CHAPTER 11

IN VIVO STUDIES

11.1 INTRODUCTION

The targeting ability and carrier properties of liposome-entrapped drugs with varying surface charge, lipid compositions and route of administration are of significant importance to alter biodistribution. The targeting ability of the formulations can well be established by carrying out the experiments in tumor and non-tumor bearing animal model. Studies of the pharmacokinetics of the formulation in suitable animal models during the later stages of development are crucial for attainment of the desired product performance. This is particularly true in case of sterically stabilized liposomes where *in vitro* testing may not necessarily indicate the *in vivo* performance of the formulation. However, in all cases, suitable *in vitro- in vivo* correlations can be established. Inclusion of these *in vitro* tests in the quality assurance program of the formulation can then be done so as to ensure that each batch will meet the criteria required for successful *in vivo* performance. These studies are also crucial for the systems, which are being investigated for the first time since new *in vivo* distribution patterns can be revealed which can pave the way for new vistas in medicine.

11.2 EXPERIMENTAL

11.2.1 SELECTION OF ANIMALS

Healthy New Zealand albino rabbits, of either sex, weighing about 2.5 kg were chosen for the blood kinetic study of the drugs and their liposomal formulations. No diet restrictions were enforced prior to studies. Three rabbits were taken for the study in each group.

Balb/c mice of either sex, of 2-3 months old, weighing between 20-25g and Balb/c mice with Ehrlich ascites tumor (EAT) developed in the left thigh region was chosen for the biodistribution and tumor uptake studies. No diet restrictions were enforced prior to studies. Four mice were taken for the study in each group.

11.2.2 TUMOR IMPLANTATION

Ehrlich ascites tumor (EAT) was maintained in the peritoneum of the mice in the ascites form by serial weekly passage. Exponentially growing EAT cells were harvested, washed and resuspended in phosphate buffered saline

and about 1.5 x 10^7 cells were injected intramuscularly in the thigh of the left hind leg of the mice of 2-3 months old weighing 25-30 gm. After 7-10 days, a palpable tumor in the volume range of 0.9 ± 0.1 cm^3 was observed and used for further studies.

11.2.3 BLOOD KINETIC STUDIES

New Zealand albino rabbits were administered with 10 MBq of the labeled complex through ear vein using a syringe with 24"gauze needle. At different time intervals, about 0.5 ml blood samples were withdrawn from the dorsal vein of the other ear and the radioactivity was measured using a gamma counter calibrated for Tc-99m energy. An amount equal to 7.3 % of the body weight was considered to represent per whole body blood and data were expressed as percent administered dose at each time interval (Wu et. al., 1981).

11.2.4 BIODISTRIBUTION STUDIES

An injected dose of 100 µl of the ^{99m}Tc- labeled complex (3.7MBq) was administered through the tail vein of each mouse using 24"gauze needle. The mice were sacrificed at different time intervals and blood was obtained by cardiac puncture. The blood was weighed and the radioactivity was measured in a shielded well gamma scintillation counter. The radioactivity present in the whole blood was calculated by keeping 7.3 % of the body weight as total blood weight. Biodistribution studies and tumor uptake studies of the ^{99m}Tc-labeled leuprolide/liposomes were studied in Ehrlich ascites tumor bearing mice. Tissues (heart, lung, liver, spleen, kidney, stomach, intestine, bone and tumor) were dissected, washed with normal saline, made free from adhering tissues, weighed and their radioactivity was measured in a shielded well gamma scintillation counter. To correct for physical decay and to calculate radiopharmaceutical uptake in each organ as a fraction of the injected dose, aliquots of the injectate, containing 2 % of the injected dose, were counted simultaneously at each time point.

11.2.5 GAMMA SCINTIGRAPHIC IMAGING

Scintigraphic study was carried out in EAT bearing mice or rabbits after administration of 3.7 MBq or 10 MBq of Tc-99m labeled complex through the tail vein of mice or ear vein of rabbit, respectively. The mice were fixed

on a board and imaging was performed using a Single Photon Emission Computerized Tomography (SPECT, LC 75-005, Diacam, Siemens, USA) gamma camera. The gamma imaging photographs showing the biodistribution of cyclosporine and its liposomal formulations, DNA and its liposomal formulations in rabbit is given in figures 11.10-11.13 and figures 11.18-11.22 respectively. The gamma imaging photograph showing the biodistribution of leuprolide and their liposomal formulations in EAT bearing mice is shown in figures 11.14-11.17.

11.2.6 DATA ANALYSIS

The data obtained above was subjected to statistical analysis using the Quickcalc software (Plexus Supporting Services, Ahmedabad). The pharmacokinetic parameters generated by the analysis of the data obtained from the blood kinetic study in rabbits and mice are tabulated in tables 11.14 and 11.41, respectively.

11.2.7 *IN VITRO* TESTING FOR DRUG RETENTION IN LIPOSOMES

The ability of the prepared liposomal formulations to withhold the drug within liposomes in presence of plasma was analyzed by the following method. The liposomal suspension containing known amount of drug (Cyclosporine, Leuprolide acetate and DNA) was diluted with human plasma in the ratio of 1:9. 0.5ml to 1ml sample of the diluted suspension in screw-cap eppendorf tube was used for each time point; samples were incubated under gentle rotating conditions using rotary shaker at 37°C. The eppendorf containing samples were taken for analysis at different time intervals (6, 12, 24 & 48h). The suspension was diluted with normal saline and liposomal pellets were then obtained by centrifuging at 15,000 rpm for 15 min. The amount of the drug present in the liposomal suspension was estimated by diluting with suitable solvent as per the methods described previously in Chapter 3, Analytical methods. The results obtained for the liposomes containing cyclosporine, leuprolide acetate and DNA are given in Tables 11.42 –11.44 respectively.

Table 11.1 Results of the blood kinetic data of cyclosporine (CsA)

Time (h)	% Injected Dose	
	Actual ± S.E.M	Calculated
0.1	20.82±1.82	20.68
0.25	4.71±0.89	4.81
0.5	3.16±0.56	2.60
1	3.04±0.67	2.41
2	2.49±0.25	2.16
4	1.91±0.2	1.75
6	0.99±0.1	1.41
24	0.24±0.01	0.21

Table 11.2 Results of the blood kinetic data of positive charged liposomes containing cyclosporine (CPL)

Time (h)	% Injected Dose	
	Actual ± S.E.M	Calculated
0.5	26.9±1.72	27.58
1	25.53±0.67	24.19
2	18.38±0.09	18.99
4	12.8±0.1	12.73
6	7.24±0.01	9.48
24	3.24±0.2	3.24

Table 11.3 Results of the blood kinetic data of negative charged liposomes containing cyclosporine (CNL)

Time (h)	% Injected Dose	
	Actual ± S.E.M	Calculated
0.5	21.48±1.98	20.69
1	17.2±0.78	18.46
2	15.7±0.1	14.90
4	11.18±0.3	10.26
6	5.02±0.5	7.57
24	2.99±0.2	1.76

Table 11.4 Results of the blood kinetic data of neutral liposomes containing cyclosporine (CL)

Time (h)	% Injected Dose	
	Actual ± S.E.M	Calculated
0.5	25.23±1.23	24.8
1	22.68±0.56	23.22
2	20.57±0.43	20.42
4	12.02±0.23	16.02
6	6.36±0.22	12.80
24	3.02±0.15	3.06

Figure 11.1 Blood kinetic study of ^{99m}Tc-cyclosporine, ^{99m}Tc-CPL, ^{99m}Tc-CNL, and ^{99m}Tc-CL in rabbits. Each value is the mean of three independent experiments

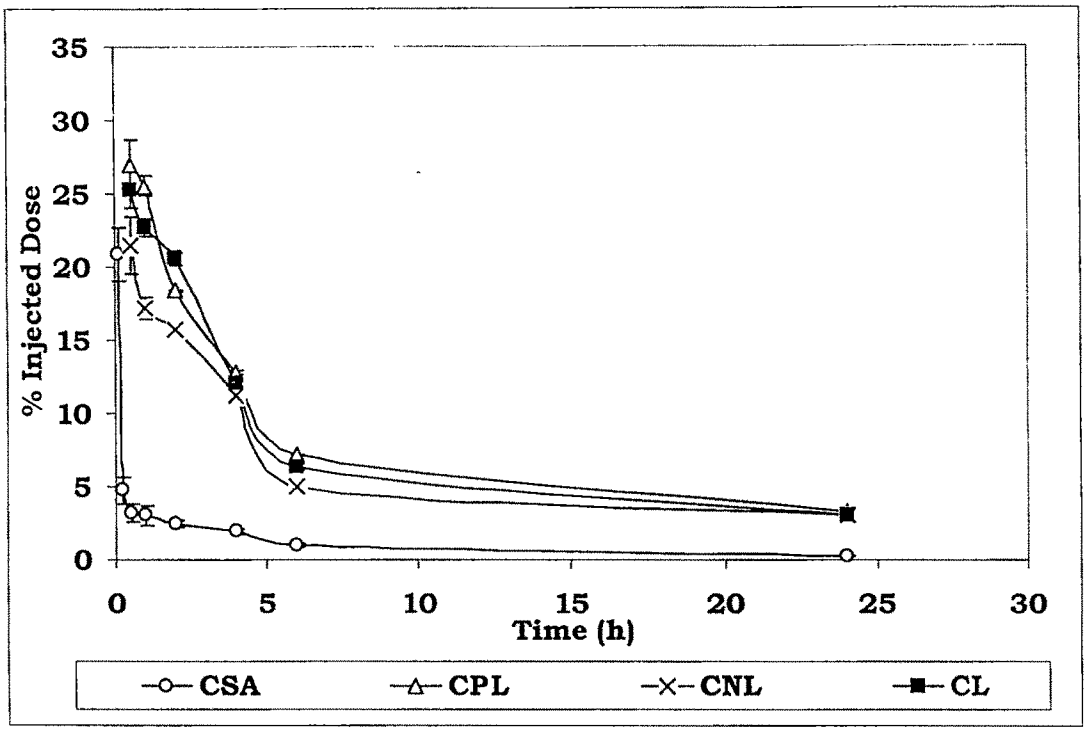

Table 11.5 Results of the blood kinetic data of leuprolide acetate (leuprolide)

Time (h)	% Injected Dose	
	Actual ± S.E.M	Calculated
0.1	11.85	11.98
0.25	8.87	8.39
0.5	5.17	5.88
1	4.67	4.36
2	3.39	3.07
4	1.08	1.56
6	0.98	0.79
24	0.12	0.001

Table 11.6 Results of the blood kinetic data of conventional liposomes containing leuprolide (LL)

Time (h)	% Injected Dose	
	Actual ± S.E.M	Calculated
0.5	23.48±1.02	22.8
1	20.12±1.23	20.84
2	17.86±0.98	17.68
4	14.98±1.65	13.59
6	7.23±0.89	11.14
24	3.89±0.96	3.77

Table 11.7 Results of the blood kinetic data of sterically stabilized liposomes containing leuprolide (SLL5000)

Time (h)	% Injected Dose	
	Actual ± S.E.M	Calculated
0.5	35.62±1.02	35.28
1	32.23±1.03	32.77
2	29.49±1.26	29.32
4	25.69±1.08	25.37
6	22.58±1.19	22.83
24	9.98±1.23	9.97

Table 11.8 Results of the blood kinetic data of sterically stabilized liposomes containing leuprolide (SLL2000)

Time (h)	% Injected Dose	
	Actual ± S.E.M	Calculated
0.5	36.24±1.23	36.18
1	34.68±1.03	34.75
2	32.56±1.32	32.48
4	28.92±1.08	29.15
6	26.71±1.19	26.54
24	12.02±1.23	12.03

Figure 11.2 Results of the blood kinetic data of leuprolide acetate and its liposomes

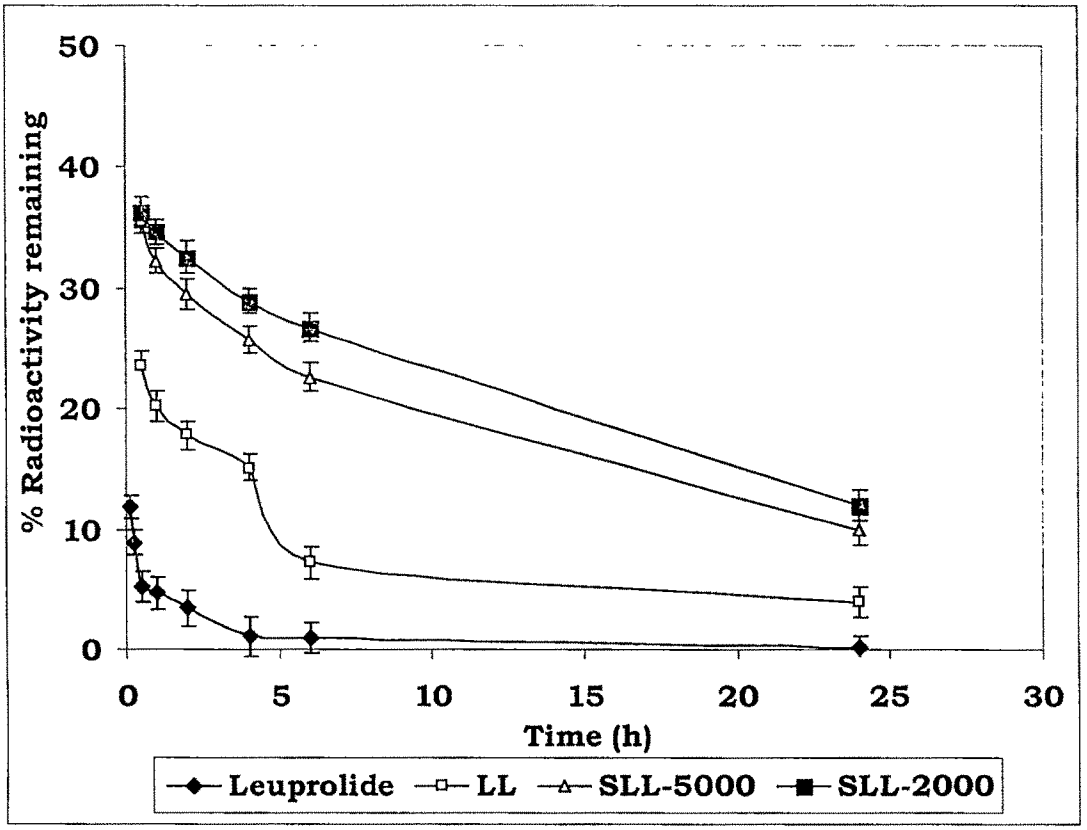

Table 11.9 Results of the blood kinetic data of DNA

Time (h)	% Injected Dose	
	Actual ± S.E.M	Calculated
0.1	12.7±0.96	12.21
0.25	11.4±0.85	11.08
0.5	8.46±0.45	9.51
1	6.99±0.59	7.25
2	5.72±1.23	4.71
4	2.37±1.36	2.47
6	1.58±1.25	1.41
24	0.52±0.99	0.01

Table 11.10 Results of the blood kinetic data of conventional liposomes containing DNA (DL)

Time (h)	% Injected Dose	
	Actual ± S.E.M	Calculated
0.5	20.68±1.04	20.99
1	19.87±1.65	19.38
2	16.58±0.87	16.67
4	12.49±0.63	12.74
6	10.23±0.36	10.06
24	2.02±0.96	2.04

Table 11.11 Results of the blood kinetic data of sterically stabilized liposomes containing DNA (SDL5000)

Time (h)	% Injected Dose	
	Actual ± S.E.M	Calculated
0.5	29.83±0.56	29.86
1	25.79±0.46	25.68
2	22.48±0.77	22.78
4	20.69±1.05	20.34
6	18.23±1.02	18.34
24	7.23±1.32	7.26

Table 11.12 Results of the blood kinetic data of sterically stabilized liposomes containing DNA (SDL2000)

Time (h)	% Injected Dose	
	Actual ± S.E.M	Calculated
0.5	30.69±0.87	28.85
1	27.92±1.23	28.16
2	25.23±1.04	26.82
4	24.29±1.23	24.34
6	22.56±0.65	22.08
24	9.82±0.56	9.82

Table 11.13 Results of the blood kinetic data of cationic sterically stabilized liposomes containing DNA (CSDL2000)

Time (h)	% Injected Dose	
	Actual ± S.E.M	Calculated
0.5	38.69±1.02	39.24
1	34.23±1.23	34.04
2	31.59±1.45	31.59
4	29.62±0.99	28.95
6	25.9±0.78	26.57
24	12.29±0.69	12.25

Figure 11.3 Results of the blood kinetic data of DNA and its liposomes

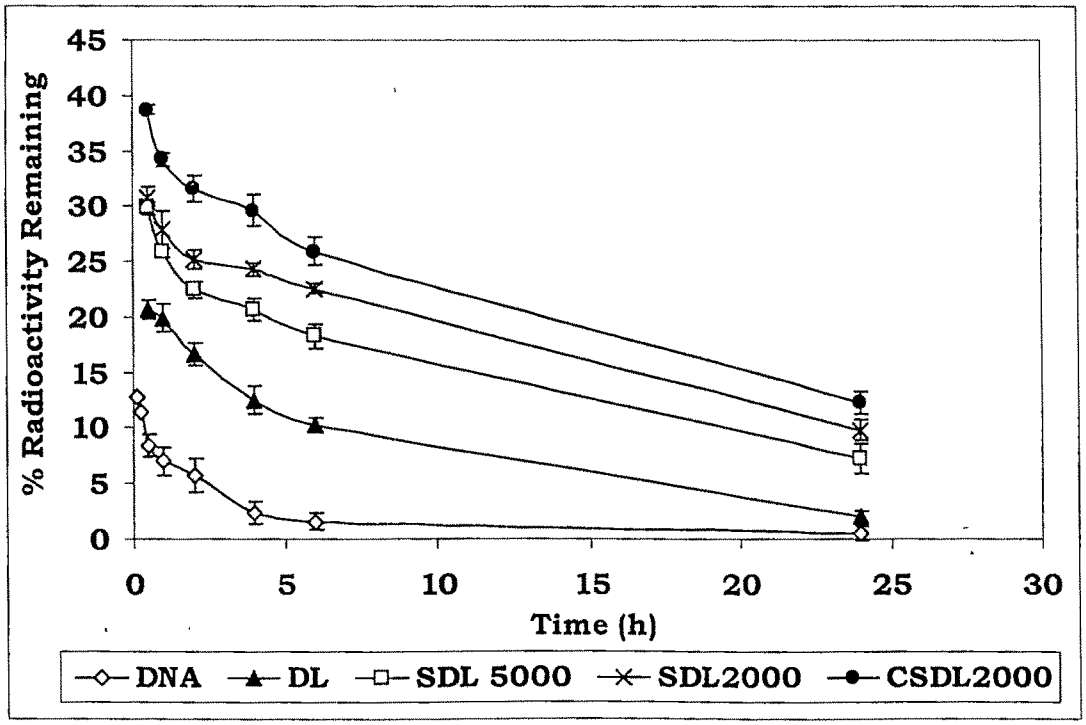

Table 11.14 Pharmacokinetic parameters for the *in vivo* studies of conventional and sterically stabilized liposomes containing cyclosporine, leuprolide acetate and DNA in rabbit

Batch	Elimination rate K_{el} (h^{-1})	Elimination half life (h)	Vd (l)	AUC
CsA	0.1067	6.49	1.32	30.31
CPL	0.0447	15.51	3.16	269.98
CL	0.0581	11.92	3.77	277.71
CNL	0.0639	10.84	4.29	172.67
Leuprolide acetate	0.3374	2.05	6.13	19.86
LL	0.0544	12.74	3.97	284.68
SLL5000	0.0457	15.15	2.59	665.51
SLL2000	0.0438	15.81	2.63	790.84
DNA	0.2729	2.54	7.65	31.29
DL	0.0811	8.55	4.39	202.87
SDL5000	0.0515	13.45	2.47	492.53
SDL2000	0.0487	14.23	3.38	606.98
CSDL2000	0.043	16.13	1.60	808.83

Table 11.15 Biodistribution of CsA in mice

Organ/ Tissue	Percent injected dose / gram of organ or tissue (± S.E.M.)			
	30 min	1 h	4 h	24 h
Blood	1.09±0.01	0.88±0.01	0.75±0.01	0.12±0.01
Heart	0.57±0.01	0.46±0.01	0.47±0.01	0.11±0.01
Lung	5.54±0.01	6.78±0.02	3.14±0.01	1.02±0.02
Liver	8.12±0.01	7.04±0.01	4.25±0.01	1.03±0.02
Spleen	8.90±0.03	6.45±0.03	5.40±0.03	0.98±0.01
Kidney	5.78±0.14	5.28±0.14	5.03±0.14	3.98±0.01
Intestine	0.23±0.14	0.15±0.14	0.12±0.14	0.36±0.10
Stomach	0.34±0.01	0.45±0.01	0.43±0.01	0.32±0.05
Bone	0.20±0.02	0.28±0.02	0.40±0.02	0.13±0.02

Table 11.16 Biodistribution of CsA in mice

Organ/ Tissue	Percent injected dose / whole organ or tissue (+ S.E.M.)			
	30 min	**1 h**	**4 h**	**24 h**
Blood	1.99±0.03	1.61±0.01	1.37±0.01	0.22±0.01
Heart	0.07±0.001	0.06±0.01	0.06±0.01	0.01±0.01
Lung	0.94±0.02	1.15±0.01	0.53±0.01	0.17±0.02
Liver	10.15±0.08	8.8±0.06	5.3±0.05	1.29±0.02
Spleen	1.33±0.01	0.97±0.01	0.81±0.02.	0.15±0.01
Kidney	2.08±0.07	1.9±0.01	1.8±001	1.43±0.01
Intestine	0.68±0.02	0.45±0.002	0.35±0.01	0.98±0.10
Stomach	0.13±0.005	0.17±0.01	0.16±0.01	0.32±0.05
Bone	-	-	-	-

Table 11.17 Biodistribution of CPL in mice

Organ/ Tissue	Percent injected dose/gram of organ or tissue (+ S.E.M.)			
	1 h	4 h	24 h	48h
Blood	8.33±0.03	6.85±0.07	2.61±0.21	0.87±0.01
Heart	1.42±0.02	1.45±0.02	0.67±0.01	0.16±0.03
Lung	4.41±0.09	3.90±0.04	1.11±0.06	0.02±0.03
Liver	21.1±0.3	18.3±0.06	15.6±0.17	10.4±0.10
Spleen	5.00±0.06	6.36±0.08	3.70±0.22	2.20±0.13
Kidney	1.72±0.18	1.13±0.07	1.03±0.02	0.05±0.04
Intestine	0.20±0.01	0.21±0.01	0.17±0.01	0.11±0.001
Stomach	0.36±0.01	0.43±0.04	0.57±0.01	0.48±0.01
Bone	1.02±0.02	6.02±0.01	2.02±0.01	0.65±0.01

Table 11.18 Biodistribution of CPL in mice

Organ/ Tissue	Percent injected dose / whole organ or tissue (+ S.E.M.)			
	1 h	4 h	24 h	48h
Blood	15.24±0.07	12.54±0.12	4.78±0.41	1.59±0.01
Heart	0.19±0.003	0.19±0.003	0.09±0.002	0.02±0.01
Lung	0.70±0.02	0.66±0.004	0.19±0.01	0.20±0.01
Liver	26.74±0.38	22.9±0.08	19.46±0.21	13.0±0.2
Spleen	0.75±0.009	0.95±0.01	0.55±0.03	0.33±0.02
Kidney	0.62±0.06	0.41±0.02	0.37±0.01	0.12±0.02
Intestine	0.59±0.01	0.61±0.02	0.51±0.001	0.42±0.01
Stomach	0.14±0.004	0.16±0.01	0.22±0.01	0.17±0.01
Bone	-	-	-	-

Table 11.19 Biodistribution of CNL in mice

Organ/ Tissue	Percent injected dose / gram of organ or tissue (+ S.E.M.)			
	1 h	4 h	24 h	48h
Blood	4.14±0.03	4.62±0.01	1.04±0.02	0.70±0.01
Heart	0.57±0.01	0.06±0.01	0.06±0.01	0.02±0.001
Lung	4.48±0.12	3.61±0.06	1.02±0.01	0.23±0.02
Liver	37.4±1.12	28.0±0.40	19.2±0.14	14.2±0.11
Spleen	5.45±0.23	10.7±0.13	8.37±0.15	3.60±0.1
Kidney	2.35±0.07	2.09±0.03	1.95±0.02	0.36±0.01
Intestine	0.20±0.01	0.20±0.01	0.14±0.01	0.36±0.12
Stomach	0.52±0.03	0.36±0.01	0.36±0.01	0.09±0.01
Bone	0.95±0.02	4.23±0.03	1.32±0.01	0.15±0.01

Table 11.20 Biodistribution of CNL in mice

Organ/ Tissue	Percent injected dose / whole organ or tissue (+ S.E.M.)			
	1 h	4h	24 h	48h
Blood	7.58±0.02	8.36±0.06	1.90±0.04	1.28±0.02
Heart	0.07±0.001	0.06±0.01	0.06±0.04	0.03±0.001
Lung	0.76±0.03	0.61±0.001	0.17±0.001	0.04±0.004
Liver	46.75±0.58	35.0±0.49	24.0±0.17	17.75±0.02
Spleen	1.32±0.01	1.6±0.02	1.26±0.02	0.63±0.02
Kidney	0.83±0.05	0.75±0.01	0.70±0.01	0.36±0.004
Intestine	0.62±0.01	0.62±0.02	0.41±0.01	0.36±0.04
Stomach	0.23±0.01	0.14±0.004	0.14±0.004	0.09±0.01
Bone	-	-	-	-

Table 11.21 Biodistribution of CL in mice

Organ/ Tissue	Percent injected dose / gram of organ or tissue (+ S.E.M.)			
	1 h	4 h	24 h	48h
Blood	6.70±0.03	6.02±0.03	1.18±0.02	0.87±0.01
Heart	1.24±0.02	1.32±0.02	0.44±0.01	0.16±0.03
Lung	1.09±0.09	4.21±0.09	1.17±0.09	0.17±0.03
Liver	27.4±0.49	25.9±1.12	17.4±0.09	11.0±0.10
Spleen	8.86±0.08	10.4±0.23	4.40±0.06	2.2±0.13
Kidney	2.32±0.15	2.24±0.07	1.84±0.02	0.23±0.04
Intestine	0.20±0.01	0.26±0.01	0.17±0.01	0.11±0.001
Stomach	0.61±0.03	0.52±0.03	0.56±0.02	0.48±0.01
Bone	0.90±0.02	3.20±0.02	1.02±0.01	0.48±0.01

Table 11.22 Biodistribution of CL in mice

Organ/ Tissue	Percent injected dose / whole organ or tissue (+ S.E.M.)			
	1 h	4h	24 h	48h
Blood	12.26±0.10	11.03±0.09	2.16±0.04	1.59±0.02
Heart	0.16±0.02	0.17±0.02	0.06±0.003	0.021±0.004
Lung	0.19±0.004	1.23±0.02	0.63±0.02	0.18±0.01
Liver	34.25±1.12	32.34±1.30	21.75±0.11	13.75±0.13
Spleen	1.33±0.03	1.56±0.03	0.66±0.01	0.33±0.002
Kidney	0.84±0.05	0.81±0.03	0.66±0.01	0.08±0.01
Intestine	0.59±0.01	0.78±0.001	0.51±0.02	0.34±0.01
Stomach	0.23±0.01	0.19±0.01	0.21±0.02	0.18±0.002
Bone	-	-	-	-

Figure 11.4 Bone/RES ratio of cyclosporine and its liposomes

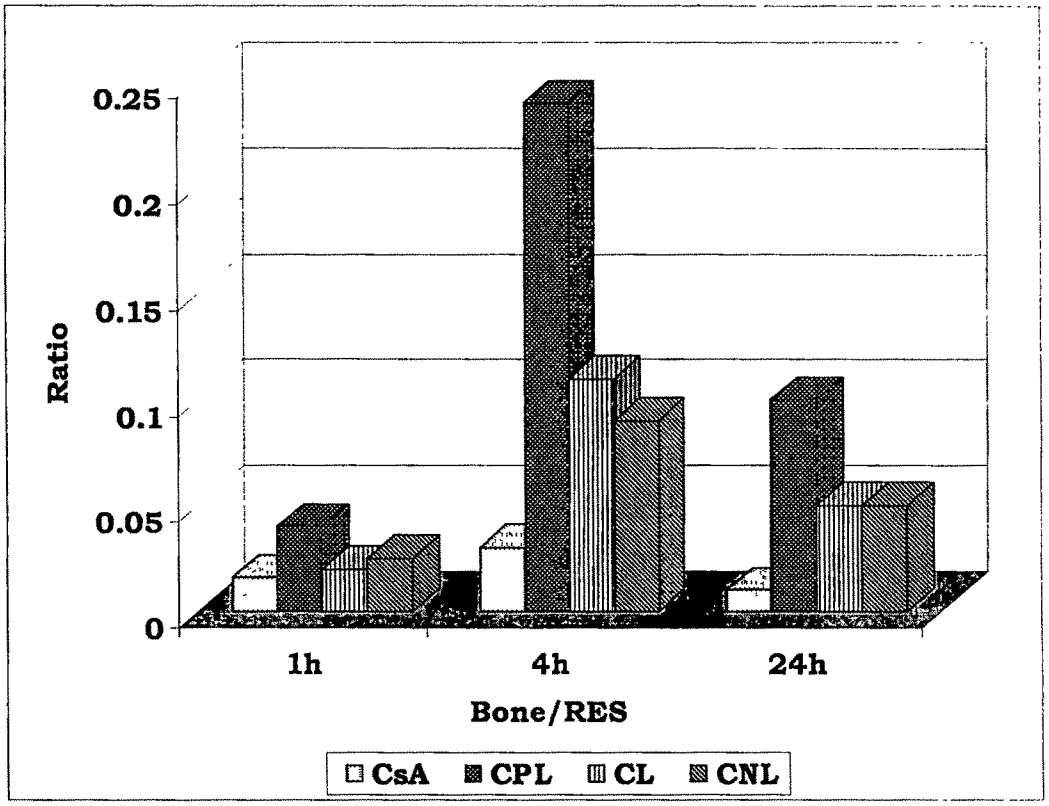

Figure 11.5 Blood/RES ratio of cyclosporine and its liposomes

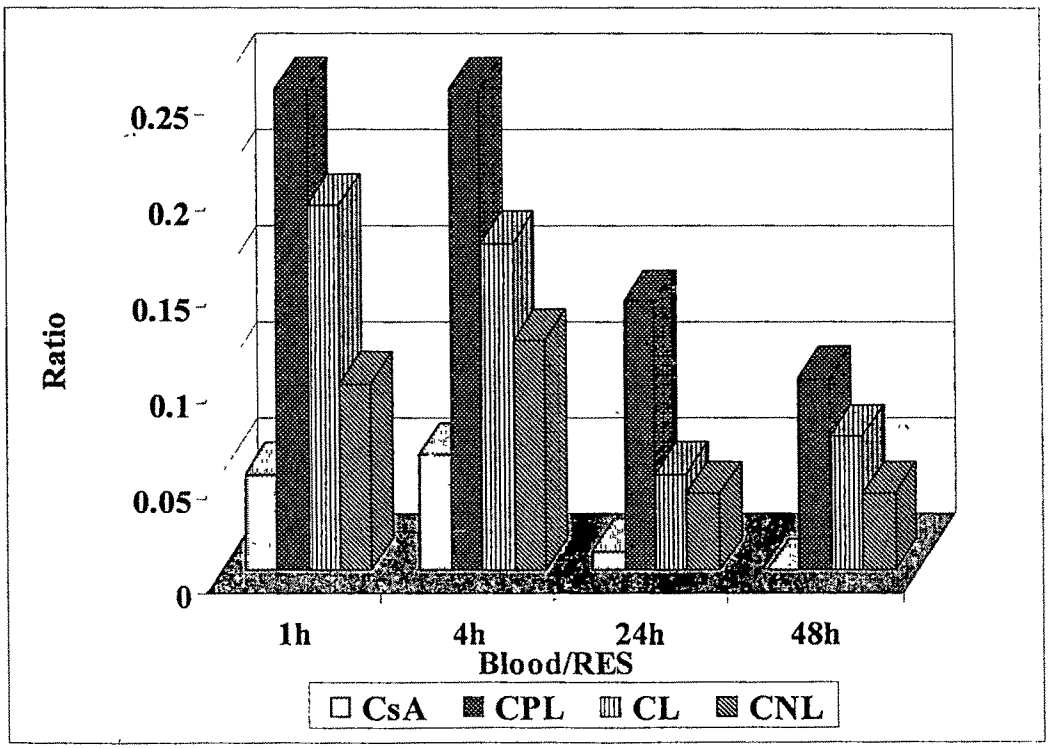

Table 11.23 Biodistribution of leuprolide acetate in EAT tumor bearing mice

Organ/ Tissue	Percent injected dose per gram of organ or tissue				
	15 min	30 min	1 hr	4 hr	24 hr
Blood	1.57±0.05	0.98±0.01	1.12±0.06	0.75±0.06	0.20±0.01
Heart	0.32±0.01	0.34±0.03	0.32±0.01	0.21±0.01	0.11±0.01
Lung	2.97±0.17	3.75±0.28	3.09±0.17	1.88±0.17	0.12±0.02
Liver	7.98±0.20	8.63±0.23	7.91±0.10	7.98±0.10	0.4±0.02
Spleen	7.01±0.20	5.82±0.08	3.03±0.06	2.90±0.06	0.30±0.01
Kidney	1.40±0.04	1.57±0.12	1.72±0.03	1.52±0.01	0.98±0.01
Intestine	0.21±0.03	0.27±0.01	0.14±0.03	0.10±0.03	0.31±0.10
Stomach	0.21±0.01	0.23±0.01	0.16±0.01	0.31±0.05	0.32±0.05
Tumor	N.D	N.D	0.12±0.01	0.14±0.01	0.02±0.02

Table 11.24 Biodistribution of leuprolide acetate in EAT tumor bearing mice

Organ/ Tissue	Percent injected dose / whole organ or tissue				
	15 min	30 min	1 hr	4 hr	24 hr
Blood	2.87±0.10	1.79±0.02	2.05±0.12	1.37±0.01	0.34±0.01
Heart	0.04±0.01	0.04±0.01	0.04±0.04	0.03±0.01	0.01±0.01
Lung	0.51±0.03	0.64±0.04	0.53±0.03	0.32±0.02	0.02±0.01
Liver	9.98±0.3	10.79±0.3	9.89±0.13	9.98±0.13	0.5±0.01
Spleen	1.05±0.03	0.87±0.01	0.45±0.01	0.44±0.01	0.05±0.01
Kidney	0.50±0.01	0.57±0.04	0.62±0.01	0.55±0.01	0.35±0.01
Intestine	0.64±0.10	0.80±0.02	0.42±0.01	0.29±0.01	0.93±0.05
Stomach	0.08±0.01	0.09±0.01	0.06±0.01	0.12±0.02	0.12±0.01
Tumor	N.D	N.D	0.12±0.01	0.14±0.01	0.02±0.01

Table 11.25 Biodistribution of LL in EAT tumor bearing mice

Organ/ Tissue	Percent injected dose / gram of organ or tissue (± S.E.M.)			
	1 h	4 h	24 h	48h
Blood	8.14±0.21	5.62±0.26	1.15±0.06	0.71±0.05
Heart	1.21±0.15	0.58±0.03	0.27±0.05	0.12±0.01
Lung	5.62±0.32	4.20±0.33	3.51±0.04	0.96±0.04
Liver	11.16±0.22	11.2±0.25	10.2±0.03	4.14±0.10
Spleen	9.93±0.03	9.93±0.04	6.78±0.10	1.63±0.22
Kidney	2.44±0.13	5.31±0.10	1.79±0.10	1.08±0.03
Intestine	0.26±0.003	0.23±0.01	0.24±0.01	0.14±0.01
Stomach	0.38±0.02	0.28±0.01	0.24±0.01	0.31±0.04
Tumor	0.12±0.01	0.38±0.07	1.28±0.01	0.47±0.01

Table 11.26 Biodistribution of LL in EAT tumor bearing mice

Organ/ Tissue	Percent injected dose / whole organ or tissue (+ S.E.M.)			
	1 h	4 h	24 h	48h
Blood	14.9±0.41	10.28±0.42	2.11±0.10	1.29±0.10
Heart	0.16±0.02	0.08±0.04	0.04±0.01	0.02±0.002
Lung	0.96±0.05	0.71±0.05	0.60±0.05	0.16±0.06
Liver	13.95±0.28	14.03±0.31	12.8±0.02	5.18±0.13
Spleen	1.94±0.05	1.49±0.01	1.02±0.04	0.24±0.03
Kidney	0.88±0.05	0.47±0.04	0.64±0.01	0.39±0.01
Intestine	0.86±0.01	0.80±0.02	0.85±0.01	0.40±0.01
Stomach	0.14±0.01	0.11±0.004	0.09±0.04	0.12±0.01
Tumor	0.09±0.1	0.31±0.06	1.02±0.01	0.78±0.07

Table 11.27 Biodistribution of SLL5000 in EAT tumor bearing mice

Organ/ Tissue	Percent injected dose / gram of organ or tissue (± S.E.M.)			
	1 h	4 h	24 h	48h
Blood	17.08±0.08	13.6±0.21	6.64±0.21	2.24±0.13
Heart	1.15±0.07	0.80±0.04	0.42±0.02	0.16±0.01
Lung	4.33±0.26	2.96±0.04	1.85±0.09	0.11±0.01
Liver	9.09±0.07	9.09±0.23	6.52±0.18	5.95±0.04
Spleen	4.94±0.04	7.57±0.14	5.20±0.10	1.04±0.04
Kidney	5.11±0.38	1.31±0.09	1.07±0.03	1.30±0.07
Intestine	0.18±0.003	0.20±0.01	0.10±0.01	0.10±0.01
Stomach	0.17±0.02	0.20±0.01	0.58±0.01	0.64±0.03
Tumor	0.10±0.1	0.58±0.01	4.00±0.02	1.23±0.02

Table 11.28 Biodistribution of SLL5000 in EAT tumor bearing mice

Organ/ Tissue	Percent injected dose / whole organ or tissue (± S.E.M.)			
	1 h	4 h	24 h	48h
Blood	31.26±0.16	24.9±0.40	12.17±0.40	4.09±0.25
Heart	0.15±0.01	0.10±0.01	0.06±0.03	0.02±0.002
Lung	0.74±0.04	0.50±0.01	0.31±0.2	0.02±0.001
Liver	11.4±0.09	11.36±0.29	8.2±0.23	7.44±0.05
Spleen	0.74±0.06	1.14±0.05	0.78±0.01	0.61±0.01
Kidney	1.84±0.14	0.47±0.02	0.39±0.01	0.16±0.03
Intestine	0.54±0.01	0.60±0.02	0.32±0.001	0.32±0.01
Stomach	0.07±0.01	0.08±0.04	0.22±0.04	0.24±0.01
Tumor	0.08±0.01	0.46±0.01	3.2±0.02	0.98±0.02

Table 11.29 Biodistribution of SLL2000 in EAT tumor bearing mice

Organ/ Tissue	Percent injected dose/gram of organ or tissue (± S.E.M.)			
	1 h	4 h	24 h	48h
Blood	21.8±0.29	16.9±0.14	8.12±0.06	4.04±0.03
Heart	0.84±0.03	0.69±0.01	0.74±0.01	0.27±0.03
Lung	3.52±0.22	3.65±0.09	1.72±0.06	1.07±0.03
Liver	8.95±0.05	8.95±0.13	5.64±0.12	5.48±0.14
Spleen	5.54±0.15	5.84±0.17	4.40±0.14	1.25±0.23
Kidney	3.39±0.14	2.84±0.09	1.63±0.22	1.19±0.05
Intestine	0.21±0.03	0.15±0.06	0.10±0.03	0.05±0.01
Stomach	0.26±0.004	0.22±0.07	0.56±0.03	0.66±0.03
Tumor	0.16±0.01	0.56±0.01	4.58±0.01	1.78±0.01

Table 11.30 Biodistribution of SLL2000 in EAT tumor bearing mice

Organ/ Tissue	Percent injected dose / whole organ or tissue (± S.E.M.)			
	1 h	4 h	24 h	48h
Blood	39.89±0.52	30.85±0.26	14.86±0.12	7.39±0.06
Heart	0.11±0.04	0.09±0.002	0.09±0.002	0.04±0.04
Lung	0.60±0.04	0.62±0.02	0.29±0.01	0.18±0.05
Liver	11.19±0.06	11.20±0.16	7.05±0.14	6.85±0.18
Spleen	0.83±0.02	0.88±0.03	0.66±0.02	0.49±0.03
Kidney	1.22±0.05	1.02±0.03	0.59±0.08	0.43±0.02
Intestine	0.64±0.1	0.45±0.2	0.32±0.01	0.14±0.01
Stomach	0.1±0.002	0.08±0.003	0.21±0.004	0.25±0.01
Tumor	0.13±0.01	0.45±0.007	3.67±0.07	1.4±0.01

Figure 11.6 Tumor/RES ratio of leuprolide acetate and its liposomes

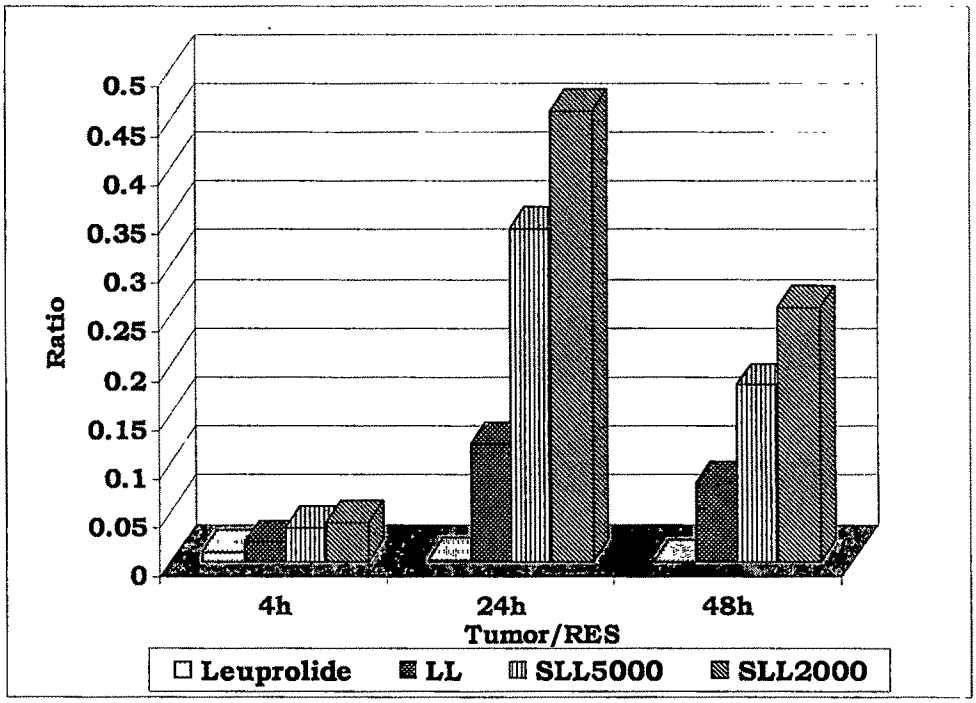

Figure 11.7 Tissue uptake ratio of leuprolide acetate and its liposomes (24 h post injection)

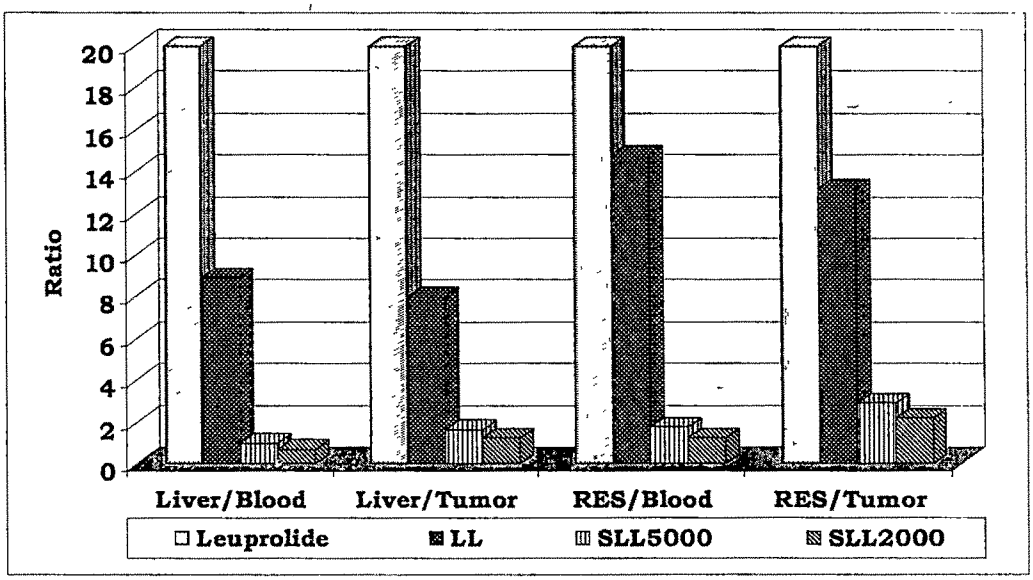

Figure 11.8 Compartmental distribution of leuprolide acetate loaded liposomes versus time after 1 h, 4 h, 24 h, and 48 h injection: a study with three liposomal formulations —◆— LL, —□— SLL5000 and —▲— SLL2000. A, B, and C represents different tissue compartments as indicated. Each value is the mean of three independent experiments. Error bars represent S.E.M.

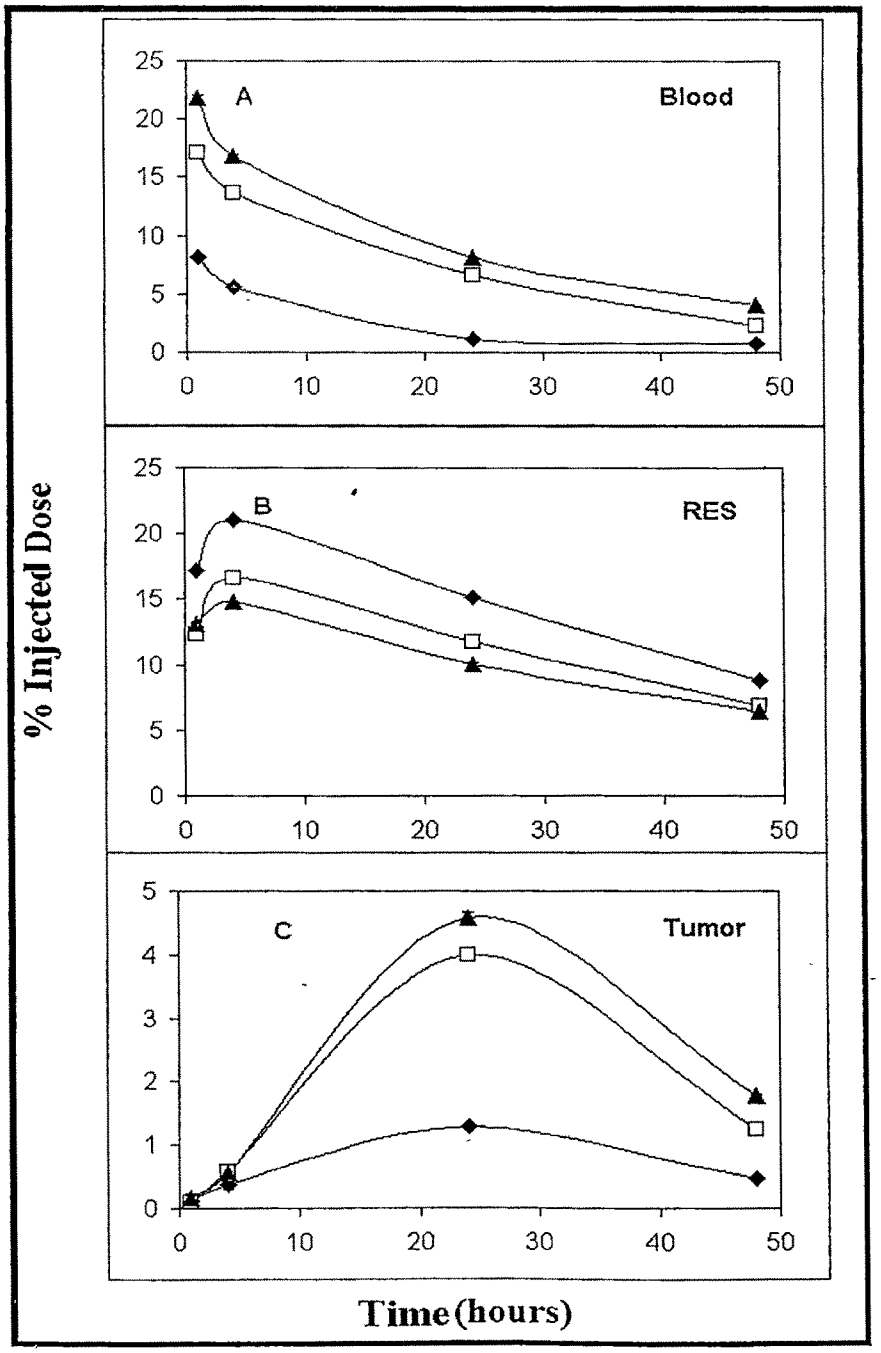

Table 11.31 Biodistribution of DNA in mice

Organ/ Tissue	Percent injected dose / gram of organ or tissue (±S.E.M.)			
	15 min	30 min	1 hr	4 hr
Blood	1.99±0.02	1.14±0.05	0.96±0.02	0.54±0.05
Heart	0.57±0.01	0.44±0.01	0.35±0.01	0.26±0.02
Lung	2.13±0.04	1.7±0.01	0.9±0.01	0.76±0.01
Liver	6.5±0.14	5.89±0.06	4.27±0.03	2.12±0.06
Spleen	4.43±0.2	3.38±0.05	1.46±0.02	1.33±0.04
Kidney	2.73±0.07	2.04±0.01	1.78±0.06	1.63±0.01
Intestine	0.14±0.01	0.33±0.03	0.24±0.001	0.13±0.03
Stomach	0.18±0.01	0.09±0.01	0.22±0.01	0.09±0.10

Table 11.32 Biodistribution of DNA in mice

Organ/Tissue	Percent injected dose / whole organ or tissue (± S.E.M.)				
	15 min	30 min	1 hr	4 hr	24 hr
Blood	3.63±0.04	1.11±0.10	1.76±0.04	0.98±0.04	0.18±0.08
Heart	0.07±0.001	0.06±0.001	0.04±0.001	0.03±0.001	0.02±0.002
Lung	0.36±0.04	0.29±0.03	0.15±0.003	0.13±0.003	0.02±0.003
Liver	08.13±0.14	7.38±0.05	5.36±0.04	2.66±0.04	1.0±0.08
Spleen	0.66±0.02	0.51±0.07	0.22±0.003	0.20±0.03	0.08±0.006
Kidney	0.98±0.07	0.73±0.04	0.64±0.02	0.59±0.02	0.32±0.004
Intestine	0.42±0.01	0.99±0.03	0.72±0.001	0.39±0.01	0.64±0.1
Stomach	0.07±0.004	0.03±0.04	0.08±0.004	0.03±0.04	0.09±0.004

Table 11.33 Biodistribution of DL in mice

Organ/ Tissue	Percent injected dose / gram of organ or tissue (± S.E.M.)			
	1 h	4 h	24 h	48h
Blood	8.07±0.03	6.07±0.03	1.58±0.01	0.76±0.01
Heart	0.76±0.02	0.69±0.01	0.45±0.02	0.18±0.01
Lung	7.1±0.05	5.12±0.02	2.16±0.03	0.84±0.03
Liver	18.62±0.06	13.24±0.02	8.13±0.06	5.59±0.02
Spleen	12.62±0.30	10.19±0.06	5.30±0.04	2.14±0.04
Kidney	3.88±0.05	2.39±0.02	1.94±0.04	1.15±0.07
Intestine	0.32±0.001	0.46±0.002	0.19±0.003	0.10±0.003
Stomach	0.42±0.03	0.52±0.02	0.64±0.01	0.81±0.01

Table 11.34 Biodistribution of DL in mice

Organ/ Tissue	Percent injected dose / whole organ or tissue (± S.E.M.)			
	1 h	4 h	24 h	48h
Blood	14.77±0.05	11.11±0.05	2.89±0.02	1.39±0.02
Heart	0.09±0.03	0.09±0.001	0.06±0.003	0.023±0.001
Lung	1.19±0.01	0.87±0.003	0.37±0.01	0.11±0.01
Liver	23.28±0.08	16.6±0.03	10.16±0.08	6.99±0.01
Spleen	1.89±0.05	1.53±0.01	0.79±0.01	0.32±0.22
Kidney	1.39±0.02	0.86±0.01	0.69±0.01	0.41±0.01
Intestine	0.95±0.04	1.47±0.05	0.58±0.01	0.32±0.03
Stomach	0.16±0.01	0.19±0.01	0.24±0.004	0.31±0.004

Table 11.35 Biodistribution of SDL5000 in mice

Organ/ Tissue	Percent injected dose / gram of organ or tissue (± S.E.M.)			
	1 h	4 h	24 h	48h
Blood	16.46±0.25	12.95±0.03	5.85±0.03	2.42±0.21
Heart	1.11±0.06	0.92±0.04	0.58±0.01	0.35±0.04
Lung	5.21±0.05	3.34±0.09	0.58±0.01	0.41±0.01
Liver	8.64±0.18	7.41±0.29	7.23±0.11	3.25±0.02
Spleen	5.14±0.06	2.91±0.03	2.32±0.15	3.18±0.03
Kidney	4.04±0.04	2.7±0.29	2.1±0.04	1.92±0.04
Intestine	0.24±0.003	0.19±0.003	0.14±0.003	0.10±0.003
Stomach	0.66±0.02	0.50±0.01	0.32±0.01	0.19±0.01

Table 11.36 Biodistribution of SDL5000 in mice

Organ/ Tissue	Percent injected dose / whole organ or tissue (± S.E.M.)			
	1 h	4 h	24 h	48h
Blood	30.12±0.46	23.7±0.06	10.71±0.06	4.42±0.40
Heart	0.14±0.01	0.12±0.004	0.08±0.001	0.05±0.01
Lung	0.89±0.01	0.57±0.02	0.19±0.002	0.05±0.002
Liver	10.8±0.23	9.26±0.36	9.03±0.14	4.1±0.03
Spleen	0.77±0.01	0.44±0.004	0.35±0.02	0.48±0.01
Kidney	1.45±0.01	0.97±0.10	0.76±0.01	0.69±0.01
Intestine	0.68±0.01	0.57±0.01	0.42±0.01	0.31±0.01
Stomach	0.25±0.08	0.19±0.004	0.12±0.004	0.07±0.003

Table 11.37 Biodistribution of SDL2000 in mice

Organ/ Tissue	Percent injected dose / gram of organ or tissue (± S.E.M.)			
	1 h	4 h	24 h	48h
Blood	20.69±0.23	16.08±0.08	9.05±0.02	3.22±0.09
Heart	1.31±0.06	1.09±0.04	0.78±0.01	0.54±0.01
Lung	4.56±0.23	3.16±0.03	1.19±0.04	0.09±0.001
Liver	7.58±0.23	6.47±0.12	4.23±0.05	3.32±0.10
Spleen	4.99±0.02	4.99±0.05	4.55±0.03	3.39±0.1
Kidney	3.26±0.07	2.19±0.003	1.18±0.09	0.93±0.03
Intestine	0.35±0.01	0.26±0.003	0.20±0.001	0.10±0.003
Stomach	0.33±0.01	0.43±0.02	0.64±0.01	0.53±0.01

Table 11.38 Biodistribution of SDL2000 in mice

Organ/ Tissue	Percent injected dose / whole organ or tissue (± S.E.M.)			
	1 h	4 h	24 h	48h
Blood	37.86±0.40	29.4±0.16	16.56±0.04	5.89±0.16
Heart	0.17±0.01	0.14±0.04	0.10±0.001	0.08±0.001
Lung	0.78±0.03	0.54±0.15	0.20±0.01	0.09±0.001
Liver	9.48±0.29	8.09±0.01	5.29±0.06	4.15±0.13
Spleen	0.75±0.002	0.75±0.01	0.68±0.003	0.51±0.01
Kidney	1.17±0.03	0.79±0.001	0.42±0.03	0.33±0.001
Intestine	1.05±0.02	0.77±0.01	0.61±0.01	0.32±0.01
Stomach	0.13±0.004	0.16±0.01	0.24±0.004	0.20±0.002

Table 11.39 Biodistribution of CSDL2000 in mice

Organ/ Tissue	Percent injected dose / gram of organ or tissue (± S.E.M.)			
	1 h	4 h	24 h	48h
Blood	21.57±0.22	17.07±0.03	10.06±0.03	4.23±0.05
Heart	1.02±0.01	0.87±0.01	0.89±0.003	0.47±0.01
Lung	7.13±0.01	5.16±0.03	3.19±0.04	1.02±.02
Liver	7.59±0.23	7.12±0.01	4.41±0.15	4.21±0.06
Spleen	4.18±0.08	2.09±0.01	3.26±0.02	1.26±0.02
Kidney	3.15±0.06	1.26±0.02	0.98±0.003	0.77±0.01
Intestine	0.37±0.01	0.33±0.003	0.22±0.003	0.18±0.002
Stomach	0.65±0.09	0.64±0.01	0.53±0.01	0.45±0.02

Table 11.40 Biodistribution of CSDL2000 in mice

Organ/ Tissue	Percent injected dose / whole organ or tissue (± S.E.M.)			
	1 h	4 h	24 h	48h
Blood	39.47±0.44	31.24±0.05	18.41±0.05	7.74±0.1
Heart	0.13±0.001	0.11±0.001	0.12±0.004	0.06±0.002
Lung	1.21±0.001	0.88±0.01	0.54±0.01	0.17±0.001
Liver	9.49±0.29	8.9±0.01	5.5±0.19	5.26±0.08
Spleen	0.63±0.01	0.31±0.002	0.49±0.003	0.19±0.003
Kidney	1.13±0.02	0.45±0.006	0.35±0.001	0.28±0.004
Intestine	1.12±0.003	0.98±0.01	0.67±0.01	0.53±0.01
Stomach	0.25±0.003	0.24±0.004	0.20±0.002	0.17±0.01

Figure 11.9 Tissue uptake ratio of DNA and its liposomes

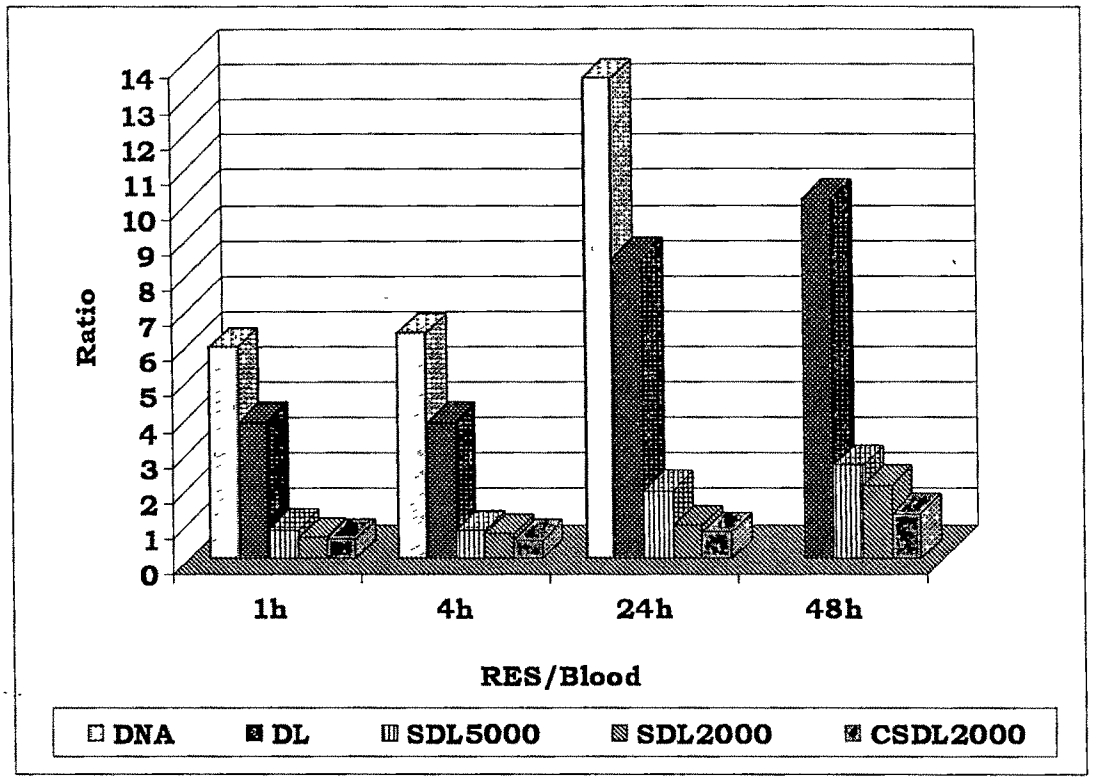

Table 11.41 Pharmacokinetic parameters for the *in vivo* studies of conventional and sterically stabilized liposomes containing cyclosporine, leuprolide acetate and DNA in mice

Batch	Elimination rate K_{el} (h^{-1})	Elimination half life (h)	Vd (l)	AUC
CsA	0.0856	8.09	1.00	28.18
CPL	0.0431	16.08	0.999	353.56
CL	0.043	16.12	1	257.81
CNL	0.0507	13.67	0.999	180.56
Leuprolide acetate	0.1898	3.65	40.15	15.84
LL	0.0715	9.689	1	214.069
SLL5000	0.0382	18.16	1	786.77
SLL2000	0.0323	21.43	0.9998	1080.54
DNA	0.1952	3.55	19.25	12.13
DL	0.0617	11.22	1	250.39
SDL5000	0.039	17.77	1	733.29
SDL2000	0.0328	21.14	1.0001	1059.51
CSDL2000	0.0295	23.49	1	1227.68

Table 11.42 *In vitro* testing for drug retention within liposomes containing cyclosporine in human plasma

Time in Hrs	% Drug Retained ± S.E.M		
	CL	CPL	CNL
6	94.0±2.02	95.0±1.64	94.2±2.23
12	90.6±2.36	91.0±2.02	90.9±2.05
24	72.0±2.45	73.3±2.32	70.5±2.68
48	60.0±3.02	62.3±2.05	60.8±2.08

Table 11.43 *In vitro* testing for drug retention within liposomes containing leuprolide acetate in human plasma

Time in Hrs	% Drug Retained ± S.E.M		
	LL	SLL5000 CC-PE	SLL2000 CC-PE
6	94.0±2.02	90.0±1.64	92.2±2.23
12	78.6±2.36	80.0±2.02	75.8±2.05
24	65.0±2.45	62.3±2.32	60.8±2.68
48	48.0±3.02	42.3±2.05	45.2±2.08

Table 10.44 *In vitro* testing for drug retention within liposomes containing DNA in human plasma

Time in Hrs	% Drug Retained (S.E.)			
	DL	SDL5000-CC-PE	SDL2000-CC-PE	CSDL5000-CC-PE
6	88.2±1.23	86.5±2.23	84.2±2.02	82.3±1.89
12	65.2±1.67	70.2±2.62	68.2±2.05	66.9±1.95
24	50.2±2.02	49.8±2.31	51.2±2.13	52.3±1.26
48	30.3±2.32	31.6±1.09	33.6±1.96	30.9±2.56

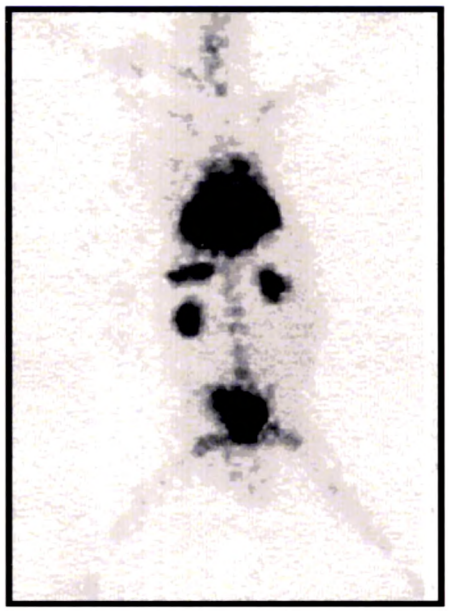

Figure 11.10 Gamma camera image of a rabbit at 4h after I.V. administration of ^{99m}Tc-CsA. Image reveals blood pool activity in liver, spleen and vertebral column

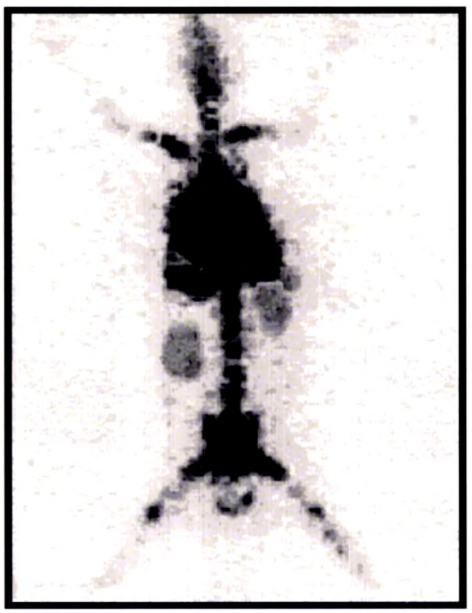

Figure 11.11 Gamma camera image of a rabbit at 4h after I.V. administration of ^{99m}Tc-CPL. Image reveals blood pool activity in liver, spleen and vertebral column

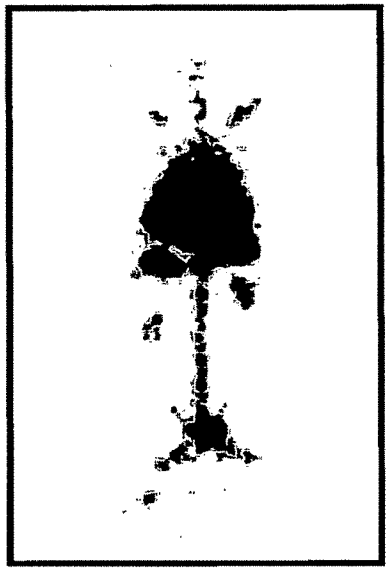

Figure 11.12 Gamma camera image of a rabbit at 4h after I.V. administration of ^{99m}Tc-CL. Image reveals blood pool activity in liver, spleen and vertebral column

Figure 11.13 Gamma camera image of a rabbit at 4h after I.V. administration of ^{99m}Tc-CNL. Image reveals blood pool activity in liver, spleen and vertebral column

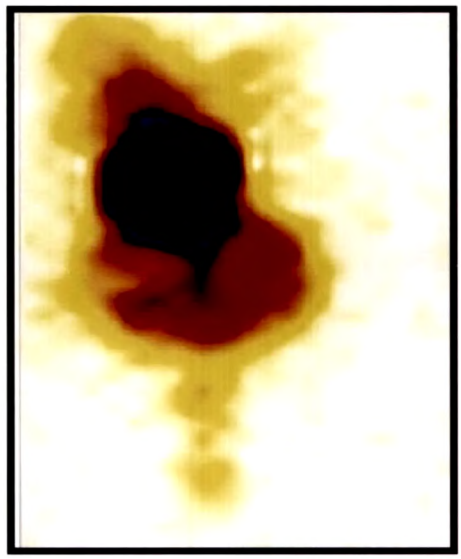

Figure 11.14 Gamma scintigraphic image of leuprolide acetate injected mice bearing EAT in the left thigh

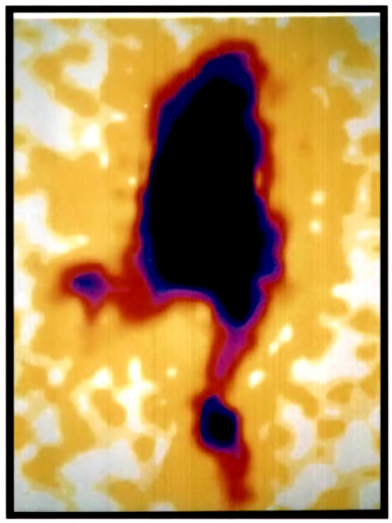

Figure 11.15 Gamma scintigraphic image of LL injected mice bearing EAT in the left thigh

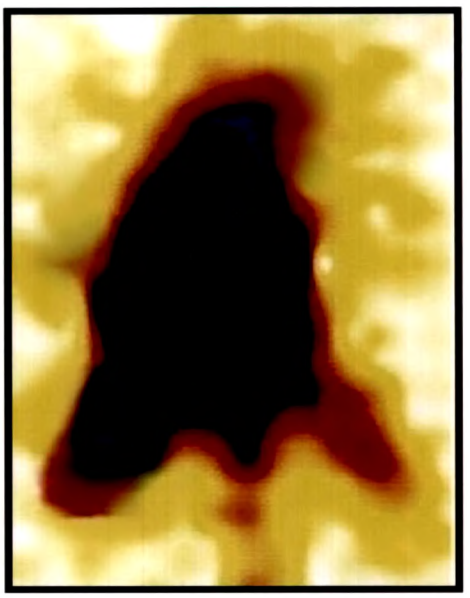

Figure 11.16 Gamma scintigraphic image of SLL5000 injected mice bearing EAT in the left thigh

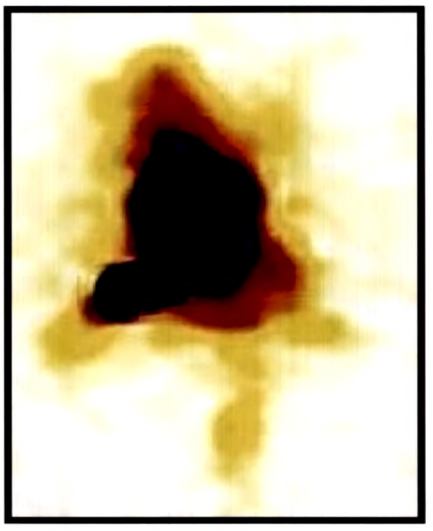

Figure 11.17 Gamma scintigraphic image of SLL2000 injected mice bearing EAT in the left thigh

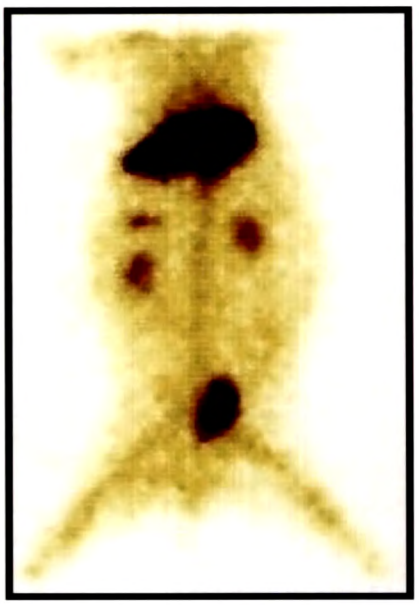

Figure 11.18 Gamma camera image of a rabbit at 1h after I.V. administration of ^{99m}Tc-DNA. Image reveals blood pool activity in liver, spleen and vertebral column.

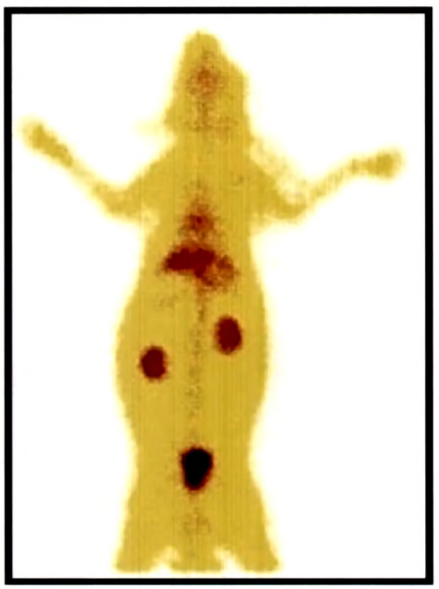

Figure 11.19 Gamma camera image of a rabbit at 4h after I.V. administration of ^{99m}Tc-DL. Image reveals blood pool activity in liver, spleen and vertebral column.

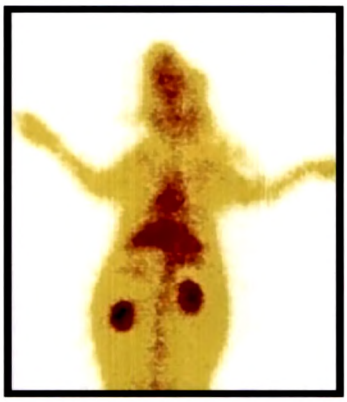

Figure 11.20 Gamma camera image of a rabbit at 4h after I.V. administration of ^{99m}Tc-SDL5000. Image reveals blood pool activity in liver, spleen and vertebral column

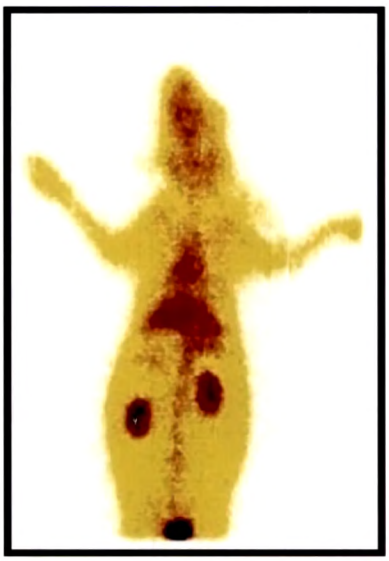

Figure 11.21 Gamma camera image of a rabbit at 4h after I.V. administration of ^{99m}Tc-SDL2000. Image reveals blood pool activity in liver, spleen and vertebral column

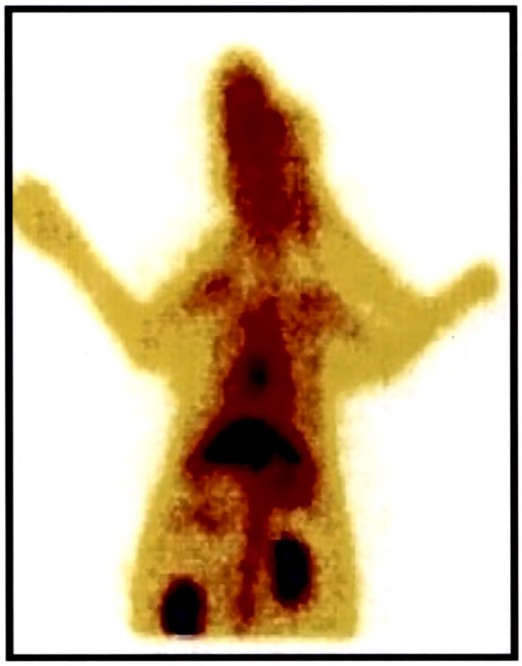

Figure 11.22 Gamma camera images of a rabbit at 4h after I.V. administration of ^{99m}Tc-CSDL2000. Images reveal blood pool activity in liver, spleen and vertebral column

11.3 RESULTS AND DISCUSSION

11.3.1 GENERAL METHODOLOGY

The ^{99m}Tc labeled drug / liposome complexes were injected into the ear vein of the rabbits and measurement of the blood levels at predetermined time intervals was performed. The data so obtained was used for the analysis using Quickcalc software to get an idea of the blood kinetic profile of the drug and the liposome formulations in rabbits. The tables 11.1 –11.13 tabulate the raw data obtained from the experiment and the calculated value from the analysis. Table 11.14 gives the pharmacokinetic parameters describing the *in vivo* behaviour of the prepared liposomes generated by the analysis of the data. Figure 11.1 –11.3 shows the comparison of the blood kinetics pattern of CsA, leuprolide and DNA and their liposomal formulations, respectively. An appropriate volume of the radiolabeled complexes was injected into the tail vein of the mice to determine the biodistribution pattern and the targetability of CsA, leuprolide and DNA, and its liposome preparations. The mice were sacrificed at different time intervals and blood was obtained by cardiac puncture. Subsequently, tissues (heart, lung, liver, spleen, kidney, stomach, intestine, bone and tumor) were dissected out and the amount of labeled complexes present in each tissue was determined. The data so obtained was treated to analysis using Quickcalc software to get an idea of the blood kinetic profile of the drug and the liposome formulations in mice. The tables 11.15 – 11.40 tabulate the biodistribution data of the drug/liposomal formulations. The percent injected dose present in the whole organ/tissue as well as percent injected dose present per gram of organ/tissue were shown in the tables 11.15 – 11.40. Table 11.41 gives the pharmacokinetic parameters describing the *in vivo* behaviour of the prepared liposomes generated by the analysis of the data obtained from the biodistribution studies. Figure 11.4 – 11.10 shows the tissue uptake ratio of various organs of CsA, leuprolide acetate, DNA and their liposomal formulations, respectively.

Scintigraphic studies were carried out in rabbit for CsA and DNA, and their liposomal formulations, and in EAT bearing mice for leuprolide and its liposomal formulation after administration of an appropriate volume of the radiolabeled complexes through the ear vein of rabbit and tail vein of mice.

The gamma imaging was performed using a Single Photon Emission Computerized Tomography (SPECT, LC 75-005, Diacam, Siemens, USA) gamma camera and are shown in figure 11.10 –11.22.

11.3.2 CYCLOSPORINE AND ITS LIPOSOMES

Cyclosporine is a neutral, extremely hydrophobic cyclic peptide composed of 11 aminoacid residues with a molecular weight of 1202; it has been reported to be suitable for encapsulation into liposomes (Stuhne-Sekalec and Stanaav, 1991; Vadiei et al., 1989). From the standpoint of biopharmaceutics, a more efficient and a safe delivery system with less systemic toxicity is needed for immunosuppressive therapy in organ transplantation. It was envisaged that liposomes are microscopic lipid spheres that accumulate preferentially at sites of infection and inflammation and also at the tumor sites after intravenous administration (Morgan et al., 1985; Ogihara et al., 1986; Ostro and Cullis, 1989). Therefore by incorporating cyclosporine into a liposomal carrier, it should be possible to achieve elevated drug levels at sites of tissue rejection, as a result of involvement of macrophages and other components of the immune response, while lowering drug exposure to sensitive organs such as kidney, and would also lead to significantly reduced side effects and an increase in therapeutic efficacy. Hence we investigated the effect of various charges on the liposome encapsulated cyclosporine on the biodistribution to various organs especially to blood, liver, spleen, kidney and bone. The particle size was maintained in a narrow range so as to have long blood circulation. Reports suggest that the high level of liposomes in the blood was only observed for small liposomes ($d \leq 200$ nm) (Klibanov et al., 1991). The use of radiolabels has proven quite useful in following the fate of liposomes *in vivo* and as diagnostic tools in nuclear medicine. The methods employed to label liposomes include entrapment of the radiolabel in the aqueous compartment, attachment of the label to the lipid components prior to liposome formulation, and the addition of label after their manufacture (Richardson et al., 1978).

The *in vivo* data reveals that the half-life of the drug, when entrapped in liposomes was greater than the drug in its free state. This observation can be corroborated based on the value of the half lives for the liposomes and

the drug in both rabbits and mice (6.49h & 8.09 for CsA, 15.51h & 16.08h for CPL, 11.92h & 16.12h for CL, 10.84h & 13.67h for CNL) as shown in table 11.14 & 11.41). Estimated pharmacokinetic parameters of CsA and its liposomes in rabbit and mice by two-compartment model using Quickcalc software are summarized in table 11.14 & 11.41. The area under the curve (AUC) values respectively for CsA, CPL and CNL were 30.31, 269.98, 277.71, and 172.69 (in rabbits) and 28.18, 353.56, 257.81 and 180.56 (in mice) thereby showing increased bioavailability, sustained release and the prolonged circulation of the liposomes. The enhanced blood circulation of the liposomal formulations containing cyclosporine may be attributed to the increase in bilayer rigidity by incorporation of charge and incorporation of high melting lipids.

The biodistribution of ^{99m}Tc-cyclosporine after 15 min, 30 min, 1 h and 4 h of intravenous injection in Balb/c mice is shown in table 11.15. The percent radioactivity was given per gram of organ or tissue and per whole organ or tissue. Blood was obtained by cardiac puncture, weighed and the radioactivity present in the whole blood was calculated by keeping 7.3 % of the body weight as total blood weight (Wu et. al., 1981). The percent-injected dose/gm and dose/whole organ in various organs of mice at different times after i.v administration of ^{99m}Tc-CL, ^{99m}Tc-CNL and ^{99m}Tc-CPL after 1h, 4h, 24h and 48h of intravenous injection in Balb/c mice were depicted in table 11.17 – 11.22. Various organs/tissues like lung, liver, spleen, kidney, stomach, intestine and bone were removed and analyzed for labeling content. In case of ^{99m}Tc-cyclosporine, the radioactivity present in whole organ, 4 h of post injection were found to be as follows: blood (0.75 %), liver (4.25 %), spleen (5.40 %) and kidney (5.03 %). In case of ^{99m}Tc-cyclosporine liposomes, liver and spleen accumulated a major portion of the administered radioactivity. The biodistribution data reveals that initial rapid uptake by liver, 27.4 %, 37.4 %, and 21.1 % at 1h and 25.9 %, 28.0 %, and 18.3 % at 4 h post injection for CL, CNL and CPL respectively. The spleen accumulates less initially, 8.86%, 5.45 %, and 5.00 % at 1 h and 10.4 %, 10.7 %, and 6.36 % at 4 h post injection for CL, CNL and CPL respectively. Hardly any activity appeared to clear via intestine. The constancy of radioactivity in stomach also pointed to the fact that there is no *in vivo* leaching of

radioactivity as free Technetium. No significant uptake of ^{99m}Tc – cyclosporine liposomes was found in the organs like heart and kidney. The radioactivity in whole blood was found to be 2.16 %, 1.90 % and 4.78 % at 24 h and 1.37 %, 1.28 % and 1.87 % at 48 h of post injection for CL, CNL and CPL respectively, which proves the enhanced circulation of liposomes in comparison to free drug. The residence time of positive liposomes in blood at 24 h was twofold greater than negative and neutral liposome, which is in agreement with the previous reports (Aoki et al., 1995, Nabar and Nadkarni, 1998). In addition to the targeting of the activity to liver and spleen, an appreciable percentage of these liposomal complexes were observed to be localized in the bone of animals. The bone accumulates 3.20 %, 4.23 %, and 6.22 % at 4 h post injection for CL, CNL and CPL respectively. From the figure 11.4 and 11.5, which depicts the tissue uptake ratio of cyclosporine and its liposomes, it was quite evident that the ratio of both Bone: RES as well as Blood: RES for positive charged liposomes were significantly high compared to free drug and CNL. When compared with free drug, positively charged liposomes, particularly showed significantly increased circulation half-life in addition to increase uptake by bones and reduced accumulation in kidney.

Through out the period of experimentation, it was found that the distribution of charged cyclosporine liposomes to liver, spleen, kidney, bone and blood varied extremely significantly ($P < 0.001$) when compared to the free drug. Analysis of variance was carried out to compare the three liposomal formulations (CL, CNL and CPL) at 4 h post injection at a significance level of $\alpha = 0.05$ and it was found that the difference between the three liposomal formulations was extremely significant in blood ($F=518.9$), liver ($F=44.44$), spleen ($F=361.88$), kidney ($F=76.27$) and bone ($F=4283.34$). The above observations have been corroborated by analyzing the gamma images of rabbit after 4 h post injection with radio labeled complexes.

Images are shown for rabbit at 4h post injection of free drug, CPL, CNL and CL respectively in figure 11.11 –11.14. There is an increasing accumulation in liver, spleen and bone marrow. No significant activity is noted in the thyroid and stomach. We confirm our studies that in general, the presence

of a surface charge induces definite changes in the organ distribution of liposomes. This demonstrates the utility of this novel cyclosporine intercalated charged liposomal systems for liver and bone marrow targeting and thereby reducing the accumulation of drug in kidney and the possibility of nephrotoxicity generally seen in free cyclosporine and prolonged circulation of positive charged liposomes.

11.3.3 LEUPROLIDE ACETATE AND ITS LIPOSOMES

Examination of the *in vivo* data for free drug and the liposomes containing leuprolide reveals that the liposomal formulations prolong the residence time of the drugs in blood. This observation is based on the value of the half-lives for the drug and the liposomes in both rabbits and mice (2.05h & 3.65h for leuprolide, 12.74h & 9.69h for LL, 15.15h & 18.17h for SLL5000-CC-PE, 15.81h & 21.43h for SLL2000-CC-PE, table 11.14 & 11.41).

The pharmacokinetic parameters of leuprolide and its liposomes in rabbit and mice were estimated by two-compartment model using Quickcalc software are summarized in table 11.14 and 11.41. The area under the curve (AUC) values respectively for leuprolide, LL, SLL5000, SLL2000 were 19.86, 284.68, 605.51 and 790.81 (in rabbits) and 15.84, 214.07, 786.77 and 1080.54 (in mice) showing increased bioavailability, sustained release and the prolonged circulation of the liposomes. The % injected dose/g of tissue and injected dose/whole organ or tissue in different organs at different time intervals for free drug and its liposomes was shown in table 11.23-11.30. Injection of the free drug resulted in only 0.02% injected dose /g of tumor after 24h of injection.

Analysis of the organ distribution of the conventional liposomes revealed that these liposomes were predominantly distributed into the liver and spleen. This type of distribution is normally found with the non sterically stabilized liposomes (Allen et. al., 1995, Allen et al., 1991). Thus conventional liposomes (LL) of leuprolide behaved as expected *in vivo*. There was an inverse relationship between liposome clearance by the reticuloendothelial system (RES) and a prolonged circulation time of liposomes. In turn, there appears to be a direct correlation between prolonged circulation time and liposome localization in tumors (Gabizon

and Papahadjopoulos, 1988). The relationship between blood clearance and uptake of liposomes by various tissues is shown in figure 11.6 and 11.7. Conventional liposomes showed a remarkable enhancement of liver uptake and minimal uptake by tumor. The liver/tumor uptake ratio of leuprolide acetate, LL, SLL5000 and SLL2000 was found to be 20, 7.99, 1.63 and 1.23 respectively which showed the increased accumulation of sterically stabilized liposomes in tumor compared to the free drug and conventional liposomes at 24 h after injection. Liver uptake of sterically stabilized liposomes was still 7 fold less than the conventional liposomes. The increase in accumulation in tumor site was mainly due to the increase in blood circulation time. MPEG coated liposomes was shown to have higher tumor targeting as compared to the free drug and conventional liposomes. MPEG coated liposomes showed highest values of tumor uptake, lowest liver/blood, RES/blood and lowest liver/tumor, RES/tumor uptake ratio. Through out the period of experimentation, it was found that the distribution of SLL5000 and SLL2000 to liver, spleen, tumor and blood varied extremely significantly ($P < 0.001$) when compared to LL. The distribution of the sterically stabilized liposomes to liver and spleen was considerably reduced as compared to the conventional liposomes. This indicates that steric stabilization of liposomes containing leuprolide acetate successfully alters the distribution pattern as compared to the conventional liposomes. The presence of very low amount of radioactivity in stomach after 24h proved the *in vivo* stability of the radiolabeled complexes (^{99m}Tc-leuprolide/liposomes). The lipid composition, the addition of sterically stabilizing agents (mPEG5000-CC-PE and mPEG2000-CC-PE), and the particle size of the liposomes, which was maintained around 200nm, played a major role in tumor accumulation in case of sterically stabilized liposomes. It has been hypothesized that the decreased uptake of sterically stabilized liposomes by MPS was possibly due to the presence of steric barrier, which decreases the adsorption of plasma proteins (opsonins) on the surface of the liposomes. The liposomes prepared using polyethylene glycol derivative of low molecular weight (mPEG2000-CC-PE) showed higher blood concentration compared to liposomes prepared using polyethylene glycol derivatives of higher molecular weight (mPEG5000-CC-PE), which

showed that the polyethylene glycol of low mol wt provides higher resistance to opsonization and also provides prolonged blood circulation. Thus the avoidance of RES and prolonged circulation time thereby increased accumulation in tumor site of sterically stabilized liposomes of leuprolide acetate is confirmed.

To facilitate a comprehensive analysis of liposome distribution, the tissue distribution data of the following 3 compartments (1) blood, (2) liver and spleen (used as an approximation of the RES), and (3) tumor are shown in figure 11.8. Figure 11.8 represents the compartmental distribution of tissues between 1 hour and 48 hours after injection in mice bearing EAT. RES continued to accumulate liposomes between 1 hour and 4 hours, followed by the slow disappearance of the label. As the time increased, the accumulation in tumor was found to increase, reaching a peak at 24 hours; after 48 hours, the concentration in tumor was decreased. The tumor accumulation after 24 hours for LL, SLL5000, and SLL2000 was found to be 1.28%, 4.00%, and 4.58%, respectively. SLL5000 and SLL2000 exhibit much higher levels in blood at all time points when compared with LL as evidenced from the figure 11.7 and figure 11.8.

The observations of the biodistribution study were supported by analysing the gamma scintigraphic images of the EAT bearing mice (figure 11.14 – 11.17). After 24 h of post injection of ^{99m}Tc labelled liposomal formulations, the gamma images were taken (gamma image was taken after 4h of post injection in case of ^{99m}Tc-leuprolide). This demonstrates increased accumulation of sterically stabilized liposomes in tumor present in the left hind leg of the mice compared to the conventional liposomes and free drug.

11.3.4 DNA AND ITS LIPOSOMES

We investigated the effect of PEG on the liposome encapsulated DNA on the biodistribution to various organs especially to blood, liver, spleen, lung, kidney, and heart. The particle size was maintained in a narrow range so as to have long blood circulation. Reports suggest that the high level of liposomes in the blood was only observed for small liposomes ($d \leq 200$ nm) (Klibanov et al., 1991). The use of radiolabels has proven quite useful in

following the fate of liposomes *in vivo* and as diagnostic tools in nuclear medicine.

The *in vivo* data reveals that the half-life of the drug, when entrapped in liposomes was greater than the drug in its free state. This observation can be corroborated based on the value of the half lives for the liposomes and the drug in both rabbits and mice (2.54h & 3.55h for DNA, 8.55h & 11.22h for DL, 13.45h & 17.77h for SDL5000, 14.23h & 21.14h for SDL2000 and 16.13h & 23.49h for CSDL2000) as shown in table 11.14 & 11.41). Estimated pharmacokinetic parameters of DNA and its liposomes in rabbit and mice by two-compartment model using Quickcalc software are summarized in table 11.14 & 11.41. The area under the curve (AUC) values respectively for DNA, DL SDL5000, SDL2000 and CSDL2000 were 31.29, 202.37, 492.53, 606.98 and 808.83 (in rabbits) and 12.13, 250.39, 733.29, 1059.51 and 1227.68 (in mice) thereby showing increased bioavailability, sustained release and the prolonged circulation of the liposomes. The enhanced blood circulation of the liposomal formulations containing DNA may be attributed to the increase in bilayer rigidity by incorporation of high melting lipids and incorporation of PEG with lipids.

The biodistribution of ^{99m}Tc-DNA after 15 min, 30 min, 1 h and 4 h of intravenous injection in Balb/c mice is shown in table 11.31and table 11.32 given as the percent radioactivity per gram of organ or tissue and per whole organ or tissue. Blood was obtained by cardiac puncture, weighed and the radioactivity present in the whole blood was calculated by keeping 7.3 % of the body weight as total blood weight. The percent-injected dose/ gm and dose/whole organ in various organs of mice at different times after i.v administration of ^{99m}Tc-DL, ^{99m}Tc-SDL5000, ^{99m}Tc-SDL2000 and ^{99m}Tc-CSDL2000 after 1h, 4 h, 24 h and 48 h of intravenous injection in Balb/c mice were depicted in table 11.33 – 11.40. Various organs / tissues like lung, liver, spleen, kidney, stomach, intestine and heart were removed and analyzed for labeling content. In case of ^{99m}Tc-DNA, the radioactivity present in whole organ, 4 h of post injection were found to be as follows: blood (0.98 %), liver (2.66 %), spleen (0.20%) and kidney (0.59%).

In case of conventional liposomes (DL), liver and spleen accumulated a major portion of the administered radioactivity. This type of distribution is

normally found with the non sterically stabilized liposomes (Allen et. al., 1995). Thus conventional liposomes of DNA behaved as expected *in vivo*. There was an inverse relationship between liposome clearance by the reticuloendothelial system (RES) and a prolonged circulation time of liposomes. The biodistribution data reveals that initial rapid uptake by liver, 23.4 %, 10.8%, 9.48 and 9.49% at 1h and 16.6%, 9.26%, 8.09% and 8.9% at 4 h post injection for DL, SDL5000, SDL2000 and CSDL2000 respectively. The spleen accumulates significant radioactivity at 1 h and 4 h post injection for DL, SDL5000, SDL2000 and CSDL2000 respectively. The distribution of the sterically stabilized liposomes to liver and spleen was considerably reduced as compared to the conventional liposomes. This indicates that steric stabilization of liposomes containing DNA successfully alters the distribution pattern as compared to the conventional liposomes. Hardly any activity appeared to clear via intestine. The constancy of radioactivity in stomach also pointed to the fact that there is no *in vivo* leaching of radioactivity as free Technetium. The radioactivity in whole blood was found to be 2.89 %, 10.71 %, 16.56% and 18.41% at 24 h and 1.39 %, 4.42% , 5.89% and 7.74% at 48 h of post injection for DL, SDL5000, SDL2000 and CSDL2000 respectively, which proves the enhanced circulation of sterically stabilized liposomes in comparison to the conventional liposomes. Through out the period of experimentation, it was found that the distribution of DNA liposomes to liver, spleen, and blood varied extremely significantly ($P < 0.001$) when compared to the free DNA. Analysis of variance was carried out to compare the four liposomal formulations (DL, SDL5000, SDL2000 and CSDL2000) at 4h and 24 h post injection at a significance level of $\alpha = 0.05$ and it was found that the difference between the four liposomal formulations was extremely significant in blood (F=7703.7 and 40364), liver (F=7016.6 and 1012.8) and spleen (F=1156.0 and 199.24). The residence time of sterically stabilized liposomes in blood at 24 h was five to nine folds greater than conventional liposomes. The lipid composition, the addition of sterically stabilizing agents (mPEG5000-CC-PE and mPEG2000-CC-PE), and the particle size of the liposomes, which was maintained around 200nm, played a major role in prolonging the blood circulation in case of sterically stabilized liposomes. It

has been hypothesized that the decreased uptake of sterically stabilized liposomes by MPS was possibly due to the presence of steric barrier, which decreases the adsorption of plasma proteins (opsonins) on the surface of the liposomes. The liposomes prepared using polyethylene glycol derivative of low molecular weight (mPEG2000-CC-PE) showed higher blood concentration compared to liposomes prepared using polyethylene glycol derivatives of higher molecular weight (mPEG5000-CC-PE), which showed that the polyethylene glycol of low mol wt provides higher resistance to opsonization and also provides prolonged blood circulation. From the figure 11.9, which depicts the tissue uptake ratio of DNA and its liposomes, it was quite evident that the ratio of RES: Blood for sterically stabilized liposomes were significantly low compared to free DNA and DL. Hence these findings indicate when compared with free DNA, PEG coated liposomes, particularly showed significantly increased circulation half-life in addition to low uptake by RES. Thus the avoidance of RES and prolonged circulation time of sterically stabilized liposomes encapsulating DNA is confirmed by the biodistribution and kinetic studies.

The above observations of long circulation have been corroborated by analyzing the gamma images of rabbit after 4 h post injection with radio labeled complexes. Images are shown for rabbit at 4h post injection of free DNA and DL, SDL5000, SDL2000 and CSDL2000 respectively in figure 11.18 –11.22. There is an increasing accumulation in liver, spleen and a clear observation of the entire blood pool. We confirm our studies that in general, the presence of a PEG along with the charged lipids induces definite changes in the organ distribution of liposomes and prolonged circulation.

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