Chapter 8

Stability Studies

8.1 INTRODUCTION

8.1.1 General

Stability testing of liquid disperse systems is one of the most difficult problems faced by formulation chemists. The first order of priority for solving stability problems of disperse systems is to define clearly the type or types of stability of concern. An understanding of the factors that lead to stability problems can help to determine which methods of testing are most likely to yield information applicable to the estimation of the product s shelf life.

Stability tests commonly stress the system to limits beyond those that the product will ever encounter. Typical examples- of stress tests include exposure of the product to high temperatures and large gravitational forces. However, for liposomes, elevated temperatures may dramatically alter the nature of the interfacial film, especially if the phase transition temperature is reached. If one expects the product to be exposed to a temperature of 45°C for an extended period of time or for short durations (shipping and warehouse storage), studies at 45-50°C (Long-term and heat-cool cycling) are quite justified. A study of a product at these temperatures determines how the product is holding up at this elevated temperature and whether the damage is reversible or irreversible when the product is brought back to room temperature. If the temperatures higher than the system will ever encounter are used, even in short term heat-cool cycling, there is a risk of irreversible damage to the bilayers so that when it is brought back to room temperature, the membrane cannot heal.

If a liposomal dispersion is partially frozen and then thawed, ice crystals nucleate and grow at the expense of water. The liposomes may then be pressed together against the ice crystals under great pressure. If the crystal grows to a size greater than the void spaces, instability is more likely. That is why a slower rate of cooling, resulting in larger ice crystals produces greater instability. Polymers may retard ice growth. These considerations, therefore, suggest that stability testing protocols should be developed for liposomal products on a case-by-case basis. One should be certain that studies are performed using all types and sizes of containers considered for packaging the product. Relevant data is then collected and analyzed so as to obtain the desired stability profile of the product under consideration

For sterically stabilized liposomes duration of steric stabilization is of paramount importance as this attribute determines *in vivo* performance of the product. Therefore, for such specialized systems, preliminary investigations should focus on

the duration of steric stabilization. Full- fledged stability studies, as mentioned above, can then be conducted on the optimized formulations.

8.1.2 Stability testing and Lyophilization

Lyophilization of liposomes is one of the most promising ways to keep the liposomes stable during long-term storage (313). The presence of cryoprotectants is necessary for protection of the membrane structure during the freeze-drying of liposomes. Disaccharides such as trehalose, and sucrose as well as mannitol have been extensively investigated as cryoprotectants of liposomes (314-317). The mechanisms of the cryoprotective effect of sugar on freeze-drying of liposomes were also investigated and two hypotheses are proposed. It is reported that in the case of fluid lipids such as egg phosphatidylcholine, or 1- palmitoyl-2-oleoylphosphatidylcholine (POPC), the damage to liposomes can be ascribed to both their fusion and phase transition from gel to liquid crystalline during the rehydration of freeze dried liposomes (316). Trehalose, a disaccharide, especially inhibits fusion and decreases the gel-to-liquid crystalline phase transition temperature of the dry lipid caused by a direct interaction between the sugar and the polar head group of the lipid (water replacement hypothesis). It is suggested that the cryoprotective effect of sugars is due to vitrified sugars, which can be ascribed to a change in the surface tension between the sugar solution and lipid (318). They indicated that the sugar that had a high glass transition temperature *(Tg)* was the most effective cryoprotectant (vitrification hypothesis).

8.2 EXPERIMENTAL

8.2.1 Reagents

Maltose, anhydrous sodium sulphate (AR grade) and sodium nitrite (LR grade) were purchased from S.D. fine Chem..Ltd., Boisar, Thane; sucrose (AR grade) was purchased from Sigma Chemical Co., St.Louis, M.O., U.S.A.

8.2.2 Apparatus

Remi cooling centrifuge C-24 (Remi Equipments, Mumbai), Shimadzu UV- 1601 UV-Visible spectrophotometer (Shimadzu corporation, Japan) and Malvern particle size analyser (Malvern Master sizer 2000 SM. U.K.).

8.2.3 Solutions

16.7% w/v solution of sucrose in distilled water was prepared by dissolving 16.7 g of sucrose in 100ml of distilled water. 0.8 and 1.6M sodium sulphate solutions in 16.7% w /v sucrose solution were prepared by dissolving the appropriate quantity of anhydrous sodium sulphate in the appropriate volume of 16.7% w/v of sucrose solution.

8.2.4 Lyophilization of FLT and 6-MP conventional and stealth liposomes

Sucrose was used for the purpose of lyophilization as a cryoprotectant. Lyophilization optimization of liposomes was performed for maximum drug retention. The use of cryoprotectants (sucrose and mannitol) during lyophilization of liposomes was evaluated and compared. Conventional liposomal suspension was centrifuged at 15, 000 RPM for 15 min after adding 50 µl of protamine and centrifuged. The total solid content present in conventional and stealth liposomes of FLT and 6-MP was calculated. The lipid: cryoprotectant ratio (1:2.5, 1:3, 1:4) was also optimized based on the drug retention capacity liposomes.

The amount of cryoprotectant and water was added in such a way that the lipid: cryoprotectant ratio would be 1:2.5, 1:3 and 1:4 and the concentration of cryoprotectant would be between 15 to 20% w/v. The prepared dispersion was taken in 5ml amber colored glass vials (USP type I glass) and the vials were stored at -70°C for 24 hr in deep freezer. The frozen vials were lyophilized in Heto Drywinner (Heto-Holten, Allerod, Denmark) for 24 hr. The lyophilized products were stored at 4°C till further use. The amount of drug retained by the liposomes following lyophilization and rehydration was determined. Table 8.1 shows the results of the drug retention in liposomes after lyophilization.

8.2.5 Stability studies of FLT and 6-MP conventional and stealth liposomes

The prepared lyophilized powder of liposomal dispersions were subjected to stability studies, in triplicate, at conditions according to ICH guidelines i.e. 2-8°C with ambient humidity and 30 ± 2°C/60 ± 5% RH as well as in human plasma at 37°C. The sealed vials with 20-mm gray bromobutyl rubber stoppers and 20-mm aluminum seals were kept at various conditioned as mentioned above. For the condition of 2-8°C with ambient humidity, the vials were placed in refrigeration. For stability studies of the vials at 30 ± 2°C/60 ± 5% RH, the vials were taken in a desicator,

containing 60% w/v sodium nitrite (318), which was then placed in an oven maintained at 30°C. 10 mg of lyophilized powder was sampled at regular time intervals (0,1,2,14,28,56 and 84 days) and diluted to 2 ml with phosphate buffer saline (PBS) pH 7.4. The liposomal dispersion was centrifuged in a laboratory centrifuge (Remi) at 3000 RPM for 15 min; in this condition the liposomes remained suspended and the leaked drug sedimented. The amount of drug in supernatant and sediment was measured spectrophotometrically at 295 nm and 325 for FLT and 6-MP in Shimadzu 1601 UV-Visible Spectrophotometer, Kyoko, Japan) and % drug retained was calculated. To evaluate drug leakage from liposomes in plasma, liposomal dispersion was diluted 1:2 with human plasma and incubated at 37°C. The leakage drug was separated, extracted with methanol and estimated by HPLC method as described above. The particle size determination and electrolyte induced flocculation test were also performed as described earlier. The results of these determinations are shown in Table 8.2.

8.3 RESULTS AND DISCUSSION

8.3.1 Lyophilization of FLT and 6-MP conventional and stealth liposomes

Sucrose alone was used for the purpose of lyophilization as a cryoprotectant in FLT liposomes and sucrose as well as maltose was used as cryoprotectants in 6-MP liposomes.

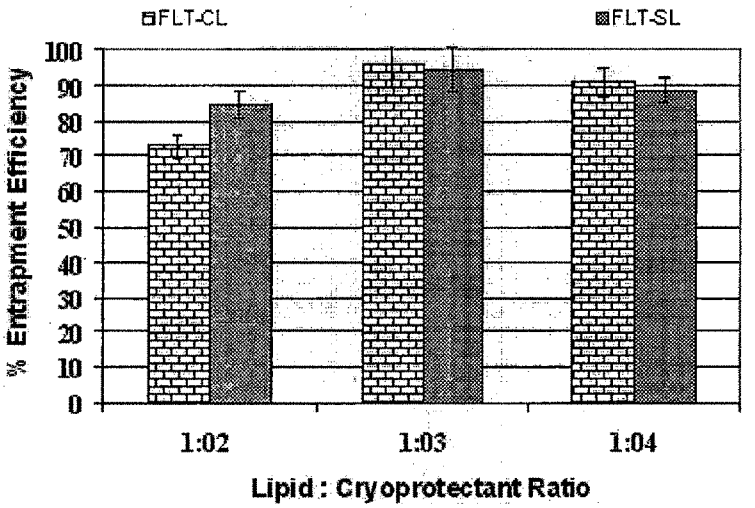

Figure 8.1 Effect of different lipid: cryoprotectant mass ratios on % EE of CL and SL of FLT

Optimization of the lyophilization process of the CL and SL was performed using various lipid: cryoprotectant ratio (1:2, 1:3 and 1:4) for getting maximum % EE (Figure 8.1 and 8.2). The data showed that no significant reduction in % EE was

found after lyophilization of CL and SL, when lipid: cryoprotectant mass ratio was 1:3.

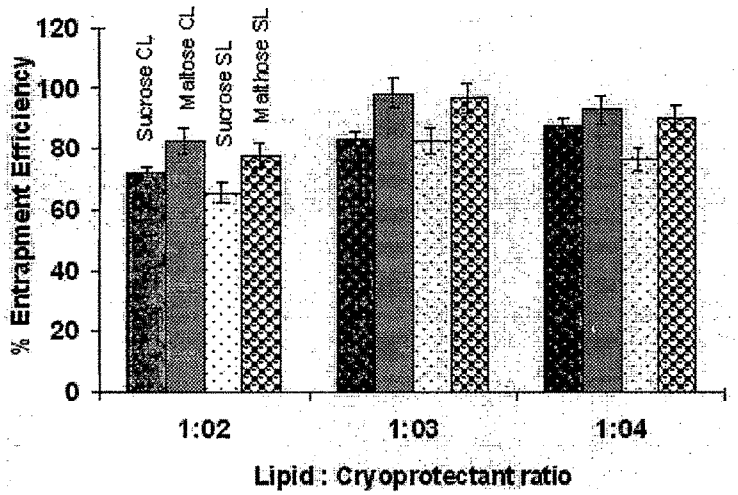

Figure 8.2: Effect of different cryoprotectants and lipid: cryoprotectant mass ratios on % EE of CL and SL of 6-MP

Sucrose (1:3 mass ratio) gave significantly higher % EE of96.03 % and 94.69% for flutamide CL and SL as compared to other mass ratio, respectively and maltose gave % EE of 97.94% and 96.59 % for 6-MP CL and SL, respectively. The results were found the same as described by Madden et al. He examined that the number of sugars in maintaining structural and functional properties of microsomal membranes at low mean liposomal size, and sucrose as well as maltose was found to be equally effective as that of trehalose for lyophilization of liposomes.

8.3.2 Stability studies of FLT and 6-MP conventional and stealth liposomes

Table 8.1 Percentage drug retained in lyophilized FLT-CL and FLT-SL stored at 2-8°C, 30 ± 2°C/60 ± 5% RH and in human plasma at 37°C

Time in days	% Drug retained (±SD)					
	Conventional liposomes			Stealth liposomes		
	2-8°C	30 ± 2°C/60 ± 5% RH	37°C in human plasma	2-8°C	30 ± 2°C/60 ± 5% RH	37°C in human plasma
0	100±2.3	100±2.8	100±1.2	100±1.5	100±1.6	100±1.9
1	100±2.6	99.72±1.8	48.12±3.4	100±1.1	100±2.8	66.38±3.6
2	100±1.1	99.21±1.2	2.3±1.2	100±1.8	99.42±2.7	5.82±3.8
14	99.38±4.2	96.32±3.9	-	99.62±1.7	98.13±1.8	-
28	98.92±4.6	93.17±4.2	-	98.4±1.1	97±3.8	-
56	98.24±4.1	88.81±3.7	-	96.92±1.7	93.48±2.7	-
84	98.08±3.9	83.02±3.7	-	96.18±1.8	89.32±3.9	-

Table 8.1 and Figure 8.3 indicate the % drug retained in lyophilized FLT-CL and FLT-SL in different conditions. It showed that liposomal preparations were physically stable for more than 3 months in lyophilized form at 2-8°C and retained 90% of their initial content over that period. Leakage of FLT from CL was faster than from SL in human blood plasma at 37°C. The % drug retained in CL (48.12 %) was less than that of SL (66.38%) after 24 hr and no more drugs was retained after 2 days. Maximum instability was observed at 37°C in presence of plasma of CL and SL. Thus, from these studies, it was observed that liposomes remained more stable at refrigeration temperature.

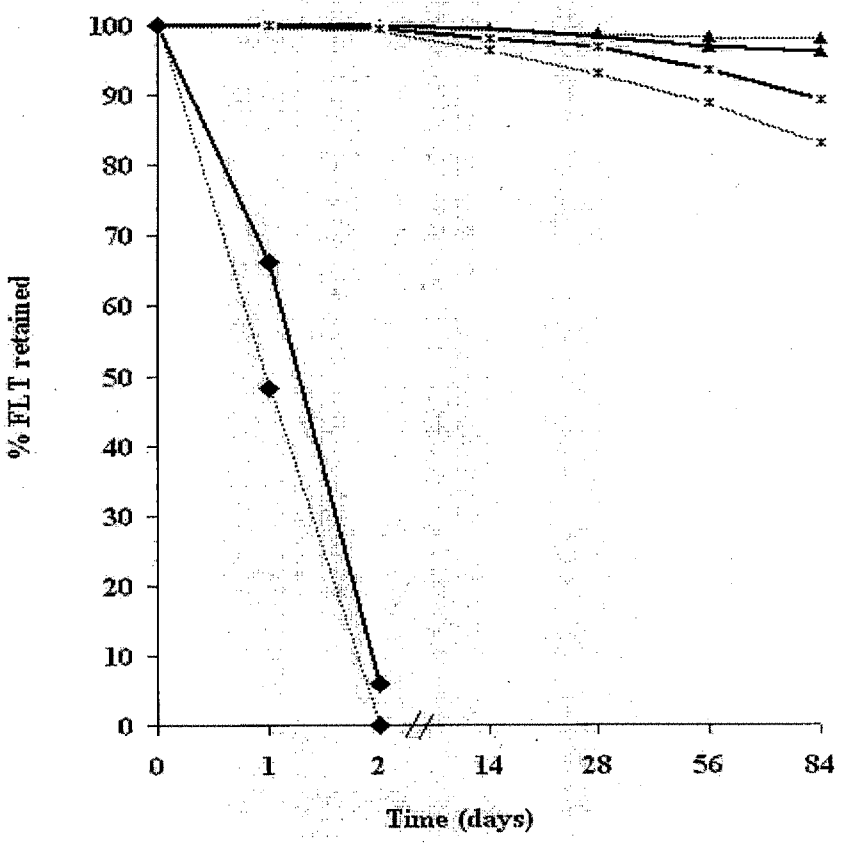

Figure 8.3: % FLT retained in CL (doted line) and SL (dark line) at 2-8°C (triangle), 30 ± 2°C/60 ± 5% RH (cross) and in human plasma at 37°C (square)

Table 8.2 and Figure 8.4 indicate the % drug retained in lyophilized 6-MP-CL and 6-MP-SL in different conditions. It showed that liposomal preparations were physically stable for more than 3 months in lyophilized form at 2-8°C and retained 90% of their initial content over that period.

Table 8.2 Percentage drug retained in lyophilized 6-MP-CL and 6-MP-SL at 2-8°C, 30 ± 2°C/60 ± 5% RH and in human plasma at 37°C

Time in days	% Drug retained					
	Conventional liposomes			Stealth liposomes		
	2-8°C	30 ± 2°C/60 ± 5% RH	37°C in human plasma	2-8°C	30 ± 2°C/60 ± 5% RH	37°C in human plasma
0	100±1.1	100±2.5	100±2.1	100±1.1	100±1.9	100±2.9
1	99.68±1.3	99.5±61.1	32.84±2.2	100±2.6	100±2.4	94.32±2.8
2	99.21±2.4	99.14±2.5	3.84±1.24	100±2.2	99.81±1.8	7.22±1.5
14	97.8±1.9	95.62±2.8	-	98.31±2.4	97.92±2.2	-
28	95.9±3.4	92.24±2.1	-	97.51±2.8	94.33±2.7	-
56	93.72±1.6	86.27±2.8	-	95.9±2.1	91.21±2.8	-
84	90.08±2.2	83.22±2.4	-	94.21±3.1	89.61±2.7	-

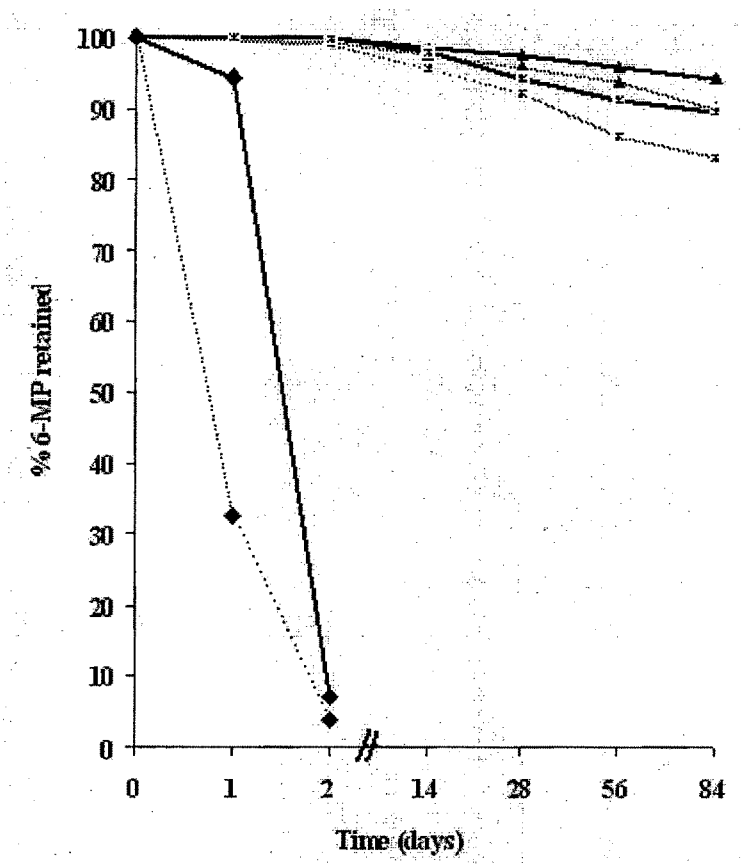

Figure 8.4: % 6-MP retained in CL (doted line) and SL (dark line) at 2-8°C (triangle), 30 ± 2°C/60 ± 5% RH (cross) and in human plasma at 37°C (square)

Leakage of 6-MP from CL was faster than from SL in human blood plasma at 37°C. The % drug retained in CL (32.48 %) was less than that of SL (94.32 %) after 24 hr and no more drugs was retained after 2 days in presence of human plasma. Maximum instability was observed at 37°C in presence of plasma of CL and SL. Thus, from these studies, it was observed that liposomes remained more stable at refrigeration temperature.

8.3.3 Particle size and stability studies

Table 8.3 shows the particle size of the rehydrated lyophilized liposomes of both drugs. The data indicated that the particle size was not altered much after storing at 2-8°C and at 30 ± 2°C/60 ± 5% RH in case of all types of liposomes.

Table 8.3: Mean Particle Size of the rehydrated lyophilized liposomal formulations of FLT and 6-MP after storing at different conditions

Time in days	Mean Particle Size in nm (±SD)					
	Conventional liposomes			Stealth liposomes		
	2-8°C	30 ± 2°C/60 ± 5% RH	37°C in human plasma	2-8°C	30 ± 2°C/60 ± 5% RH	37°C in human plasma
Flutamide						
0	136.14±5.8	136.14±5.8	136.14 ±5.8	158.26±6.3	158.26±6.3	158.26 ±6.3
1	136.28±1.8	136.84±2.1	916.18±16.4	159.54±2.1	160.12±2.6	301.89±3.4
2	136.92±1.4	137.14±2.4	2018.45±34.6	160.14±1.8	160.84±2.1	981.64±24.8
7	144.7±2.4	142.14±1.4	-	162.74±1.6	172.1±1.7	
14	150.54±1.5	150.17±1.9	-	165.92±2.3	183.15±1.2	-
28	159.72±1.6	161.68±2.6	-	168.98±1.6	194.24±1.6	-
56	162.9±2.1	175.57±2.3	-	171.15±2.3	202.38±2.1	-
84	165.26±0.8	189.1±0.9	-	174.25±2.1	214.17±2.3	-
6-Mercaptopurine						
0	120.24±2.4	120.24±1.0	120.24±1.7	132.26±2.1	132.26±1.7	132.26±1.6
1	122.14±1.4	124.21±1.3	1540.3±2.4	134.2±2.6	137.21±2.4	172.33±2.9
2	121.5±1.4	131.54±1.7	3041.22±48.5	133.62±1.4	139.58±1.4	587.61±10.5
7	124.27±2.1	139.17±1.6	-	136.81±2.1	151.7±1.1	-
14	128.51±1.2	148.6±2.1	-	139.48±1.8	169.88±1.8	-
28	131.25±2.6	152.31±2.1	-	146.21±1.5	182.48±2.1	-
56	136.5±2.3	169.24±1.3	-	152.34±2.1	201.54±2.3	-
84	141.27±1.7	186.14±2.4	-	169.41±2.6	224.2±1.4	-

The liposomal preparations stored at 2-8°C were little more stable as compared to the liposomes stored at 30 ± 2°C/60 ± 5% RH. The particle size of conventional

liposomes increased extremely with incubation in the presence of human plasma at 37°C, which suggested the occurrence of aggregation and/or fusion of liposomes. The aggregation or fusion can be inhibited by coating liposomes with $mPEG_{2000}$-PE. The effectiveness of polymer was optimum at 5-mole% of $mPEG_{2000}$-PE of total lipid. Stealth liposomes with 5-mole % of $mPEG_{2000}$-PE may effectively prevent adsorption of aggregation promoting components such as proteins in plasma as well as leak less amount of drug.

8.3.4 Steric stabilization and stability studies

The duration of steric stabilization of the prepared liposomes was evaluated by subjecting the liposomal dispersions to the electrolyte induced flocculation test at regular intervals of time after storing at different above-mentioned conditions. Concurrence of the absorbance obtained after adding 0.8M and 1.6M sodium sulphate in 16.7% w/v sucrose solutions indicated that the liposomal preparation was sterically stabilized.

Table 8.5 shows that the steric stability of conventional liposomal preparations is maintained for 14 days when stored at both conditions. Examination data indicated that the steric stabilizing property of the liposomes was also preserved in the lyophilized form in case of stealth liposomes. The results obtained from the electrolyte-induced flocculation tests proved that the presence of steric stabilizing agent in the bilayer is in the lyophilized form and provided excellent results when rehydrated with the distilled water. The uniformity of stability profile of different liposomal preparations of FLT and 6-MP is expected since the basic lipid composition of all FLT liposomes is PC/cholesterol and HSPC/cholesterol in case of all 6-MP liposomal preparations. The moderate rigidity of the liposomal membrane is not adequate for keeping the steric stabilizing agents at the membrane surface for long period of time since all these agents are also having affinity for the external aqueous phase.

Table 8.5: Steric stability studies of rehydrated lyophilized liposomal formulations at different storage conditions

Time days	Absorbance (Mean ± SD)							
	Conventional liposomes				Stealth liposomes			
	2-8°C		30 ± 2°C/60 ± 5% RH		2-8°C		30 ± 2°C/60 ± 5% RH	
	0.8M	1.6M	0.8M	1.6M	0.8M	1.6M	0.8M	1.6M
Flutamide								
0	0.631 ± 0.016	0.728 ± 0.017	0.631 ± 0.016	0.728 ± 0.017	0.546 ± 0.011	0.565 ± 0.015	0.546 ± 0.011	0.565 ± 0.015
14	0.644 ± 0.015	0.768 ± 0.012	0.681 ± 0.026	0.781 ± 0.022	0.549 ± 0.019	0.568 ± 0.023	0.551 ± 0.019	0.562 ± 0.019
28	0.678 ± 0.016	0.782 ± 0.024	0.705 ± 0.022	0.802 ± 0.031	0.551 ± 0.026	0.574 ± 0.012	0.567 ± 0.014	0.579 ± 0.021
56	0.741 ± 0.02	0.801 ± 0.018	0.768 ± 0.015	0.831 ± 0.027	0.549 ± 0.025	0.569 ± 0.02	0.572 ± 0.012	0.583 ± 0.025
84	0.768 ± 0.018	0.832 ± 0.038	0.811 ± 0.020	0.881 ± 0.037	0.562 ± 0.029	0.571 ± 0.027	0.581 ± 0.018	0.589 ± 0.022
6-Mercaptopurine								
0	0.676 ± 0.023	0.783 ± 0.023	0.676 ± 0.023	0.783 ± 0.023	0.588 ± 0.019	0.592 ± 0.015	0.588 ± 0.019	0.592 ± 0.015
14	0.691 ± 0.020	0.799 ± 0.015	0.686 ± 0.021	0.809 ± 0.034	0.596 ± 0.031	0.593 ± 0.018	0.594 ± 0.027	0.599 ± 0.018
28	0.714 ± 0.013	0.811 ± 0.018	0.702 ± 0.021	0.826 ± 0.032	0.601 ± 0.028	0.598 ± 0.019	0.603 ± 0.021	0.604 ± 0.017
56	0.725 ± 0.024	0.824 ± 0.024	0.721 ± 0.028	0.842 ± 0.028	0.610 ± 0.021	0.604 ± 0.027	0.612 ± 0.022	0.611 ± 0.029
84	0.744 ± 0.027	0.839 ± 0.029	0.740 ± 0.039	0.851 ± 0.031	0.618 ± 0.021	0.618 ± 0.021	0.624 ± 0.019	0.619 ± 0.021

8.3.5 Stability studies of Microbubbles

The prepared microbubble dispersions were subjected to stability studies, in triplicate, at conditions according to ICH guidelines i.e. 2-8°C with ambient humidity and 30 ± 2°C/60 ± 5% RH as well as in human plasma at 37°C. The sealed vials with 20-mm gray bromobutyl rubber stoppers and 20-mm aluminum seals were kept at various conditioned as mentioned above. For the condition of 2-8°C with ambient humidity, the vials were placed in refrigeration. For stability studies of the vials at 30 ± 2°C/60 ± 5% RH, the vials were stored in a desiccator, containing 60% w/v sodium nitrite (6), which was then placed in an oven maintained at 30°C. If the microbubble size distribution varies, the microbubble number dose may not be equally good descriptors of the efficacy of the agents. Therefore, in this study, the particle size determination and particle number were selected as evaluation parameters. Table 8.6

shows the stability data of selected batch (AAL2) of FLT and 6-MP at different storage conditions. The data indicated that AALs showed initial particle size of 154±3.1 μm and $1.26*10^7$ particle number per ml. The data indicated that microbubble preparation (AALs) was physically stable for more than 3 months and no significant difference in particle size and particle number was found at 2-8°C. The microbubbles stored at 2-8°C were little more stable as compared to the microbubbles stored at 30 ± 2°C/60 ± 5% RH. The microbubbles could remain stable for 48 hr in presence human plasma. The particle size increased extremely with incubation of microbubbles in the presence of human plasma at 37°C after 48 hr. The presence of $mPEG_{2000}$-PE may effectively prevent adsorption of aggregation promoting components such as proteins in plasma and change in particle size.

Table 8.6: Stability data of FLT and 6-MP AALs

Time in days	Particle size in nm (Mean±SD)			Particle number/ml	
	2-8°C	30 ± 2°C/60 ± 5% RH	37°C in human plasma	2-8°C	30 ± 2°C/60 ± 5% RH
FLT AALs					
0	154±3.1	154±3.1	154±3.1	$1.26*10^7$	$1.26*10^7$
1	155 ±3.3	168±3.8	348±12.7	$1.24*10^7$	$1.18*10^7$
2	154±2.9	187±5.2	764±13.8	$1.21*10^7$	$1.07*10^7$
7	161±3.1	594±10.7	-	$1.16*10^7$	$0.82*10^7$
14	169±4.0	843±21.5	-	$1.10*10^7$	$3.4*10^6$
28	181±6.4	2048±107.8	-	$1.06*10^7$	$8.6*10^3$
56	197±5.9	-	-	$0.84*10^7$	-
84	219±6.3	-	-	$0.76*10^7$	-
6-MP AALs					
0	139±2.8	139±2.8	139±2.8	$1.17*10^7$	$1.17*10^7$
1	141±3.1	145±2.4	401±10.2	$1.13*10^7$	$1.09*10^7$
2	143±3.7	282±3.1	869±82.6	$1.08*10^7$	$1.0*10^7$
7	151±4.8	478±6.1	-	$1.0*10^7$	$0.71*10^7$
14	174±3.4	889±43.4	-	$0.81*10^7$	$4.8*10^6$
28	204±8.5	3027±121.6	-	$0.59*10^7$	$2.7*10^3$
56	251±8.0	-	-	$2.3*10^6$	-
84	289±12.5	-	-	$1.1*10^6$	-

8.4 CONCLUSION

The liposomal preparations showed suitable stability studies, which can enable them to be commercialized. The process of lyophilization was used to increase stability of

liposomes. The lyophilization of the liposomal preparations using sucrose and maltose as cryoprotectants were found to be highly successful in providing excellent stability to the liposomal preparations and desired shelf life required for commercial purposes. Stability data showed that AALs for both drugs showed stability less than 4 weeks at 30 ± 2°C/60 ± 5% RH and of three months at 2-8°C. AALs were stable for 24 hr and then after the change in particle size and number was observed in presence of human plasma at 37°C.