Chapter 10

In vivo studies

10.1 INTRODUCTION

The study of the *in vivo* behaviour is very critical to the success of the development of liposome formulations. The tumour targeting ability of the formulations can well be established by carrying out the experiments in tumour 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 the cancer therapy.

10.2 EXPERIMENTAL

10.2.1 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 restriction was 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 with Ehrlich ascites tumour (EAT) developed in the left thigh region were chosen for the biodistribution and tumour uptake studies. No diet restriction was enforced prior to studies. Four mice were taken for the study in each group.

10.2.2 TUMOUR IMPLANTATION

Ehrlich ascites tumour (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 tumour in the volume range of 0.9 ± 0.1 cm^3 was observed and used for further studies.

10.2.3 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).

10.2.4 BIODISTRIBUTION AND TUMOUR UPTAKE STUDIES

Biodistribution and tumour uptake of the ^{99m}Tc-labeled complex was studied in Ehrlich ascites tumour bearing mice. 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. Tissues (heart, lung, liver, spleen, kidney, stomach, intestine and tumour) 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.

10.2.5 GAMMA SCINTIGRAPHIC IMAGING

Scintigraphic study was carried out in EAT bearing mice after administration of 3.7 MBq of Tc-99m labeled complex through the tail vein of mice. 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 photograph showing the

biodistribution of MTX, TMX, CYT and their liposomal formulations in EAT bearing mice is given in Figure 10.7-10.21.

10.2.6 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 Table 10.16 and 10.32, respectively.

10.2.7 *IN VITRO* TESTING FOR DRUG RETENTION IN LIPOSOMES

The ability of the prepared liposomal formulations to retain the drug within liposomes in presence of plasma was analyzed by the following method. The liposomal suspension containing known amount of drug (MTX, TMX and CYT) was diluted with human plasma in the ratio of 1:9. 1ml 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 then centrifuged at 15,000rpm for 15min and the liposomal pellet was separated out. The amount of the drug present in the liposomal pellet was estimated by diluting with methanol as per the methods described previously in Chapter 3, Analytical methods. The supernatant was also diluted with methanol, kept for 10min, and then centrifuged at 5,000rpm for 15min. The plasma proteins were precipitated out by the addition of methanol. The amount of drug present in the methanolic layer was estimated. The amounts of the drug retained in the liposomes containing MTX, TMX and CYT are given in Tables 10.33 –10.35 respectively.

Table 10.1 Blood kinetic study of ^{99m}Tc labeled methotrexate (MTX)

Time (h)	% Injected Dose	
	Actual ± S.E.	Calculated
0.25	9.99 ± 1.26	10.38
0.5	5.25 ± 0.71	4.81
1	1.67 ± 0.23	1.78
2	1.22 ± 0.11	1.08
4	0.98 ± 0.09	0.74
6	0.42 ± 0.03	0.52
24	0.02 ± 0.01	0.02

Table 10.2 Blood kinetic study of ^{99m}Tc labeled conventional liposomes containing methotrexate (ML)

Time (h)	% Injected Dose	
	Actual ± S.E.	Calculated
0.5	22.46 ± 2.36	23.27
1	20.52 ± 2.01	20.86
2	16.21 ± 1.65	16.78
4	13.95 ± 2.31	10.92
6	6.64 ± 1.06	7.20
24	3.16 ± 0.87	3.15

Table 10.3 Blood kinetic study of ^{99m}Tc labeled sterically stabilized liposomes containing methotrexate (SML5000-CC-PE)

Time (h)	% Injected Dose	
	Actual ± S.E.	Calculated
0.5	38.53 ± 2.98	38.91
1	34.38 ± 3.10	33.71
2	27.01 ± 2.49	27.30
4	21.62 ± 1.89	21.67
6	19.16 ± 2.06	19.08
24	8.32 ± 1.06	8.32

Table 10.4 Blood kinetic study of ^{99m}Tc labeled sterically stabilized liposomes containing methotrexate (SML2000-CC-PE)

Time (h)	% Injected Dose	
	Actual ± S.E.	Calculated
0.5	36.83 ± 3.22	37.12
1	33.21 ± 2.87	33.17
2	30.62 ± 2.65	29.78
4	28.46 ± 2.91	26.78
6	22.61 ± 1.96	24.44
24	10.93 ± 1.44	10.81

Table 10.5 Blood kinetic study of ^{99m}Tc labeled sterically stabilized liposomes containing methotrexate (SML5000-IC-PE)

Time (h)	% Injected Dose	
	Actual ± S.E.	Calculated
0.5	35.24 ± 3.26	35.39
1	33.22 ± 2.44	33.56
2	30.94 ± 2.35	30.22
4	24.63 ± 1.54	24.65
6	20.22 ± 1.86	20.31
24	9.16 ± 1.62	9.15

Figure 10.1 Blood kinetic study of ^{99m}Tc labeled methotrexate and its liposomes

Error bars denote S.E.

Table 10.8 Blood kinetic study of ^{99m}Tc labeled sterically stabilized liposomes containing tamoxifen citrate (STL5000-CC-PE)

Time (h)	% Injected Dose	
	Actual ± S.E.	Calculated
0.5	36.47 ± 3.22	36.46
1	34.12 ± 2.94	34.10
2	31.28 ± 3.08	30.83
4	26.52 ± 2.66	26.84
6	22.19 ± 1.82	24.05
24	9.83 ± 1.09	9.69

Table 10.9 Blood kinetic study of ^{99m}Tc labeled sterically stabilized liposomes containing tamoxifen citrate (STL2000-CC-PE)

Time (h)	% Injected Dose	
	Actual ± S.E.	Calculated
0.5	33.63 ± 3.16	33.28
1	32.51 ± 2.95	32.19
2	29.63 ± 3.00	30.26
4	27.52 ± 2.59	27.07
6	24.13 ± 2.34	24.50
24	11.08 ± 1.16	11.05

Table 10.10 Blood kinetic study of ^{99m}Tc labeled sterically stabilized liposomes containing tamoxifen citrate (STL5000-IC-PE)

Time (h)	% Injected Dose	
	Actual ± S.E.	Calculated
0.5	37.63 ± 3.49	37.66
1	35.46 ± 2.89	35.82
2	32.19 ± 3.14	32.43
4	28.62 ± 2.64	26.74
6	21.46 ± 1.98	22.26
24	9.63 ± 1.08	9.61

Figure 10.2 Blood kinetic study of ^{99m}Tc labeled tamoxifen citrate and its liposomes

Error bars denote S.E.

Table 10.11 Blood kinetic study of ^{99m}Tc labeled cytarabine (CYT)

Time (h)	% Injected Dose	
	Actual ± S.E.	Calculated
0.25	7.60 ± 1.09	7.63
0.5	5.30 ± 0.84	5.26
1	5.25 ± 0.57	4.47
2	4.40 ± 0.37	3.58
4	2.49 ± 0.19	2.30
6	0.85 ± 0.23	1.47
24	0.03 ± 0.01	0.03

Table 10.12 Blood kinetic study of ^{99m}Tc labeled conventional liposomes containing cytarabine (CL)

Time (h)	% Injected Dose	
	Actual ± S.E.	Calculated
0.5	26.52 ± 3.04	26.52
1	23.28 ± 2.59	23.22
2	19.46 ± 2.20	20.68
4	17.63 ± 1.69	16.82
6	14.13 ± 1.24	13.69
24	2.12 ± 0.39	2.14

Table 10.13 Blood kinetic study of ^{99m}Tc labeled sterically stabilized liposomes containing cytarabine (SCL5000-CC-PE)

Time (h)	% Injected Dose	
	Actual ± S.E.	Calculated
0.5	34.29 ± 3.26	34.70
1	33.81 ± 3.13	33.21
2	30.42 ± 2.89	30.56
4	26.83 ± 2.34	26.21
6	19.56 ± 1.62	22.78
24	7.83 ± 1.02	7.75

Table 10.14 Blood kinetic study of ^{99m}Tc labeled sterically stabilized liposomes containing cytarabine (SCL2000-CC-PE)

Time (h)	% Injected Dose	
	Actual ± S.E.	Calculated
0.5	36.52 ± 3.28	36.50
1	31.63 ± 2.99	31.72
2	30.14 ± 3.10	29.39
4	27.32 ± 2.49	26.75
6	23.28 ± 2.35	24.37
24	10.63 ± 1.21	10.56

Table 10.15 Blood kinetic study of ^{99m}Tc labeled sterically stabilized liposomes containing cytarabine (SCL5000-IC-PE)

Time (h)	% Injected Dose	
	Actual ± S.E.	Calculated
0.5	36.46 ± 3.36	36.98
1	34.68 ± 3.15	33.79
2	29.46 ± 2.78	29.58
4	24.32 ± 2.26	25.04
6	22.81 ± 1.94	22.21
24	8.56 ± 1.06	8.58

Figure 10.3 Blood kinetic study of ^{99m}Tc labeled cytarabine and its liposomes

Error bars denote S.E.

Table 10.16 Pharmacokinetic parameters for the *in vivo* studies of ^{99m}Tc labeled conventional and sterically stabilized liposomes containing methotrexate, tamoxifen citrate and cytarabine in rabbits

Batch	Dose (μg)	Elimination rate K_{el} (h^{-1})	Elimination half life (h)	Vd (l)	AUC
MTX	150	0.1826	3.79	4.02	14.51
ML	150	0.1105	6.27	3.85	113.98
SML5000-CC-PE	150	0.0454	15.25	2.16	573.34
SML2000-CC-PE	150	0.0453	15.29	2.21	716.02
SML5000-IC-PE	150	0.0556	12.47	2.68	274.59
TMX	300	0.2191	3.16	7.45	34.96
TL	300	0.0790	8.77	3.31	230.69
STL5000-CC-PE	300	0.0503	13.76	2.53	653.58
STL2000-CC-PE	300	0.0433	16.00	2.90	730.69
STL5000-IC-PE	300	0.0447	15.51	2.52	281.51
CYT	750	0.2219	3.12	4.34	27.30
CL	750	0.1031	6.72	2.26	251.04
SCL5000-CC-PE	750	0.0573	12.09	2.75	554.98
SCL2000-CC-PE	750	0.0465	14.91	1.72	700.87
SCL5000-IC-PE	750	0.0526	13.17	2.42	590.31

Table 10.17 Biodistribution of ^{99m}Tc - MTX in EAT bearing mice

Organ / Tissue	% Injected dose per whole organ / tissue (S.E.)			
	15min	30min	1h	4h
Blood	4.53 (0.23)	2.77 (0.12)	2.18 (0.05)	1.60 (0.05)
Heart	0.06 (0.01)	0.06 (0.01)	0.04 (0.01)	0.03 (0.01)
Lung	1.26 (0.03)	0.75 (0.11)	0.41 (0.07)	0.59 (0.10)
Liver	11.52 (0.58)	11.20 (0.38)	10.85 (0.94)	9.16 (0.15)
Spleen	2.09 (0.08)	2.12 (0.11)	1.58 (0.12)	1.33 (0.08)
Kidney	0.56 (0.03)	0.39 (0.04)	0.38 (0.08)	0.75 (0.06)
Intestine	0.51 (0.03)	0.62 (0.06)	0.57 (0.04)	0.48 (0.12)
Stomach	0.05 (0.01)	0.06 (0.02)	0.05 (0.01)	0.10 (0.01)
Tumour	0.01 (0.01)	0.04 (0.01)	0.12 (0.03)	0.17 (0.02)

Table 10.18 Biodistribution of ^{99m}Tc - ML in EAT bearing mice

Organ / Tissue	% Injected dose per whole organ / tissue (S.E.)			
	1h	4h	24h	48h
Blood	19.56 (0.21)	12.76 (0.25)	3.90 (0.07)	1.01 (0.09)
Heart	0.07 (0.01)	0.04 (0.02)	0.03 (0.03)	0.01 (0.01)
Lung	0.79 (0.06)	0.55 (0.05)	0.26 (0.02)	0.03 (0.01)
Liver	11.44 (0.16)	13.90 (0.34)	14.1 (0.13)	3.48 (0.23)
Spleen	1.68 (0.06)	1.21 (0.07)	0.81 (0.11)	0.50 (0.01)
Kidney	0.90 (0.01)	0.76 (.01)	0.70 (0.03)	0.68 (0.02)
Intestine	0.74 (0.22)	0.63 (0.06)	1.66 (0.41)	1.48 (0.24)
Stomach	0.11 (0.01)	0.13 (0.01)	0.17 (0.04)	0.16 (0.01)
Tumour	0.17 (0.02)	0.35 (0.03)	1.82 (0.11)	0.96 (0.12)

Table 10.19 Biodistribution of ^{99m}Tc - SML5000-CC-PE in EAT bearing mice

Organ / Tissue	% Injected dose per whole organ / tissue (S.E.)			
	1h	4h	24h	48h
Blood	30.47 (1.01)	23.7 (1.51)	10.89 (0.18)	3.62 (0.05)
Heart	0.06 (0.01)	0.05 (0.04)	0.03 (0.01)	0.02 (0.01)
Lung	0.65 (0.06)	0.47 (0.11)	0.26 (0.06)	0.02 (0.01)
Liver	7.12 (0.61)	9.05 (0.21)	11.70 (0.37)	9.54 (0.13)
Spleen	1.08 (0.06)	0.77 (0.08)	0.62 (0.02)	0.35 (0.03)
Kidney	0.79 (0.02)	0.64 (0.06)	0.24 (0.04)	0.35 (0.08)
Intestine	0.60 (0.3)	0.44 (0.04)	0.37 (0.03)	0.54 (0.09)
Stomach	0.18 (0.01)	0.17 (0.02)	0.21 (0.01)	0.26 (0.01)
Tumour	0.21 (0.01)	0.56 (0.05)	4.11 (0.15)	1.98 (0.16)

Table 10.20 Biodistribution of ^{99m}Tc - SML2000-CC-PE in EAT bearing mice

Organ / Tissue	% Injected dose per whole organ / tissue (S.E.)			
	1h	4h	24h	48h
Blood	35.81 (0.24)	25.29 (0.51)	10.93 (0.40)	5.03 (0.26)
Heart	0.05 (0.02)	0.03 (0.02)	0.04 (0.02)	0.01 (0.01)
Lung	0.51 (0.14)	0.34 (0.02)	0.12 (0.03)	0.12 (0.02)
Liver	6.48 (0.44)	9.20 (0.31)	11.82 (0.32)	8.84 (0.12)
Spleen	0.92 (0.08)	0.76 (0.11)	0.73 (0.01)	0.49 (0.07)
Kidney	1.23 (0.07)	1.47 (0.03)	0.87 (0.07)	0.87 (0.01)
Intestine	0.52 (0.18)	0.48 (0.16)	0.60 (0.08)	0.24 (0.06)
Stomach	0.17 (0.01)	0.22 (0.01)	0.20 (0.02)	0.25 (0.01)
Tumour	0.21 (0.03)	0.99 (0.12)	4.43 (0.12)	2.06 (0.08)

Table 10.21 Biodistribution of ^{99m}Tc - SML5000-IC-PE in EAT bearing mice

Organ / Tissue	% Injected dose per whole organ / tissue (S.E.)			
	1h	4h	24h	48h
Blood	24.5 (0.49)	17.86 (0.16)	10.34 (0.26)	2.53 (0.11)
Heart	0.12 (0.01)	0.10 (0.02)	0.08 (0.03)	0.03 (0.02)
Lung	0.61 (0.02)	0.40 (0.03)	0.20 (0.03)	0.17 (0.01)
Liver	6.08 (0.17)	9.35 (0.14)	7.79 (0.18)	6.16 (0.21)
Spleen	1.04 (0.05)	0.63 (0.03)	0.68 (0.06)	0.67 (0.02)
Kidney	1.12 (0.01)	0.99 (0.03)	0.62 (0.01)	0.64 (0.03)
Intestine	0.58 (0.02)	0.73 (0.15)	0.69 (0.11)	0.52 (0.09)
Stomach	0.08 (0.01)	0.12 (0.01)	0.15 (0.02)	0.28 (0.01)
Tumour	0.27 (0.02)	1.08 (0.16)	4.09 (0.11)	1.82 (0.12)

Figure 10.4 Tissue uptake ratio of various organs of the ^{99m}Tc labeled methotrexate and its liposomes

Table 10.22 Biodistribution of ^{99m}Tc - TMX in EAT bearing mice

Organ / Tissue	% Injected dose per whole organ / tissue (S.E.)			
	15min	**30min**	**1h**	**4h**
Blood	4.30 (0.37)	3.73 (0.18)	3.15 (0.21)	1.67 (0.09)
Heart	0.02 (0.01)	0.08 (0.01)	0.08 (0.02)	0.06 (0.02)
Lung	0.85 (0.03)	0.52 (0.04)	0.27 (0.04)	0.13 (0.01)
Liver	9.80 (0.09)	8.92 (0.19)	10.66 (0.39)	6.14 (0.28)
Spleen	1.30 (0.06)	1.17 (0.08)	1.14 (0.04)	0.81 (0.06)
Kidney	0.85 (0.05)	0.64 (0.01)	0.60 (0.01)	0.49 (0.02)
Intestine	0.62 (0.03)	0.46 (0.03)	0.45 (0.09)	0.38 (0.05)
Stomach	0.05 (0.01)	0.06 (0.01)	0.08 (0.01)	0.09 (0.01)
Tumour	0.08 (0.02)	0.09 (0.03)	0.11 (0.02)	0.16 (0.03)

Table 10.23 Biodistribution of ^{99m}Tc - TL in EAT bearing mice

Organ / Tissue	% Injected dose per whole organ / tissue (S.E.)			
	1h	**4h**	**24h**	**48h**
Blood	15.85 (0.62)	11.66 (0.24)	3.26 (0.19)	1.43 (0.03)
Heart	0.15 (0.02)	0.12 (0.02)	0.03 (0.01)	0.01 (0.01)
Lung	0.69 (0.03)	0.50 (0.01)	0.16 (0.02)	0.02 (0.01)
Liver	9.56 (0.24)	13.46 (0.21)	10.75 (0.13)	4.37 (0.18)
Spleen	1.48 (0.08)	1.39 (0.05)	1.11 (0.07)	0.26 (0.05)
Kidney	0.65 (0.01)	1.70 (0.06)	1.17 (0.04)	0.41 (0.02)
Intestine	0.93 (0.11)	0.78 (0.21)	0.82 (0.10)	0.92 (0.16)
Stomach	0.15 (0.03)	0.16 (0.01)	0.20 (0.01)	0.28 (0.19)
Tumour	0.18 (0.03)	0.32 (0.04)	1.36 (0.12)	0.88 (0.19)

Table 10.24 Biodistribution of ^{99m}Tc - STL5000-CC-PE in EAT bearing mice

Organ / Tissue	% Injected dose per whole organ / tissue (S.E.)			
	1h	4h	24h	48h
Blood	30.23 (0.27)	23.83 (0.22)	15.77 (0.19)	5.38 (0.09)
Heart	0.08 (0.01)	0.08 (0.03)	0.04 (0.01)	0.01 (0.02)
Lung	0.42 (0.01)	0.41 (0.02)	0.21 (0.01)	0.01 (0.005)
Liver	6.12 (0.13)	8.34 (0.10)	7.36 (0.29)	6.85 (0.23)
Spleen	1.02 (0.08)	1.22 (0.02)	0.93 (0.04)	0.53 (0.02)
Kidney	1.45 (0.01)	1.38 (0.02)	0.80 (0.03)	0.37 (0.02)
Intestine	1.21 (0.16)	0.72 (0.12)	0.92 (0.14)	0.31 (0.03)
Stomach	0.05 (0.01)	0.05 (0.01)	0.12 (0.01)	0.17 (0.03)
Tumour	0.36 (0.04)	0.78 (0.11)	4.82 (0.13)	2.53 (0.14)

Table 10.25 Biodistribution of ^{99m}Tc - STL2000-CC-PE in EAT bearing mice

Organ / Tissue	% Injected dose per whole organ / tissue (S.E.)			
	1h	4h	24h	48h
Blood	36.58 (0.33)	29.92 (0.32)	22.04 (0.29)	6.46 (0.29)
Heart	0.13 (0.02)	0.10 (0.02)	0.10 (0.02)	0.03 (0.01)
Lung	0.55 (0.06)	0.41 (0.04)	0.31 (0.02)	0.15 (0.02)
Liver	5.69 (0.20)	7.13 (0.12)	7.05 (0.12)	5.46 (0.10)
Spleen	0.92 (0.03)	1.01 (0.02)	0.62 (0.01)	0.42 (0.01)
Kidney	1.44 (0.01)	0.94 (0.01)	0.74 (0.01)	0.52 (0.03)
Intestine	1.14 (0.24)	0.98 (0.19)	0.63 (0.05)	0.43 (0.09)
Stomach	0.05 (0.01)	0.07 (0.01)	0.12 (0.01)	0.17 (0.02)
Tumour	0.17 (0.02)	0.85 (0.08)	5.02 (0.06)	2.88 (0.05)

Table 10.26 Biodistribution of ^{99m}Tc - STL5000-IC-PE in EAT bearing mice

Organ / Tissue	% Injected dose per whole organ / tissue (S.E.)			
	1h	4h	24h	48h
Blood	29.24 (0.26)	22.18 (0.40)	14.52 (0.23)	5.92 (0.10)
Heart	0.09 (0.01)	0.08 (0.02)	0.06 (0.01)	0.03 (0.01)
Lung	0.66 (0.02)	0.44 (0.05)	0.19 (0.02)	0.15 (0.03)
Liver	6.52 (0.23)	7.22 (0.13)	6.98 (0.25)	5.66 (0.21)
Spleen	0.88 (0.08)	1.13 (0.05)	1.09 (0.11)	0.90 (0.02)
Kidney	0.97 (0.02)	0.95 (0.02)	0.75 (0.01)	0.67 (0.01)
Intestine	0.86 (0.12)	0.89 (0.05)	0.60 (0.05)	0.70 (0.02)
Stomach	0.05 (0.01)	0.12 (0.02)	0.21 (0.02)	0.28 (0.01)
Tumour	0.17 (0.01)	0.91 (0.06)	4.02 (0.09)	1.92 (0.03)

Figure 10.5 Tissue uptake ratio of various organs of the ^{99m}Tc labeled tamoxifen citrate and its liposomes

Table 10.27 Biodistribution of ^{99m}Tc - CYT in EAT bearing mice

Organ / Tissue	% Injected dose per whole organ / tissue (S.E.)			
	15min	**30min**	**1h**	**4h**
Blood	3.57 (0.16)	2.98 (0.24)	2.49 (0.14)	0.95 (0.09)
Heart	0.05 (0.01)	0.04 (0.01)	0.04 (0.01)	0.03 (0.01)
Lung	0.27 (0.01)	0.14 (0.01)	0.14 (0.02)	0.12 (0.01)
Liver	4.33 (0.37)	7.24 (0.34)	8.44 (0.39)	5.13 (0.38)
Spleen	0.48 (0.02)	0.87 (0.01)	1.29 (0.05)	0.83 (0.02)
Kidney	1.06 (0.02)	0.86 (0.02)	0.63 (0.04)	0.58 (0.01)
Intestine	0.30 (0.06)	0.46 (0.07)	0.52 (0.18)	0.47 (0.03)
Stomach	0.06 (0.01)	0.04 (0.01)	0.03 (0.01)	0.03 (0.01)
Tumour	-	0.02 (0.01)	0.12 (0.07)	0.18 (0.06)

Table 10.28 Biodistribution of ^{99m}Tc - CL in EAT bearing mice

Organ / Tissue	% Injected dose per whole organ / tissue (S.E.)			
	1h	**4h**	**24h**	**48h**
Blood	14.27 (1.13)	9.41 (1.06)	4.25 (0.2)	2.25 (0.08)
Heart	0.15 (0.01)	0.12 (0.01)	0.07 (0.01)	0.04 (0.01)
Lung	0.73 (0.02)	0.50 (0.01)	0.27 (0.05)	0.12 (0.01)
Liver	14.05 (0.25)	13.01 (1.09)	11.16 (0.23)	4.46 (0.21)
Spleen	2.13 (0.03)	1.83 (0.02)	1.51 (0.02)	0.92 (0.01)
Kidney	1.13 (0.02)	1.01 (0.03)	0.75 (0.01)	0.68 (0.02)
Intestine	0.93 (0.24)	0.72 (0.18)	0.58 (0.06)	0.63 (0.24)
Stomach	0.05 (0.02)	0.08 (0.01)	0.16 (0.01)	0.22 (0.02)
Tumour	0.13 (0.02)	0.52 (0.02)	1.52 (0.18)	0.98 (0.06)

Table 10.29 Biodistribution of ^{99m}Tc - SCL5000-CC-PE in EAT bearing mice

Organ / Tissue	% Injected dose per whole organ / tissue (S.E.)			
	1h	4h	24h	48h
Blood	27.34 (0.12)	19.82 (0.10)	9.74 (0.34)	5.12 (0.09)
Heart	0.09 (0.01)	0.08 (0.01)	0.06 (0.02)	0.04 (0.01)
Lung	0.80 (0.03)	0.52 (0.01)	0.18 (0.01)	0.04 (0.01)
Liver	5.69 (0.23)	6.88 (0.17)	8.72 (0.11)	7.66 (0.32)
Spleen	1.20 (0.02)	1.02 (0.02)	0.95 (0.05)	0.84 (0.01)
Kidney	0.61 (0.05)	0.84 (0.06)	0.47 (0.01)	0.39 (0.02)
Intestine	0.81 (0.11)	0.92 (0.18)	0.92 (0.08)	0.68 (0.15)
Stomach	0.11 (0.01)	0.14 (0.01)	0.26 (0.02)	0.25 (0.01)
Tumour	0.23 (0.11)	1.62 (0.14)	4.18 (0.11)	1.91 (0.07)

Table 10.30 Biodistribution of ^{99m}Tc - SCL2000-CC-PE in EAT bearing mice

Organ / Tissue	% Injected dose per whole organ / tissue (S.E.)			
	1h	4h	24h	48h
Blood	31.53 (0.47)	24.21 (0.18)	13.78 (0.18)	6.82 (0.07)
Heart	0.08 (0.04)	0.08 (0.01)	0.05 (0.01)	0.02 (0.01)
Lung	0.65 (0.02)	0.45 (0.01)	0.21 (0.06)	0.14 (0.03)
Liver	6.02 (0.31)	7.44 (0.23)	7. 25 (0.12)	6.11 (0.11)
Spleen	0.89 (0.02)	0.97 (0.03)	0.78 (0.01)	0.62 (0.02)
Kidney	0.56 (0.03)	1.01 (0.05)	0.82 (0.08)	0.35 (0.03)
Intestine	0.42 (0.11)	0.86 (0.18)	0.84 (0.11)	0.38 (0.03)
Stomach	0.08 (0.01)	0.11 (0.01)	0.18 (0.12)	0.25 (0.01)
Tumour	0.13 (0.01)	1.04 (0.05)	5.13 (0.08)	2.11 (0.02)

Table 10.31 Biodistribution of ^{99m}Tc - SCL5000-IC-PE in EAT bearing mice

Organ / Tissue	% Injected dose per whole organ / tissue (S.E.)			
	1h	4h	24h	48h
Blood	27.21 (2.24)	22.34 (2.38)	12.49 (1.29)	6.11 (0.15)
Heart	0.12 (0.02)	0.11 (0.01)	0.09 (0.02)	0.05 (0.04)
Lung	0.52 (0.03)	0.42 (0.03)	0.27 (0.03)	015 (0.01)
Liver	6.20(0.80)	7.64 (1.02)	8.14 (0.41)	7.62 (0.36)
Spleen	0.94 (0.02)	1.13 (0.03)	1.01 (0.03)	1.06 (0.04)
Kidney	0.96 (0.02)	0.81 (0.06)	0.70 (0.03)	0.53 (0.04)
Intestine	0.96 (0.14)	0.93 (0.10)	0.76 (0.06)	1.11 (0.14)
Stomach	0.07 (0.02)	0.11 (0.01)	0.18 (0.02)	0.19 (0.02)
Tumour	0.23 (0.03)	0.72 (0.02)	4.62 (0.13)	1.96 (0.07)

Figure 10.6 Tissue uptake ratio of various organs of the ^{99m}Tc labeled cytarabine and its liposomes

Table 10.32 Pharmacokinetic parameters for the *in vivo* studies of ^{99m}Tc labeled conventional and sterically stabilized liposomes containing methotrexate, tamoxifen citrate and cytarabine in mice

Batch	Dose (μg)	Elimination rate K_{el} (h^{-1})	Elimination half life (h)	Vd (l)	AUC
MTX	50	0.1318	5.26	1.00	26.55
ML	50	0.0576	12.03	1.00	304.87
SML5000-CC-PE	50	0.0439	15.78	1.00	694.46
SML2000-CC-PE	50	0.0323	21.44	2.38	785.91
SML5000-IC-PE	50	0.0291	23.77	4.49	789.19
TMX	100	0.2053	3.38	18.59	18.97
TL	100	0.0343	20.18	5.64	276.45
STL5000-CC-PE	100	0.0207	33.42	3.53	1442.33
STL2000-CC-PE	100	0.0133	52.28	2.89	2796.41
STL5000-IC-PE	100	0.0155	44.66	3.72	2145.13
CYT	250	0.321	2.16	16.01	11.05
CL	250	0.0323	21.47	1.00	342.24
SCL5000-CC-PE	250	0.0268	25.87	3.14	728.63
SCL2000-CC-PE	250	0.0288	24.06	0.93	976.14
SCL5000-IC-PE	250	0.0291	23.83	3.29	861.90

Table 10.33 *In vitro* testing for drug retention within liposomes containing methotrexate in human plasma

Time in Hrs	% Drug Retained (S.E.)			
	ML	SML5000-CC-PE	SML2000-CC-PE	SML5000-IC-PE
6	94.5 (1.14)	95.6 (2.06)	94.6 (2.32)	95.1 (2.19)
12	81.9 (1.45)	83.6 (1.48)	80.2 (1.46)	83.9 (1.64)
24	67.2 (1.22)	72.3 (1.66)	69.4 (2.10)	70.4 (1.71)
48	39.4 (1.06)	49.3 (1.52)	51.2 (0.94)	47.5 (1.17)

Table 10.34 *In vitro* testing for drug retention within liposomes containing tamoxifen citrate in human plasma

Time in Hrs	% Drug Retained (S.E.)			
	TL	STL5000-CC-PE	STL2000-CC-PE	STL5000-IC-PE
6	96.2 (1.64)	97.1 (1.54)	96.4 (1.22)	96.1 (1.93)
12	84.5 (1.16)	85.2 (1.33)	82.1 (1.29)	83.5 (1.20)
24	69.8 (1.64)	73.3 (1.49)	69.1 (1.17)	72.1 (1.21)
48	51.4 (1.60)	54.2 (1.39)	55.3 (1.25)	53.5 (1.45)

Table 10.35 *In vitro* testing for drug retention within liposomes containing cytarabine in human plasma

Time in Hrs	% Drug Retained (S.E.)			
	CL	SCL5000-CC-PE	SCL2000-CC-PE	SCL5000-IC-PE
6	83.2 (1.34)	86.4 (1.75)	82.6 (1.50)	85.6 (1.23)
12	67.9 (1.95)	71.2 (1.52)	72.0 (2.03)	73.2 (2.10)
24	54.2 (1.16)	53.1 (1.29)	49.2 (1.94)	51.2 (1.27)
48	32.4 (1.49)	30.4 (1.46)	29.7 (2.04)	31.9 (1.56)

Figure 10.7 Gamma scintigraphic image of ^{99m}Tc - MTX injected mouse bearing EAT in the left thigh.

Figure 10.8 Gamma scintigraphic image of ^{99m}Tc - ML injected mouse bearing EAT in the left thigh.

Figure 10.9 Gamma scintigraphic image of ^{99m}Tc - SML5000-CC-PE injected mouse bearing EAT in the left thigh.

Figure 10.10 Gamma scintigraphic image of ^{99m}Tc - SML2000-CC-PE injected mouse bearing EAT in the left thigh.

Figure 10.11 Gamma scintigraphic image of ^{99m}Tc - SML5000-IC-PE injected mouse bearing EAT in the left thigh.

Figure 10.12 Gamma scintigraphic image of ^{99m}Tc - TMX injected mouse bearing EAT in the left thigh.

Figure 10.13 Gamma scintigraphic image of ^{99m}Tc - TL injected mouse bearing EAT in the left thigh.

Figure10.14 Gamma scintigraphic image of ^{99m}Tc - STL5000-CC-PE injected mouse bearing EAT in the left thigh.

Figure 10.15 Gamma scintigraphic image of ^{99m}Tc - STL2000-CC-PE injected mouse bearing EAT in the left thigh.

Figure 10.16 Gamma scintigraphic image of ^{99m}Tc - STL5000-IC-PE injected mouse bearing EAT in the left thigh.

Figure 10.17 Gamma scintigraphic image of ^{99m}Tc - CYT injected mouse bearing EAT in the left thigh.

Figure 10.18 Gamma scintigraphic image of ^{99m}Tc - CL injected mouse bearing EAT in the left thigh.

Figure 10.19 Gamma scintigraphic image of ^{99m}Tc - SCL5000-CC-PE injected mouse bearing EAT in the left thigh.

Figure 10.20 Gamma scintigraphic image of ^{99m}Tc - SCL2000-CC-PE injected mouse bearing EAT in the left thigh.

Figure 10.21 Gamma scintigraphic image of ^{99m}Tc - SCL5000-IC-PE injected mouse bearing EAT in the left thigh.

10.3 RESULTS AND DISCUSSION

10.3.1 GENERAL METHODOLOGY

Sterically stabilized liposomes of methotrexate, tamoxifen citrate and cytarabine were prepared with the aim of increasing blood circulation time and thereby increasing the tumour targetability. It was envisaged that this type of drug delivery would lead to drug action only at the sites required and would also lead to significantly reduced side effects. Thus, an increase in therapeutic efficacy of these drugs would be achieved.

In order to ascertain whether these objectives have been achieved, *in vivo* testing of the prepared liposomes was performed by administration of an appropriate volume of the liposomes labeled with technetium-99m. The particle size was maintained in a narrow range so as to have long blood circulation (between 200-400nm). Reports suggest that the high level of liposomes in the blood was only observed for small liposomes ($d \leq 200$ nm); larger liposomes ($d > 200$ nm) showed high level of accumulation in spleen and low concentration in blood (Klibanov *et. al.,* 1991). The small liposomes ($d < 100$ nm) might be expected to penetrate through the fenestrations and gain access to hepatocytes, because the endothelial lining of the liver sinusoids includes fenestrae with an average diameter of 100 nm (Litzinger *et. al.,* 1994; Wisse, 1970). The ^{99m}Tc labelled 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 treated to analysis using Quickcalc software to get an idea of the blood kinetic profile of the drug and the liposome formulations in rabbits. The Tables 10.1 – 10.15 tabulate the raw data obtained from the experiment and the calculated value from the analysis. Table 10.16 gives the pharmacokinetic parameters describing the *in vivo* behaviour of the prepared liposomes generated by the analysis of the data. Figure 10.1 – 10.3 shows the comparison of the blood kinetics pattern of MTX, TMX and CYT and their liposomal formulations, respectively.

An appropriate volume of the radiolabeled complexes was injected into the tail vein of the mice bearing ehrlich ascites tumour (EAT) to determine the biodistribution pattern and the tumour targetability of the 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 and tumour) were dissected out, washed in saline, dried using filter paper and the amount of labelled complexes present in each tissue was determined using gamma counter. 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 10.17 – 10.31 tabulate the biodistribution data of the drug/liposomal formulations. The percent injected dose of the drugs and its liposomal formulations present in the whole organ/tissue is shown in the Tables 10.17 –10.31. Table 10.32 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. Figures 10.4 – 10.6 shows the comparison of the tissue uptake ratio of various organs of MTX, TMX and CYT and their liposomal formulations, respectively.

Scintigraphic studies were carried out in EAT bearing mice after administration of an appropriate volume of the radiolabeled complexes through the tail vein. 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 Figures 10.7 –10.21.

10.3.2 METHOTREXATE AND ITS LIPOSOMES

Examination of the *in vivo* data for free drug and the liposomes containing MTX 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 (3.79h & 5.26h for MTX, 12.03h & 6.27h for ML, 15.78h & 15.25h for SML5000-CC-PE, 21.44h & 15.29h for SML2000-CC-PE and 23.77h & 12.47h for SML5000-IC-PE, Tables 10.16 & 10.32).

Estimated pharmacokinetic parameters of MTX and its liposomes in rabbit and mice by two-compartment model using Quickcalc software are summarized in Tables 10.16 & 10.32. The area under the curve (AUC) values respectively for ML, SML5000-CC-PE, SML2000-CC-PE and SML5000-IC-PE were 7.86, 39.51, 49.35 & 19.06 (in rabbits) and 11.48,

26.16, 19.60 & 29.72 (in mice) times greater than free drug showing increased bioavailability, which may be ascribed to the sustained release and the prolonged circulation of the liposomes. The reverse was observed for elimination rate constant (K_{el}) and volume of distribution (V_d).

The biodistribution studies of free drug (MTX) were carried out in mice for a period of 4h, as the drug was eliminated from the blood circulation and untraceable amount of drug was found after 4h. The amount of drug accumulated at the tumour site after 4h was found to be as less as 0.17% (Table 10.17).

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). Thus conventional liposomes (ML) of methotrexate behaved as expected *in vivo.* As reported previously (Gabizon and Papahadjopoulos, 1988), 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 tumours.

Examination of the results of *in vivo* investigations of liposomes containing methotrexate sterically stabilized using polyethylene glycol derivates showed extremely encouraging results with high elimination half-lives and high bioavailability (Tables 10.16 & 10.32). This is in conjunction with reports, which acknowledge the superiority of methoxy polyethylene glycol over the other agents for steric stabilization of liposomes (Section 2.3.5, Literature review). The sterically stabilized liposomes were present in the blood for an extended period of time as compared to the corresponding conventional liposomes (3.9% for ML compared to 10.89%, 10.93% and 10.34% for SML5000-CC-PE, SML2000-CC-PE and SML5000-IC-PE respectively after 24h of post injection, Tables 10.18-10.21). 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 methotrexate successfully alters the distribution pattern as compared to the conventional liposomes.

After 24h, the sterically stabilized liposomes achieved about 3 times more concentration in circulation as that of conventional methotrexate liposomes (ML). The presence of very low amount of radioactivity in stomach after 24h proved the *in vivo* stability of the radiolabeled complexes (^{99m}Tc-ML / SML5000-CC-PE / SML2000-CC-PE / SML5000-IC-PE). As the time increases, the accumulation of liposomes in tumour was found to be increasing until 24h and the concentration in tumour was decreased at 48h. The tumour accumulation after 24h for ML, SML5000-CC-PE, SML2000-CC-PE and SML5000-IC-PE was found to be 1.82%, 4.11%, 4.43% and 4.09% respectively. The increase in accumulation in tumour site was also due to the increase in blood circulation time. Through out the period of experimentation, it was found that the distribution of sterically stabilized methotrexate liposomes to liver, spleen, tumour and blood varied extremely significantly ($P<0.001$) when compared to conventional methotrexate liposomes (ML). The lipid composition, the addition of sterically stabilizing agents (mPEG5000-CC-PE, mPEG2000-CC-PE and mPEG5000-IC-PE), and the particle size of the liposomes played a major role in tumour accumulation in case of sterically stabilized liposomes. It has been postulated 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 derivative of higher molecular weight (mPEG5000-CC-PE), which showed that the polyethylene glycol of low mol wt provides equally, or little higher resistance to opsonization and also provides prolonged blood circulation. The results obtained were in agreement with the previous report (Allen and Hansen, 1991). From the Figure 10.4, which depicts the tissue uptake ratio of methotrexate and its liposomes, it was quite evident that the ratio of both Blood: RES as well as Tumour: RES for sterically stabilized liposomes were significantly high compared to MTX and ML. Thus the avoidance of RES and prolonged circulation time thereby increased accumulation in tumour site of sterically stabilized liposomes of methotrexate is proved. The observation

was supported by analysing the γ scintigraphic images of the EAT bearing mice (Figures 10.7 – 10.11). After 24 h of post injection of ^{99m}Tc labeled liposomal formulations, the γimages were taken (gamma image was taken after 4h of post injection in case of ^{99m}Tc-MTX). This demonstrates increased accumulation of sterically stabilized liposomes in tumour present in the left hind leg of the mice compared to the conventional liposomes and free drug.

10.3.3 TAMOXIFEN CITRATE AND ITS LIPOSOMES

Examination of the *in vivo* data for free drug and the liposomes containing TMX 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 (3.16h & 3.38h for TMX, 8.77h & 20.18h for TL, 13.76h & 33.42h for STL5000-CC-PE, 16.00h & 52.28h for STL2000-CC-PE and 15.51h & 44.66h for STL5000-IC-PE, Tables 10.16 & 10.32).

The pharmacokinetic parameters of TMX and its liposomes in rabbit and mice were estimated by two-compartment model using Quickcalc software and are summarized in Table 10.16 & 10.32. The area under the curve (AUC) values respectively for TL, STL5000-CC-PE, STL2000-CC-PE and STL5000-IC-PE were 6.60, 18.70, 20.90, 8.05 (in rabbits) and 14.57, 76.03, 147.41 & 113.08 (in mice) times greater than free drug showing increased bioavailability, which may be ascribed to the sustained release and the prolonged circulation of the liposomes. The reverse was observed for elimination rate constant (K_{el}) and volume of distribution (V_d). The difference in the biodistribution pattern of TMX and liposomes in rabbit and mice may be due to the species variation.

The biodistribution studies of free drug (TMX) were carried out in mice for a period of 4h, as the drug was eliminated from the blood circulation and untraceable amount of drug was found after 4h. The amount of drug accumulated in tumour site after 4h of post injection was found to be 0.16%, which is very less compared to the liposomes (Table 10.22).

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). Thus conventional liposomes (TL) of tamoxifen citrate 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 tumours (Gabizon and Papahadjopoulos, 1988).

Examination of the results of *in vivo* investigations of liposomes containing tamoxifen citrate sterically stabilized using polyethylene glycol derivates showed extremely encouraging results with high elimination half-lives and high bioavailability (Tables 10.16 & 10.32). This is in conjunction with reports, which recognize the advantage of methoxy polyethylene glycol over the other agents for steric stabilization of liposomes (Section 2.3.5, Literature review). The sterically stabilized liposomes were present in the blood for an extended period of time as compared to the corresponding conventional liposomes (3.26% for TL compared to 15.77%, 22.04% and 14.52% for STL5000-CC-PE, STL2000-CC-PE and STL5000-IC-PE respectively after 24h of post injection, Tables 10.23-10.26). 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 tamoxifen citrate successfully alters the distribution pattern as compared to the conventional liposomes.

After 24h, the sterically stabilized liposomes achieved about 5-7 times more concentration in circulation as that of conventional tamoxifen citrate liposomes (TL). The presence of very low amount of radioactivity in stomach after 24h proved the *in vivo* stability of the radiolabeled complexes (^{99m}Tc-TL / STL5000-CC-PE / STL2000-CC-PE / STL5000-IC-PE). As the time increases, the accumulation of liposomes in tumour was found to be increasing until 24h and the concentration in tumour was decreased at 48h. The tumour accumulation after 24h for TL, STL5000-CC-PE, STL2000-CC-PE and STL5000-IC-PE was found to be 1.36 %, 4.82 %, 5.02 and 4.02 respectively. The increase in accumulation in tumour site was also due to the increase in blood circulation time. Through out the period of

experimentation, it was found that the distribution of sterically stabilized tamoxifen citrate liposomes to liver, spleen, tumour and blood varied extremely significantly ($P<0.001$) when compared to conventional tamoxifen citrate liposomes (TL). The lipid composition, the addition of sterically stabilizing agents (mPEG5000-CC-PE, mPEG2000-CC-PE and mPEG5000-IC-PE), and the particle size of the liposomes, which was maintained around 200nm, played a major role in tumour 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 & mPEG5000-IC-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 10.5, which depicts the tissue uptake ratio of tamoxifen citrate and its liposomes, it was apparent that the ratio of both Blood: RES as well as Tumour: RES for sterically stabilized liposomes were significantly high compared to TMX and TL. Thus the avoidance of RES and prolonged circulation time thereby increased accumulation in tumour site of sterically stabilized liposomes of tamoxifen citrate is confirmed.

The observations of the biodistribution study were supported by analysing the γ scintigraphic images of the EAT bearing mice (Figures 10.12 –10.16). After 24 h of post injection of ^{99m}Tc labelled liposomal formulations, the γ images were taken (gamma image was taken after 4h of post injection in case of ^{99m}Tc-TMX). This demonstrates increased accumulation of sterically stabilized liposomes in tumour present in the left hind leg of the mice compared to the conventional liposomes and free drug.

10.3.4 CYTARABINE AND ITS LIPOSOMES

Investigation of the *in vivo* data for free drug and the liposomes containing CYT 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 (3.12h & 2.16h for CYT, 6.72h & 21.47h for CL, 12.09h & 25.87h for SCL5000-CC-PE, 14.91h & 24.06h for SCL2000-CC-PE and 13.17h & 23.83h for SCL5000-IC-PE, Tables 10.16 & 10.32).

The pharmacokinetic parameters of CYT and its liposomes in rabbit and mice were estimated by two-compartment model using Quickcalc software and are summarized in Tables 10.16 & 10.32. The area under the curve (AUC) values respectively for CL, SCL5000-CC-PE, SCL2000-CC-PE and SCL5000-IC-PE were 9.20, 20.33, 25.67, 21.62 (in rabbits) and 30.97, 65.94, 88.34 & 78.00 (in mice) times greater than free drug showing increased bioavailability, which may be ascribed to the sustained release and the prolonged circulation of the liposomes. The reverse was observed for elimination rate constant (K_{el}) and volume of distribution (V_d). The difference in the biodistribution pattern of CYT and liposomes in rabbit and mice may be due to the species variation.

The biodistribution studies of free drug (CYT) were carried out in mice for a period of 4h, as the drug was eliminated from the blood circulation and untraceable amount of drug was found after 4h. The amount of drug accumulated in tumour site after 4h of post injection was found to be 0.18%, which is very less compared to the liposomes (Table 10.27).

Biodistribution of the conventional liposomes of cytarabine (CL) revealed that these liposomes were predominantly distributed into the liver and spleen. 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 tumours (Gabizon and Papahadjopoulos, 1988).

Examination of the results of *in vivo* investigations of liposomes containing cytarabine sterically stabilized using polyethylene glycol derivates showed extremely encouraging results with high elimination half-lives and high bioavailability (Tables 10.16 & 10.32). This is in concurrence with reports, which recognize the advantage of methoxy polyethylene glycol over the other agents for steric stabilization of liposomes (Section 2.3.5, Literature review).

The sterically stabilized liposomes were present in the blood for an extended period of time as compared to the corresponding conventional liposomes (4.25% for CL compared to 9.74%, 13.78% and 12.49% for SCL5000-CC-PE, SCL2000-CC-PE and SCL5000-IC-PE respectively after 24h of post injection, Tables 10.28-10.31). The distribution of the sterically stabilized liposomes to liver and spleen was considerably reduced as compared to the conventional liposomes, which indicates that steric stabilization of liposomes containing cytarabine successfully alters the distribution pattern as compared to the conventional liposomes.

The presence of very low amount of radioactivity in stomach after 24 h proved the *in vivo* stability of the radiolabeled complexes (^{99m}Tc-CL / SCL5000-CC-PE / SCL2000-CC-PE / SCL5000-IC-PE). As the time increases, the accumulation of liposomes in tumour was found to be increasing until 24h and the concentration in tumour was decreased at 48h. The tumour accumulation after 24h for CL, SCL5000-CC-PE, SCL2000-CC-PE and SCL5000-IC-PE was found to be 1.52 %, 4.18 %, 5.13% and 4.62% respectively. The increase in accumulation in tumour site was also due to the increase in blood circulation time. Through out the period of experimentation, it was found that the distribution of sterically stabilized cytarabine liposomes to liver, spleen, tumour and blood varied extremely significantly ($P<0.001$) when compared to conventional cytarabine liposomes (CL). The lipid composition, the addition of sterically stabilizing agents (mPEG5000-CC-PE, mPEG2000-CC-PE and mPEG5000-IC-PE), and the particle size of the liposomes, which was maintained around 200nm, played a major role in tumour accumulation in case of sterically stabilized liposomes. It has been conjectured 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 enhanced blood circulation compared to liposomes prepared using polyethylene glycol derivatives of higher molecular weight (mPEG5000-CC-PE & mPEG5000-IC-PE). This demonstrates that the polyethylene glycol of low mol wt provides higher resistance to opsonization and also provides

prolonged blood circulation. From the Figure 10.6, which depicts the tissue uptake ratio of cytarabine and its liposomes, it was apparent that the ratio of both Blood: RES as well as Tumour: RES for sterically stabilized liposomes were significantly high compared to free drug (CYT) and conventional liposomes containing cytarabine (CL). Thus the avoidance of RES and prolonged circulation time thereby increased accumulation in tumour site of sterically stabilized liposomes of cytarabine is proved.

The observations of biodistribution study were supported by analysing the γ scintigraphic images of the EAT bearing mice (Figures 10.17 – 10.21). After 24 h of post injection of ^{99m}Tc labelled liposomal formulations, the γ images were taken (gamma image was taken after 4h of post injection in case of ^{99m}Tc-CYT). This demonstrates increased accumulation of sterically stabilized liposomes containing cytarabine in tumour present in the left hind leg of the mice compared to the conventional liposomes containing cytarabine and free drug.

In order to prove the stability of the liposomes to retain the drug inside the bilayer in presence of plasma, the liposomes containing the drugs (MTX, TMX and CYT) were incubated with human plasma at 37°C. The analysis of the liposomal pellets for the drugs after the specific time points using methanol as per the methods described in Chapter 3, Analytical methods, showed that the release of the drugs from the liposomes was increasing gradually. The liposomes retained around 70% of drug in case of methotrexate and tamoxifen citrate liposomes and around 50% of drug in case of cytarabine (Tables 10.33 - 10.35). The reason for faster release of drug from the liposomes containing cytarabine could be due to the higher aqueous solubility of cytarabine compared to methotrexate and tamoxifen citrate.

10.3.5 CONCLUSIONS

The rapid elimination of free drugs and the conventional liposomes containing the anticancer drugs proved their inability to remain in the circulation for a longer period of time thereby increase the tumour accumulation. All the agents used in these studies are capable of imparting steric stabilization to the liposomes. These studies are preliminary

investigations showing the potential of the agents used for steric stabilization of liposomes containing anticancer drugs. Further studies need to be performed in order to investigate the effect of particle size and increased bilayer rigidity on the *in vivo* performance of these liposomes. The enhanced circulation time and the increased tumour accumulation of the sterically stabilized liposomes containing anticancer drugs will greatly help to fight the dreadful disease, cancer.

HANSA MEHTA LIBRARY

10.4 REFERENCES

Allen TM and Hansen C. Pharmacokinetics of stealth vs. conventional liposomes: Effect of dose. Biochim. Biophys. Acta. 1991; 1068: 133-141.

Allen TM, Hansen C, Lopez de Menezes D. Pharmacokinetics of long circulating liposomes. Adv. Drug Deliv Rev. 1995; 16: 267-284.

Gabizon A, Papahadjopoulos D. Liposome formulations with prolonged circulation time in blood and enhanced uptake by tumours. Proc. Natl. Acad. Sci USA 1988; 85: 6949-6953.

Klibanov AL, Maruyama K, Beckerleg AM, Torchilin VP, Huag L. Activity of amphipathic poly (ethylene glycol) 5000 to prolong the circulation time of liposomes depends on the liposome size and is unfavourable for immuno liposome binding to target. Biochim Biophys Acta 1991; 1062: 142-148.

Litzinger DC, Buiting AMJ, Rooijen NV, Huag L. Effect of liposome size on the circulation time and intra organ distribution of amphipathic poly (ethylene glycol)- containing liposomes. Biochim Biophys Acta 1994; 1190: 99-107.

Wisse E. An electron microscopic study of the fenestrated endothelial lining of rat liver sinusoids. J Ultrastruc. Res. 1970; 31: 125-150.

Wu, MS, Robbins JC, Ponpipom MM, Shen, TV. Modified *in vivo* behaviour of liposomes containing synthetic glycolipids. Biochim Biophys Acta. 1981; 674: 19-29.