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COMMUNICATION

Biodistribution of Liposomes of Terbutaline Sulfate in Guinea Pigs

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ABSTRACT

A series of liposomes was prepared with various lipid (egg phosphatidyl choline [egg PC], phosphatidyl glycerol [PG], dipalmitoyl phosphatidyl choline [DPPC], distearoyl phosphatidyl choline [DSPC], dipalmitoyl phosphatidyl glycerol [DPPG], phosphatidyl ethanolamine [PE], cholesterol [CH], and stearylamine [SA]) compositions, such as egg PC:PG:CH (55:5:40), DPPC:PG:CH (55:5:40), DSPC:DSPG:CH (55:5:40) egg PC:SA:CH (55:5:40), DSPE:DSPG:CH (55:5:40) in molar ratio. Liposomal formulations were administered to guinea pigs intravenously; 3 hr after the treatment, serum samples and various organs (e.g., liver, spleen, lung) were removed and analyzed for drug concentration by a high-performance liquid chromatographic (HPLC) method. Based on the above study, a liposomal preparation with better lung specificity was selected, and the time profile of these liposomes was determined in guinea pigs. Three hours postadministration, a significant difference in blood levels was observed between free terbutaline sulfate and the various liposomal formulations. Localization of the drug in the lungs increased considerably when encapsulated drug was used, and the highest percentage localization was observed with DSPC:DSPG:CH (55:5:40) liposomes. The percentage recovery of the drug in the lungs with egg PC:CH:SA (55:40:5) liposomes did not change significantly when compared with egg PC:CH:PG (55:40:5) liposomes. To establish the time course of disposition of the liposomes, DSPC:DSPG:CH (55:5:40) liposomes were selected. Terminal half-life $t_{1/2}$ *of the drug in blood with free drug solution was about 12 hr, whereas with liposomes, a twofold increase*

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in $t_{1/2}$ *was observed. The disposition data indicated that the clearance of the drug was delayed by 1.5 times when incorporated into liposomes*.
Key Words: *Biodisposition; Clearance; Half-life; Liposomes; Plasma.*

INTRODUCTION

Liposomes are phospholipid-based vesicles consisting of lipid bilayers that enclose an aqueous compartment. Both hydrophilic and lipophilic molecules can be incorporated into the liposomes in the aqueous compartment and lipid bilayer, respectively. As a drug carrier system, liposomes have attracted attention in the areas of cancer chemotherapy, inflammatory diseases, vaccine therapy, and immunological infectious diseases because the vesicles may be manipulated in terms of size and composition, and certain ligands may be attached for targeted delivery of bioactive molecules. These carriers were observed to change the pharmacodynamic and pharmacokinetic fates of the active molecules when administered in vivo (1). Apart from the targeting potential, they delay the loss of rapidly cleared drugs, enhance absorption of topically applied drugs, reduce toxicity, and potentiate therapeutic efficacy and immunogenicity (2,3)

Asthma is a multifactorial condition in which there is airway hyperresponsiveness with a propensity for widespread, reversible airway narrowing on exposure to diverse inciting factors (triggers). Several hypotheses have been proposed to explain the mechanism by which β_2 agonists might be harmful to subjects with asthma. Although clinically important tachyphylaxis to the bronchodilating effects of β-agonists does not occur in asthmatic subjects, one hypothesis is that the development of tolerance to the nonbronchodilating effects of β-agonists could result in loss of asthma control over time. Alternatively, by keeping the bronchi dilated, regular β-agonist treatment may increase allergen load in the lungs. This increased allergen load is the main cause behind the massive inflammatory response seen in those with asthma (4–6).

Lung function has a diurnal rhythm both in normal individuals and individuals with asthma. The best lung function occurs around 16:00, and the worst lung function occurs around 04:00, with about 8% change in the normal population (7). However, those with asthma can have much more dramatic decrements overnight in lung function than the normal population. Therefore, chemotherapy of asthma demands a carrier system that gives a quick onset of action and lasts for a prolonged period, which ultimately prevents the tolerance and increases the therapeutic index. Liposomes were assumed to be the better choice as the drug carrier because of the flexibility and the advantages associated with the system. In the present work, we prepared liposomes of terbutaline sulfate, and the effects of lipid composition on encapsulation efficiency, targeting potential of the carrier, and the disposition profile of the carrier were studied in comparison to free terbutaline sulfate.

EXPERIMENTAL

Materials

Egg phosphatidyl choline (egg PC), phosphatidyl glycerol (PG), dipalmitoyl phosphatidyl choline (DPPC), distearoyl phosphatidyl choline (DSPC), dipalmitoyl phosphatidyl glycerol (DPPG), phosphatidyl ethanolamine (PE), cholesterol (CH), and stearylamine (SA) were purchased from Sigma Chemical Company (St. Louis, MO). Terbutaline sulfate was a gift sample from Astra IDL Limited (Bangalore, India). Acetonitrile, methanol, and water were high-performance liquid chromatograph (HPLC) grade (Qualigens, Mumbai, Maharashtra, India), and all other reagents were analytical grade.

Methods

Preparation of Liposomes

Various lipid compositions (egg PC:PG:CH 55:5:40; egg PC:SA:CH 55:5:40; DPPC:PG:CH 55:5:40; DSPC:DSPG:CH 55:5:40; DSPE:DSPG:CH 55:5:40) were used for the preparation of liposomes by a proliposome method (8). The lipid mixture was thoroughly dried and dissolved in warm ethanol (80 mg) along with drug (terbutaline sulfate), and phosphate buffered saline (PBS) at pH 7.4 (200 mg) was added to yield a lipid:ethanol:water (100:80:20 w/w/w) mixture. This mixture was heated to 60°C for a few minutes and then allowed to cool to room temperature to facilitate the formation of a proliposome mixture. The proliposome mixture was finally converted to a liposome suspension by dropwise addition of the buffer containing the drug with continuous stirring. The multilamellar vesicles (MLVs) were treated with a bath sonicator for 5 min. Unentrapped terbutaline sulfate was removed by washing the liposomes

twice in PBS at pH 7.4 and pelleting at 50,000*g* for 30 min using a Beckman ultracentrifuge (Palo Alto, CA).

Characterization

Polydispersity

The prepared MLVs were observed under an optical microscope (Biomed, Heerbrugg, Switzerland) for the determination of the average diameter of the vesicles. In each study, 100 vesicles were observed for the size determination.

Drug-to-Lipid Ratio

A volume of 100 μl of stock liposomal dispersion was taken in a standard flask, and 10 ml of methanol were added, vortexed thoroughly, filtered through a 0.4-μm membrane filter, and injected into the HPLC system to determine the drug-to-lipid ratio.

In Vivo Disposition Studies

All experiments used male guinea pigs weighing 350–370 g. At the end of an experimental period, guinea pigs were sacrificed, and blood was collected by cardiac puncture. The liver, spleen, and lung were removed, weighed, and stored at −20°C until further analysis.

Experiment 1

To assess the effect of lipid composition on disposition, five batches of liposomes of terbutaline sulfate were used. The control group animals ($n = 3$) were administered terbutaline sulfate solution (900 μg/guinea pig), and five other groups ($n = 3$) were administered liposomes at an average lipid dose of 7 μmol/kg by intravenous injection and were sacrificed 3 hr later.

Experiment 2

To establish the time course for the disposition of liposomes (DSPC:DSPG:CH 55:5:40). Six groups ($n = 3$) of guinea pigs received 0.5 L of liposomes by intravenous injection (7 μmol of lipid/kg) and were killed after 2, 3, 4, 8, 12, and 24 hr.

High-Performance Liquid Chromatography Analysis of the Samples

To a volume of 100 μl of the serum, 100 μl of standard samples of terbutaline sulfate in methanol were added at various concentrations (20 μg/ml to 160 μg/ml) and then vortexed for a few seconds, and the volume of the solution was made up to 1 ml. The solution was vortexed for 2 min and centrifuged at 15,000 rpm for 15 min, and 0.5 ml of the supernatant was collected, evaporated under N_2 gas, and reconstituted with 1 ml of mobile phase and injected for HPLC (Shimadzu LC-10 Ai, Kyoto, Japan). Acetonitrile:methanol:water (95:5:5) was used as the mobile phase at a flow rate of 3 ml/min, and the wavelength was fixed at 225 nm. Tissue samples were homogenized with the required amount of methanol and centrifuged, and the supernatant was assayed for terbutaline sulfate the same as for serum samples. The calibration curve was found to be linear between 2 μg/ml and 16 μg/ml, with the following regression equation: $rsp = 4.62e + 004\ (amt) + 9.26e + 004$. Disposition of the drug as percentage of the administered dose was calculated in various organs and the blood. Statistical significance of the data was analyzed using analysis of variance (ANOVA).

RESULTS AND DISCUSSION

A series of liposomal formulations containing various phospholipid mixtures (egg PC:PG:CH 55:5:40; egg PC:SA:CH 55:5:40; DPPC:PG:CH 55:5:40; DSPC:DSPG:CH 55:5:40; DSPE:DSPG:CH 55:5:40) were prepared by the proliposome method, followed by bath sonication for 5 min. The size of the MLVs was observed under an optical microscope. The average diameter of the liposomes was calculated by measuring the diameter of 100 liposomes in each case, and the average diameter of PC:PG:CH (55:5:40) liposomes was 3.22 μm. Incorporation of stearylamine considerably increased the average diameter of the liposomes to 6.1 μm. Inclusion of charged lipids such as stearylamine and phosphatidyl glycerol resulted in repulsive separation of the phospholipid bilayers, and thus an increase in size was observed. When egg PC was substituted with long-chain phospholipids such as DSPC, DPPC, DSPE, and the like, the average diameter of the vesicles was found to be higher than the vesicles based on egg PC. These long-chain phospholipids are high glass transition temperature lipids ($T_c >$ 40°C) and require higher temperatures and high energy for reduction of the vesicle size. The average diameter of the liposomes prepared with these long-chain phospholipids was in the range 6–8 μm.

It is well documented that liposomes in the size range 5–8 μm are more associated with the lung because of trapping in the capillaries. This is found to be augmented by the incorporation of certain negatively charged lipids in the membrane (9). Therefore, the proliposome method

Table 1

Effect of Lipid Composition on Disposition of Terbutaline Sulfate (Percentage of Administered Dose) in Various Organs Following Intravenous Administration of Free and Liposomal Terbutaline Sulfate in Guinea Pigs Dose 2.6 mg/kg

	Disposition, % of Administered Dose 3 hr After Administration			
Composition	Blood	Liver	Spleen	Lung
Terbutaline sulfate	36.16 ± 2.99	9.91 ± 0.73	0.31 ± 0.12	3.47 ± 0.39
Egg PC:PG:CH (55:5:40)	23.81 ± 5.13	53.74 ± 1.75	14.63 ± 1.22	4.87 ± 0.03
DPPC:PG:CH (55:5:40)	31.08 ± 1.27	48.08 ± 2.64	9.58 ± 0.76	8.30 ± 1.60
DSPC:DSPG:CH (55:5:40)	34.35 ± 2.41	42.27 ± 0.48	8.74 ± 1.15	12.10 ± 1.11
Egg PC:SA:CH (40:10:50)	24.89 ± 1.22	55.41 ± 2.98	13.54 ± 0.82	6.11 ± 0.22
DSPE:DSPG:CH (55:5:40)	36.95 ± 1.31	44.82 ± 6.89	8.25 ± 0.48	6.33 ± 0.56

was adopted to prepare the vesicles of the required size range.

A comparative biodisposition study was conducted between free terbutaline sulfate and encapsulated terbutaline sulfate to understand the magnitude of change in pharmacokinetic profile of the drug when administered in liposomal form. With egg PC:PG:CH (55:5:40) liposomes, 24% of the injected dose was recovered in blood 3 hr after administration; when a positively charged lipid was incorporated (egg PC:SA:CH 50:5:40), about 25% of the injected dose was estimated in blood (Table 1). Thus, no significant difference in blood levels was observed between these two preparations ($p > .05$). However, drug levels with those liposomal formulations prepared by long-chain phospholipids such as DPPC, DSPC, and DSPE were higher compared to liposomes based on egg PC.

Localization of the drug in lungs with free drug solution was around 3.5% of the injected dose 3 hr postadministration. With liposomes based on DSPC and DPPC, the percentage of the injected dose localized in the lungs was significantly higher than for the other liposomal preparations; in general, percentage localization in the lungs with all the liposomal formulations was higher than for the free drug (Table 1). The high localization of the drug in lungs with liposomal formulations may be explained by the physical trapping of the liposomes in the vascular network of the lung. However, the reason for the higher localization observed with liposomes based on DSPC, DPPC, and DSPE over the other liposomes is not known, but it may be assumed that the increase in chain length of the phospholipid increases the localization in the lung.

Liver and spleen are considered to be the RES-rich organs, and they play an important role in the disposition of colloidal drug delivery systems. The disposition profile in RES-rich organs (liver and spleen) was parallel to that of the disposition in lungs with various liposomal formulations used in the present study. However, between various liposomal formulations, an increase in the uptake by RES-rich organs (liver and spleen) was accompanied by a decrease in the percentage deposition in the lungs. This may be taken into account by less trapping of liposomes in the vascular network of the lungs, re-

Table 2

Pharmacokinetic Parameters of Terbutaline Sulfate Following Intravenous Injection of Free Terbutaline Sulfate and Liposomal Terbutaline Sulfate in Guinea Pigs (Dose 900 μg/Guinea Pig)

Parameters	Free Terbutaline Sulfate	Liposomal Terbutaline Sulfate
$AUC_{0-\infty}$ (μg/ml/hr)	218.08 ± 0.8	330.15 ± 38.46
$t_{1/2}$ (hr)	12.01 ± 0.09	22.48 ± 1.11
Clearance (ml/hr)	4.15 ± 0.35	2.75 ± 0.34
MRT (hr)	17.31 ± 0.11	32.15 ± 1.52

Table 3

Disposition Profile of Terbutaline Sulfate (Percentage of Administered Dose) in Various Organs Following Intravenous Injection of Free Terbutaline Sulfate in Guinea Pigs (Dose 2.6 mg/kg)

	Disposition, % of Administered Dose			
Time	Blood	Liver	Spleen	Lung
1 hr	63.11 ± 4.07	10.18 ± 1.86	0.98 ± 0.36	4.03 ± 0.65
2 hr	50.20 ± 4.80	9.17 ± 2.13	0.76 ± 0.28	3.51 ± 0.88
4 hr	28.91 ± 1.13	8.48 ± 2.35	0.37 ± 0.19	3.19 ± 1.23
8 hr	22.17 ± 2.46	6.12 ± 1.12	0.31 ± 0.10	2.01 ± 0.55
24 hr	10.20 ± 1.26	3.18 ± 0.56	0.26 ± 0.12	ND

ND = not detectable.

sulting in their increased availability for systemic circulation and eventually for selective RES uptake.

The effect of cholesterol was not studied in the present work as it has already been proved that cholesterol improves the stability of the liposomes by increasing the rigidity of the lipid bilayers; therefore, it was incorporated into all the liposomal formulations. From experiment 1, it was observed that preparations based on saturated, long-chain phospholipids are more suitable for lung targeting than the formulations based on unsaturated, short-chain phospholipids.

Based on the results of experiment 1 (percentage localization in lungs), the liposomal formulation with DSPC:DSPG:CH 55:5:40 was selected for studying the time course of the disposition of liposomal terbutaline sulfate in guinea pigs. About 63% of the injected dose was found in blood 1 hr after administration of these liposomes, whereas 70% of the injected dose was found with the free terbutaline preparation.

As shown in Table 2, the elimination half-life of free terbutaline sulfate was half that of the liposomal terbutaline sulfate and clearance was about 1.5-fold higher, indicating extended availability of the drug in blood. Mean residence time (MRT) with liposomes was more than two times that of the free drug. With free drug, a maximum of 11% of the injected dose was recovered in the liver in 1 hr, and from 1 to 24 hr, the concentration of the drug decreased gradually, with about 3% of the injected dose recovered after 24 hr. A similar trend was observed in the case of the spleen. About 5.7% of the injected dose was found in the spleen in 1 hr, and then the concentration of the drug declined gradually to 1.5% in 24 hr. About 4% of the injected dose was found in the lung 1 hr after administration of the free drug, and the concentration decreased gradually; detectable levels of the drug were not found after 24 hr. However, with liposomes, about 13% of the administered dose was found in the lung 1 hr after administration, and the drug levels decreased slowly up to 24 hr (Table 3 and 4).

The disposition profile of the drug in the liver with liposomes was parallel to that in the spleen; C_{max} in the liver and spleen were achieved in 1 hr. A significant dif-

Table 4

Disposition Profile of Terbutaline Sulfate (Percentage of Administered Dose) in Various Organs Following Intravenous Injection of Liposomes of Terbutaline Sulfate (DSPC:DSPG:CH 55:5:40) in Guinea Pigs

Time (hr)	Blood	Liver	Spleen	Lung
1	32.53 ± 2.20	13.70 ± 3.10	5.71 ± 1.13	13.09 ± 0.63
2	27.76 ± 1.15	22.90 ± 3.82	7.93 ± 0.48	11.48 ± 0.44
4	23.82 ± 0.87	53.51 ± 2.99	11.63 ± 0.25	8.95 ± 0.38
6	21.17 ± 0.46	41.08 ± 3.11	9.14 ± 0.20	7.25 ± 0.36
8	18.96 ± 0.91	32.21 ± 1.28	6.25 ± 0.84	5.96 ± 0.40
12	16.04 ± 0.41	19.18 ± 3.25	3.61 ± 0.11	4.83 ± 0.25
24	14.39 ± 0.86	11.55 ± 2.09	1.48 ± 0.19	4.08 ± 0.38

Table 5

Disposition Kinetics of Terbutaline Sulfate in Blood and Various Organs Following Intravenous Injection of Free and Liposomal Terbutaline Sulfate in Guinea Pigs

Formulation	Parameters	Blood	Liver	Spleen	Lung
Free terbutaline sulfate	AUC_{0-t}	154.19	127.32	157.63	78.58
	$t_{1/2}$ (hr)	13.57	14.03	14.92	7.17
	Clearance (ml/hr)	4.24	4.79	3.42	5.99
	MRT (hr)	17.78	20.41	24.07	10.68
Liposomes of terbutaline sulfate	AUC_{0-t}	126.53	479.07	1995.49	487.09
	$t_{1/2}$ (hr)	29.59	9.33	6.67	13.73
	Clearance (ml/hr)	2.95	1.46	0.40	1.18
	MRT (hr)	43.44	15.57	10.80	21.99

AUC_{0-t} (μg/ml/hr) for blood; AUC_{0-t} (μg/g/hr) for organs.

ference was observed in the liver drug concentration between the liposomes and the free drug at 1 hr ($p < .01$). However, the rate of clearance of the drug from the liver with liposomes was slower compared to the free drug, as evident from Table 2. With liposomes, AUC_{0-t} (330 μg/ml/hr) was around 1.5 times higher and the clearance about 1.5 times slower than the free drug. The data in Tables 3 and 4 indicate that liposomes were largely taken up by the liver cells, and the subsequent slow release of the drug from liposomes may be the reason for very slow clearance of the drug.

The disposition kinetics of the free terbutaline sulfate and liposomal terbutaline sulfate preparations in various organs are presented in Table 5. The area under the concentration-time curve AUC_{0-t} in the lungs with free terbutaline sulfate was 78.58 μg/g/hr, whereas with liposomes, it was around sixfold higher (487 μg/g/hr). Mean residence time of the drug in the lungs with liposomes was around 22 hr, whereas with free drug, it was only about 11 hr. Thus, liposomes were found to offer a sustained drug concentration profile.

The above studies demonstrate that the kinetics of liposome-associated drugs are sensitive to lipid composition when the size of the liposomes is maintained constant. The presence of saturated phospholipids such as DSPC increased the residence time of the drug in the lungs. The saturated phospholipids are known to decrease liposome membrane permeability to the incorporated molecules and to protect liposomes from in vivo destabilization (10). Time-averaged relative drug exposure to a particular tissue r_e was calculated as explained in an earlier report (11). The r_e value (time-averaged relative drug exposure) with liposomes in the lungs was 6.19, whereas in the liver, it was calculated as only 3.76 (Table 6). Thus, the targeting potential to the lung was significantly high with the liposomes.

Table 6

Time-Averaged Relative Drug Exposure r_e *to Various Organs of Guinea Pigs Following Intravenous Administration of Free and Liposomal Terbutaline Sulfate*

Tissue	Free	Liposomal	r_e
Liver	127.32	479.07	3.76
Spleen	157.63	1995.49	2.19
Lung	78.58	487.09	6.19

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