CHAPTER 7

STABILITY TESTING

OF

LIPOSOMES

7.1 INTRODUCTION

The problem of long-term stability of liposomes has been addressed in recent years through the development of methods for preserving unilamellar vesicles, including the materials trapped inside, as freeze dried powders. As mentioned before (Section 2.2.8, Literature review) the stability of liposomes can be discussed under broad categories of physical and chemical stability. 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. 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 products 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.

Lyophilisation of liposomes is one of the most promising ways to keep the liposomes stable during long-term storage (Shulkin et al., 1984). Preservation of liposomes during drying has its basis in the observation that many organisms that survive dehydration (a phenomenon known as anhydrobiosis) accumulate large quantities of disaccharides, the most common of which are sucrose. 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 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. 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 (*T*g) was the most effective cryoprotectant (vitrification hypothesis).

7.2 EXPERIMENTAL

7.2.1 REAGENTS

Trehalose purchased from Himedia, Bangalore, Lactose (AR grade), 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 2.0M 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 CYCLOSPORINE (CsA) LOADED LIPOSOMAL FORMULATIONS

The CsA loaded liposomal dispersions were stored at 4°C, 30°C and 45°C for 30 days and were followed by visual and microscopic observation. The particle size determination of the liposomal dispersions before lyophilisation after storing at different temperatures for a period of 30 days was shown in table 7.1. Entrapment Efficiency (EE) was determined at the end of 7 days, 15 days and 30 days as shown in table 7.2.

Liposomal dispersions were prepared by hydrating the thin lipid film with PBS (pH 7.8). Unencapsulated drug was removed by centrifugation at 15,000 rpm for 30 minutes using a Remi centrifuge. The pellet was resuspended in PBS containing different ratios of lactose and sucrose on mass basis (g of lipid / g of the cryoprotectant). The mixture was frozen overnight at -40°C. Freeze drying was performed in Lyophiliser (Heto-Holten A/S, Denmark), for 24 h to yield the product in a dry powder form.

The % retention of the drug was calculated as follows:

$$\% \text{ Retention} = EE_{(a)} / EE_{(b)} * 100$$

where $EE_{(b)}$ is the entrapment efficiency before lyophilisation and $EE_{(a)}$ is the entrapment efficiency after lyophilisation.

Various cryoprotectants like sucrose and lactose were used for the purpose of lyophilisation. Lyophilisation optimization of positively charged liposomes was performed for maximum drug retention. The use of cryoprotectants (sucrose and lactose) during lyophilisation of liposomes was evaluated and compared. Liposomal suspension containing cyclosporine (CsA) was diluted with hydration medium containing lactose or sucrose and the amount of cyclosporine retained by the liposomes following lyophilisation 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). With the optimised lipid: cryoprotectant ratio the negative and neutral charged liposomes were lyophilised in the similar manner. 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 PBS (pH 7.8) to form liposomal suspension and subjected for the analysis of the size distribution and drug retention efficiency, the results of these determinations are shown in tables 7.3 and 7.4 respectively.

7.2.5 STABILITY STUDIES OF CONVENTIONAL AND STERICALLY STABILIZED LIPOSOMES CONTAINING LEUPROLIDE ACETATE

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 after storing in sealed glass vials (USP Type I glass). 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, entrapment efficiency and electrolyte induced flocculation test described earlier (Section 4.2.7, Chapter 4). The results of these determinations are shown in tables 7.5, 7.6 and 7.7, for the samples placed in refrigeration and at 30°C / 60% R.H.

Liposomal dispersions were prepared by reverse phase evaporation method. Unencapsulated drug was removed by ficoll centrifugation at 15,000 rpm for 30 minutes using a Remi centrifuge. The pellet was resuspended in PBS containing different ratios of trehalose and sucrose on mass basis (g of lipid / g of the cryoprotectant). The mixture was frozen overnight at -40°C. Freeze drying was performed in Lyophiliser (Heto-Holten A/S, Denmark), for 24 h to yield the product in a dry powder form.

The % retention of the drug was calculated as follows:

$$\% \text{ Retention} = EE_{(a)} / EE_{(b)} * 100$$

where $EE_{(b)}$ is the entrapment efficiency before lyophilisation and $EE_{(a)}$ is the entrapment efficiency after lyophilisation.

Lyophilisation optimisation of liposomes was performed for maximum drug retention. The use of cryoprotectants (sucrose and trehalose) during lyophilisation of liposomes was evaluated and compared. Conventional liposomal suspension containing leuprolide acetate was diluted with PBS containing trehalose or sucrose and the amount of leuprolide acetate retained by the liposomes following lyophilisation and rehydration was determined. The lipid: cryoprotectant ratio was also optimized based on the

drug retention capacity of the rehydrated lyophilised liposomal powder (figure 7.2). 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 two month for a period of 6 months, rehydrated using PBS 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, 7.8 and 7.9 respectively.

7.2.6 STABILITY STUDIES OF CONVENTIONAL AND STERICALLY STABILIZED LIPOSOMES CONTAINING DNA

Freeze dried empty liposomes (FDELs) technique was used for the incorporation of DNA into liposomes using sucrose as the cryoprotectant. The prepared FDELs 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 after storing in sealed glass vials (USP Type I glass). 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. The lyophilised conventional and sterically stabilized FDELs were withdrawn from the vials at the intervals of every two months for a period of 6 months, rehydrated using DNA aqueous solution to form liposomal suspension and subjected for the analysis of the size distribution, entrapment efficiency and electrolyte induced flocculation test and the results of these determinations are shown in tables 7.4, 7.9 and 7.10 respectively. The FDELs was found to be excellent during storage. The rehydrated liposomal DNA suspension was subjected to drug retention efficiency, particle size and steric stabilization after 7 days and 15 days and the results are shown in tables 7.11, 7.12 and 7.13.

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

Batch	Average Mean Particle Size ± S.E.M (in nm)						
		2-8°C			30±2°C		
	0 days	7 days	15 days	30 days	7 days	15 days	30 days
CL	198.2 ± 54.6	190.5 ±24.8	195.8 ±50.6	187.6 ± 24.5	206.9 ±25.6	475.8 ±40.8	700.5 ±24.8
CPL	185.3 ± 32.6	180.6 ±32.5	187.6 ±30.5	190.5 ±22.5	190.8 ±35.6	356.8 ±20.5	586.5 ±40.2
CNL	177.4 ± 27.4	172.6 ±30.6	178.5 ±42.5	180.2 ±32.6	180.8 ±28.8	325.8 ±30.5	586.5 ±32.6

n=3

Table 7.2 % Entrapment Efficiency of the liposomal dispersion before lyophilisation after storage of 7 days, 15 days and 1 month at various temperatures

	CPL			CNL			CL		
Initial EE	**96.6 ± 2.02**			**93.52 ± 1.97**			**92.13 ± 1.50**		
	4°C	**30°C**	**45°C**	**4°C**	**30°C**	**45°C**	**4°C**	**30°C**	**45°C**
After 7 days	96.6% ± 1.28	94.0% ± 1.86	90.2% ± 2.21	93.1% ± 1.56	91.3% ± 1.98	89.2% ± 1.02	92.5% ± 1.68	91.2% ± 1.46	85.2% ± 2.03
After 15 days	95.9% ± 2.23	93.2% ± 1.42	87.2% ±1.23	92.9% ± 1.28	91.0% ± 1.88	86.9% ± 1.23	92.1% ± 2.12	90.6% ± 1.69	80.1% ± 3.05
After 1 month	95.1% ± 1.59	90.1% ± 3.23	82.1% ± 1.68	92.1% ± 2.31	90.9% ± 3.02	82.2% ± 1.68	92.9% ± 2.92	89.0% ± 2.57	78.2% ± 1.39

n=3

Table 7.3 Percentage drug retention of the rehydrated lyophilized cyclosporine loaded liposomal formulations after storing at different temperatures for a period of 6 months

Batch	% Drug Retention ± S.E.M						
		2-8°C			30±2°C		
	0 month	2 month	4 month	6 month	2 month	4 month	6 month
CPL	96.22 ±1.5	96.12 ±1.2	95.9 ±1.2	95.6 ±1.6	96.02 ±2.2	95.9 ±1.6	96.02 ±1.5
CNL	93.10 ±1.3	93.17 ±1.2	92.9 ±1.2	92.81 ±1.3	93.0 ±1.2	93.02 ±1.3	92.92 ±1.2
CL	93.50 ±1.3	93.02 ±1.3	93.48 ±1.2	92.60 ±2.1	92.42 ±2.1	92.6 ±1.8	92.54 ±2.1

Table 7.4 Particle size determination of the lyophilized liposomal formulations containing cyclosporine, leuprolide acetate and DNA after storing at different temperatures for a period of 6 months

Batch	Average Mean Particle Size ± S.E.M (in nm)						
	0 month	2-8°C			30±2°C		
		2 month	4 month	6 month	2 month	4 month	6 month
CPL	198.2 ± 54.6	190.2 ± 26.3	195.6 ± 20.6	191.6 ± 28.6	186.2 ± 32.6	185.6 ± 25.6	190.8 ± 20.5
CNL	185.3 ± 32.6	178.8 ± 20.6	186.2 ± 18.6	185.3 ± 23.6	188.9 ± 25.0	190.2 ± 26.8	185.6 ± 20.0
CL	177.4 ± 27.4	180.6 ± 26.5	188.6 ± 27.0	172.5 ± 15.6	180.5 ± 20.5	185.6 ± 20.6	182.3 ± 21.0
LL	188 ± 27.4	180.4 ± 22.4	183.4 ±16.9	179.5 ±21.7	182.6 ±22.5	185.2 ±23.1	178.1 ±22.5
SLL5000	175 ± 22.3	202.3 ± 21.3	189.5 ±23.4	212.3 ±30.4	191.9 ±23.4	206.7 ±24.8	214.3 ±23.6
SLL2000	179 ± 30.2	182.6 ±26.1	179.5 ±26.5	190.4 ±21.1	184.6 ±26.7	192.5 ±31.4	186.2 ±21.9
DL	182 ±33.8	176.9 ±23.0	186.3 ±16.7	191.2 ±19.8	185.4 ±20.6	192.3 ±28.6	194.5 ±16.4
SDL5000	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
SLL2000	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
CSDL 2000	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

Table 7.5 Particle size determination of the leuprolide acetate loaded liposomal dispersions after storing at different temperatures for a period of 30 days

Batch	Average Mean Particle Size ± S.E.M (in nm)						
		2-8°C			30±2°C		
	0 days	10 days	20 days	30 days	10 days	20 days	30 days
LL	188 ± 54.6	190.5 ±24.8	195.8 ±50.6	187.6 ± 24.5	200.3 ±25.6	350.5 ±40.8	600.8 ±24.8
SLL5000	175 ± 32.6	180.6 ±32.5	187.6 ±30.5	190.5 ±22.5	220.8 ±35.6	356.8 ±20.5	586.5 ±40.2
SLL2000	179 ± 27.4	172.6 ±30.6	178.5 ±42.5	180.2 ±32.6	240.8 ±28.8	325.8 ±30.5	586.5 ±32.6

Table 7.6 % Entrapment Efficiency of the leuprolide acetate loaded liposomal dispersions after storage of 7 days, 15 days and 1 month at various temperatures.

	LL		SLL5000		SLL2000	
Initial EE	**47.12 ± 2.64**		**37.8 ± 3.46**		**33.03 ± 4.02**	
	2-8°C	**30°C**	**2-8°C**	**30°C**	**2-8°C**	**30°C**
After 10 days	47.12% ± 2.64	42.12% ± 2.64	37.8% ± 3.46	35.9% ± 3.46	33.02% ± 4.02	30.92% ± 2.65
After 20 days	40.28% ± 2.32	35.28% ± 2.32	33.65% ± 3.06	30.25% ± 3.06	31.23% ± 2.86	26.38% ± 1.89
After 30 days	35.26% ± 1.02	31.26% ± 1.02	30.68% ± 2.08	25.23% ± 2.08	30.68% ± 1.23	20.23% ± 1.68

n=3

Table 7.7 Steric stability studies of the leuprolide acetate loaded 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	2.0M	0.8M	2.0M	0.8M	2.0M	0.8M	2.0M	0.8M	2.0M	0.8M	2.0M	0.8M	2.0M
LL	0.552 (0.02)	0.612 (0.02)	0.564 (0.02)	0.620 (0.02)	0.567 (0.02)	0.621 (0.02)	0.556(0.01)	0.618 (0.03)	0.558 (0.01)	0.625 (0.01)	0.558 (0.02)	0.630 (0.02)	0.720 (0.03)	0.798 (0.02)
SLL5000 PE	0.526 (0.02)	0.530 (0.02)	0.535 (0.024)	0.549 (0.016)	0.525 (0.014)	0.552(0.016)	0.534 (0.016)	0.550 (0.025)	0.532 (0.023)	0.538 (0.033)	0.538 (0.013)	0.540 (0.023)	0.616 (0.010)	0.640 (0.014)
SLL2000 PE	0.538 (0.01)	0.578 (0.02)	0.540 (0.015)	0.590 (0.022)	0.542 (0.017)	0.582 (0.013)	0.540 (002)	0.586 (0.02)	0.546 (0.03)	0.580 (0.01)	0.548 (0.026)	0.582 (0.028)	0.612 (0.016)	0.628 (0.027)

Table 7.8 Percentage drug retention of the rehydrated lyophilized leuprolide acetate loaded liposomal formulations after storing at different temperatures for a period of 6 months

Batch	% Drug Retention ± S.E.M						
	0 month	2-8°C			30±2°C		
		2 month	4 month	6 month	2 month	4 month	6 month
LL	92.7 ±1.3	92.0 ±1.6	92.1 ±1.5	89.6 ±2.0	89.3 ±2.1	90.2 ±2.1	88.2 ±2.2
SLL5000	89.2 ±2.0	89.2 ±1.8	88.2 ±1.6	87.9 ±1.5	88.1 ±2.2	87.3 ±2.3	87.2 ±2.4
SLL2000	86.3 ±1.8	86.5 ±1.7	85.3 ± 1.5	84.9 ±1.6	85.4 ±1.8	85.6 ±1.9	85.7 ±2.0

Table 7.9 Percentage entrapment efficiency of the rehydrated lyophilized DNA loaded liposomal formulations after storing at different temperatures for a period of 6 months

Batch	% Entrapment Efficiency ± S.E.M						
	0 month	2-8°C			30±2°C		
		2 month	4 month	6 month	2 month	4 month	6 month
DL	49.56 ±0.96	49.62 ±1.2	49.02 ±1.5	49.23 ±1.3	48.98 ±1.9	49.05 ±1.8	48.79 ±1.9
SDL5000 CC-PE	45.34 ±1. 6	45.32 ±2.1	45.62 ±1.1	45.32 ±1.2	45.02 ±1.3	45.23 ±2.1	45.23 ±1.3
SDL2000 CC-PE	46.23 ±1.9	46.29 ±1.1	46.69 ±1.3	46.02 ±1.2	46.99 ±1.3	45.08 ±1.1	46.20 ±1.2
CSDL 2000-CC-PE	75.62 ±1.5	75.08 ±2.2	75.23 ±2.3	75.62 ±2.2	75.36 ±1.7	75.99 ±1.8	74.78 ±2.2

Table 7.10 Steric stability studies of rehydrated lyophilized leuprolide acetate and DNA loaded 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	2.0M	0.8M	2.0M	0.8M	2.0M	0.8M	2.0M	0.8M	2.0M	0.8M	2.0M	0.8M	2.0M
LL	0.552 (0.01)	0.612 (0.02)	0.555 (0.02)	0.614 (0.05)	0.550 (0.03)	0.618 (0.02)	0.542 (0.05)	0.610 (0.02)	0.558 (0.01)	0.608 (0.06)	0.548 (0.02)	0.610 (0.03)	0.556 (0.02)	0.618 (0.01)
SLL5000 PE	0.526 (0.01)	0.542 (0.01)	0.530 (0.01)	0.546 (0.02)	0.532 (0.01)	0.548 (0.03)	0.524 (0.03)	0.540 (0.06)	0.528 (0.02)	0.550 (0.03)	0.530 (0.03)	0.546 (0.01)	0.528 (0.03)	0.550 (0.02)
SLL2000 PE	0.538 (0.01)	0.578 (0.02)	0.530(0.06)	0.580 (0.01)	0.542 (0.01)	0.582 (0.02)	0.536 (0.02)	0.580 (0.03)	0.532 (0.03)	0.572 (0.01)	0.539 (0.01)	0.578 (0.02)	0.532 (0.01)	0.580 (0.02)
DL	0.526 (0.02)	0.579 (0.01)	0.530 (0.02)	0.582 (0.01)	0.528 (0.02)	0.586 (0.02)	0.532 (0.01)	0.580 (0.03)	0.529 (0.02)	0.576 (0.01)	0.536 (0.02)	0.582 (0.05)	0.528 (0.02)	0.579 (0.05)
SDL5000 CC-PE	0.580 (0.01)	0.598 (0.02)	0.582 (0.04)	0.594 (0.01)	0.576 (0.02)	0.599 (0.02)	0.580 (0.03)	0.596 (0.03)	0.582 (0.03)	0.599 (0.03)	0.578 (0.02)	0.586 (0.04)	0.580 (0.02)	0.596 (0.03)
SDL2000 PE	0.518 (0.02)	0.530 (0.01)	0.520 (0.02)	0.532 (0.01)	0.516 (0.01)	0.530 (0.01)	0.518 (0.01)	0.532 (0.01)	0.522 (0.01)	0.531 (0.01)	0.518 (0.04)	0.530 (0.03)	0.520 (0.04)	0.532 (0.03)
CSDL 5000PE	0.520 (0.04)	0.536 (0.02)	0.522 (0.02)	0.540 (0.01)	0.520 (0.03)	0.538 (0.02)	0.522 (0.02)	0.540 (0.02)	0.521 (0.02)	0.539 (0.02)	0.520 (0.01)	0.543 (0.01)	0.521 (0.03)	0.538 (0.01)

Table 7.11 % drug retention of the DNA loaded liposomal suspension after rehydration and storage at 7 days and 15 days at various temperatures

Batch	% Drug retention ± S.E.M			
	2-8°C		30±2°C	
	7 days	15 days	7 days	15 days
DL	94.6 ±2.1	85.62 ±1.8	90.2 ±1.7	76.3 ±1.8
SDL5000	91.6 ±2.1	82.3 ±1.3	90.1 ±1.4	74.2 ±1.5
SDL2000	94.3 ±2.0	82.7 ±1.5	91.3 ±1.6	70.6 ±2.2
CSDL2000	96.7 ±2.3	86.2 ±1.8	92.5 ±1.9	80.2 ±1.4

Table 7.12 Particle size determination of the DNA loaded liposomal dispersions after rehydration and storing at different temperatures for a period of 15 days

Batch	Average Mean Particle Size ± S.E.M (in nm)				
	0 days	2-8°C		30±2°C	
		7 days	15 days	7 days	15 days
DL	182 ±33.6	184.6 ±30.2	412.5 ±25.6	269.5 ±36.8	601.2 ±30.9
SDL5000	190.6 ±25.6	196.5 ±33.6	402.8 ±24.6	246.5 ±23.6	588.6 ±32.6)
SDL2000	236.2 ±28.3	201.3 ±29.7	378.1 ±30.5	236.5 ±27.8	564.8 ±30.7
CSDL2000	205.6 ±25.6	190.4 ±32.5	369.8 ±38.6	274.6 ±33.6	557.1 ±36.9

Table 7.13 Steric stability studies of the DNA loaded liposomal dispersions after rehydration and storing at different temperatures for a period of 15 days

Batch			2-8°C				30±2°C			
	0 days		7 days		15 days		7 days		15 days	
	0.8M	2.0M	0.8M	2.0M	0.8M	2.0M	0.8M	2.0M	0.8M	2.0M
DL	0.526 ±0.02	0.579 ±0.01	0.530 ±0.03	0.582 ±0.02	0.642 ±0.01	0.702 ±0.03	0.579 ±0.03	0.632 ±0.01	0.665 ±0.02	0.756 ±0.02
SDL5000 CC-PE	0.580 ±0.01	0.598 ±0.02	0.582 ±0.01	0.602 ±0.04	0.643 ±0.02	0.669 ±0.02	0.618 ±0.01	0.628 ±0.03	0.670 ±0.04	0.702 ±0.02
SDL2000 PE	0.518 ±0.02	0.530 ±0.01	0.520 ±0.01	0.532 ±0.01	0.586 ±0.03	0.612 ±0.02	0.546 ±0.02	0.576 ±0.01	0.605 ±0.01	0.629 ±0.05
CSDL 5000PE	0.520 ±0.04	0.536 ±0.02	0.524 ±0.03	0.540 ±0.03	0.592 ±0.03	0.599 ±0.03	0.562 ±0.02	0.576 ±0.03	0.604 ±0.01	0.619 ±0.03

Figure 7.1 Effect of sucrose and lactose on the % retention of CsA loaded positive charged liposomes

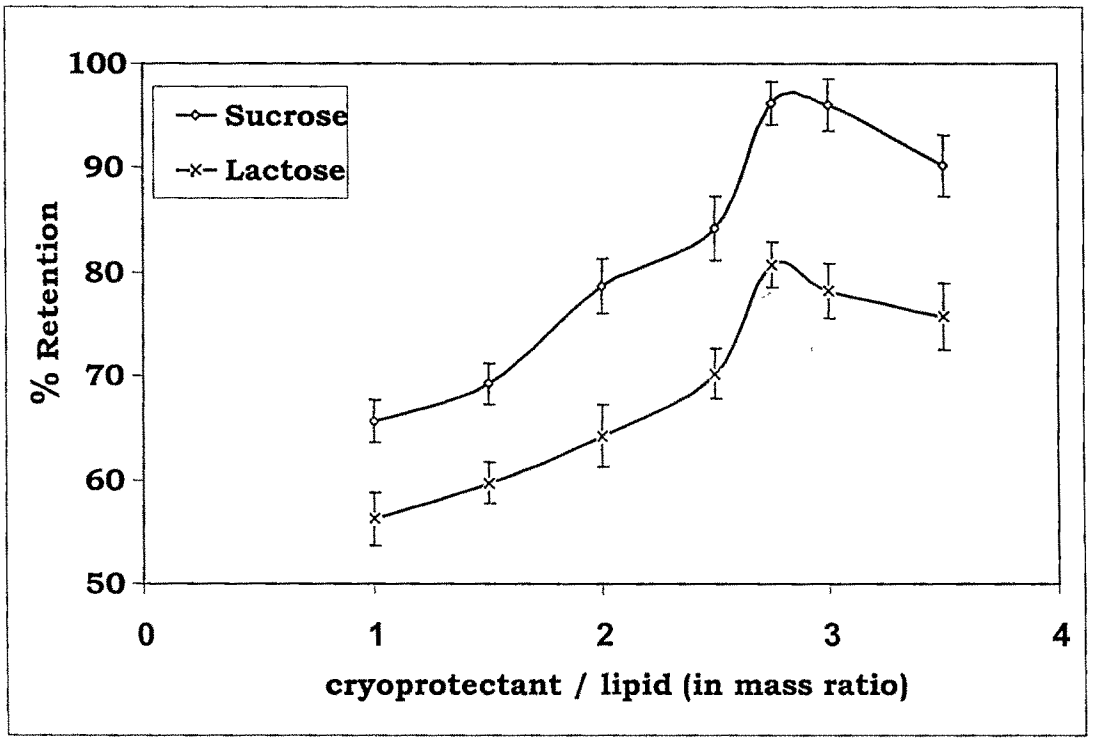
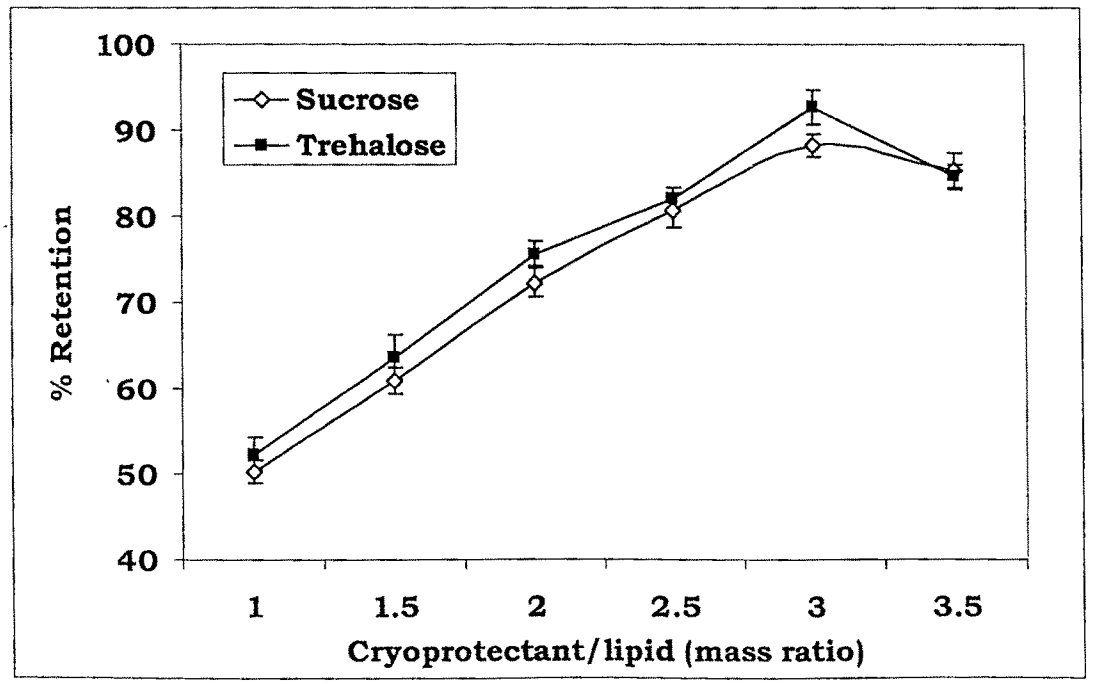


Figure 7.2 Effect of sucrose and trehalose on the % retention of leuprolide acetate loaded conventional liposomes



7.3 RESULTS AND DISCUSSION

7.3.1 CYCLOSPORINE LOADED LIPOSOMES

Pharmaceutically acceptable liposome formulation should retain encapsulated drug for long periods, so that it can be stored in a form ready for injection. The particle size distribution of the cyclosporine loaded liposomal dispersions after storing at different temperatures for a period of 30 days was shown in table 7.1, which proved that there was no significant change in vesicle size or aggregation for 1 month at 2-8°C but a significant change in the vesicle size at 30°C was observed after 15 days. After 7 days of storage the percentage of the drug retained in drug loaded liposomes were 96.6%, 93.1% and 92.5% at 4°C, 94.0%, 91.3% and 91.2% at 25°C and 90.2%, 89.2% and 85.2% at 45°C for CPL, CNL and CL respectively. After 15 days of storage the percentage of the drug retained in drug loaded liposomes were 95.9%, 92.9% and 92.1% at 4°C, 93.2%, 91.0% and 90.63 at 25°C and 87.2% , 86.9% and 80.1% at 45°C for CPL, CNL and CL respectively. After 30 days of storage, the percentage of the drug retained in drug loaded liposomes were 95.1%, 92.1% and 92.9% at 4°C, 90.1%, 90.9% and 89.0% at 25°C and 82.1%, 82.2% and 78.2% at 45°C for CPL, CNL and CL respectively. These results indicate that maximum reduction in entrapment efficiency was observed following storage at 45°C as shown in table 7.2, nearly 90% of the encapsulated drug was retained inside the liposomes for a period of 30 days in PBS (pH 7.8) at 2-8°C. This stability of formulation can be attributed to 15mol % of cholesterol and the presence of charged lipids included in the formulation.

Lyophilisation has a great potential as a method to solve long-term stability problems of liposomes. Liposomal dispersions when freeze dried without cryoprotectant gave rise to a pasty mass, while the use of lactose and sucrose gave free flowing powder. The use of cryoprotectants during lyophilisation of liposomes was evaluated and compared. Liposomal suspension was diluted with PBS containing sucrose or lactose and the amount of CsA retained by the liposomes following lyophilisation and rehydration was determined as shown in figure 7.1. The data reveals that sucrose gave significantly higher percentage of CsA retention compared to lactose. This is in agreement with the findings of Madden et al, who

examined the effectiveness of number of sugars in maintaining structural and functional properties of microsomal membranes at low mean liposomal size and found trehalose to be the most effective one and sucrose at higher concentrations was found to be equally effective for liposomes. Liposomes containing entrapped CsA were lyophilized in the presence of varying concentrations of sucrose. The % of CsA retained by the lyophilized liposomes upon rehydration was found to be dependent upon the sucrose concentration. With the increase in the concentration of sucrose from 1.5 to 2.75 (mass ratio with respect to lipids) the % CsA retained within the liposomes was increased and was found to be maximum at 2.75 mass ratio as shown in figure 7.1. A mass ratio of 2.75 times of lipid was found to be the most effective and gave more than 90% of drug retention. This is in agreement with the previous studies, which have shown that liposomes can retain greater than 90% of lipid soluble drugs, such as doxorubicin, on reconstitution (Van Bommel and Crommelin, 1984). The amount retained depends on the use of cryoprotectants and lipid composition (Lasic et al., 1998). When the concentration was further increased to 3.5 mass ratio, there was a significant decrease in the % CsA retention. This may be due to the fact that in the process of dehydration, the liposomal vesicles get constricted and coated on the surface of internally crystallized sugar. This stabilization by coating is in addition to stabilization by the hydration of polar head groups with hydroxyl group of sucrose, which replaces the lyophilizing water molecules. If the concentration of sucrose is less than optimum, the internally crystallized sugar does not provide adequate surface for the adherence of constricted bilayers. Similarly if the concentration of the sucrose is high, the large sized crystals pierce out the bilayers. Lactose is not particularly effective like sucrose, which retains only 80% at 1:2.75 compared to sucrose, as shown in figure 7.1.

Thus with the optimized cryoprotectant ratio (1: 2.75), CNL and CL were lyophilized. The freeze-dried powder was stable for a period of 6 months and the size of the liposomes was maintained as shown in table 7.3 and 7.4. The process of lyophilisation-rehydration did not significantly alter the entrapment levels of CsA indicating that 100 % of the entrapped drug remains associated with liposomal membranes.

7.3.2 LEUPROLIDE ACETATE LOADED LIPOSOMES

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 2.0M 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 particle size distribution as shown in table 7.5 reveals that the size is maintained at 2-8°C for a period of 30 days and entrapment efficiency as shown in table 7.6 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. The increase in mean particle size of all liposomal formulations stored at 30°C proved the instability of the liposomes in dispersion form. Table 7.7 reveals that the steric stability of all the liposomal dispersions is maintained for a period of 30 days (stored at 2-8°C) and less than 20 days (stored at 30°±2°C / 60 ± 5% R.H). 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 trehalose were used for the purpose of lyophilisation. Optimization of the lyophilisation process of the conventional liposomes containing leuprolide acetate was performed using various lipid : sugar (trehalose/sucrose) mass ratio (1: 1, 1: 1.5, 1: 2, 1: 2.5,

1: 3 and 1:3.5) for maximum drug retention (figure 7.2). The data reveals that sucrose and trehalose (1: 3 mass ratio) gave significantly higher percentage of drug retention (92.7%) and proved to be better cryoprotectant for the lyophilisation 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 lyophilisation 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 PBS 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 7.8 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 7.10 revealed that the steric stabilizing property of the liposomes was also preserved in the lyophilised form.

7.3.3 DNA LOADED LIPOSOMES

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 DNA aqueous solution and evaluated for the particle size distribution, steric stabilization and percent entrapment efficiency. 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 7.9 showed the entrapment efficiency of the prepared liposomal formulations and no significant reduction in the entrapment efficiency was observed and proved to be highly stable during storage. Examination of the data included in the

table 7.10 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.

The particle size distribution of the prepared liposomes after rehydration was ascertained by subjecting the 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 particle size distribution as shown in table 7.12 reveals that the size is maintained at 2-8°C for a period of 7 days and % drug retention as shown in table 7.11 of the liposomal dispersions showed that liposomal formulations stored at 2-8°C was little more stable for a period of 7 days compared to the liposomes stored at 30°±2°C. 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. Table 7.13 reveals that the steric stability of all the liposomal dispersions is maintained for a period of 7 days (stored at 2-8°C) and less than 7 days (stored at 30°±2°C / 60 ± 5% R.H).

7.4 CONCLUSIONS

Inclusion of high melting lipids like hydrogenated soya phosphatidyl choline, stearyl amine and Distearoyl phosphatidyl glycerol in the bilayer helped to preserve the stability of the liposomal dispersions. The process of lyophilisation was used as an alternate method for providing the stability to the liposomes. The lyophilisation of the liposomal preparations using sucrose, trehalose and lactose as cryoprotectant was found to be highly successful in providing excellent stability to the liposomal preparations and desired shelf life required for commercial purposes.

REFERENCES

Crowe, J.H., Crowe, L.M., Carpenter, J.F. (1988). Interaction of sugars with membranes. *Biochim. Biophys. Acta.*, 947, 367–384.

Koster, K.L., Webb, M.S., Bryant, G., Lynch, D.V. (1994). Interactions between soluble sugars and POPC (1-palmitoyl-2-oleoylphosphatidilcholine) during dehydration, vitrification of sugars alters the phase behavior of the phospholipid. *Biochim. Biophys. Acta.*, 1193, 143–150.

Dean, J.A. (1999). (ed.). Lange's Handbook of Chemistry. 15th edn., McGraw Hill Inc., New York, 116.

Madden, T.D., Cullis, P.R., Hope, M.J., Bally, M.B., Schieren, H.P., Janoff , A.S. (1985). Protection of large unilamellar vesicles by trehalose during dehydration retention of vesicle contents. *Biochim. Biophys. Acta.*, 817, 67–74.

Mobley, W.C., Schreier, H. (1994). Phase transition temperature and glass formation in dehydroprotectant lyophilized liposomes. *J. Control Rel.*, 31, 73–87.

Shulkin, P.M., Selter, S.E., Davis, M.A., Adams, D.F. (1984). Lyophilized liposomes: a new method for long-term vesicular storage. *J. Microencapsulation.*, 1, 73–80.

Tanaka, K., Takeda, T., Fujii, K., Miyajima, K. (1992). Cryoprotective mechanism of saccharides on freeze-drying of liposome. *Chem. Pharm. Bull.*, 40, 1–5.