pharmacia. Reagent-grade methanol and acetonitrile were obtained from Burdick and Jackson; ether for anesthesia and high-performance liquid chromatographic (HPLC)-grade, t-butyl methyl ether were supplied by Mallinkrodt. Aquasol LSC scintillation cocktail was supplied by NEN Research Products.

Animals

Male Sprague–Dawley rats weighing approximately 400 g were obtained from Bantum Kingman.

Gel Filtration

Sepharose 6B gel was packed into a Pharmacia column (1.6 × 40 cm) as recommended by the manufacturer. After column packing, the gel was presaturated (17,18) with an aliquot of empty lipid vesicles (DOPC and DOPG). The buffer system was composed of the following ingredients: 0.14% sodium phosphate (dibasic), 0.89% sodium chloride, 0.02% potassium chloride, and 0.005% Thimerosal.

Liposome Preparation

All components of the liposome formulation, DOPC, DOPG, RS-93522 (dissolved in chloroform), and ^{14}C-DOPC (used as a liposome structural marker for the *in vitro* studies, only), were combined. The organic solvent was evaporated using low heat (60°C) and nitrogen. The remaining film was further dried by vacuum overnight to remove any residual organic solvent. The lipid layer was subsequently hydrated with a 5% sorbitol solution containing 0.01% Thimerosal. Once hydrated, the mixture was sonicated for 1 hr using a Branson cell disrupter with a water-jacketed cup horn, thus allowing for sonication of a sealed vial. The final concentration of each excipient, unless otherwise specified, was as follows: DOPC, 21.0 mg/ml; DOPG, 9.0 mg/ml; RS-93522, 2.0 mg/ml; and ^{14}C-DOPC, trace (used for *in vitro* studies only).

Particle Size Analysis

Light-scattering measurements were performed with a Coulter submicron particle analyzer (Model N4 MD). Immediately prior to analysis, the liposome preparations were filtered through a 0.2-μm polycarbonate membrane, then diluted 1:20 with filtered Dulbeco's phosphate-buffered saline (per liter: 2 g KC1, 2 g KH_2PO_4, 80 g NaCl, 11.44 g Na_2HPO_4; adjusted to pH 7.4 with NaOH). The following instrument settings were used: temperature, 20°C; viscosity, 0.01 P; refractive index, 1.333; scattering angle, 90°; run time, 300 sec; and range, 10–1000 nm.

Procedure for *In Vivo* Comparison of the Liposome to the Conventional Formulation

Male Sprague–Dawley rats weighing approximately 400 g were anesthetized with ether. Under anesthesia, the rat was given an iv injection of either the liposome formulation or a conventional formulation [ethanol, 10%; polyethylene glycol 400, 40%; RS-93522, 0.2% (2 mg/ml); qs with phosphate buffer]. The delivered dose was approximately 1.0 mg/kg. The rats recovered from anesthesia and were placed in their cages until blood collection. At designated time points, each rat was again ether anesthetized, and blood was collected via terminal bleed from the descending aorta into a citrated vacutainer tube. The blood was centrifuged and the plasma was stored frozen until assay.

Analytical Procedures

HPLC. For *in vitro* the mobile phase was composed of methanol:water:triethylamine (60:39.8:0.2) which was adjusted to pH 3.0 with phosphoric acid. The equipment included an IBM LC/9523 variable UV detector, an IBM pump, a Micromeretics 728 autosampler, a Spectra Physics SP 4270 integrator, and an Alltech Spherisorb ODS 5-μ column. The injection volume was 10 μl; the mobile phase flow rate was 0.6 ml/min; and the absorbance wavelength was 280 nm. Solutions of known RS-93522 concentration was used as external standards. For *in vivo* studies, the mobile phase was composed of acetonitrile:water (45:55). The injection volume was 50 μl; the flow rate of the mobile phase was 1.4 ml/min and the absorbance wavelength was 229 nm. An ether extraction method (19) was used to recover RS-93522 from rat plasma. An internal standard, RS-71645 (an analogue of RS-93522), was included to ensure accurate and reliable quantitation.

Sample Preparation. Prior to injection on the HPLC column the samples were prepared accordingly: (a) for gel filtration fractions, a 500-μl aliquot of the sample was diluted with 500 μl of mobile phase; and (b) for *in vivo* plasma samples, a 1.0-ml aliquot of plasma was spiked with 0.2 μg of

internal standard. The sample was washed twice with 4.0 ml of t-butyl methyl ether. The 8.0 ml of ether was collected and dried under nitrogen and gentle heat (60°C). After ether evaporation, 200 μl of mobile phase reconstituted the dried film.

Scintillation Counting (Used for In Vitro *Studies Only).* In order to determine the ^{14}C-isotope in each gel filtration fraction, a 0.5-ml aliquot of sample was placed in a scintillation vial with 12.0 ml of Aquasol scintillation cocktail. The samples were then placed in a Beckman LS 8100 scintillation counter.

RESULT

Solubilization Optimization

The optimum quantity of lipid required to solubilize RS-93522 was determined by mixing increasing amounts of drug into a fixed quantity of lipid (30 mg; 7:3, DOPC/DOPG, w/w) dissolved in chloroform. After liposome preparation, each sample was visually inspected for clarity and analyzed by quasielastic laser light scattering to determine size and polydispersity.

Preparations containing up to 6 mg/ml of RS-93522 were clear after sonication and remained physically stable for more than 4 weeks. The 12- and 18-mg/ml samples were clear after sonication but developed a precipitate after overnight storage at 4°C.

Light-scattering data are summarized in Table I. Samples containing up to 6 mg/ml of drug gave a bimodal size distribution. The smaller population predominated (>88%), having a diameter of 20–25 nm. The larger population (<12%) was 50–90 nm in diameter. Figure 2 shows a representative plot of differential weight versus particle diameter. The values presented were generated from filtered samples; unfiltered samples were also evaluated and gave very similar results.

The 12-mg/ml sample results showed that approximately 29% of the population was greater than 1.0 μm; the sample was filtered (0.2 μm) just prior to light-scattering analysis. These findings indicate that 12 mg/ml of RS-93522 is beyond the loading capacity of a 30-mg/ml phospholipid concentration. Maximum loading for this system is 6 mg/ml of drug. At this concentration, 3.5 mol of phospholipid is present per 1 mol of drug; this represents a 500-fold increase over the drugs' aqueous solubility.

Table I. Particle Size Evaluation of Various RS-93522 Concentrations in the Liposome Formulation[a]

RS-93522 concentration (mg/ml)	Smaller population (nm)	Larger population (nm)	Percentage greater than 1.0 μm
1.5	25.4 ± 6 (88%)	62.2 ± 15 (12%)	0.1
2.0	23.3 ± 9 (98%)	81.2 ± 10 (2%)	1.0
3.0	23.9 ± 9 (93%)	55.5 ± 13 (7%)	2.3
6.0	20.1 ± 5 (89%)	51.0 ± 11 (11%)	1.3
12.0	—	—	29.0
18.0	Precipitated (not evaluated)		

[a] Liposomes were prepared incorporating 1.5 to 18.0 mg/ml of RS-93522; the total final lipid concentration was fixed for all samples at 30 mg/ml. Each preparation was sonicated, filtered, then analyzed by quasielastic laser light scattering, as described in Materials and Methods. The size distribution analysis was done by deconvoluting the autocorrelation curve with a computer program supplied by the manufacturer. Error data are expressed as the standard deviation. The numbers in parentheses refer to the percentage of mass within that size population.

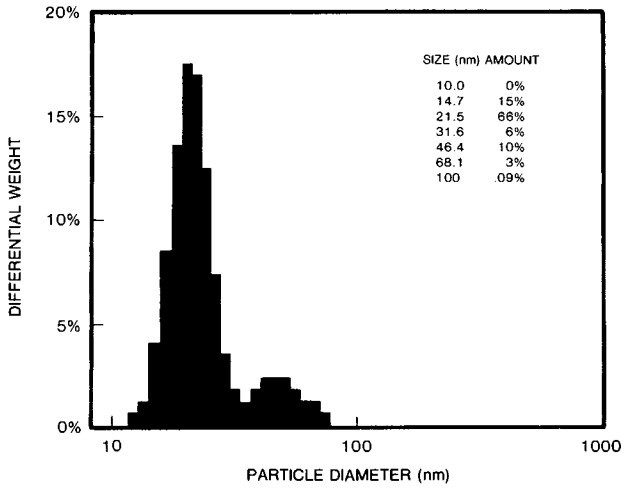

Fig. 2. Particle sizing by quasielastic laser light scattering. A sonicated liposome preparation containing 6 mg/ml of RS-93522 was analyzed with a Coulter submicron particle analyzer according to Materials and Methods.

Gel Filtration of Lipid–Drug Complexes

Further characterization of the liposome system was performed by gel filtration. ^{14}C-Labeled DOPC was incorporated into the liposome to allow observation of liposome behavior. Figure 3 depicts the liposomal–drug formulation; the drug elutes similarly and concomitantly with the lipid

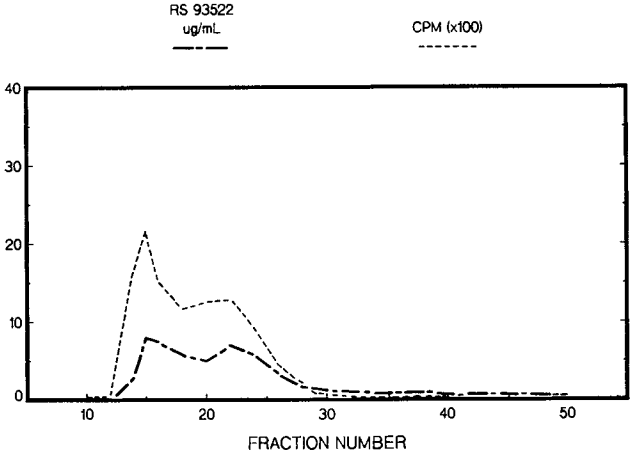

Fig. 3. Gel filtration elution profile of ^{14}C-liposomes containing RS-93522. A 150-μl aliquot of the ^{14}C-liposome–drug preparation was applied to the gel filtration column and 1.25-ml fractions were collected. The molar ratio of phospholipid to RS-93522 in the starting material was 9.6 to 1; the molar ratio in the higher molecular weight material (fractions 12–19) was 32.7 and that of the lower molecular weight material (fractions 20–28) was 9.1. (--------) ^{14}C-Lipid (cpm); (— - — - —) RS-93522 concentration (μg/ml).

peaks, confirming drug entrapment within the liposome. The elution profile shows two peaks for the liposome–drug formulation, which is consistent with the bimodal size distribution observed by laser light scattering. The higher RS-93522/lipid ratio observed in the second peak, corresponding to the smaller vesicle population, indicated that the drug preferentially partitions into smaller vesicles. The elevated drug baseline observed in the higher fraction numbers indicated that the drug, but not the lipid, partitioned to some extent into the gel filtration matrix or perhaps into the gel matrix-bound empty liposomes originally used to saturate the column (see Materials and Methods).

Data generated from particle size analysis show that the majority (>88%) of the liposomes is within the smaller of the two populations. The gel filtration elution profile suggests that the majority of liposomes is of the larger size. These studies were not performed sequentially using the same liposome preparation. The instrument conditions for sonication differed for the two sets of liposomes prepared; the liposomes made for size analysis received more extensive sonication to produce the greater number of small-size liposomes.

The Interaction of Drug with Albumin

Gel filtration of the free drug in buffer was not possible due to low aqueous solubility. However, the drug could be solubilized in a 4% albumin solution without liposomes. this was accomplished by combining 1 vol of a concentrated ethanol solution of RS-93522 at 6 mg/ml with 14 vol of a 4% human serum albumin solution. This procedure produced a physically stable, clear solution of drug at 0.4 mg/ml. The gel filtration elution profile is shown in Fig. 4. Recovery of drug by this method was 91%. The drug and protein peaks coincided with each other exactly, clearly indicating that the drug was binding to albumin. Thus albumin, in addition to lipids, may be used to solubilize RS-93522. Drug introduced into serum in the same way demonstrated the same elution profile (Fig. 4). This implies that the drug interacted preferentially with albumin in serum rather than interacting with other serum components.

Interaction of the Lipid–Drug Complexes with Serum

After serum incubation, the lipid-solubilized drug was evaluated by gel filtration chromatography. The resulting elution profile, shown in Fig. 5, indicates that, in less than 5 min, all of the drug is recovered in the albumin peak rather than the lipid peaks. Thus, upon exposure to serum the drug moved rapidly into the same serum pool as it would if it were solubilized by the conventional formulation.

The chromatogram shown in Fig. 6 illustrates that the lipid profile was also affected by serum incubation. This is consistent with published results which indicate that liposomes are often destabilized by serum components (20,21).

In Vivo Evaluation

The liposome formulation characterized above was compared *in vivo* with a conventional vehicle formulation containing 10% ethanol and 40% PEG 400 in a phosphate buffer. The data presented in Table II, and the pharmacokinetic parameters in Table III, indicate that liposomal RS-93522 follows the same kinetic profile as the conventional RS-93522 formulation.

DISCUSSION

Developing an intravenous injectable solution for lipophilic drugs generally involves using a cosolvent to achieve solubility at the required dose concentration. For example, to solubilize RS-93522, at least 40% of a cosolvent (w/v) is necessary. It became apparent that this compound would be a suitable candidate for studies involving solubilization by liposomes. The rationale and benefits for using a

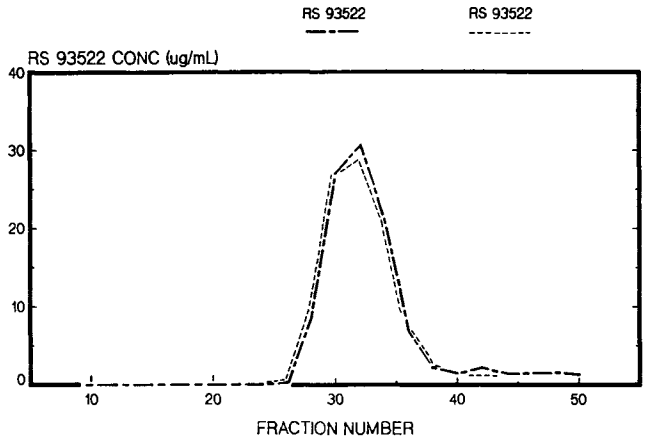

Fig. 4. Gel filtration profile of serum albumin-solubilized RS-93522. Seventy-five microliters of a 6-mg/ml RS-93522 solution (50% aqueous ethanol) was combined with 925 μl of either whole serum or a 4% human serum albumin solution. The 1.0-ml mixture was placed on the gel filtration column after a 5-min incubation at 37°C; 1.25-ml fractions were collected and assayed for RS-93522 content by HPLC. RS-93522 (μg/ml) incubated in whole serum (— - — - —) or 4% human serum albumin (--------).

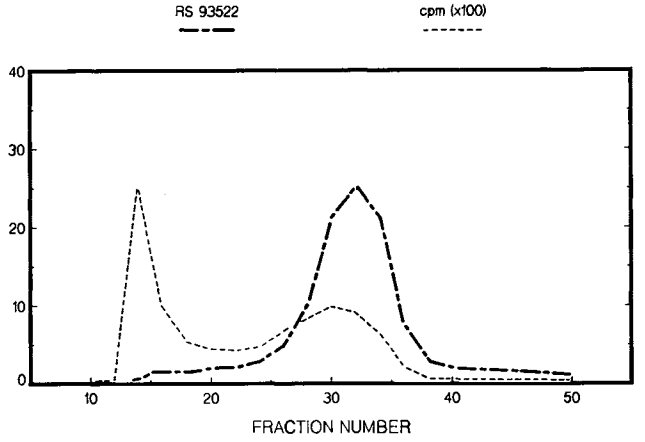

Fig. 5. Serum incubation of liposome-solubilized RS-93522; gel filtration profile. A 150-μl aliquot of liposome (containing ^{14}C tracer)-solubilized RS-93522 was mixed with 850 μl of fresh human serum. This mixture was incubated at 37°C for 5 min. The sample was applied to the gel filtration column, and 1.25-ml fractions were collected. Each fraction was assayed for both RS-93522 and the radioactive phospholipid. (--------) ^{14}C-Lipid (cpm); (— - — - —) RS-93522 concentration (μg/ml).

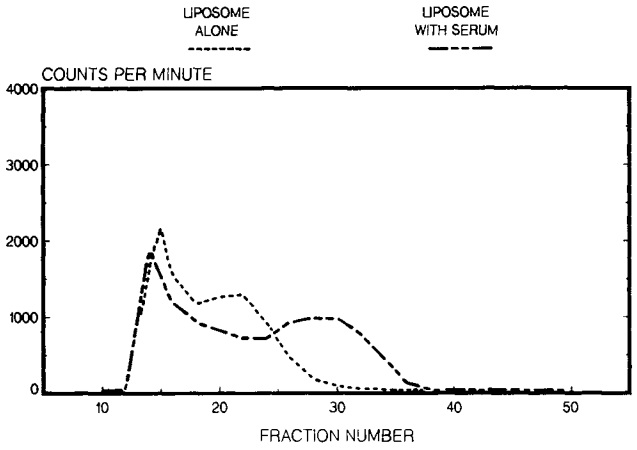

Fig. 6. Empty liposome incubation with and without serum; gel filtration elution profile. ^{14}C-Labeled liposomes were prepared according to Materials and Methods without RS-93522 (empty liposomes). A 150-μl aliquot was diluted with either aqueous buffer (850 μl; ------) or human serum (850 μl; — - —) and incubated for 15 min at 37°C. The samples were applied to the gel filtration column; 1.25-ml fractions were collected and analyzed for radioactivity (cpm).

liposome to solubilize a lipophilic drug include (1) eliminating organic solvent from the formulation; (2) producing a biocompatible formulation; and (3) producing a vehicle that can be used for routine toxicity screening or as a vehicle for iv administration. In order to accomplish these goals satisfactorily, the liposome system must behave similarly to conventionally formulated vehicles by exhibiting complete and immediate release of the drug compound upon introduction into the circulatory system. Further, the drug released from the liposome–drug formulation must demonstrate an identical pharmacokinetic profile when compared *in vivo*.

Preparation and screening of a suitable liposome formulation for solubilizing RS-93522 entailed evaluation of solubility limits within the lipid bilayer and characterizing the resulting physical properties and stability of the drug–lipid system. Physical stability of this liposome–drug system can be attributed, in part, to the hydrophobicity of the drug. The nonpolar compound should intercalate or solubilize within the liposome membrane (3).

It is well established that solubilization of lipophilic molecules by surfactants is dependent upon the molar ratio of micellar surfactant to the agent solubilized (7,22). Since the critical micellar concentrations of phospholipids, such as those used in this study, are very low ($<10^{-9}$ *M*), solubilization is essentially determined by the ratio of the total molar phospholipid concentration to the drug concentration. The quantity of phospholipid required to solubilize a drug varies widely depending on its specific physical properties, hydrogen bonding potential, and three-dimensional geometry (23–25).

Table II. Plasma Profile of RS-93522[a]

Time (min)	Formulation (ng/ml ± SD)	
	Conventional	Liposome
5.0	492 ± 59	490 ± 21
15.0	361 ± 117	346 ± 117
30.0	129 ± 25	129 ± 26
60.0	62 ± 11	61 ± 17
120.0	32 ± 23	25 ± 16

[a] For each time point, groups of six rats, each rat weighing approximately 400 g, were dosed (iv) under anesthesia with 200 μl of a 2.0-mg/ml RS-93522 formulation (apparent pH of 5.0). Three rats were given the liposome formulation and three rats received the conventional (10% ethanol, 40% polyethylene glycol 400) formulation. Blood was collected by terminal bleed and samples were analyzed for RS-93522 by HPLC according to the text.

Table III. Pharmacokinetic Parameters for RS-93522[a]

Parameter	Formulation	
	Conventional	Liposome
Half-life (hr)	0.78	0.65
Apparent volume of distribution ($V_{d\beta}$, liters/kg)	3.96	3.55
Systemic clearance rate (CL_s, liters/hr/kg)	3.52	3.79
AUC (ng/ml) (hr)	241	248
Cmax (ng/ml)	492	490

[a] The pharmacokinetic parameters were generated from the plasma profile shown in Table II, using a BIOP program on an HP 9816 computer. Equations used were (1) apparent volume of distribution, $V_{d\beta}$ = dose/(AUC · β); and (2) systemic clearance, Cl_s = dose/AUC.

The results with RS-93522 are notable for the relatively large quantity of drug which could be solubilized by the phospholipid. For the purposes of this study we evaluated a relatively low phospholipid concentration (30 mg/ml). Since phospholipid suspensions can be made at a concentration of 200 to 300 mg/ml (or more), we would expect to be able to obtain solubilized drug levels of at least 40 mg/ml.

Three observations from the gel filtration studies were of particular interest. First, we were able to show that the drug bound to albumin. A solution of drug and albumin produced a physically stable, apparently solubilized formulation; the drug comigrated with albumin over the column. For our studies, approximately 1 mol of albumin was used to solubilize each mole of RS-93522. Second, liposome-solubilized drug could be shown to comigrate with the lipid on the gel filtration column. This result indicated that the mechanism of drug solubilization involved drug and lipid forming into the same structure. Finally, the drug was not retained in the liposome after incubation of drug with serum. Within 5 min of incubation in serum, essentially all of the drug was recovered in the albumin fraction. Although we did not investigate the details of this mechanism, published results suggest that high-density lipoprotein is involved in the destabilization of liposomes in serum (1,20,21,26). The data in Fig. 6 showing changes in the gel filtration profile of empty liposomes after serum incubation are consistent with these published results. Although specific liposome compositions can overcome this destabilization (12), we intentionally avoided those formulations for the purposes of this study. Based on our observations, once the liposome drug formulation is combined with serum, drug transfers from the liposomes and binds with a high affinity to albumin.

Comparing the half-lives and AUCs for liposome-

solubilized vs. cosolvent-solubilized drug revealed no differences (Table II), indicating that the liposome formulation did not impede drug delivery to the serum pool. The drug diminished from the plasma at an equivalent rate, further implying that all liposomal drug was immediately freed. If drug remained liposome bound, a different plasma profile would have emerged; specifically, drug protection from metabolism would have led to sustained blood levels of RS-93522 over time (11).

Analysis of this liposomal drug formulation has established that a liposome vehicle can solubilize a hydrophobic compound up to 500 times above its intrinsic aqueous solubility. The drug formulated in this way is rapidly released from the liposome vehicle, and the hydrophobic drug compound behaves identically (both *in vitro* and *in vivo*) to a conventionally formulated vehicle.

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