Chapter 7

Stability testing of Liposomes

7.1 INTRODUCTION

As industrially produced liposomes will reach the patient only after a prolonged period of time, the liposome formulation should not change its characteristics or lose the associated drug during storage or transport. In general, a shelf life of at least one year is a minimum prerequisite for a commercial product.

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. As mentioned before (Section 2.2.10, Literature review) the stability of liposomes can be discussed under broad categories of physical and chemical stability. 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 analysed 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.

Lyophilization of liposomes is one of the most promising ways to keep the liposomes stable during long-term storage (Shulkin *et. al.,* 1984). The presence of cryoprotectants is necessary for protection of the membrane structure during the freeze-drying of liposomes. Saccharides, especially disaccharides such as trehalose and sucrose, have been extensively investigated as cryoprotectants of liposomes (Crowe *et. al.,* 1988; Tanaka *et. al.,* 1992; Koster *et. al.,* 1994; Mobley and Schreier, 1994). The mechanisms of the cryoprotective effect of sugar on freeze-drying of liposomes were also investigated and two hypotheses are proposed. Crowe et al. (1988) reported that in the case of fluid lipids such as egg phosphatidyl choline, 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. Trehalose, a disaccharide, especially inhibits fusion and decreases the gel-to-liquid crystalline phase transition temperature (*T*m) of the dry lipid caused by a direct interaction between the sugar and the polar head group of the lipid (water replacement hypothesis). Koster *et. al.,* (1994) 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. They indicated that the sugar that had a high glass transition

temperature (*Tg*) was the most effective cryoprotectant (vitrification hypothesis).

7.2 EXPERIMENTAL

7.2.1 REAGENTS

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

7.2.2 APPARATUS

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

7.2.3 SOLUTIONS

16.7% w/v solutions of sucrose in distilled water were prepared by dissolving 16.7g 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 sucrose solution.

7.2.4 STABILITY STUDIES OF CONVENTIONAL AND STERICALLY STABILIZED LIPOSOMES CONTAINING METHOTREXATE, TAMOXIFEN CITRATE AND CYTARABINE

The prepared 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. The prepared dispersions were taken in 5ml amber colored glass vials (USP Type I glass) and the vials were purged with nitrogen. The vials were then sealed using 20-mm grey bromobutyl rubber stoppers and 20-mm aluminium seals. 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 desiccator, containing 60% w/v sodium nitrite (Dean, 1999), which was then placed in an oven maintained at 30°C. At the intervals of 10, 20 and 30 days, liposomal dispersion samples were withdrawn from vials placed at

both the conditions and subjected for the analysis of the size distribution, drug retention efficiency and electrolyte induced flocculation test described earlier (Section 4.2.8, Chapter 4). The results of these determinations are shown in Tables 7.1-7.3, for the samples placed in refrigeration and at 30°C/60% R.H.

Various cryoprotectants like sucrose and maltose were used for the purpose of lyophilisation. Lyophilization optimisation of liposomes was performed for maximum drug retention. The use of cryoprotectants (sucrose and maltose) during lyophilization of liposomes was evaluated and compared. Conventional liposomal suspension containing methotrexate (ML) was diluted with distilled water containing maltose or sucrose and the amount of MTX retained by the liposomes following lyophilization and rehydration was determined. The lipid: cryoprotectant ratio was also optimized based on the drug retention capacity of the rehydrated lyophilised liposomal powder (Figure 7.1). The lyophilised samples were also subjected to stability studies, in triplicate, at the conditions according to ICH guidelines i.e. 2-8°C with ambient humidity and 30 ± 2°C/60 ± 5% RH after storing in sealed glass vials (USP Type I glass). The lyophilised samples were withdrawn from the vials at the intervals of every two months for a period of 6 months, rehydrated using distilled water to form liposomal suspension and subjected for the analysis of the size distribution, drug retention efficiency and electrolyte induced flocculation test and the results of these determinations are shown in Tables 7.4-7.6, respectively.

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Table 7.1 Particle size determination of the liposomal dispersions after storing at different temperatures for a period of 30 days

Batch	Average Mean Particle Size (S.E.) (in nm)						
		2-8°C			30±2°C		
	0 days	10 days	20 days	30 days	10 days	20 days	30 days
ML	182.3 (34.6)	191.2 (22.6)	185.3 (16.2)	192.4 (24.7)	245.6 (19.4)	475.3 (36.4)	654.2 (21.2)
SML5000-CC-PE	165.4 (24.5)	174.5 (32.0)	178.4 (22.9)	212.3 (22.4)	229.7 (23.7)	512.3 (19.4)	622.4 (26.1)
SML2000-CC-PE	173.3 (27.6)	169.2 (18.6)	176.8 (29.4)	185.6 (32.8)	263.2 (29.1)	398.2 (26.5)	548.9 (31.2)
SML5000-IC-PE	172.1 (22.5)	176.5 (26.4)	183.4 (19.4)	174.0 (38.4)	196.5 (22.6)	378.4 (23.6)	565.7 (33.5)
TL	197.2 (41.0)	210.6 (12.5)	199.5 (29.7)	232.5 (29.4)	278.6 (31.5)	442.6 (27.4)	754.8 (31.6)
STL5000-CC-PE	176.5 (22.6)	182.4 (22.4)	185.6 (34.7)	224.3 (26.8)	267.9 (26.7)	331.2 (33.1)	532.1 (34.8)
STL2000-CC-PE	183.2 (26.7)	178.5 (29.7)	185.6 (30.4)	194.2 (34.1)	248.3 (28.2)	374.9 (36.7)	521.3 (26.4)
STL5000-IC-PE	190.6 (24.5)	184.6 (27.4)	178.2 (34.8)	196.3 (27.4)	269.5 (36.4)	412.5 (38.4)	601.2 (29.7)
CL	236.2 (25.3)	221.3 (29.4)	246.3 (22.4)	228.3 (36.4)	294.6 (41.6)	468.4 (29.6)	645.6 (26.4)
SCL5000-CC-PE	205.6 (22.9)	196.5 (32.1)	201.7 (37.4)	213.1 (32.7)	246.5 (23.6)	402.8 (22.9)	588.6 (31.6)
SCL2000-CC-PE	185.1 (29.7)	201.3 (26.7)	184.6 (20.1)	195.4 (21.4)	236.5 (22.8)	378.1 (31.5)	564.8 (34.7)
SCL5000-IC-PE	195.0 (31.1)	190.4 (29.7)	201.3 (26.7)	221.6 (31.8)	274.6 (26.8)	369.8 (28.5)	557.1 (24.9)

Table 7.2 Percentage drug retention of the liposomal dispersions after storing at different temperatures for a period of 30 days

Batch	% Drug Retention (S.E.)					
		2-8°C			30±2°C	
	0 days	10 days	20 days	30 days	10 days	20 days
ML	95.8 (1.4)	92.4 (0.9)	83.6 (2.3)	79.2 (2.1)	55.6 (2.0)	32.1 (0.6)
SML5000-CC-PE	89.5 (2.6)	87.6 (1.2)	79.8 (1.4)	77.5 (2.5)	62.4 (1.6)	43.9 (0.9)
SML2000-CC-PE	88.6 (1.7)	84.6 (2.3)	81.4 (0.9)	79.1 (1.5)	74.4 (0.9)	49.7 (2.1)
SML5000-IC-PE	92.7 (2.0)	89.4 (1.6)	86.4 (1.2)	81.5 (1.4)	68.4 (1.7)	43.9 (1.6)
TL	89.0 (0.6)	86.1 (1.0)	85.7 (2.3)	79.4 (2.4)	76.8 (1.2)	54.7 (1.9)
STL5000-CC-PE	84.4 (1.0)	82.2 (0.7)	81.6 (0.7)	75.9 (1.6)	71.7 (1.6)	59.4 (1.3)
STL2000-CC-PE	85.3 (1.6)	84.6 (1.3)	82.9 (1.8)	76.1 (1.1)	69.8 (2.3)	56.2 (0.9)
STL5000-IC-PE	83.5 (2.4)	81.9 (2.1)	79.6 (1.6)	74.1 (2.1)	70.5 (1.6)	51.4 (1.0)
CL	83.5 (1.6)	82.2 (1.5)	79.4 (1.7)	74.1 (2.6)	54.2 (2.4)	35.9 (1.5)
SCL5000-CC-PE	84.2 (1.2)	83.7 (1.6)	80.6 (2.3)	78.4 (2.2)	63.5 (2.1)	39.6 (1.8)
SCL2000-CC-PE	83.8 (1.4)	81.6 (2.1)	82.4 (2.1)	78.4 (2.0)	66.4 (1.0)	42.1 (2.0)
SCL5000-IC-PE	82.2 (2.1)	81.4 (1.3)	80.3 (0.8)	74.5 (1.6)	61.4 (2.6)	40.7 (1.3)

Table 7.3 Steric stability studies of the liposomal dispersions after storing at different temperatures for a period of 30 days

Batch			2-8°C						30±2°C					
	0 days		10 days		20 days		30 days		10 days		20 days		30 days	
	0.8M	1.6M	0.8M	1.6M	0.8M	1.6M	0.8M	1.6M	0.8M	1.6M	0.8M	1.6M	0.8M	1.6M
ML	0.694 (0.021)	0.801 (0.018)	0.698 (0.022)	0.812 (0.029)	0.722 (0.013)	0.815 (0.019)	0.768 (0.026)	0.877 (0.021)	0.774 (0.019)	0.864 (0.024)	0.864 (0.009)	0.947 (0.014)	0.892 (0.022)	0.962 (0.034)
SML5000-CC-PE	0.588 (0.012)	0.592 (0.011)	0.579 (0.023)	0.597 (0.022)	0.592 (0.025)	0.602 (0.015)	0.635 (0.015)	0.657 (0.034)	0.637 (0.016)	0.644 (0.023)	0.712 (0.013)	0.763 (0.016)	0.754 (0.022)	0.748 (0.016)
SML2000-CC-PE	0.613 (0.035)	0.619 (0.019)	0.616 (0.012)	0.629 (0.015)	0.637 (0.016)	0.645 (0.034)	0.685 (0.026)	0.701 (0.020)	0.698 (0.026)	0.706 (0.012)	0.749 (0.026)	0.742 (0.022)	0.811 (0.011)	0.834 (0.017)
SML5000-IC-PE	0.545 (0.023)	0.558 (0.011)	0.541 (0.010)	0.564 (0.033)	0.559 (0.022)	0.571 (0.016)	0.583 (0.021)	0.604 (0.012)	0.589 (0.018)	0.603 (0.038)	0.654 (0.020)	0.681 (0.015)	0.718 (0.026)	0.743 (0.031)
TL	0.644 (0.021)	0.741 (0.015)	0.649 (0.023)	0.749 (0.018)	0.667 (0.016)	0.775 (0.018)	0.721 (0.012)	0.849 (0.031)	0.694 (0.010)	0.788 (0.009)	0.752 (0.016)	0.824 (0.016)	0.781 (0.031)	0.855 (0.024)
STL5000-CC-PE	0.559 (0.018)	0.578 (0.013)	0.566 (0.024)	0.591 (0.016)	0.582 (0.014)	0.591 (0.016)	0.655 (0.016)	0.674 (0.025)	0.646 (0.023)	0.631 (0.033)	0.722 (0.013)	0.731 (0.023)	0.733 (0.010)	0.746 (0.014)
STL2000-CC-PE	0.603 (0.015)	0.614 (0.011)	0.612 (0.015)	0.618 (0.022)	0.629 (0.017)	0.633 (0.013)	0.679 (0022)	0.702 (0.024)	0.655 (0.026)	0.679 (0.012)	0.706 (0.026)	0.726 (0.028)	0.745 (0.016)	0.769 (0.027)
STL5000-IC-PE	0.641 (0.035)	0.652 (0.015)	0.651 (0.034)	0.663 (0.013)	0.645 (0.033)	0.661 (0.025)	0.689 (0.031)	0.705 (0.033)	0.687 (0.027)	0.697 (0.013)	0.756 (0.029)	0.771 (0.011)	0.792 (0.024)	0.811 (0034)
CL	0.694 (0.031)	0.774 (0.017)	0.689 (0.033)	0.782 (0.022)	0.712 (0026)	0.803 (0.028)	0.762 (0.034)	0.854 (0.012)	0.744 (0.013)	0.823 (0.024)	0.789 (0.015)	0.855 (0.034)	0.806 (0.023)	0.887 (0.016)
SCL5000-CC-PE	0.621 (0.013)	0.638 (0.030)	0.633 (0.016)	0.649 (0.043)	0.611 (0.015)	0.642 (0.016)	0.668 (0.016)	0.674 (0.033)	0.655 (0.013)	0.689 (0.026)	0.705 (0.016)	0.712 (0.016)	0.744 (0.034)	0.739 (0.032)
SCL2000-CC-PE	0.613 (0.018)	0.624 (0.022)	0.603 (0.018)	0.622 (0.016)	0.622 (0.022)	0.638 (0.014)	0.655 (0.020)	0.677 (0.016)	0.658 (0.020)	0.671 (0.018)	0.687 (0.031)	0.701 (0.021)	0.722 (0.031)	0.741 (0.025)
SCL5000-IC-PE	0.576 (0.035)	0.573 (0.015)	0.581 (0.031)	0.579 (0.033)	0.586 (0.019)	0.591 (0.036)	0.622 (0.022)	0.639 (0.013)	0.608 (0.013)	0.611 (0.019)	0.657 (0.038)	0.669 (0.013)	0.687 (0.011)	0.705 (0.015)

Figure 7.1 Effect of different ratios of cryoprotectants on efficiency of lyophilization of liposomal formulation

Table 7.4 Particle size determination of the rehydrated lyophilized liposomal formulations after storing at different temperatures for a period of 6 months

Batch	Average Mean Particle Size (S.E.) (in nm)						
		2-8°C			30±2°C		
	0 mon	2 mon	4 mon	6 mon	2 mon	4 mon	6 mon
ML	182.3 (34.6)	179.3 (32.4)	184.5 (18.4)	190.4 (19.4)	183.2 (16.4)	188.6 (18.5)	186.4 (19.7)
SML5000-CC-PE	165.4 (24.5)	168.7 (26.7)	162.5 923.1)	171.3 (23.6)	168.4 (24.0)	172.8 (22.4)	174.6 (16.4)
SML2000-CC-PE	173.3 (27.6)	178.3 (19.6)	180.4 (16.8)	178.6 (31.6)	190.4 (26.9)	178.4 (16.9)	183.2 (30.1)
SML5000-IC-PE	172.1 (22.5)	180.4 (22.4)	183.4 (16.9)	179.5 (21.7)	182.6 (22.5)	185.2 (23.1)	178.1 (22.5)
TL	197.2 (41.0)	202.3 (21.3)	189.5 (23.4)	212.3 (30.4)	191.9 (23.4)	206.7 (24.8)	214.3 (23.6)
STL5000-CC-PE	176.5 (22.6)	182.6 (26.1)	179.5 (26.5)	190.4 (21.1)	184.6 (26.7)	192.5 (31.4)	186.2 (21.9)
STL2000-CC-PE	183.2 (26.7)	176.9 (23.0)	186.3 (16.7)	191.2 (19.8)	185.4 (20.6)	192.3 (28.6)	194.5 (16.4)
STL5000-IC-PE	190.6 (24.5)	186.5 (19.4)	193.5 (31.5)	201.3 (22.3)	196.4 (31.4)	206.4 (25.7)	215.3 (29.7)
CL	236.2 (25.3)	242.3 (34.5)	249.7 (37.4)	240.9 (26.7)	246.1 (33.6)	245.3 (18.6)	247.6 (31.5)
SCL5000-CC-PE	205.6 (22.9)	209.3 (29.0)	199.6 (16.3)	210.6 (34.0)	218.4 (34.5)	211.6 (20.1)	220.1 (26.4)
SCL2000-CC-PE	185.1 (29.7)	190.2 (26.7)	188.9 (19.7)	196.4 (36.4)	199.6 (26.7)	194.5 (28.4)	190.4 (30.6)
SCL5000-IC-PE	195.0 (31.1)	194.3 (30.4)	193.2 (20.7)	206.4 (26.9)	210.3 (22.1)	211.5 (22.6)	207.9 (25.8)

Table 7.5 Percentage drug retention of the rehydrated lyophilized liposomal formulations after storing at different temperatures for a period of 6 months

Batch	% Drug Retention (S.E.)						
		2-8°C			30±2°C		
	0 mon	2 mon	4 mon	6 mon	2 mon	4 mon	6 mon
ML	95.8 (1.4)	95.8 (1.2)	93.1 (1.1)	93.9 (1.5)	94.6 (2.6)	92.4 (2.8)	90.1 (1.5)
SML5000-CC-PE	89.5 (2.6)	89.0 (1.5)	90.4 (0.8)	88.1 (1.6)	88.2 (2.1)	85.6 (0.9)	86.4 (1.6)
SML2000-CC-PE	88.6 (1.7)	89.1 (2.4)	88.3 (1.4)	86.9 (1.1)	86.4 (1.8)	87.2 (2.1)	83.8 (2.3)
SML5000-IC-PE	92.7 (2.0)	91.4 (2.4)	92.0 (1.5)	89.3 (2.3)	92.3 (1.5)	90.4 (1.6)	86.1 (1.6)
TL	89.0 (0.6)	89.6 (1.8)	88.6 (1.5)	86.9 (2.1)	88.1 (2.6)	86.4 (1.4)	86.2 (2.1)
STL5000-CC-PE	84.4 (1.0)	84.9 (1.3)	85.1 (1.7)	82.6 (1.6)	83.7 (2.1)	81.6 (2.1)	80.4 (1.3)
STL2000-CC-PE	85.3 (1.6)	85.3 (0.9)	84.2 (1.4)	84.6 (1.9)	84.9 (1.7)	83.1 (2.0)	81.3 (1.6)
STL5000-IC-PE	83.5 (2.4)	84.1 (1.4)	83.2 (1.4)	82.6 (2.1)	81.6 (1.2)	82.0 (2.6)	80.6 (2.3)
CL	83.5 (1.6)	83.8 (2.1)	82.4 (2.3)	82.0 (1.0)	82.9 (1.6)	81.6 (1.1)	81.2 (1.0)
SCL5000-CC-PE	84.2 (1.2)	84.6 (2.5)	83.5 (2.1)	83.9 (2.1)	84.1 (2.7)	82.6 (1.3)	82.0 (3.1)
SCL2000-CC-PE	83.8 (1.4)	84.1 (1.1)	83.6 (1.3)	82.2 (1.3)	83.1 (2.5)	82.4 (2.6)	80.2 (2.2)
SCL5000-IC-PE	82.2 (2.1)	82.1 (1.8)	81.5 (1.6)	81.6 (1.7)	81.3 (2.1)	80.4 (2.3)	80.7 (1.7)

Table 7.6 Steric stability studies of rehydrated lyophilized liposomal formulations after storing at different temperatures for a period of 6 months

Batch			2-8°C						30±2°C					
	0 months		2 months		4 months		6 months		2 months		4 months		6 months	
	0.8M	1.6M	0.8M	1.6M	0.8M	1.6M	0.8M	1.6M	0.8M	1.6M	0.8M	1.6M	0.8M	1.6M
ML	0.694 (0.021)	0.801 (0.018)	0.689 (0.016)	0.806 (0.015)	0.699 (0.033)	0.796 (0.012)	0.704 (0.035)	0.806 (0.012)	0.694 (0.011)	0.810 (0.026)	0.703 (0.012)	0.807 (0.030)	0.711 (0.022)	0.815 (0.015)
SML5000-CC-PE	0.588 (0.012)	0.592 (0.011)	0.596 (0.011)	0.596 (0.016)	0.599 (0.011)	0.601 (0.013)	0.590 (0.031)	0.591 (0.016)	0.593 (0.028)	0.588 (0.010)	0.602 (0.033)	0.599 (0.012)	0.607 (0.031)	0.607 (0.012)
SML2000-CC-PE	0.613 (0.035)	0.619 (0.019)	0.607 (0.026)	0.620 (0.012)	0.610 (0.015)	0.627 (0.025)	0.620 (0.020)	0.631 (0.026)	0.618 (0.030)	0.629 (0.013)	0.625 (0.015)	0.629 (0.026)	0.619 (0.016)	0.634 (0.027)
SML5000-IC-PE	0.545 (0.023)	0.558 (0.011)	0.551 (0.022)	0.552 (0.013)	0.559 (0.022)	0.565 (0.029)	0.554 (0.011)	0.566 (0.034)	0.552 (0.022)	0.563 (0.027)	0.561 (0.018)	0.568 (0.025)	0.557 (0.030)	0.561 (0.036)
TL	0.644 (0.021)	0.741 (0.015)	0.649 (0.027)	0.746 (0.019)	0.645 (0.016)	0.752 (0.018)	0.651 (0.026)	0.756 (0.017)	0.658 (0.019)	0.755 (0.026)	0.652 (0.022)	0.759 (0.034)	0.649 (0.016)	0.752 (0.031)
STL5000-CC-PE	0.559 (0.018)	0.578 (0.013)	0.562 (0.016)	0.581 (0.018)	0.569 (0.018)	0.588 (0.013)	0.574 (0.018)	0.583 (0.018)	0.575 (0.014)	0.591 (0.013)	0.579 (0.034)	0.589 (0.033)	0.587 (0.034)	0.594 (0.026)
STL2000-CC-PE	0.603 (0.015)	0.614 (0.011)	0.599 (0.023)	0.609 (0.011)	0.605 (0.032)	0.624 (0.026)	0.610 (0.026)	0.618 (0.016)	0.597 (0.025)	0.616 (0.024)	0.603 (0.016)	0.620 (0.019)	0.609 (0.026)	0.618 (0.014)
STL5000-IC-PE	0.641 (0.035)	0.652 (0.015)	0.633 (0.033)	0.655 (0.031)	0.646 (0.011)	0.659 (0.011)	0.649 (0.015)	0.661 (0.030)	0.651 (0.034)	0.657 (0.026)	0.658 (0.025)	0.663 (0.028)	0.654 (0.014)	0.661 (0.028)
CL	0.694 (0.031)	0.774 (0.017)	0.687 (0.011)	0.770 (0.037)	0.697 (0.016)	0.776 (0.028)	0.696 (0.014)	0.772 (0.026)	0.703 (0.016)	0.778 (0.031)	0.699 (0.011)	0.781 (0.019)	0.710 (0.011)	0.785 (0.024)
SCL5000-CC-PE	0.621 (0.013)	0.638 (0.030)	0.629 (0.015)	0.641 (0.029)	0.625 (0.022)	0.639 (0.034)	0.630 (0.038)	0.645 (0.014)	0.628 (0.18)	0.642 (0.022)	0.631 (0.030)	0.644 (0.014)	0.633 (0.033)	0.650 (0.029)
SCL2000-CC-PE	0.613 (0.018)	0.624 (0.022)	0.618 (0.032)	0.622 (0.018)	0.609 (0.014)	0.629 (0.016)	0.611 (0.011)	0.634 (0.019)	0.615 (0.011)	0.628 (0.031)	0.619 (0.022)	0.622 (0.026)	0.620 (0.019)	0.629 (0.014)
SCL5000-IC-PE	0.576 (0.035)	0.573 (0.015)	0.579 (0.024)	0.570 (0.027)	0.580 (0.033)	0.576 (0.018)	0.577 (0.017)	0.580 (0.018)	0.583 (0.032)	0.588 (0.036)	0.578 (0.019)	0.591 (0.10)	0.586 (0.024)	0.596 (0.037)

7.3 RESULTS AND DISCUSSION

The particle size distribution of the prepared liposomes was ascertained by subjecting samples of liposomal dispersions to 2-8°C with ambient humidity and 30°±2°C/60 ± 5% R.H. and measuring the particle size of the liposomes by particle size analyzer at regular time intervals. The percentage drug retained in the liposomes was also determined to check the stability of the prepared liposomal formulations. 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 the temperatures and conditions mentioned above. Concurrence of the absorbance readings obtained after addition of 0.8M and 1.6M sodium sulphate in 16.7% sucrose solutions indicated that the liposomal preparation was sterically stabilized. It was envisaged that such preliminary studies would help in development of products with stability profiles that will meet commercial requirements.

The evaluation of the particle size distribution, drug retention and steric stabilization studies of the liposomal dispersions showed that liposomal formulations stored at 2-8°C was little more stable compared to the liposomes stored at 30°±2°C. Table 7.1 shows that the liposomal dispersions stored at both the temperatures (2-8°C & 30°±2°C) were not stable for a period of 1 month. The increase in mean particle size of all liposomal formulations proved the instability of the liposomes in dispersion form. Table 7.2 shows the gradual leakage of the drugs from the liposomal formulations during storage and none of the liposomal formulations could retain the drug within themselves over a period of 1 month. Examination of the data included in Table 7.3 reveals that the steric stability of all the liposomal dispersions is maintained for a period of 20 days (stored at 2-8°C) and less than 10 days (stored at 30°±2°C/60 ± 5% R.H). The uniformity of stability profile of different liposomal preparations is expected since the basic lipid composition of all these preparations is the same. The moderate rigidity of the liposomal membrane is not adequate for keeping the steric stabilizing agents within the membrane for long periods of time since all these agents are also having affinity for the external aqueous phase. From the above observations, it can be concluded that all the prepared liposomal

formulations lacked suitable stability profiles, which indicates that freeze-drying was the only alternative to formulate stable liposomal products.

Various cryoprotectants like sucrose and maltose were used for the purpose of lyophilisation. Optimization of the lyophilization process of the conventional liposomes containing methotrexate was performed using various lipid : sugar (maltose/ sucrose) mass ratio (1: 0, 1: 1, 1: 2, 1: 3, 1: 4 and 1:5) for maximum drug retention (Figure 7.1). The data reveals that sucrose (1: 3 mass ratio) gave significantly higher percentage of drug retention (95.2%) as compared to maltose (1:4 mass ratio) (88.9%) and proved to be better cryoprotectant for the lyophilization of liposomes. This is in agreement with the findings of previous report (Madden *et. al.,* 1985), which examined the effectiveness of number of sugars in maintaining structural and functional properties of microsomal membranes at low mean liposomal size, and sucrose was found to be equally effective as that of trehalose for lyophilization of liposomes.

The liposomal suspensions were freeze dried along with the sucrose (1:3 mass ratio of lipid/sucrose) overnight at -20°C, lyophilized for 24 h. The lyophilized liposomal products were also tested for its stability at two different temperatures viz. refrigerator (2-8°C) and room temperature (30°±2°C/60±5% R.H) stored for a period of 6 months. At predetermined time points, samples were removed; rehydrated using distilled water and evaluated for the particle size distribution, steric stabilization and percent drug retained.

Table 7.4 showed that the particle size of the rehydrated lyophilised liposomes was not altered much after storing at different temperatures over a period of 6 months. Table 7.5 showed that no significant reduction in the percentage drug retained after rehydration of the lyophilised liposomes and proved to be highly stable during storage. Examination of the data included in the Table 7.6 revealed that the steric stabilizing property of the liposomes was also preserved in the lyophilised form. The results obtained from the electrolyte induced flocculation tests proved that the presence of steric stabilizing agents in the lipid bilayer in the lyophilised form and provides excellent results when rehydrated with the distilled water.

7.3.1 CONCLUSIONS

The prepared liposomal suspensions lacked suitable stability profiles, which can enable these preparations to be commercialised. Inclusion of high melting lipids in the bilayer after ascertaining the *in vivo* drug release rate requirements can help in developing products with better stability. The process of lyophilization was used as an alternate method for providing the stability to the liposomes. The lyophilization of the liposomal preparations using sucrose as cryoprotectant was found to be highly successful in providing excellent stability to the liposomal preparations and desired shelf life required for commercial purposes.

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