Chapter 10

Pharmacokinetics and Biodistribution Studies

Pharmacokinetic and biodistribution studies of iv administered flutamide liposomes for possible reduction in hepatotoxicity, **Online Journal of Pharmacokinetics**, 2005, Vol 3, 1-15.

And

Development of new parenteral formulation of 6-Mercaptopurine entrapped stealth liposomes for improvement of therapeutic efficacy in leukemia and reduction of hepatotoxicity and nephrotoxicity, **Cancer Investigation** (Under communication)

10.1 INTRODUCTION

The study of the in vivo behavior is very critical to the success of the development of novel drug delivery systems. Studies of the pharmacokinetics of the new formulation in suitable animal models during the later stages of development are crucial to attain the desired product performance. This is particularly true in case of sterically stabilized liposomes and microbubbles 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

Wistar-Albino Rats of both sex weighing 200-300 gm obtained from Zydus Research Center, Gujarat, India. The animals were housed in departmental animal house under natural light conditions, fed a standard pellet diet and water ad libidium. Their care and handling were in accordance with the provisions of Social Justice and Empowerment Committee as recognized and adopted by the Ministry of Government of India, New Delhi. Institute's Animal Ethics Committee approved the project. No diet restriction was enforced prior to studies. Three rats were taken for the study in each group.

10.2.2 Pharmacokinetic studies

10.2.2.1 Pharmacokinetic studies of pure flutamide and its formulations

Pharmacokinetic studies were performed as described elsewhere (332) using either sex albino wistar rats. The animals were treated with either conventional, stealth liposomes or AALs and compared with the free drug solution (drug dissolved in mixture of 0.9%w/v of NaCl-ethanol-PEG-200 [2/0.18/3.82, v/v/v]) (333). The different formulations were injected through the tail vein at the FLT dose of 25

mg/kg in rat (groups of three animals per group). The tail vein was exposed to ultrasound for 30 sec using 0.5 MHz ultrasound transducer to burst microbubbles (AALs) in blood vessels. Blood samples were taken from the retro-orbital plexus at various time intervals (15 min, 30 min, 1, 2, 4, 6, 8, 12, 24 and 36 hr) in centrifuge tube containing 0.1 ml of 3.3 % w/v of sodium citrate solution as an anti-coagulant and centrifuged immediately at 5000 RPM for 10 min to separate plasma. The plasma samples were spiked with internal standard (6-MP), diluted to 5 ml with methanol, vortexed (10 min) and centrifuged at 8000 RPM for 20 min. Supernatant was collected and 20 µl was injected in to HPLC column as described above. One compartmental analysis was used to calculate the pharmacokinetic parameters from mean plasma concentration-time data. Pharmacokinetic parameters were calculated using the software Winlonlin® (Version 3.0, Pharsight Corporation Ltd., USA). Elimination, distribution and disposition were represented by the following parameters: Area under curve (AUC); mean residence time (MRT); total body clearance (Clt); volume of distribution at steady state (Vss); elimination half life ($t_{1/2}$).

10.2.2.2 Pharmacokinetic studies of pure 6-Mercaptopurine and its formulations

Plasma levels of 6-MP, its liposomal and microbubble formulations were examined in albino-wistar rats of both sexes weighing 200 to 300 gm. The animals were treated with intravenous injection of conventional, stealth liposomes or AALs and compared with the free drug solution of 6-MP (5 mg of 6-MP dissolved in 5 ml of mixture of polyethylene glycol-400, ethanol, dimethyl sulphoxide and 0.9% w/v saline, 0.75:0.75:0.15:3.35, v/v/v/v) via tail vein at dose of 5 mg/kg. The tail vein of rat was exposed to ultrasound for 30 sec using 0.5 MHz ultrasound transducer to burst the microbubbles traveling in blood vessels. At various time intervals after drug administration (30 min, 1, 2, 4, 6, 8, 12, 24, and 36 h) animals were anesthetized by diethyl ether and blood samples (0.5 ml) were collected from retro orbital plexus in centrifuge tube containing 0.1 ml of 3.3 % w/v of sodium citrate solution as an anti-coagulant and centrifuged immediately at 5000 RPM for 10 min to separate plasma and stored at –20°C until analysis. 0.2 ml of the plasma samples were deproteinised with methanol and acetonitrile mixture (2 ml, 1:1,v/v), vortexed for 5 min, centrifuged at 6000 RPM for 15 min, supernatants were collected and assayed as described above. One compartmental analysis was used to calculate the pharmacokinetic parameters from mean plasma concentration-time data. The area

under curve between the first and last sampling times (AUC), Cmax, Tmax and total body clearance (Clt) were calculated by QUICKCALC® Software. The elimination half-life ($T_{1/2}$) was estimated by linear regression analysis of the terminal phase of the plasma concentration-time profile. Volume of distribution at steady-state (Vss), AUC_{∞}(infinity), $AUMC_{\infty}$, and mean residence time (MRT) calculated from standard formulas (334).

10.2.3 Biodistribution studies

10.2.3.1 Biodistribution studies of flutamide and its formulations

Conventional, stealth liposomes or microbubble dispersion (AALs) equivalent to 25 mg of FLT/kg was injected through tail vein to rats. The respective organs of rat were exposed to 0.5 MHz ultrasound frequency from ultrasound transducer to burst the microbubbles in respective organ. The rats were sacrificed and organs (liver, prostate, heart, lung, kidney, and spleen) were collected at different time intervals (30 min, 4, 8 and 24 hr) after i.v injection of all formulations. The organs were washed twice with 0.9 % w/v of NaCl, wiped and weighed. Approximately 500 mg or less depending on total weight of organ slices were excised, minced, homogenized with 10 ml of methanol in a tissue homogenizer (Ultra-Turrax, T25, Germany) and centrifuged immediately at 8000 RPM for 20 min. The supernatant was transferred and evaporated to dryness. The residues were reconstituted with 1 ml of mobile phase and 20 µl of sample was injected in to HPLC column as described above.

10.2.3.2 Biodistribution studies of 6-Mercaptopurine and its formulations

Either conventional or stealth liposomes and microbubble dispersion equivalent to 5 mg of 6-MP/kg was injected through tail vein to rats and tissue distribution parameters were compared with that of the same dose of free drug. The animals were anaesthetized, sacrificed (3/ time point) after different time intervals (1, 4, 8, and 24 hr) and different tissues (lung, liver, kidney, heart and spleen) were collected. These time points were selected with the aim to identifying peak tissue concentrations as well as to measure the rapid elimination of 6-MP. The organs were washed twice with 0.9% w/v saline, wiped and the weight of each tissue was recorded. Approximately 500 mg of organ slices or less were excised, minced, homogenized with 5 ml of methanol and centrifuged at 6000 RPM for 20 min. The drug content

was determined in supernatant by HPLC method. AUC, Cmax and Tmax of 6-MP in each organ for all formulations were calculated using NCSS® Software.

10.3 RESULTS AND DISCUSSION

10.3.1 Pharmacokinetic studies of pure flutamide and its liposomal formulations

With novel drug delivery systems, drug release mechanisms often remain undefined but circulation profile and drug pharmacokinetics are often comparable (335,336). Figure 10.1 shows the plasma concentration-time profile of FLT after i.v injection of free drug, CL, SL and AALs in a dose equivalent to 25 mg/kg/day of FLT in rats which indicated that there was a rapid elimination of the free drug from the blood circulation and only 3.6 % of initial drug level per ml in blood was remaining after 4 hr (Table 10.1). The plasma concentration of FLT after 24 hr was significantly high in case of SL (1.908 µg/ml) and AALs (0.764 µg/ml) as compared to CL (0.129µg/ml) (Table 10.1).

Table 10.1 Mean plasma concentration of flutamide after i.v injection of free drug, CL and SL containing 25 mg/kg/day of dose to rats

Time in hr	Plasma concentration in µg/ml (Mean ± SD)			
	FLT-AALs	FLT-SL	FLT-CL	Pure FLT
0.25	9.626±1.143	11.33±1.16	5.434± 0.78	5.066± 0.634
0.5	8.243±1.013	8.26±1.64	3.746± 0.324	4.68 ±0.36
1	7.826±0.949	7.92± 1.28	3.55 ±0.128	3.12± 0.74
2	6.791±0.803	6.18±0.92	2.043 ±0.723	1.74 ±0.16
4	5.442±0.633	5.088± 0.76	1.459± 0.48	0.331± 0.12
6	4.561±0.552	3.166± 0.179	0.843 ±0.268	0.18± 0.092
8	3.602±0.432	2.477± 0.62	0.640± 0.109	0.014± 0.002
12	2.863±0.272	2.108± 0.463	0.268± 0.126	-
24	1.209±0.258	1.908 ±0.124	0.129± 0.078	-
36	0.764±0.246	0.94 ±0.28	-	-

The pharmacokinetic parameters of free drug, CL, SL and AALs are represented in Table 10.2. There was marked increase in Cmax of FLT in blood following of the administration of SL (15.54 µg/ ml) relative to AALs (8.89 µg/ml), CL (7.88 µg/ml) and free drug (5.48 µg/ml).

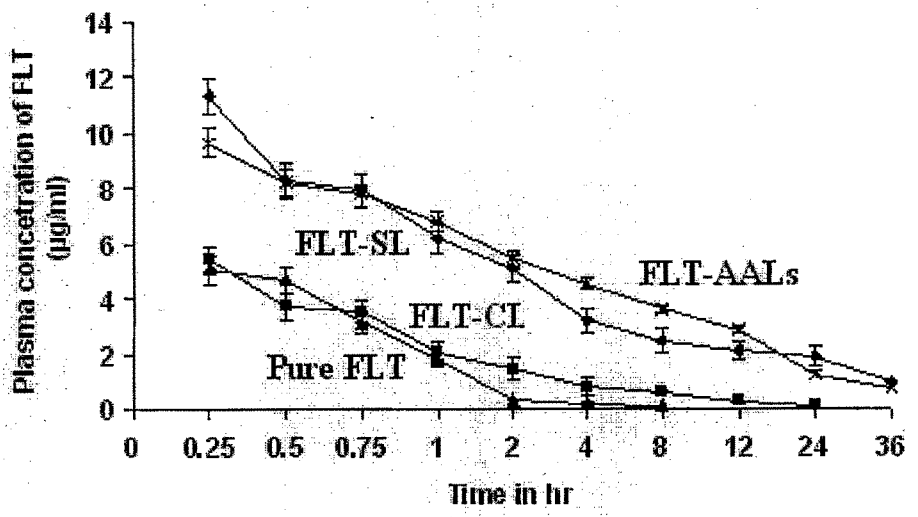


Figure 10.1: plasma concentration-time profile of FLT after i.v injection of free drug (triangle), CL (rectangle), SL (round) and AALs (cross)

Table 10.2: One-compartmental pharmacokinetic parameters of FLT after i.v administration of free drug, CL, SL and AALs containing 25 mg/kg/day of dose to rats*

Pharmacokinetic parameters	FLT-AALs	FLT-SL	FLT-CL	Pure FLT
Cmax (µg/ml)	8.89	15.54	7.88	5.48
AUC (µg h ml^{-1})	83.09	85.12	16.41	6.08
AUC∞ (µg h ml^{-1})	166.17	108.72	16.85	6.08
AUMC∞ (µg h^2 ml^{-1})	1324.64	2536.19	122.42	6.20
MRT (hr)	7.97	23.33	7.27	1.02
Clt (l h^{-1} kg^{-1})	0.298	0.05	0.30	0.82
Vss (l kg^{-1})	2.36	1.15	2.78	1.51
$t_{1/2}$ (hr)	6.47	17.40	6.50	1.28
Kel (/hr)	0.107	0.04	0.11	0.54

* The values are arithmetic means of three experiments (n=3 animals per group). Standard Deviations are less than 5% (Not reported)

It indicated that the SL showed much longer circulation time with half-life of about 17.4 hr after i.v administration comparatively, CL was distributed to the tissue in few short times and was cleared from circulation with in 24 hr. Although AALs showed

short less elimination half-life of 7.97 hr, it remained in blood circulation for 36 hr. As reported previously, there was an inverse relationship between liposome clearance by the RES and prolonged circulation time of liposomes, which may reduce side effects associated with RES system (337). The denser hydrophobic core yields vesicles that will (338) retain their content in plasma for longer time (339). The increase in circulation time may also be due to the addition of $mPEG_{2000}$-CC-PE in stealth liposomes and AALs or particle size less than 200 nm. It is reported that bare liposomes circulate for only about few hrs half-life in rats. With small amounts of mPEG-2000 (7-10%), liposome circulation half-life can be extended to about 10-15 hr (340,341). The AUC of SL and AALs was 85.12 (μg h ml^{-1}) 83.09 (μg h ml^{-1}), which was comparatively higher than CL (16.41 μg h ml^{-1}) and free drug (6.08 μg h ml^{-1}), respectively. The mean clearance value of FLT was 3, 3 and 16 folds greater than AALs, CL and SL, respectively. It, therefore, appears that the longer half-life of SL and a pronounced increase in the blood residence time was the results of a reduced clearance rate.

10.3.2 Pharmacokinetic studies of pure 6-Mercaptopurine and its liposomal formulations

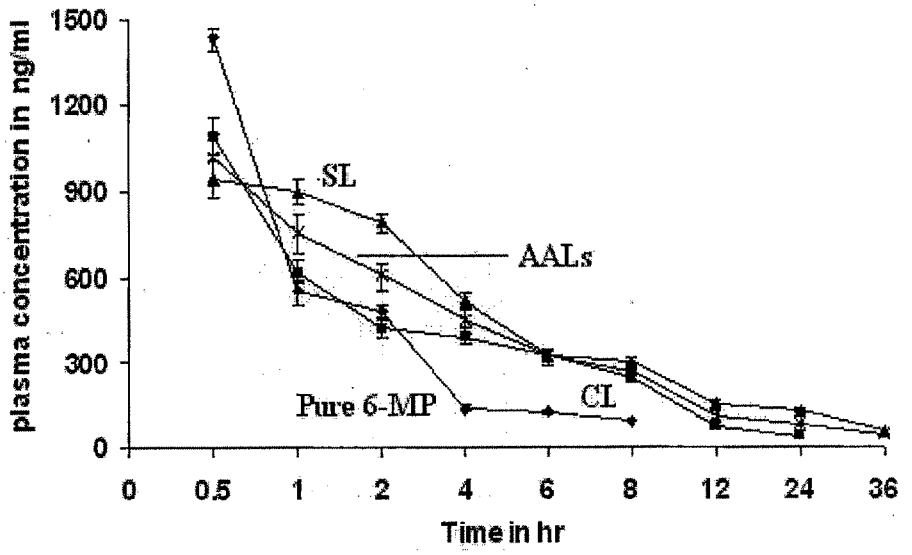


Figure 10.2: plasma concentration-time profile of 6-MP after i.v injection of free drug (round), CL (rectangle), SL (triangle) and AALs (cross)

The free drug, CL, SL and AALs containing 6-MP were injected in to rat tail vein and resultant plasma concentration-time profile of 6-MP at 5 mg/kg of dose showed in Figure 10.2. The administration of 6-MP orally showed Cmax of 158.1±27.6 ng/ml and AUC_{0-4} hr of 147.4±24.3 hr*ng/ml (342). This limited bioavailability is the result of first-pass hepatic metabolism and variable absorption and presystemic metabolism of the drug by the intestine in to the inactive metabolite 6-thiouric acid (343) so the importance of optimizing 6-MP therapy and achieving high systemic drug exposure, it has encouraged the use of parenteral (i.v) 6-MP in patients with acute lymphoblastic leukemia. There was marked increase in Cmax of 6-MP (10 folds than oral dose) 1437±74.3 ng/ml in blood after intravenous administration of 6-MP of the same dose was found (Table 10.3).

Table 10.3 Mean plasma concentration of 6-MP after i.v injection of free drug, CL, SL and AALs containing 5 mg/kg/day of dose to rats

Time	Plasma concentration in ng/ml (Mean ± SD)			
in hr	Pure 6-MP	6-MP-CL	6-MP-SL	6-MP-AALs
0.5	1437.27±110.32	1088.67±86.92	943.75±93.47	1016.21±123.35
1	552.75±32.21	616.35±68.61	899.24±63.43	757.80±93.31
2	482.13±39.65	422.34±49.65	791.57±44.47	606.96±74.35
4	134.30±15.61	390.71±28.32	517.32±30.48	454.01±53.02
6	121.75±10.64	322.43±29.32	321.46±28.15	321.94±54.01
8	89.55±9.34	242.98±19.34	299.48±18.17	271.23±34.02
12	-	73.36±16.3	151.53±17.49	112.45±31.04
24	-	36.69±9.31	125.44±10.45	81.06±24
36	-	-	52.39±9.35	40.87±27.04

Table 10.3 indicates that there was a rapid elimination of the free drug from the blood circulation and only 16 % of initial drug level per ml in blood was remaining after 8 hr. The limitation of short biological half-life of parenteral form of 6-MP resulted in an inconveniently high dosing frequency. To prolong the biological half-life of the drug and to reduce the frequency of the dose, drug was encapsulated in liposomes and pharmacokinetic parameters of free drug compared with conventional liposomes and stealth liposomes (Table 10.4).

Table 10.4: One-compartmental pharmacokinetic parameters of 6-MP after i.v administration of free drug, CL, SL and AALs containing 5 mg/kg/day of dose to rats*

Pharmacokinetic parameters	Pure 6-MP	6-MP-CL	6-MP-SL	6-MP- AALs
AUC (hr*µg/ml)	2.181±0.151	4.196±0.457	6.275±0.826	5.240.13±0.84
Cmax (µg/ml)	1.437±0.214	1.088±0.147	0.943±0.079	1.016±0.14
Kel hr^{-1}	1.0135	0.2305	0.1668	0.1907
AUC∞ (hr*µg/ml)	4.383	8.402	12.552	10.478
AUMC∞(h^2*µg/ml)	7.519	31.54	93.536	68.332
MRT (hr)	1.715	3.75	7.45	6.52
Elimination $t_{1/2}$ (hr)	0.6844	3	4.15	3.634
Vss ml/kg	0.394	0.0429	0.00729	0.0114
Total body Clearance (ml/hr/kg)	0.023	0.012	0.0008	0.001

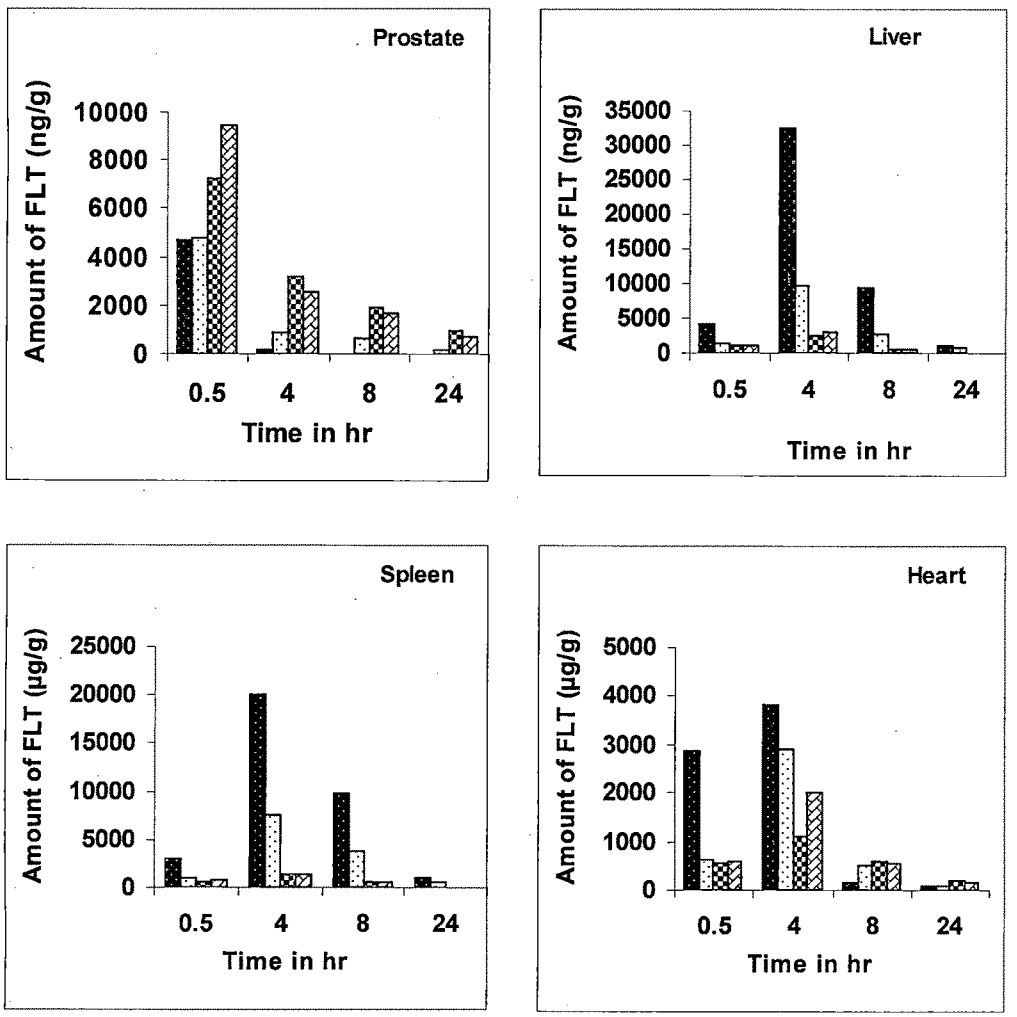
* The values are arithmetic means of three experiments (n=3 animals per group). Standard Deviations are less than 5% (Not reported).

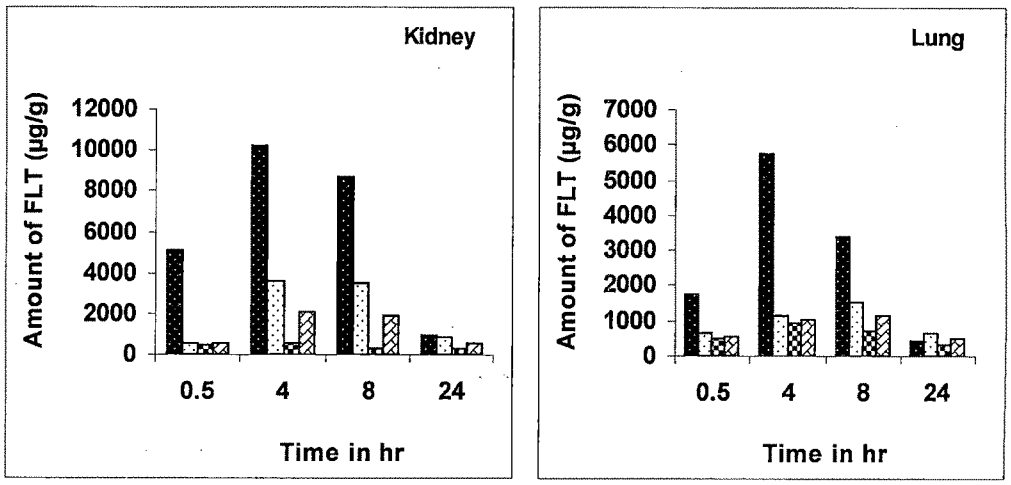
SL and AALs showed Cmax of 943±39 ng/ml and 1.016±0.14 µg/ml as well as AUC of 6.275±0.826 hr*µg/ml and 5.240.13±0.84 hr*µg/ml. The plasma concentration of 6-MP after i.v administration of SL (125.44±10.45 ng/ml) and AALs (81.06±24 ng/ml) was significantly high even after 24 hr as compared to CL (36.69±9.31ng/ml). It indicated that the SL and AALs showed much longer circulation time with elimination half-life of about 4.15 hr and 3.634 hr, respectively after i.v administration. Comparatively, CL were distributed to the tissue in few short times and cleared from circulation with in 24 hr. Although the antileukemic and cytotoxic effects of 6-MP have been related to the generation of intracellular nucleotides derived from 6-MP rather than to plasma 6-MP concentration (344-346). After first-pass metabolism through the liver, the remaining 6-MP is taken up by blood cells from plasma and converted by two major pathways into its active metabolites, therefore, higher plasma concentration of 6-MP is advantageous to generate more intracellular nucleotides and to achieve better antileukemic effects. The mean clearance value of 6-MP was 2,29, and 23 folds greater than CL, AALs and SL respectively. It, therefore, appears that the longer half-life of SL and AALs was the result of a reduced clearance rate. High Cmax, long circulating capacity in blood and biological half-life may help SL and AALs to increase therapeutic efficacy of 6-MP and to reduce amount as well as frequency of the dose.

10.3.3 Biodistribution studies of flutamide, its liposomes and microbubble formulations

Hepatotoxicity is the limitation of therapeutic potential of FLT (347). In this study, it was proven that SL and AALs were shown fewer uptakes by RES (liver and spleen) as compared to CL and free drug as well as exhibited longer half-life. To facilitate a comprehensive analysis of liposomes, microbubbles and free drug, the distribution of FLT in different organs at different time intervals after intravenous injections of free drug, CL, SL and AALs are shown in Figure 10.3.

Figure 10.3: The concentration of FLT in various organs after i.v administration of free drug (black), CL (white), SL (lined) and AALs (cross line) of 25 mg/kg of dose.





Standard deviations were below 5% of the mean values (Not reported). Histograms represent the arithmetic means of three determinations (n=3 animals per group).

It indicated that the distributions of SL and AALs in liver, spleen and prostate were significantly different form those of CL and free drug (p>0.05). It showed that FLT was more concentrated in liver and spleen as compared to other organs in case of free drug than that of CL, SL and AALs. RES also continued to accumulate liposomes and AALs with in 4 hr and then after the biodistribution of SL and AALs showed considerable decrease in drug uptake by liver in comparison to CL and free drug.

The ratio of uptake between free drug: CL: SL: AALs was found to be (1: 0.298: 0.07: 0.09) at 4 hr, (1: 0.297: 0.05: 0.07) at 8 hr and (1: 0.766: 0.106: 0.03) at 24 hr in liver and was at (1:0.375:0.06: 0.07) 4hr, (1:0.39:0.05: 0.06) at 8 hr and (1: 0.479: 0.081: 0.08) at 24 hr in case of spleen. It has been postulated that the decreased uptake of SL and AALs by RES is possibly due to the presence of steric barrier, which decreases the adsorption of plasma proteins (opsonins) on the surface of the SL and AALs (348). Initial uptake of SL and AALs in liver, together with increase in AUC and the decreased in Cl_t (Table 10.2) suggest rapid uptake of SL and AALs by the liver may be due to extravasation to interstitial spaces that will re-enter in to blood stream with initial decrease of the blood level (349). In contrast, CL was accumulated intensely (9.06 µg/g) in liver and (7.51 µg/g) in spleen, though the size of liposomes was only 150 nm. It indicated that SL and AALs could remain in blood for prolonged time and reduce the uptake of liposomes to RES and thereby reduce the possibility of the risk of toxicity to RES generally seen with free FLT. The

localization of FLT in lung is less as compared to other organs and may be due to endocytosis mechanism in all formulation (350).

10.3.4 Biodistribution studies of 6-MP and its liposomal formulations

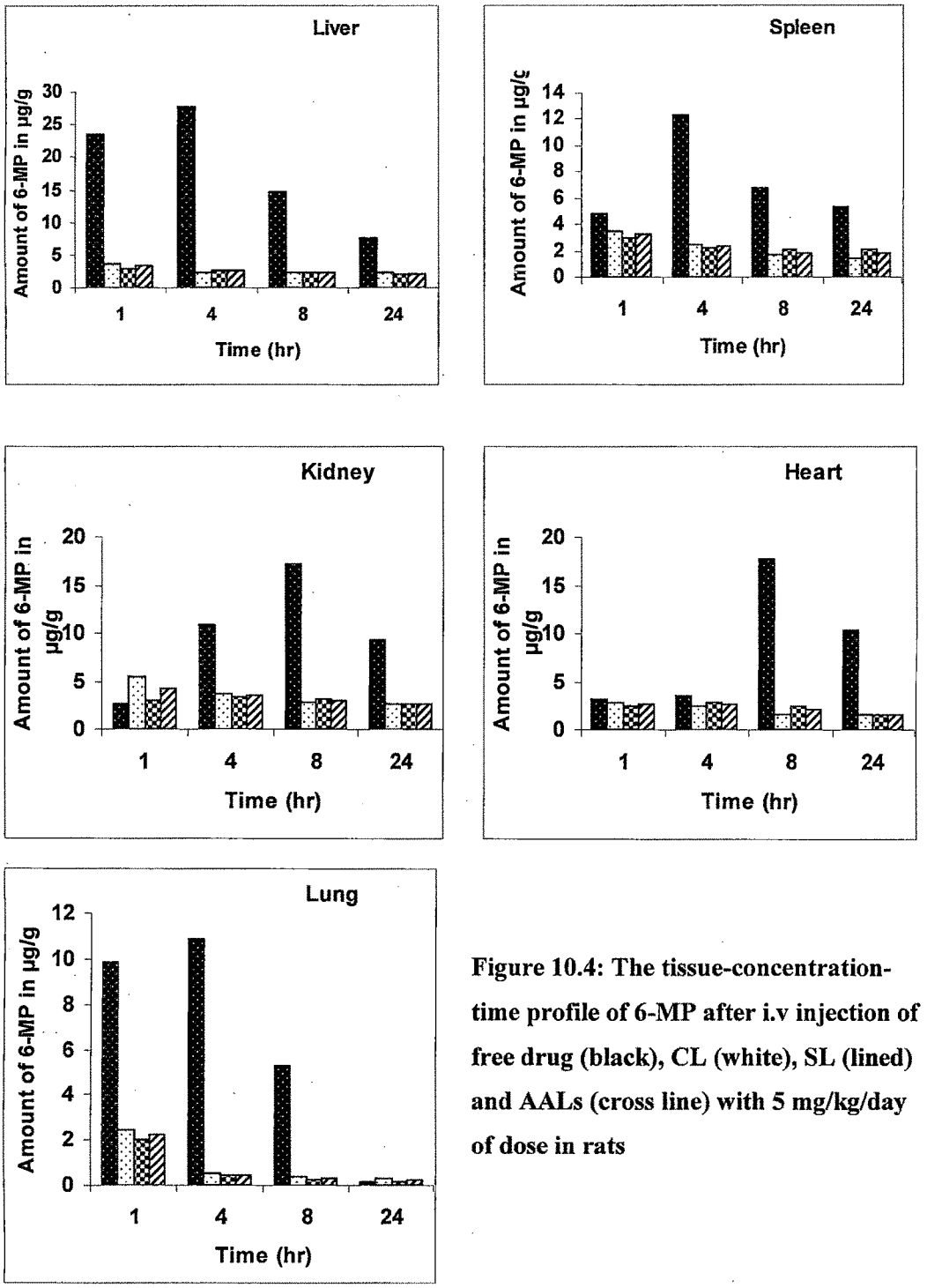


Figure 10.4: The tissue-concentration-time profile of 6-MP after i.v injection of free drug (black), CL (white), SL (lined) and AALs (cross line) with 5 mg/kg/day of dose in rats

To reduce the side effects associated with 6-MP mainly hepatotoxicity, vesicular formulations are thought to be useful where it is necessary to compare the in vivo tissue distribution (amount of 6-MP present in each organs) of free drug with CL, SL and AALs after administration of 5 mg/kg dose of 6-MP intravenously (Figure 10.4). For CL, 6-MP was rapidly taken up to considerable extent by the liver and spleen. It also showed that there was considerable decrease in 6-MP uptake in the reticulo endothelial system (RES)-containing organs (liver and spleen) found after 1 hr of i.v injection of SL and AALs.

Table 10.5: Tissue distribution parameters of different 6-MP formulations in rats at dose of 5 mg/kg* (n=3)

Biodistribution parameters	Liver	Heart	Kidney	Lung	Spleen
6-MP					
AUC_{0-24} (hr*μg/ml)	338.44±21.3	237.45±18.45	257.95±17.62	107.04±10.6	160.19±13.81
Cmax (μg/ml)	27.55±5.7	13.72±3.9	14.13±3.62	10.90±2.84	12.28±3.05
Tmax (hr)	4	8	8	4	4
Conventional liposomes					
AUC_{0-24} (hr*μg/ml)	56.55±9.64	42.29±7.88	71.07±10.2	11.55±2.36	41.8±8.21
Cmax (μg/ml)	3.76±0.45	2.79±0.33	5.37±0.38	2.45±0.12	3.45±0.24
Tmax (hr)	1	1	1	1	1
Stealth liposomes					
AUC_{0-24} (hr*μg/ml)	53.59±8.89	50.49±9.4	68.6±11.2	7.93±1.26	49.97±5.66
Cmax (μg/ml)	2.91±0.23	2.74±0.21	3.41±0.18	2.00±0.11	2.94±0.16
Tmax (hr)	1	4	4	1	1
AALs					
AUC_{0-24} (hr*μg/ml)	54.97±7.62	46.29±6.89	69.84±11.24	9.705±0.24	45.86±7.54
Cmax (μg/ml)	3.33±0.15	2.64±0.18	4.19±0.26	2.23±0.12	3.2±0.21
Tmax (hr)	1	4	1	1	1

*The values are arithmetic means ±SD of three experiments (n=3 animals per group)

It indicated that the distribution of SL and AALs in liver and spleen was significantly different from that of CL and free drug (p>0.05) throughout the period of experimentation. It has been postulated that the decreased uptake of SL and AALs by RES is possibly due to the presence of steric stabilizing agent, which decreases the adsorption of plasma proteins (opsonins) on the surface of the SL and AALs (351).

Table 10.5 showed that free 6-MP was more concentrated in each organ specifically in liver with Cmax of 27.55±5.7 μg/ml and AUC of 338.44±21.3 hr*μg/ml. CL were accumulated intensely (3.768μg/g) in liver and (3.45 μg/g) in spleen, though the size of liposomes was only 120 nm (Table 8.5). It indicated that SL and AALs could remain in blood for prolonged time and reduce the uptake to RES thereby reduce the possibility of the risk of toxicity to RES generally seen with free 6-MP.

10.4 CONCLUSION

In conclusion, the pharmacokinetic parameters and biodistribution in different organs of sterically stabilized liposomes are significantly different than that of conventional liposomes and the free drug. Stealth liposomes of flutamide exhibited long circulation and fewer uptakes by RES, which may help to reduce side effects associated particularly with liver and improve efficacy of the flutamide. This study indicates that liposomes containing 6-MP show different pharmacokinetics and biodistribution from free drug. Furthermore, these results shows that stealth liposomes of 6-MP may serve as suitable intravenous dosage form of 6-MP and replace oral and parenteral drug formulations which have poor bioavailability and short biological half life as well as need more frequency of dosing, respectively. Stealth liposomes may also reduce side effects related to liver and kidney associated with 6-MP.