Chapter 5
Preparation and Characterization of Liposomes

5.1. **Preparation of Liposomes**

5.1.1 INTRODUCTION

There are a large variety of methods for preparing liposomes. From a pharmaceutical point of view, the three most important factors to be evaluated before selecting the method of preparation are the entrapping efficiency, drug retention property and drug/lipid ratio (Betagiri et. al., 1993)

Trapping efficiency is one of the important parameters in selecting a method of preparation of liposomes. An optimum loading procedure would achieve trapping efficiency of 90% or more. This obviates the need for removal of unentrapped drug because loading doses of 10 % or less of free drug can usually be tolerated. The procedures, such as dialysis and passage through exclusion columns for removal of unentrapped drug are often time consuming, tedious, expensive and recovery of unentrapped drug is usually difficult.

Considering drug retention it is unlikely that most drug-liposome formulations can exhibit sufficiently low leakage rates to allow retention times of one year or more. However if the entrapment efficiencies are sufficiently high (e.g. 90 % or more), unentrapped drug need not be removed. No leakage of drug would then occur, on extended storage because of the absence of transmembrane drug concentration gradients. The optimum drug: lipid ratio of a liposomal formulation will likely be dictated by the biological efficacy and toxicity of the preparation. From a pharmaceutical point of view, high drug: lipid ratios are obviously more economical.

In summary, optimum liposomal formulations will exhibit drug trapping efficiencies in excess of 90%, use of inexpensive and relatively saturated lipids and exhibit the highest possible drug: lipid ratio which is consistent with maintained efficacy of the preparation. Apart from these factors, other factors which need to be considered in selection of the methods of preparation include selection of methods which would avoid the use of organic solvents and detergents (which are difficult to remove), yield well defined and

reproducible liposomes which are rapid and amenable to scale up procedures. Any special applications of the liposomes to be prepared also may contribute in the selection of the appropriate method.

5.1.2 EXPERIMENTAL

Material:

Dioleoylphosphatidylethanolamine (DOPE) is the generous gift from Lipoid (Lipoid GmbH, Ludwigshafen, Germany) 1,2 Dimyristoyl-sn-glycero-3-phosphoglycerol, sodium salt (DMPG) was the kind gift from Genzyme Corporation, MA, USA. Hydrogenated soy phosphatidylcholine (HSPC) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*n*-[methoxy(polyethylene glycol)-2000] ($mPEG_{2000}$-DSPE) and Cholesterol (CHOL) were purchased from Avanti polar lipids. Cholesteryl hemisuccinate (CHEMS) was purchased from Sigma Chemical (MO, USA). 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES) was purchased from Himedia Labs, Mumbai. Paclitaxel (PCL) was generously gifted by Xechem Pharmaceuticals NJ, USA. Irinotecan Hydrochloride Trihydrate was the generous gift from Sun Pharma Advanced Research Centre (SPARC, Baroda). Sephadex G-50 & Sephadex G-25 were purchased from Sigma Chemical (MO, USA). All lipids were used without further purification. All other reagents were of analytical grade.

Apparatus

Rotary evaporator with vacuum pump and thermostatically controlled water bath and nitrogen purging facility(Superfit equipments, India), Probe sonicator (Sartorius), Remi cooling centrifuge, Sigma centrifuge, Shimadzu UV-1601 UV–Visible spectrophotometer (Shimadzu Corporation , Japan).

5.1.3 Preparation of conventional liposomes of Paclitaxel

Paclitaxel containing conventional liposomes were prepared by lipid film hydration technique (New, 1990). Briefly, DMPG, HSPC and Cholesterol in the molar ratio of 6:2:2 along with drug were dissolved in a mixture of Chloroform and methanol (ratio 7:3

by volume) in a 250 ml round bottom flask (Quick fit neck B-24) and the solvent was evaporated using a rotary evaporator. The flask was rotated in a rotary flash evaporator at 100 rpm for 45 minutes in a thermostatically controlled water bath at 57°C under vacuum (600mm of Hg) with nitrogen as bleed. The thin dry lipid film formed was hydrated using 2 ml of PBS pH 7.4 and the flask was rotated once again at the speed of 80 rpm at the same temperature as above for 45 min. The liposomal suspension so formed was then transferred to a suitable glass container and sonicated for 3 min using a probe sonicator in an ice batch for heat dissipation. The sonicated dispersion was then allowed to stand undisturbed for about 2 hours at room temperature for the annealing to be completed. This liposomal suspension was then sequentially extruded through a series of polycarbonate membrane filters (Nucleopore, CA, USA) with pore sizes of 400–100 nm, using an Extruder. The unentrapped drug was removed from the liposomal suspension by using centrifugation at 5000rpm. The unentrapped drug settles down on centrifugation which was analysed and supernatant suspension was separated. The Sephadex G-50 column method did not give expected separation. The entrapment efficiency (EE) is defined as the ratio of the amount of the paclitaxel encapsulated in liposome to that of the total paclitaxel in liposomal dispersion. The amount of Paclitaxel encapsulated in liposomes was measured following the method in the literature with slight modification (Shieh et. al., 1997). Briefly, aliquots (0.1mL each) of liposomal dispersion diluted to 1.1mL by PBS (pH 7.4) immediately after preparation was centrifuged at 1000 rpm for 10 min to remove any Paclitaxel particle already released from the liposomes. Then, 1.0mL of the liposome supernatant was centrifuged at 25,000 rpm for 30 min (Sigma 3K30, USA). After removing the supernatant by aspiration, the precipitate (*i.e.*, liposome pellet) was washed twice with PBS (pH 7.4). The liposome pellet was dissolved organic solvents mixture of Methanol and chloroform (9:1 v/v). The concentration of Paclitaxel was determined by spectrophotometrically at 231 nm after appropriate dilution with the mixed solvent that destroyed the liposome pellet. The liposomal suspension was freeze dried over night at -20 °C, lyophilized for 24 hours using sucrose as cryoprotectant (3 times the weight of total lipids used) and stored in vials at 2-8 °C. A flow chart depicting the above process is shown in figure 5.1.1. The major process parameters were optimized using the percentage drug entrapment as the response parameter. The observations of the

optimization process are tabulated in table 5.1.2. Comparison of these liposomes was made with pH sensitive liposomes containing Paclitaxel.

Table 5.1.1. Optimization parameters for preparation of conventional liposomes containing paclitaxel (CLPT)

Batch No.	Drug: Lipid (molar ratio) X_1	[(DMPG:HSPC): Chol] (% of total lipids) X_2	Hydration volume (ml) X_3	Drug entrapment (% ± S.E.)*
CLPT1	1:5	60:40	1	26.32 ± 1.45
CLPT2	1:10	60:40	1	33.14 ± 2.01
CLPT3	1:15	60:40	1	46.26 ± 1.25
CLPT4	1:5	70:30	1	27.43 ± 1.92
CLPT5	1:10	70:30	1	36.22 ± 1.48
CLPT6	1:15	70:30	1	55.75 ± 1.74
CLPT7	1:5	80:20	1	41.32 ± 2.31
CLPT8	1:10	80:20	1	60.45 ± 2.11
CLPT9	1:15	80:20	1	72.58 ± 2.15
CLPT10	1:5	60:40	2	53.18 ± 1.77
CLPT11	1:10	60:40	2	60.86 ± 1.86
CLPT12	1:15	60:40	2	70.22 ± 2.22
CLPT13	1:5	70:30	2	57.02 ± 1.94
CLPT14	1:10	70:30	2	69.58 ± 1.57
CLPT15	1:15	70:30	2	71.48 ± 2.24
CLPT16	1:5	80:20	2	68.41 ± 2.19
CLPT17	**1:10**	**80:20**	**2**	**84.56 ± 1.38**
CLPT18	1:15	80:20	2	82.45 ± 1.13
CLPT19	1:5	60:40	3	47.16 ± 2.13
CLPT20	1:10	60:40	3	61.82 ± 1.99
CLPT21	1:15	60:40	3	67.57 ± 2.05
CLPT22	1:5	70:30	3	56.68 ± 1.42
CLPT23	1:10	70:30	3	72.09 ± 2.01
CLPT24	1:15	70:30	3	76.91 ± 1.93
CLPT25	1:5	80:20	3	59.52 ± 2.08
CLPT26	1:10	80:20	3	70.17 ± 2.26
CLPT27	1:15	80:20	3	79.14 ± 2.82

***n=3**

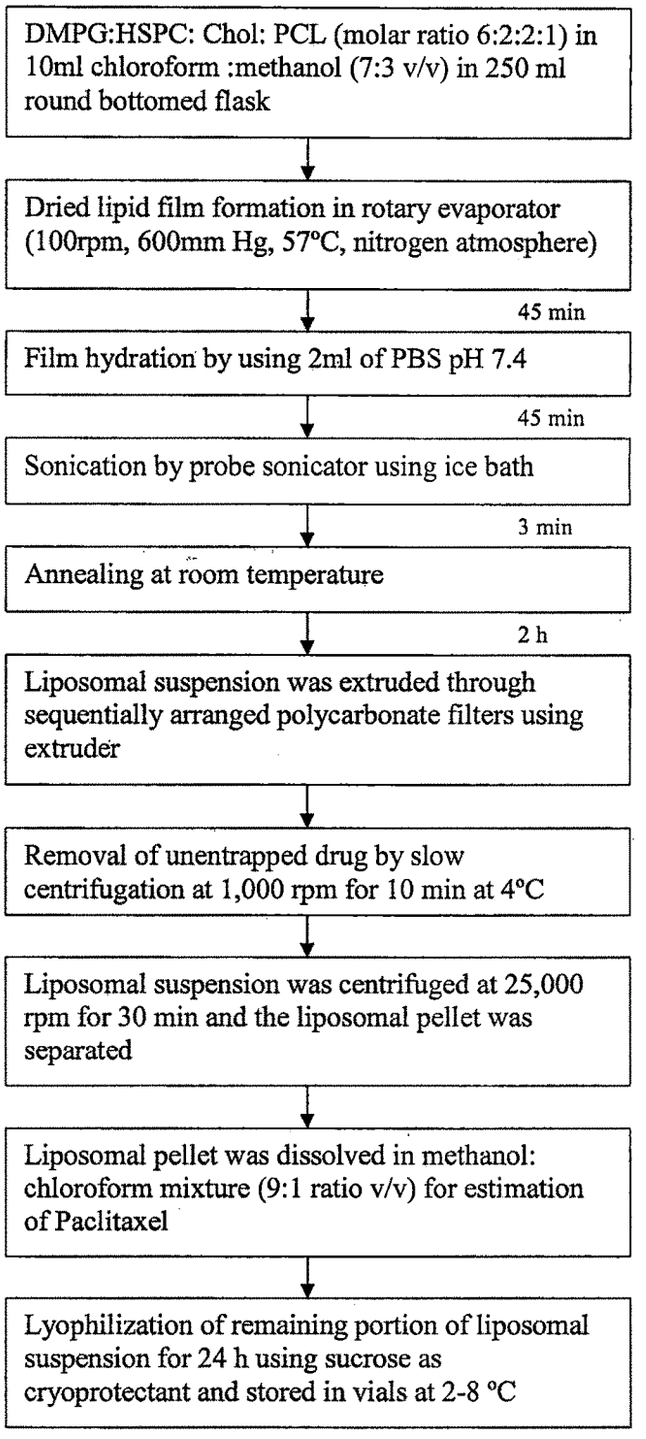


Figure 5.1.1. Flow chart for the preparation of conventional liposomes containing Paclitaxel.

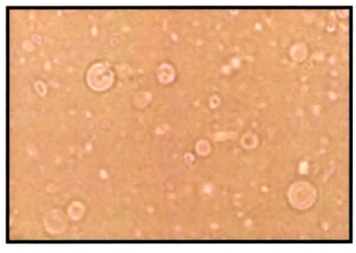


Figure 5.1.2. Photomicrograph of conventional paclitaxel liposomes before extrusion

Table 5.1.2. Characterization of Conventional liposomes of Paclitaxel

Formulation	Size (nm)	PDI (Uniformity)	Zeta potential	Encapsulation efficiency
CLPT	128 ± 8.65	0.189	-12.8 ± 2.54	86.56 ± 1.38

5.1.4 Preparation of pH sensitive, Serum stable, long circulating liposomes

Different methods have been reported to achieve long circulation of liposomes *in vivo*, including coating the liposome surface with inert, biocompatible polymers, such as PEG, which form a protective layer over the liposome surface and delay the liposome recognition by opsonins and therefore subsequent clearance of liposomes (Blume and Cevc, 1993).

5.1.5 METHODS

5.1.6 Preparation of pH sensitive Paclitaxel Liposomes:

The liposomes were composed of DOPE: HSPC: CHEMS: CHOL at various molar ratios either with or without $mPEG_{2000}$-DSPE at 5 mol % to phospholipids. The $mPEG_{2000}$-DSPE is used for preparing sterically stabilized liposomes. Small unilamellar vesicles were prepared as previously described (Ishida et. al., 2003). Briefly, lipids were mixed and dissolved in organic solvents like chloroform-methanol mixture along with drug in a 250 ml round bottom flask (Quick fit neck B-24) and the solvent was evaporated using a rotary evaporator; residual solvent was removed under high vacuum. The dried lipid films were hydrated with an appropriate buffer and sequentially extruded through a series of polycarbonate membrane filters (Nucleopore, CA, USA) with pore sizes of 400–100

nm, using an Extruder. All process and formulation parameters were optimized to achieve the desired properties like size and encapsulation efficiency. The mean diameters of the resulting liposomes were determined by Nano-ZS Particle size analyzer (Malvern Instruments, UK). The unentrapped drug was separated by ultracentrifugation/ Sephadex column method. Other methods of liposomal preparation like Ethanol injection and reverse phase evaporation were also tried for encapsulation of Paclitaxel. Due to low encapsulation of drug, these methods were not adopted further.

Table 5.1.3. Optimization of preparation of pH sensitive liposomes of Paclitaxel.

Batch No.	Drug: Lipid (molar ratio) X_1	[(DOPE:HSPC: CHEMS): Chol] (% of total lipids) X_2	Hydration volume (ml) X_3	Drug entrapment (% ± S.E.)*
PSPT1	1:5	60:40	1	27.46 ± 1.23
PSPT 2	1:10	60:40	1	34.47 ± 1.48
PSPT 3	1:15	60:40	1	48.78 ± 1.89
PSPT 4	1:5	70:30	1	30.12 ± 1.97
PSPT 5	1:10	70:30	1	38.23 ± 1.57
PSPT 6	1:15	70:30	1	56.48 ± 1.98
PSPT 7	1:5	80:20	1	41.34 ± 2.01
PSPT 8	1:10	80:20	1	52.46 ± 2.43
PSPT 9	1:15	80:20	1	64.75 ± 1.84
PSPT 10	1:5	60:40	2	58.45 ± 1.46
PSPT 11	1:10	60:40	2	61.08 ± 0.98
PSPT 12	1:15	60:40	2	66.38 ± 2.12
PSPT 13	1:5	70:30	2	60.21 ± 1.68
PSPT 14	1:10	70:30	2	72.58 ± 1.75
PSPT 15	1:15	70:30	2	71.75 ± 2.16
PSPT 16	1:5	80:20	2	69.57 ± 1.92
PSPT 17	**1:10**	**80:20**	**2**	**87.13 ± 1.49**
PSPT 18	1:15	80:20	2	84.69 ± 1.17
PSPT 19	1:5	60:40	3	58.14 ± 1.76
PSPT 20	1:10	60:40	3	64.80 ± 2.01
PSPT 21	1:15	60:40	3	65.15 ± 2.15
PSPT 22	1:5	70:30	3	62.64 ± 1.37
PSPT 23	1:10	70:30	3	74.17 ± 1.64
PSPT 24	1:15	70:30	3	78.89 ± 1.39
PSPT 25	1:5	80:20	3	63.48 ± 1.85
PSPT 26	1:10	80:20	3	76.86 ± 1.73
PSPT 27	1:15	80:20	3	80.07 ± 2.31

Freeze-drying of Liposomal formulation

A freeze-dried liposomal formulation should have certain desirable characteristics, including: i) the preservation of the primary physical and chemical characteristics of the product (elegant cake appearance, short reconstitution time, an acceptable suspension and low or unmodified particle size distribution of liposomal suspensions, unchanged activity of encapsulated drug), ii) an acceptable relative humidity, and iii) long-term stability (peptides, or colloidal carriers :liposomes, nanoparticles, nanoemulsions). This process is relatively slow and expensive, it is especially applies only for products having a high added value. Freeze-drying cycle can be divided into three steps: freezing (solidification), primary drying (ice sublimation) and secondary drying (desorption of unfrozen water).

Freeze-drying may generate many stresses that could destabilize colloidal suspensions, especially, the stress of freezing and dehydration. It is well known that during the freezing of a sample there is a phase separation into ice and cryo-concentrated solution. This high concentration of particulate system may induce aggregation and in some cases irreversible fusion of colloidal carriers. Furthermore, the crystallization of ice may exercise a mechanical stress on liposomes leading to their destabilization. For these reasons, special excipients must be added to the suspension of nanoparticles before freezing to protect these fragile systems (Abdelwahed et. al., 2006: Abdelwahed et. al., 2006a). These excipients are usually added in order to protect the product from freezing stress (cryoprotectant) or drying stress (lyoprotectant) and also to increase its stability upon storage. The most popular cryoprotectants encountered in the freeze-drying process of pharmaceutical products in literature are sugars: trehalose, sucrose, glucose and mannitol. These sugars are known to vitrify at a specific temperature denoted Tg′ (Pikal, 1999: Franks, 1982). The immobilization of liposomes/nanoparticles within a glassy matrix of cryoprotectant can prevent their aggregation and protect them against the mechanical stress of ice crystals. Generally, freezing must be carried out below Tg′ of a frozen amorphous sample or below Teu (eutectic crystallization temperature) which is the crystallization temperature of soluble component as a mixture with ice, if it is in a

crystalline state in order to ensure the total solidification of the sample (Tang and Pikal, 2004).

Lyophilization optimization of liposomes was performed for maximum drug retention. The use of cryoprotectants (Sucrose, Trehalose and Maltose) during lyophilization of liposomes was evaluated and compared. The liposomal suspension containing Paclitaxel was diluted with distilled water containing Sucrose, Trehalose or Maltose and the amount of drug retained by liposomes following lyophilization and rehydration was determined. The lipid: cryoprotectant ratio was also optimized based on the drug retention capacity of the rehydrated lyophilized liposomal powder. The lyophilized 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/ 65 % RH after storing in sealed USP type I glass vials. The lyophilized samples were withdrawn from the vials at predetermined time intervals for a period of 6 months rehydrated using water for injection to form liposomal suspension and subjected for the different analysis.

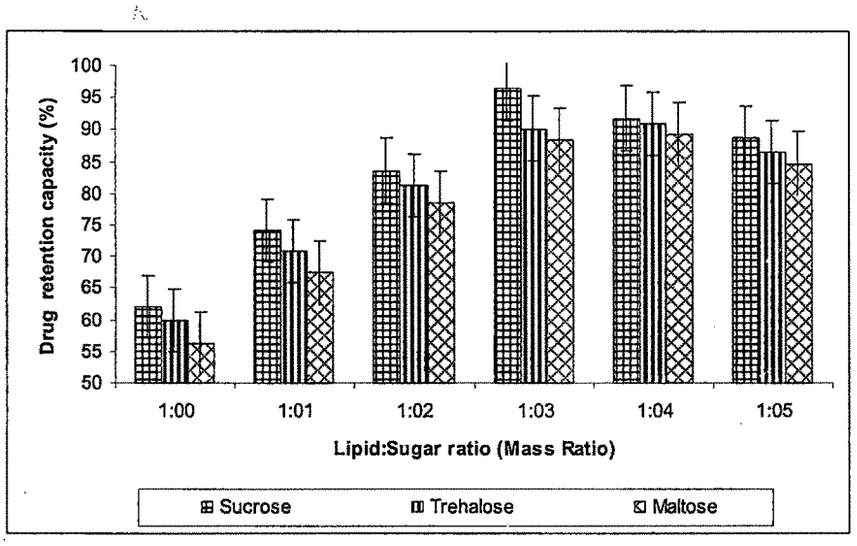


Figure 5.1.3. Effect of different ratios of lipid to cryoprotectants on drug retention efficiency in freeze dried Paclitaxel liposomal formulation.

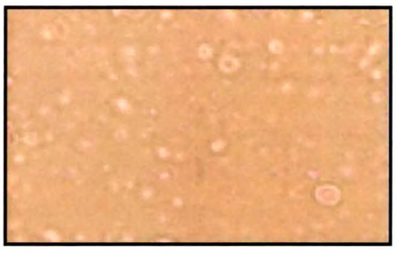


Figure 5.1.4. Photomicrograph of Paclitaxel pH sensitive liposomes before extrusion.

Electrolyte induced flocculation test

Sodium sulphate solutions ranging from 0 M to 2 M were prepared in 16.7 % sucrose solution. An appropriate volume of liposome formulation, which gives a final concentration of 1mg/ml of lipid, was taken and the volume was made up to 5ml using the sodium sulphate solutions of various concentrations. The resulting dispersions were mixed and the absorbances were measured within 5 min at 400 nm on Shimadzu 1601 UV-Visible spectrophotometer against respective blank (Subramanian and Murthy, 2003).

Table 5.1.4. Results of electrolyte induced flocculation test on conventional liposomes of Paclitaxel and pH sensitive liposomes of Paclitaxel containing different concentrations of mPEG-DSPE.

Concentration of Sodium sulphate (in M)	Mean absorbance ± S.E at 400 nm of Paclitaxel liposomes containing mPEG-DSPE				
	CLPT	PSPT (3 mol%)	PSPT (5 mol%)	PSPT (7 mol%)	PSPT (9 mol%)
0.0	0.596 ± 0.012	0.566 ± 0.016	0.571 ± 0.029	0.583 ± 0.026	0.592 ± 0.017
0.4	0.648 ± 0.023	0.604 ± 0.031	0.582 ± 0.031	0.589 ± 0.034	0.584 ± 0.034
0.8	0.699 ± 0.021	0.635 ± 0.022	0.601 ± 0.012	0.608 ± 0.041	0.600 ± 0.046
1.2	0.748 ± 0.036	0.684 ± 0.016	0.614 ± 0.038	0.619 ± 0.025	0.604 ± 0.029
1.6	0.791 ± 0.018	0.715 ± 0.036	0.626 ± 0.011	0.627 ± 0.019	0.611 ± 0.016
2.0	0.838 ± 0.041	0.796 ± 0.010	0.641 ± 0.046	0.655 ± 0.026	0.621 ± 0.031

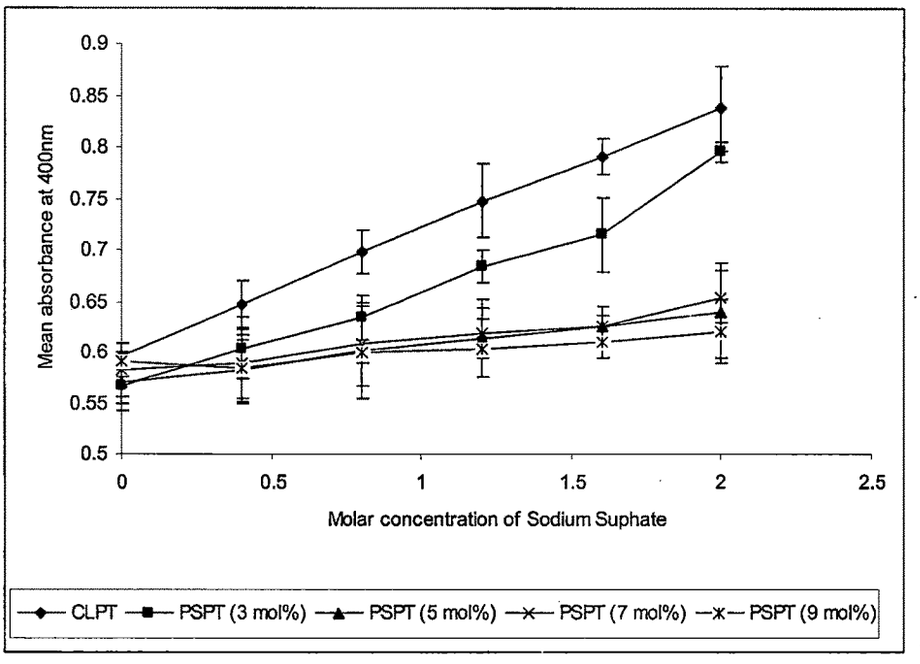


Figure 5.1.5. Results of electrolyte induced flocculation test on conventional liposomes of Paclitaxel and pH sensitive liposomes of Paclitaxel containing different concentrations of mPEG-DSPE.

5.1.7 Preparation of conventional liposomes of Irinotecan Hydrochloride

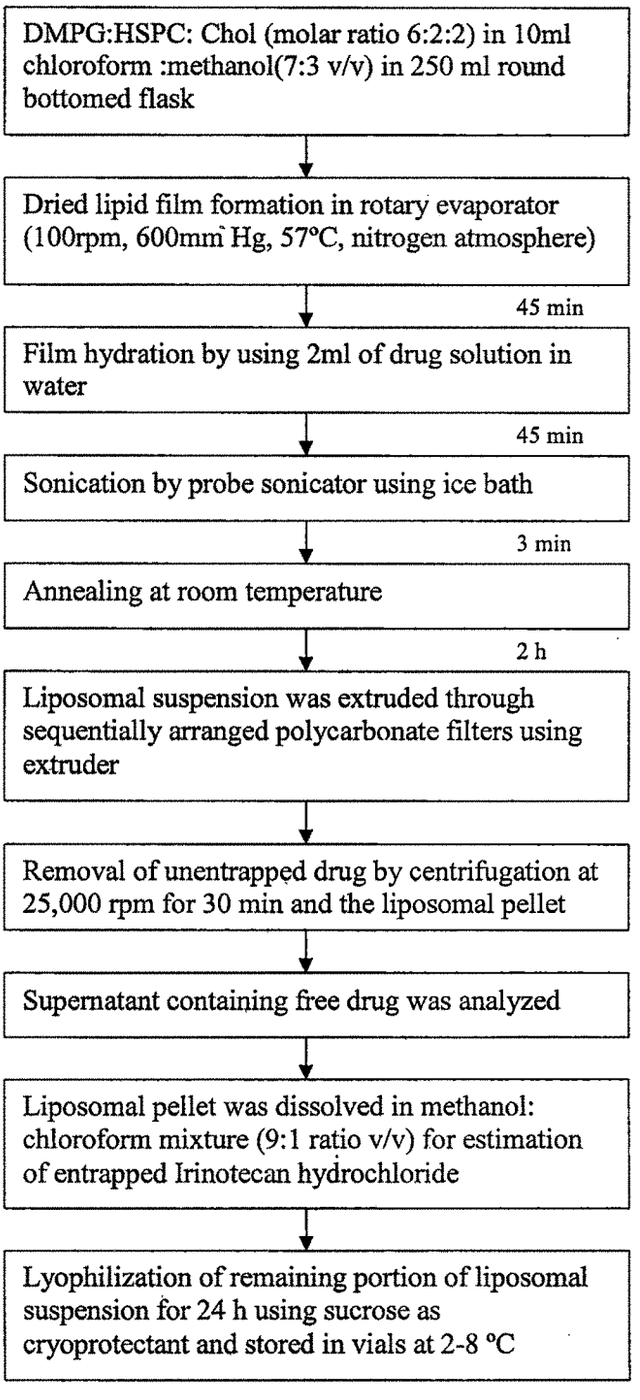
Conventional liposomes of Irinotecan liposomes were prepared by the lipid film hydration technique similar to the preparation of conventional liposomes containing Paclitaxel except the composition of drug lipid ratio. Other procedures remain the same except the free drug separation. A flow chart depicting the above process is shown in Figure 5.1.6. The major process parameters were optimized using the percentage drug entrapment as the response parameter. The observations of the optimization process are tabulated in Table 5.1.6. Comparisons of these liposomes are made with pH sensitive liposomes of Irinotecan hydrochloride.

Table 5.1.5. Optimization parameters for preparation of conventional liposomes containing Irinotecan hydrochloride (CLIH).

Batch No.	Drug: Lipid (molar ratio) X_1	DMPG: Chol (% of total lipids) X_2	Hydration volume (ml) X_3	Drug entrapment (% ± S.E.)*
CLIH1	1:5	60:40	1	20.32 ± 1.03
CLIH2	1:10	60:40	1	31.65 ± 2.12
CLIH3	1:15	60:40	1	45.19 ± 1.56
CLIH4	1:5	70:30	1	25.42 ± 1.87
CLIH5	1:10	70:30	1	33.64 ± 1.42
CLIH6	1:15	70:30	1	54.56 ± 1.58
CLIH7	1:5	80:20	1	37.52 ± 1.23
CLIH8	1:10	80:20	1	60.79 ± 2.28
CLIH9	1:15	80:20	1	70.64 ± 2.42
CLIH10	1:5	60:40	2	50.15 ± 1.47
CLIH11	1:10	60:40	2	59.65 ± 1.18
CLIH12	1:15	60:40	2	68.21 ± 2.31
CLIH13	1:5	70:30	2	55.24 ± 1.68
CLIH14	1:10	70:30	2	67.62 ± 1.55
CLIH15	1:15	70:30	2	71.30 ± 2.34
CLIH16	1:5	80:20	2	56.41 ± 2.09
CLIH17	1:10	80:20	2	71.18 ± 1.13
CLIH18	**1:15**	**80:20**	**2**	**73.56 ± 1.28**
CLIH19	1:5	60:40	3	45.16 ± 2.26
CLIH20	1:10	60:40	3	60.14 ± 2.01
CLIH21	1:15	60:40	3	65.16 ± 2.11
CLIH22	1:5	70:30	3	46.17 ± 1.09
CLIH23	1:10	70:30	3	66.05 ± 2.08
CLIH24	1:15	70:30	3	69.39 ± 1.63
CLIH25	1:5	80:20	3	54.34 ± 1.38
CLIH26	1:10	80:20	3	68.12 ± 1.76
CLIH27	1:15	80:20	3	69.14 ± 2.62

***n=3**

Figure 5.1.6. Flow chart for the preparation of conventional liposomes containing Irinotecan hydrochloride



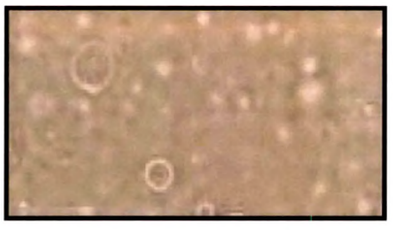


Figure 5.1.7. Photomicrograph of Irinotecan conventional liposomes before extrusion

Table 5.1.6. Characterization of Conventional liposomes of Irinotecan

Formulation	Size (nm)	PDI (Uniformity)	Zeta potential	Encapsulation efficiency
CLIH	131 ± 6.50	0.154	-11.7 ± 2.20	74.56 ± 1.26

5.1.8 Preparation of pH sensitive Irinotecan Hydrochloride Liposomes:

The liposomes were composed of DOPE: HSPC: CHEMS: CHOL at various molar ratios either with or without $mPEG_{2000}$-DSPE at 5 mol % to phospholipids. The $mPEG_{2000}$-DSPE is used for preparing sterically stabilized liposomes. Small unilamellar vesicles were prepared as previously described (Ishida et. al., 2003). Briefly, lipids were mixed and dissolved in organic solvents like chloroform-methanol mixture in a 250 ml round bottom amber coloured flask (Quick fit neck B-24) and the solvent was evaporated using a rotary evaporator; residual solvent was removed under high vacuum. The dried lipid films were hydrated with an appropriate buffer and sequentially extruded through a series of polycarbonate membrane filters (Nucleopore, CA, USA) with pore sizes of 400–100 nm, using an Extruder. For preparation of Irinotecan liposomes the drug was dissolved in buffer. All process and formulation parameters were optimized to achieve the desired property. The mean diameters of the resulting liposomes were determined by Nano-ZS Particle size analyzer (Malvern Instruments, UK). The unentrapped drug was separated by ultracentrifugation/ Sephadex column method.

Other methods of liposomal preparation like Ethanol injection and reverse phase evaporation were also tried for encapsulation of Irinotecan HCl.

Table 5.1.7. Optimization of preparation of pH sensitive liposomes containing Irinotecan hydrochloride.

Batch No.	Drug: Lipid (molar ratio) X_1	[(DOPE:HSPC: CHEMS): Chol] (% of total lipids) X_2	Hydration volume (ml) X_3	Drug entrapment (% ± S.E.)*
PSIH1	1:5	60:40	1	21.34 ± 1.24
PSIH 2	1:10	60:40	1	32.08 ± 1.56
PSIH 3	1:15	60:40	1	45.26 ± 1.47
PSIH 4	1:5	70:30	1	26.41 ± 1.34
PSIH 5	1:10	70:30	1	35.18 ± 1.82
PSIH 6	1:15	70:30	1	56.43 ± 1.02
PSIH 7	1:5	80:20	1	40.78 ± 1.63
PSIH 8	1:10	80:20	1	62.14 ± 1.27
PSIH 9	1:15	80:20	1	71.05 ± 1.54
PSIH 10	1:5	60:40	2	51.34 ± 1.74
PSIH 11	1:10	60:40	2	60.61 ± 1.89
PSIH 12	1:15	60:40	2	69.15 ± 2.22
PSIH 13	1:5	70:30	2	56.24 ± 1.28
PSIH 14	1:10	70:30	2	68.09 ± 1.59
PSIH 15	1:15	70:30	2	72.87 ± 1.46
PSIH 16	1:5	80:20	2	59.22 ± 0.78
PSIH 17	1:10	80:20	2	72.16 ± 1.83
PSIH 18	**1:15**	**80:20**	**2**	**75.18 ± 1.62**
PSIH 19	1:5	60:40	3	46.18 ± 2.13
PSIH 20	1:10	60:40	3	64.13 ± 2.45
PSIH 21	1:15	60:40	3	66.33 ± 2.02
PSIH 22	1:5	70:30	3	53.97 ± 1.99
PSIH 23	1:10	70:30	3	67.76 ± 2.12
PSIH 24	1:15	70:30	3	70.42 ± 1.44
PSIH 25	1:5	80:20	3	57.21 ± 1.78
PSIH 26	1:10	80:20	3	68.15 ± 1.65
PSIH 27	1:15	80:20	3	69.44 ± 1.98

Lyophilization optimization of liposomes was performed for maximum drug retention. The use of cryoprotectants (Sucrose, Trehalose and Maltose) during lyophilization of liposomes was evaluated and compared. The liposomal suspension containing Irinotecan was diluted with distilled water containing Sucrose or Trehalose or Maltose and the

amount of drug retained by liposomes following lyophilization and rehydration was determined. The lipid: cryoprotectant ratio was also optimized based on the drug retention capacity of the rehydrated lyophilized liposomal powder. The lyophilized samples were also subjected to stability studies, in triplicate, at the conditions according to ICH guidelines i.e. 2-8 ºC with ambient humidity and 30 ± 2 ºC/ 65 % RH after storing in sealed USP type-I amber coloured glass vials. The lyophilized samples were withdrawn from the vials at predetermined time intervals for a period of 6 months rehydrated using water for injection to form liposomal suspension and subjected for the different analysis.

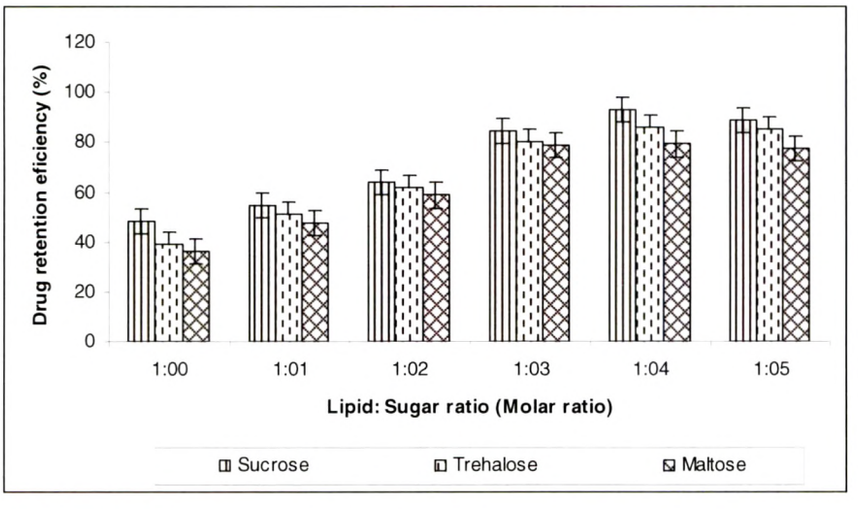


Figure 5.1.8. Effect of different ratios of lipid to cryoprotectants on drug retention efficiency in freeze dried Irinotecan liposomal formulation

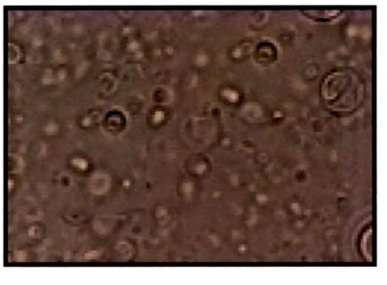


Figure 5.1.9. Photomicrograph of pH sensitive Irinotecan liposomes before extrusion.

Electrolyte induced flocculation test

Sodium sulphate solutions ranging from 0 M to 2 M were prepared in 16.7 % sucrose solution. An appropriate volume of liposome formulation, which gives a final concentration of 1mg/ml of lipid, was taken and the volume was made up to 5ml using the sodium sulphate solutions of various concentrations. The resulting dispersions were mixed and the absorbances were measured within 5 min at 400 nm on Shimadzu 1601 UV-Visible spectrophotometer against respective blank (Subramanian and Murthy, 2003)

Table 5.1.8. Results of electrolyte induced flocculation test on conventional liposomes of Irinotecan Hydrochloride and pH sensitive liposomes of Irinotecan Hydrochloride containing different concentrations of mPEG-DSPE.

Concentration of Sodium sulphate (in M)	Mean absorbance ± S.E at 400 nm of Irinotecan liposomes containing mPEG-DSPE				
	CLIH	PSIH (3 mol%)	PSIH (5 mol%)	PSIH (7 mol%)	PSIH (9 mol%)
0.0	0.615 ± 0.007	0.592 ± 0.021	0.596 ± 0.027	0.598 ± 0.021	0.604 ± 0.031
0.4	0.667 ± 0.018	0.625 ± 0.028	0.603 ± 0.029	0.612 ± 0.031	0.609 ± 0.028
0.8	0.713 ± 0.028	0.676 ± 0.024	0.635 ± 0.034	0.631 ± 0.036	0.628 ± 0.033
1.2	0.787 ± 0.034	0.718 ± 0.031	0.664 ± 0.037	0.648 ± 0.039	0.641 ± 0.046
1.6	0.823 ± 0.028	0.779 ± 0.019	0.689 ± 0.021	0.674 ± 0.041	0.661 ± 0.037
2.0	0.894 ± 0.047	0.816 ± 0.043	0.711 ± 0.026	0.702 ± 0.029	0.689 ± 0.034

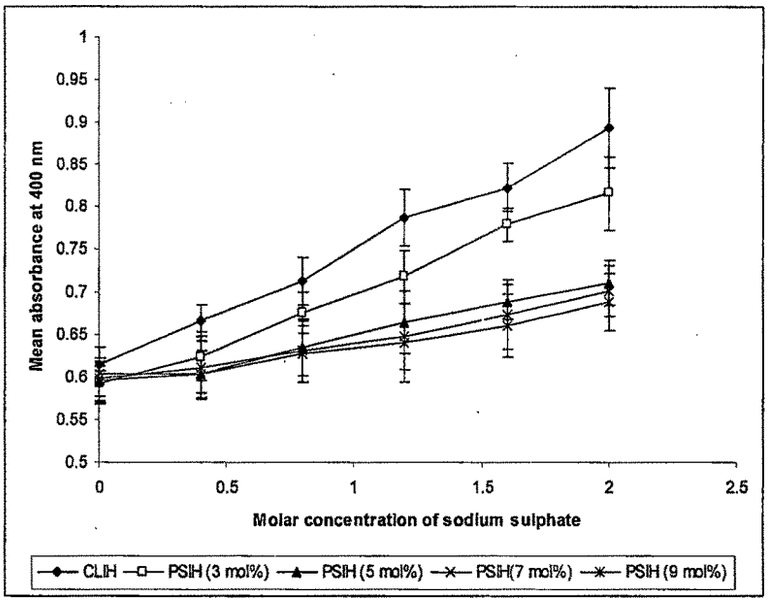


Figure 5.1.10. Results of electrolyte induced flocculation test on conventional liposomes of Irinotecan Hydrochloride and pH sensitive liposomes of Irinotecan Hydrochloride containing different concentrations of mPEG-DSPE.

5.1.9 RESULTS AND DISCUSSION

For conventional liposomal preparation:

Conventional liposomal formulation was optimized before proceeding to the formulation of sterically stabilized liposomes. Accordingly, conventional liposomes containing Paclitaxel and Irinotecan were prepared. Combination of lipids (DMPG and HSPC) with cholesterol in different molar ratios were tried to get an optimum encapsulation. Small unilamellar vesicles were prepared containing Paclitaxel or Irinotecan by film hydration method. This method was adopted over other methods like ethanol injection and reverse phase evaporation due to better encapsulation efficiency, ease of preparation, mechanical stability of the liposomes and ease of scale up using microfluidizer (New, 1990). The required mechanical stability and rigidity was fulfilled by incorporating cholesterol which is well documented as being able to decrease the fluidity or micro viscosity of the bilayer, to reduce the permeability of the membrane for water soluble molecules and stabilize the membranes in presence of biological fluids such as plasma (Lasic et. al., 1998).

A mixture of chloroform and methanol in a ratio (7:3) by volume was used to dissolve the lipid mixture because solubility of these lipids is more in this solvent blend in comparison to their solubility in individual solvents (New, 1990). 5ml of this solvent system was found suitable to dissolve the lipid mixture based on the rate of formation of uniform film of satisfactory thickness.

The speed of rotation of rotary evaporator was kept at a maximum of 100 rpm because rapid rotation increases the surface area for evaporation of the solvent thereby reducing the time required for the film formation process (New, 1990).Uniformity of heating during evaporation by use of water bath was found to be a critical factor for uniform film formation. Change in temperature caused non-uniformity in formation of thin film there by improper hydration. Temperature in the vicinity of 58 °C ± 2 °C gave even films which were almost transparent in all regions of round bottom flask. Vacuum was maintained at about 600 mm of mercury by means of nitrogen introduction via bleed valve. Lower values of vacuum (≈ 200 mm of mercury) increased the time of dry film formation.

Speed of rotation was kept at 80 rpm while hydrating the lipid film. Phosphate buffer saline (PBS pH 7.4) and double distilled water was tried as hydration medium for conventional liposomes of both Paclitaxel and Irinotecan. Finally PBS pH 7.4 was finalized as hydrating medium for conventional liposomes containing Paclitaxel and Irinotecan due to better encapsulation at 58 °C ± 2 °C. A period of 45 minutes was found to be adequate for complete film removal and dispersion in most cases. In case of paclitaxel formulations some film remained adhere to walls of the flask, so few glass beads (0.3cm) were introduced into the flask and rotated gently to effect film removal and uniform dispersion (Dhanikula et. al., 2005).

Hydrated lipid film in suspension form was sonicated for 2 minutes using probe sonicator. An ice batch was used to dissipate heat generated in the process (New, 1990). The suspension after sonication was allowed to stand undisturbed at room temperature for 2 hours for annealing which ensured complete hydration of the lipid film. Later on the

suspension was sequentially extruded through a series of polycarbonate membrane filters (Nucleopore, CA, USA) with pore sizes of 400 –100 nm, using an Extruder to get the liposomes in a required size range.

The unentrapped drug separation was carried out by minicolumn centrifugation method using Sephadex G-50 (New, 1990a) and ultracentrifugation methods. Sephadex G-50 mini column method failed to give satisfactory results due to the presence of insoluble, unentrapped Paclitaxel crystals. Centrifugation at 1,000 rpm for 10 minutes at 4 °C was reported to cause settling of unentrapped Paclitaxel which could be separated from the suspension (Yang et. al, 2007). After removal of the Paclitaxel crystals by this method, the liposomal pellet was then separated by centrifugation at 25,000 rpm for 30 minutes. The unentrapped Paclitaxel and encapsulated paclitaxel was determined by methods explained earlier in Analytical development section. Entrapment of about 86.56 ± 1.38 % was obtained for Paclitaxel.

For separation of unentrapped Irinotecan, the liposomal suspension was centrifuged at 25,000 rpm for 30 minutes and the supernatant was analysed for unentrapped drug in it. The liposomal pellet was also analysed simultaneously to determine the encapsulated Irinotecan. Entrapment of about 73.56 ± 1.26% was obtained for Irinotecan.

Conditions for preparation of PCL conventional liposomes:

The following conditions were optimized for PCL containing conventional liposomes (CLPT). Encapsulating PCL into liposomes is highly sensitive towards formulation parameters. Formulation variables like Drug / Lipid molar ratio, percent ratio of total lipids with cholesterol and hydration volume have been predicted to play a significant role in enhancing the Percent Drug entrapment (PDE) are taken as variable parameters keeping other variables like temperature, vacuum and hydration time constant. 3^3 factorial design was used to study the main and interaction effects of the variables on PDE. Based on factorial design 27 batches of PCL conventional liposomes were prepared and evaluated for drug entrapment. Mathematical modeling was carried out to obtain a

second order polynomial equation (full model, equation 1) (Anthony Armstrong et. al., 1996).

A substantial high drug entrapment achieved in liposomes by lipid film hydration was 84.56 % at X_1 (1:10), X_2 (80:20) and X_3 (2 ml). The PDE (dependent variable) obtained at various levels of three independent variables (X_1, X_2 and X_3) were subjected to multiple regression to yield a second order polynomial equation.

$$\mathbf{Y_{EE} = 68.6241 + 10.2956\ X_1 + 8.4483\ X_2 + 10.6439\ X_3 - 2.1322\ X_1^2 + 2.1561\ X_2^2 - 13.6106\ X_3^2 + 0.6275\ X_1X_2 - 1.605\ X_1X_3 - 3.0291\ X_2X_3 - 1.5138\ X_1X_2X_3} \quad \mathbf{(1)}$$

The main effect of X_1, X_2 and X_3 represent the average result of changing one variable at a time from its low to high value. The interactions (X_1X_2, X_1X_3, X_2X_3 and $X_1X_2X_3$) show how the PDE changes when two or more variables were changed simultaneously. The encapsulation efficiency for the prepared 27 batches showed a wide variation from 26.32% to 84.56 % (Table 5.1.1). Small values of the coefficient of the terms X_1^2, X_2^2, X_1X_2, X_1X_3, X_2X_3 and $X_1X_2X_3$ in equation 1 are regarded as least contributing in the preparation of conventional liposomes. Therefore, these terms are neglected from the full model considering non-significance and a reduced polynomial equation (equation 2) obtained following multiple regression of PDE and significant terms ($p < 0.05$) of equation 1.

$$\mathbf{Y_{EE} = 68.64 + 10.2956\ X_1 + 8.4483\ X_2 + 10.6439\ X_3 - 13.6106\ X_3^2} \quad \mathbf{(2)}$$

The significance of each coefficient of the equation 1 was determined by student 't' test and p-value, which showed that the quadratic main effects of Drug / Lipid ratio (p value = 0. 00000005) and total lipids / Chol percent ratio (p value = 0.0000007) and hydration volume (p value = 0.00000003) are significant. The interaction between X_3^2 was found to be significant with the p value of 0.0000016.

ANOVA between the full model and reduced model was performed. F-Statistic of the results of ANOVA of full and reduced model confirmed omission of non-significant terms of equation 1. Since the calculated F value (1.7757) is less than the tabulated F

value (2.74), it was concluded that the neglected terms do not significantly contribute in the prediction of PDE. When the coefficients of the three independent variables in equation 1 were compared the value for the variable X_3 (10.6439) and X_1 (10.2956) were found to be higher than X_2 (8.4483) and hence, volume of hydration (X_3) and Drug: Lipid ratio (X_1) was considered to be the major contributing variables for PDE of PCL conventional liposomes. The Fisher "F" test with a very low probability value ($P_{model} > F = 0.00000001$) demonstrates a very high significance for the regression model. The goodness of fit for the model was checked by the determination coefficient (R^2). In this case R^2 values obtained for full model (0.9522) and reduced model (0.9204) indicated that over 90% of the total variations are explained by the model. The values of adjusted determination coefficients for full model (0.9224) and reduced model (0.9059) are also very high which indicates a high significance of the model. A higher value of correlation coefficient 'R' for full model (0.9758) and reduced model (0.9594) signifies an excellent correlation between the independent variables (Box et. al., 1978). All the above considerations indicate an excellent adequacy of the regression model (Adinarayana et. al., 2002: Box et. al., 1978: Cochran and Cox, 1992: Yee et. al., 1993).Thus from the above study it was found that the entrapment of Paclitaxel in liposomes primarily based on volume of hydration, ratio between drug and lipids.

Conditions for preparation of IH conventional liposomes:

3^3 factorial design was used to study the main and interaction effects of the variables on PDE of Irinotecan. Based on factorial design 27 batches of IH conventional liposomes (CLIH) were prepared and evaluated for drug entrapment. Mathematical modeling was carried out to obtain a second order polynomial equation (full model, equation 1) (Anthony Armstrong et. al., 1996) similarly, as in the case of PCL conventional liposome.

A substantial high drug entrapment achieved in liposomes by lipid film hydration was 73.56 % at X_1 (1:15), X_2 (80:20) and X_3 (2 ml). The PDE (dependent variable) obtained at various levels of three independent variables (X_1, X_2 and X_3) were subjected to multiple regression to yield a second order polynomial equation.

$$Y_{EE} = 64.8596 + 10.9122\ X_1 + 6.4483\ X_2 + 9.1077\ X_3 - 3.322\ X_1^2 + 1.5861\ X_2^2 - 12.4022\ X_3^2 + 0.1783\ X_1X_2 - 2.4258\ X_1X_3 - 4.2208\ X_2X_3 - 1.6813\ X_1X_2X_3 \quad (1)$$

The main effect of X_1, X_2 and X_3 represent the average result of changing one variable at a time from its low to high value. The interactions (X_1X_2, X_1X_3, X_2X_3 and $X_1X_2X_3$) show how the PDE changes when two or more variables were changed simultaneously. The encapsulation efficiency for the prepared 27 batches showed a wide variation from 20.32% to 73.56 % (Table 5.1.5). Small values of the coefficient of the terms X_1^2, X_2^2, X_1X_2, X_1X_3 and $X_1X_2X_3$ in equation 1 are regarded as least contributing in the preparation of conventional liposomes. Therefore, these terms are neglected from the full model considering non-significance and a reduced polynomial equation (equation 2) obtained following multiple regression of PDE and significant terms ($p < 0.05$) of equation 1.

$$Y_{EE} = 63.7022 + 10.9122\ X_1 + 6.4483\ X_2 + 9.1077\ X_3 - 12.4022\ X_3^2 - 4.2208\ X_2X_3 \quad (2)$$

The significance of each coefficient of the equation 1 was determined by student 't' test and p-value, which showed that the quadratic main effects of Drug / Lipid ratio (p value = 0. 00000002) and total lipids / Chol percent ratio (p value = 0.000014) and hydration volume (p value = 0.00000018) are significant. The interaction between X_3^2 (p value = 0. 0000041) and X_2X_3 (p value = 0. 0046) was found to be significant.

ANOVA between the full model and reduced model was performed. F-Statistic of the results of ANOVA of full and reduced model confirmed omission of non-significant terms of equation 1. Since the calculated F value (1.7678) is less than the tabulated F value (2.85), it was concluded that the neglected terms do not significantly contribute in the prediction of PDE. When the coefficients of the three independent variables in equation 1 were compared the value for the variable X_1 (10.9122) and X_3 (9.1077) were found to be more than X_2 (6.4483) and hence, Drug: Lipid ratio (X_1) and volume of hydration (X_3) was considered to be the major contributing variables for PDE of IH

conventional liposomes (CLIH). The Fisher "F" test with a very low probability value ($P_{model} > F = 0.00000002$) demonstrates a very high significance for the regression model. The goodness of fit for the model was checked by the determination coefficient (R^2). In this case R^2 values obtained for full model (0.9473) and reduced model (0.9183) indicated that over 90 % of the total variations are explained by the model. The values of adjusted determination coefficients for full model (0.9144) and reduced model (0.8988) are also very high which indicates a high significance of the model. A higher value of correlation coefficient 'R' for full model (0.9733) and reduced model (0.9583) signifies an excellent correlation between the independent variables (Box et. al., 1978). All the above considerations indicate an excellent adequacy of the regression model (Adinarayana et. al., 2002: Box et. al., 1978: Cochran and Cox, 1992: Yee et. al., 1993). Thus from the above study it was found that the entrapment of Irinotecan in liposomes primarily based on ratio between drug and lipids and volume of hydration.

Morphology and lamellarity of the Paclitaxel and Irinotecan conventional liposomes were ascertained from photomicrographs taken using an Olympus BX40 microscope at a magnification of 1000X. Figures 5.1.2 and 5.1.7 show the photomicrograph of the liposomes before extrusion. The close observation of the Photomicrographs of the prepared liposomes indicates that the majority of the prepared liposomes were spherical and combination of multi and unilamellar structures. The unilamellar structures observed may be attributed to the sonication process used before extrusion.

Conventional liposomes of Paclitaxel and Irinotecan were also analysed for size and zeta potential using Malvern particle size analyzer after proper dilution. Particle size of 128 ± 8.65 nm with uniformity value of 0.189 and zeta potential of -12.8 ± 2.54 mV with 86.56% ± 1.38 % of encapsulation efficiency was obtained for Paclitaxel conventional liposomes (Table 5.1.2) where as Particle size of 131 ± 6.50nm with uniformity value of 0.154 and zeta potential of -11.7 ± 2.20 mV with 74.56 % ± 1.26 % of encapsulation efficiency for Irinotecan conventional liposomes (Table 5.1.6).

The liposomal suspension was then freeze dried at -20 °C overnight and lyophilized for 24 hours using sucrose as cryoprotectant The lyophilization process is discussed in detail further while discussing pH sensitive liposomal formulations.

Electrolyte induced flocculation study revealed that, as the concentration of electrolyte increased flocculation of the liposomes took place due to the absence of hydrophilic material coating like $mPEG_{2000}$-DSPE in conventional liposomes. Conventional liposomes of both the drugs could not oppose the electrolyte flocculation effectively due to this reason.

Results of *in-vitro* release studies of the conventional liposomal preparations carried out in PBS 7.4 and buffer pH 5.0 are discussed in detail further while discussing pH sensitive liposomal formulations.

The above discussed parameters in the preparation of conventional liposomes indicated the vulnerability of liposomes containing Paclitaxel or Irinotecan to the preparation conditions of the drug encapsulated. So, optimization was necessary for reproducibility of drug entrapment.

For Serum stable, long circulating, pH sensitive liposomal formulations:

Liposomal drugs have been suggested to be the long awaited "magic bullet" cancer therapy due to their ability to accumulate selectively in the tumor. However, the problem remains that not all the cancers and not all patients respond to the "bullet" equivalently. Considerable advances have been made in the design of pH sensitive liposomal drug delivery systems. Increased cytoplasmic delivery, rapid and pH optimal content release along with improvements in the types of liposomes that may be modified for pH dependent content release have been achieved with a certain degree of success. The drug being delivered by liposomes plays an important role in the response achieved. pH sensitive liposomal drugs have the advantage of "trigger release" of neoplastic drugs at tumor site with low doses of standard chemotherapy for a sustained period of time without harming other tissues.

A systematic attempt was made to make the anticancer therapy more effective and safe by incorporating the anticancer drugs in pH sensitive, serum stable, long circulating liposomes. It was hypothesized that pH sensitive, sterically stabilized liposomes will provide a prolonged systemic circulation and enhanced tumor accumulation of the drug. This combination of sustained and targeted delivery would help to reduce side effects of these drugs and would also lead to a significantly lower dose being required for achieving therapeutic efficacy. The anticancer agents, Paclitaxel and Irinotecan Hydrochloride were chosen as the drugs for investigation due to their frequency of use and the range of side effects associated with their use. Unilamellar vesicles of these drugs were prepared using the lipid film hydration technique. The other methods adopted for preparing liposomes like ethanol injection and reverse phase evaporation didn't gave good encapsulation. The important process parameters for the preparation of liposomes were optimized as explained. It was found that the molar ratio of the components of the liposomal system played an important role in determining the percentage drug entrapped.

The parameters such as speed of rotary flash evaporator, and vacuum applied for drying was important in the formation of thin dry film during the preparation of liposomes which is already discussed above. The selection of hydration medium was critical in the preparation of pH sensitive liposomes. The use of HEPES buffer (pH 8.2) as the hydration medium gave a good entrapment for both the drugs. The higher pH was necessary for the DOPE formulations as DOPE does not hydrate at acidic pH to self assemble into bilayers (Ellens et al., 1984; Ellens et al., 1985; Ishida et al., 2006).

The pH sensitive liposomes were prepared by using pH sensitive lipid DOPE along with other lipids. For providing long circulatory effect $mPEG_{2000}$-DSPE was incorporated in the system. The presence of negatively charged lipids at a basic pH as well as a low transition temperature of the lipid membrane might drive the release of encapsulated drug from pH sensitive liposomes. DOPE is negatively charged at a pH around 9.0 (Stollery and Vail, 1977) and CHEMS, an anionic Cholesterol ester, when added to the pH sensitive formulation to stabilize DOPE vesicles at neutral pH (Ellens et al., 1984; Lai et al., 1985), is also negatively charged at pH above 7.5 (Ellens et al.,

1984). The presence of different lipids led to significant difference in the entrapment of the drugs as compared to that in the conventional liposomes.

Conditions for preparation of PCL pH sensitive liposomes:

3^3 factorial design was used to study the main and interaction effects of the variables on PDE of Paclitaxel. Based on factorial design 27 batches of PCL pH sensitive liposomes (PSPT) were prepared and evaluated for drug entrapment. Mathematical modeling was carried out to obtain a second order polynomial equation (full model, equation 1) (Anthony Armstrong et. al., 1996) similarly, as in the case of conventional liposomes.

A substantial high drug entrapment achieved in liposomes by lipid film hydration was 87.13 % at X_1 (1:10), X_2 (80:20) and X_3 (2 ml). The PDE (dependent variable) obtained at various levels of three independent variables (X_1, X_2 and X_3) were subjected to multiple regression to yield a second order polynomial equation.

$$Y_{EE} = 70.9560 + 8.085\,X_1 + 7.5356\,X_2 + 12.7839\,X_3 - 1.9561\,X_1^2 + 0.8289\,X_2^2 - 13.6328\,X_3^2 + 1.5717\,X_1X_2 - 2.6033\,X_1X_3 - 1.2933\,X_2X_3 + 0.9363\,X_1X_2X_3 \quad (1)$$

The main effect of X_1, X_2 and X_3 represent the average result of changing one variable at a time from its low to high value. The interactions (X_1X_2, X_1X_3, X_2X_3 and $X_1X_2X_3$) show how the PDE changes when two or more variables were changed simultaneously. The encapsulation efficiency for the prepared 27 batches showed a wide variation from 27.46% to 87.13 % (Table 5.1.3). Small values of the coefficient of the terms X_1^2, X_2^2, X_1X_2, X_2X_3 and $X_1X_2X_3$ in equation 1 are regarded as least contributing in the preparation of pH sensitive liposomes. Therefore, these terms are neglected from the full model considering non-significance and a reduced polynomial equation (equation 2) obtained following multiple regression of PDE and significant terms ($p < 0.05$) of equation 1.

$$Y_{EE} = 70.2044 + 8.085\,X_1 + 7.5356\,X_2 + 12.7839\,X_3 - 13.6328\,X_3^2 - 2.6033\,X_1X_3 \quad (2)$$

The significance of each coefficient of the equation 1 was determined by student 't' test and p-value, which showed that the quadratic main effects of Drug / Lipid ratio (p value = 0. 00000044) and total lipids / Chol percent ratio (p value = 0.0000011) and hydration volume (p value = 0.0000000007) are significant. The interaction between X_3^2 (p value = 0. 0000006) and X_1X_3 (p value = 0. 0479) was found to be significant.

ANOVA between the full model and reduced model was performed. F-Statistic of the results of ANOVA of full and reduced model confirmed omission of non-significant terms of equation 1. Since the calculated F value (0.7880) is less than the tabulated F value (2.85), it was concluded that the neglected terms do not significantly contribute in the prediction of PDE. When the coefficients of the three independent variables in equation 1 were compared the value for the variable X_3 (12.7839) and X_1 (8.085) were found to be more than X_2 (7.5356) and hence, volume of hydration (X_3) and Drug: Lipid ratio (X_1) was considered to be the major contributing variables for PDE of PCL pH sensitive liposomes (PSPT). The Fisher "F" test with a very low probability value ($P_{model} > F = 0.000000004$) demonstrates a very high significance for the regression model. The goodness of fit for the model was checked by the determination coefficient (R^2). In this case R^2 values obtained for full model (0.9577) and reduced model (0.9452) indicated that over 90 % of the total variations are explained by the model. The values of adjusted determination coefficients for full model (0.9313) and reduced model (0.9322) are also very high which indicates a high significance of the model. A higher value of correlation coefficient 'R' for full model (0.9786) and reduced model (0.9722) signifies an excellent correlation between the independent variables (Box et. al., 1978). All the above considerations indicate an excellent adequacy of the regression model (Adinarayana et. al., 2002: Box et. al., 1978: Cochran and Cox, 1992: Yee et. al., 1993).

Thus from the above study it was found that the entrapment of PCL in liposomes primarily based on volume of hydration and ratio between drug and lipids.

Conditions for preparation of Irinotecan pH sensitive liposomes:

3^3 factorial design was used to study the main and interaction effects of the variables on PDE of Irinotecan. Based on factorial design 27 batches of Irinotecan pH sensitive liposomes (PSIH) were prepared and evaluated for drug entrapment. Mathematical modeling was carried out to obtain a second order polynomial equation (full model, equation 1) (Anthony Armstrong et. al., 1996) similarly, as in the case of conventional liposomes.

A substantial high drug entrapment achieved in liposomes by lipid film hydration was 75.18 % at X_1 (1:15), X_2 (80:20) and X_3 (2 ml). The PDE (dependent variable) obtained at various levels of three independent variables (X_1, X_2 and X_3) were subjected to multiple regression to yield a second order polynomial equation.

$$\mathbf{Y_{EE} = 66.2722 + 10.1911\ X_1 + 6.6061\ X_2 + 9.6066\ X_3 - 2.8766\ X_1^2 + 0.945\ X_2^2 - 11.97\ X_3^2 - 0.285\ X_1X_2 - 2.9483\ X_1X_3 - 4.7608\ X_2X_3 - 1.7837\ X_1X_2X_3} \quad \mathbf{(1)}$$

The main effect of X_1, X_2 and X_3 represent the average result of changing one variable at a time from its low to high value. The interactions (X_1X_2, X_1X_3, X_2X_3 and $X_1X_2X_3$) show how the PDE changes when two or more variables were changed simultaneously. The encapsulation efficiency for the prepared 27 batches showed a wide variation from 21.34% to 75.18 % (Table 5.1.7). Small values of the coefficient of the terms X_1^2, X_2^2, X_1X_2 and $X_1X_2X_3$ in equation 1 are regarded as least contributing in the preparation of pH sensitive liposomes. Therefore, these terms are neglected from the full model considering non-significance and a reduced polynomial equation (equation 2) obtained following multiple regression of PDE and significant terms ($p < 0.05$) of equation 1.

$$\mathbf{Y_{EE} = 64.9844 + 10.1911\ X_1 + 6.6061\ X_2 + 9.6066\ X_3 - 11.97\ X_3^2 - 2.9483\ X_1X_3 - 4.7608\ X_2X_3} \quad \mathbf{(2)}$$

The significance of each coefficient of the equation 1 was determined by student 't' test and p-value, which showed that the quadratic main effects of Drug / Lipid ratio (p value = 0. 000000013) and total lipids / Chol percent ratio (p value = 0.000004) and hydration

volume (p value = 0.00000003) are significant. The interaction between X_3^2 (p value = 0. 0000023), X_1X_3 (p value = 0. 0239) and X_2X_3 (p value = 0.00098) was found to be significant.

ANOVA between the full model and reduced model was performed. F-Statistic of the results of ANOVA of full and reduced model confirmed omission of non-significant terms of equation 1. Since the calculated F value (1.2133) is less than the tabulated F value (3.01), it was concluded that the neglected terms do not significantly contribute in the prediction of PDE. When the coefficients of the three independent variables in equation 1 were compared the value for the variable X_1 (10.1911) and X_3 (9.6066) were found to be more than X_2 (6.6061) and hence, Drug: Lipid ratio (X_1) and volume of hydration (X_3) was considered to be the major contributing variables for PDE of PSIH. The Fisher "F" test with a very low probability value ($P_{model} > F = 0.000000008$) demonstrates a very high significance for the regression model. The goodness of fit for the model was checked by the determination coefficient (R^2). In this case R^2 values obtained for full model (0.9545) and reduced model (0.9407) indicated that over 90 % of the total variations are explained by the model. The values of adjusted determination coefficients for full model (0.9261) and reduced model (0.9229) are also very high which indicates a high significance of the model. A higher value of correlation coefficient 'R' for full model (0.9770) and reduced model (0.9699) signifies an excellent correlation between the independent variables (Box et. al., 1978). All the above considerations indicate an excellent adequacy of the regression model (Adinarayana et. al., 2002: Box et. al., 1978: Cochran and Cox, 1992: Yee et. al., 1993). Thus from the above study it was found that the entrapment of IH in liposomes primarily based on the ratio between drug and lipids and volume of hydration.

Morphology and lamellarity of the sterically stabilized pH sensitive liposomes was ascertained from photomicrographs taken using an Olympus BX40 microscope at a magnification of 1000X. Figure 5.1.4 and 5.1.9 shows the photomicrographs of the liposomes before extrusion. The close observation of the Photomicrographs of the prepared liposomes indicates that the majority of the prepared liposomes were spherical

and combination of multi and unilamellar structures. The unilamellar structures observed may be attributed to the sonication process used before extrusion.

Lyophilization process optimization for liposomes was performed for maximum drug retention. The use of different cryoprotectants like Sucrose, Trehalose and Maltose during lyophilization of Paclitaxel and Irinotecan liposomal formulations was evaluated and compared by the amount of drug retained by liposomes following lyophilization and rehydration. The lipid: cryoprotectant (Sucrose/Trehalose/Maltose) mass ratio (1:0, 1:1, 1:2, 1:3, 1:4, and 1:5) was also optimized based on the drug retention capacity of the rehydrated lyophilized liposomal powder (Figure 5.1.3 and 5.1.8).

The data reveals that sucrose (1:3 mass ratio) gave significantly higher percentage of drug retention (96.45%) for Paclitaxel pH sensitive liposomes as compared to trehalose (90.86% for 1:4 mass ratio) or maltose (89.18% for 1:4 mass ratio). But in case of Irinotecan pH sensitive liposomes sucrose (1:4 mass ratio) gave significantly higher percentage of drug retention (93.17 %) as compared to trehalose (85.86% for 1:4 mass ratio) or maltose (79.18 % for 1:4 mass ratio). 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.

Sterically stabilized pH sensitive liposomes of Paclitaxel and Irinotecan were prepared with the aim of altering the pharmacokinetics of these formulations inside the body and thereby increasing the tumor accumulation of the drugs. The change in pharmacokinetic properties, particularly plasma residence time might lead to better efficacy of these carrier systems in treatment of cancer due to increased accumulation in tumor sites owing to EPR effect. Literature revealed that hydrophilic polymeric materials with highly flexible main chain as if in Polyethylene Glycol (PEG) derivatives could be used for steric stabilization (Torchilin and Trubetskoy, 1995). PEG grafting has been widely used as one of the effective method to reduce the rapid clearance of liposomes from circulation

by RES system. Therefore, $mPEG_{2000}$-DSPE was incorporated in the formulation for steric stabilization.

The concentration of the polymer necessary for steric stabilization was optimized using electrolyte flocculation test. This is a standard test to investigate whether a dispersed system is sterically stabilized or not. The physical stability of the dispersion mainly depends upon the competitive forces of attraction (van der Waals forces) and repulsion (either electrostatic repulsive forces or steric stabilizing barrier or both) (Lin et. al., 1994). In addition to this, a number of other interactions (depletion and steric interactions) could play an important role in colloidal system stability (Tadros and Vincent, 1983). Steric stabilization occurs due to the presence of steric barriers from the adsorbed non-ionic molecules on particles that prevent the particles from coming close enough to allow van der Walls attractive forces between the particles to dominate (Tadros, 1986).

The conventional liposomes are predominantly electrostatically stabilized. Addition of an electrolyte will be sufficient to compress the electrostatic double layer surrounding the liposomes and results in the aggregation leading to flocculation with a corresponding increase in optical turbidity. But if the liposomes are mainly stabilized by hydrated steric stabilized barrier, which is produced by the surface modification due to the polymer incorporation, the system should be stable even if the electrostatic double layers have been compressed. The flocculation even occurs in sterically stabilized systems on addition of certain amount of an electrolyte, due to dehydration of the hydrated steric stabilized barriers. Thus, if optical turbidity of the liposomal dispersion is measured at 400 nm after addition of different concentrations of electrolyte, one can assess steric stabilization capacity by measuring minimum electrolyte concentration required to bring about significant change in optical turbidity. The lipid concentration was kept at 1mg/ml and sucrose, a density neutralizing agent, at a concentration of 16.7 % w/v, was included in the electrolyte solution to prevent settling of liposomes. The scattering of the sample increase by the inverse 4^{th} power of the wavelength of the incident light, hence a lower wavelength of 400nm was used for measurements (Betagiri et. al., 1993).

Figure 5.1.5 and Figure 5.1.10 show the plots of absorbance against the concentration of electrolyte added for conventional as well as pH sensitive liposomes of Paclitaxel and Irinotecan respectively (data in Table 5.1.5 and Table 5.1.10). The conventional liposomes of Paclitaxel and Irinotecan showed a gradual increase in flocculation as the concentration of the Sodium sulphate increases from 0M to 2M. The investigation has been carried out on $mPEG_{2000}$-DSPE as sterically stabilizing agent in case of pH sensitive liposomes of Paclitaxel and Irinotecan respectively. In both the cases 3 mol % ratio of lipid was insufficient to provide protection against electrolyte induced flocculation probably due to insufficient coverage at the surface of the prepared liposomes. 5 mol % on molar basis of this reagent was found to provide steric stability to pH sensitive liposomes of Paclitaxel and Irinotecan. Further increase in $mPEG_{2000}$-DSPE concentration (7 mol % and 9 mol % ratio) does not produce much significant increase in protection to the liposomes prepared with 5 mol % of the reagent.

5.2. **Characterization of Liposomes.**

5.2.1 Characterization of pH sensitive Liposomes of Paclitaxel:

Characterization of the prepared pH sensitive, serum stable, long circulating liposomes for its physicochemical characteristics such as

i) **Particle size:**

This was performed using Nano-ZS particle size analyzer (Malvern Instruments, UK) which uses New Dynamic Light Scattering Technique or Photon Correlation spectroscopy to analyze the sample. The sample was filled in glass cuvette with round aperture after proper dilution and the readings were taken by maintaining the temperature at 25° C. Uniformity value was also noted down for the sample.

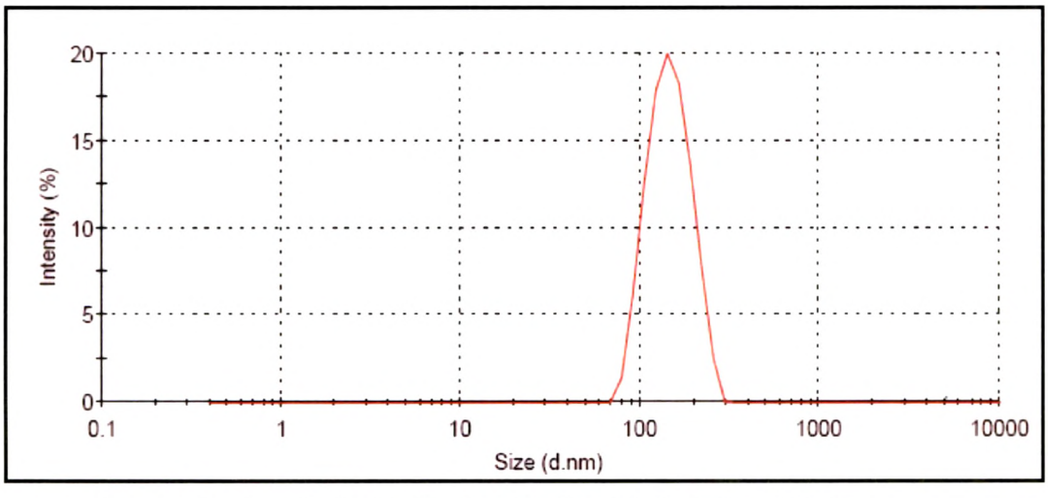


Figure 5.2.1. Particle size distribution of Paclitaxel loaded pH sensitive liposomes.

ii) **Zeta potential:**

This was performed using Nano-ZS particle size analyzer (Malvern Instruments, UK). The sample was filled in unique disposable folded capillary cell and the readings were taken by maintaining the sample at 25° C temperature. Zeta potentials were calculated from the mean electrophoretic mobility by applying the Smoluchowski equation. The results are the means of 3 determinations ± standard deviation.

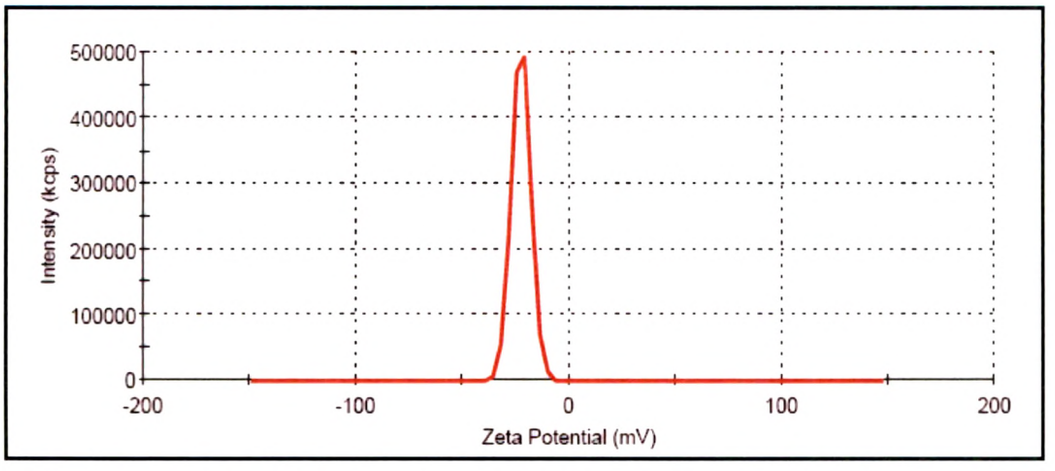


Figure 5.2.2. Zeta Potential of Paclitaxel loaded pH sensitive liposomes.

iii) **Entrapment efficiency of Paclitaxel in liposomes**:

The entrapment efficiency (EE) is defined as the ratio of the amount of the paclitaxel encapsulated in liposome to that of the total paclitaxel in liposomal dispersion. The amount of Paclitaxel encapsulated in liposomes was measured following the method in the literature with slight modification (Shieh et. al., 1997: Yang et. al., 2007)). Briefly, aliquots (0.1mL each) of liposomal dispersion diluted to 1.1mLby PBS (pH 7.4) immediately after preparation was centrifuged at 1000 rpm for 10 min to remove any Paclitaxel particle already released from the liposomes. Then, 1.0mL of the liposome supernatant was centrifuged at 25,000 rpm for 30 min (Sigma 3K30, USA). After removing the supernatant by aspiration, the precipitate (*i.e.*, liposome pellet) was washed twice with PBS (pH 7.4). The liposome pellet was dissolved organic solvents mixture of Methanol and chloroform (9:1, v/v). The concentration of Paclitaxel was determined spectrophotometrically at 227 nm after appropriate dilution with the mixed solvent that destroyed the liposome pellet. Even method developed with high performance liquid chromatography (HPLC) for Paclitaxel estimation. An aliquot (0.1mL each) of the liposome suspension was also dissolved with the same mixed solvent to determine the total amount of paclitaxel in the liposome suspension, after which the EE was calculated from the following equation:

$$EE(\%) = \frac{\text{amount of paclitaxel in liposome pellet } (\mu g)}{\text{amount of paclitaxel in liposomal dispersion } (\mu g)} \times 100$$

An aliquot (50 mg each) of the freeze-dried liposome powder was dissolved with the same mixed solvent (4 ml) to determine the content of paclitaxel in the freeze-dried liposome powder using the following equation after appropriate dilution with the same mixed solvent:

$$\text{Content} = \frac{\text{amount of paclitaxel in freeze-dried liposome } (\mu g)}{\text{amount of freeze-dried liposome (mg)}}$$

The EE and paclitaxel content were determined from three separately prepared liposome suspensions, and were expressed as the mean value with standard deviation.

iv) **Solid-state analysis (DSC and XRD):**

Differential Scanning Calorimetry (DSC) studies were conducted for optimum batches having minimum particle size and maximum entrapment efficiency using Diamond Differential Scanning Calorimeter (Mettler, Star SW 8.10). These included studies on pure drug, bulk lipids, and lyophilized liposomal preparations. The analysis was performed at a rate 10 °C/ min from 35.0°C to 350 °C temperature range under inert nitrogen atmosphere at flow rate of 50 ml/min. The main objective of these studies is to determine the melting behaviour of lipids in the liposomal dispersions and possible various polymorphic modifications that the lipid may assume during the process of liposomal preparation.

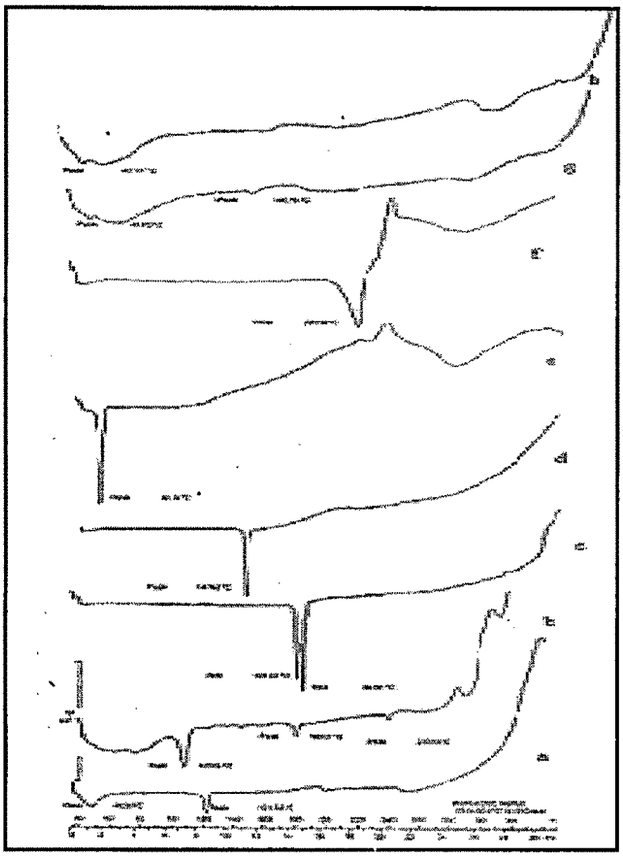


Figure 5.2.3. Differential Scanning Calorimetry thermograms of (a) DOPE, (b) HSPC, (c) CHEMS, d) Cholesterol, (e) mPEG-DSPE, (f) Paclitaxel, (g) Placebo liposomes and (h) pH sensitive Paclitaxel Liposomes.

X-ray Diffraction studies (XRD): Powder X-ray diffraction patterns were obtained using an X-ray diffractometer (Philips PW 1710) with Cu Kα radiation generated at 30 mA and 40 kV. The source of X-ray was copper anode with a wavelength of 1.54060 A.

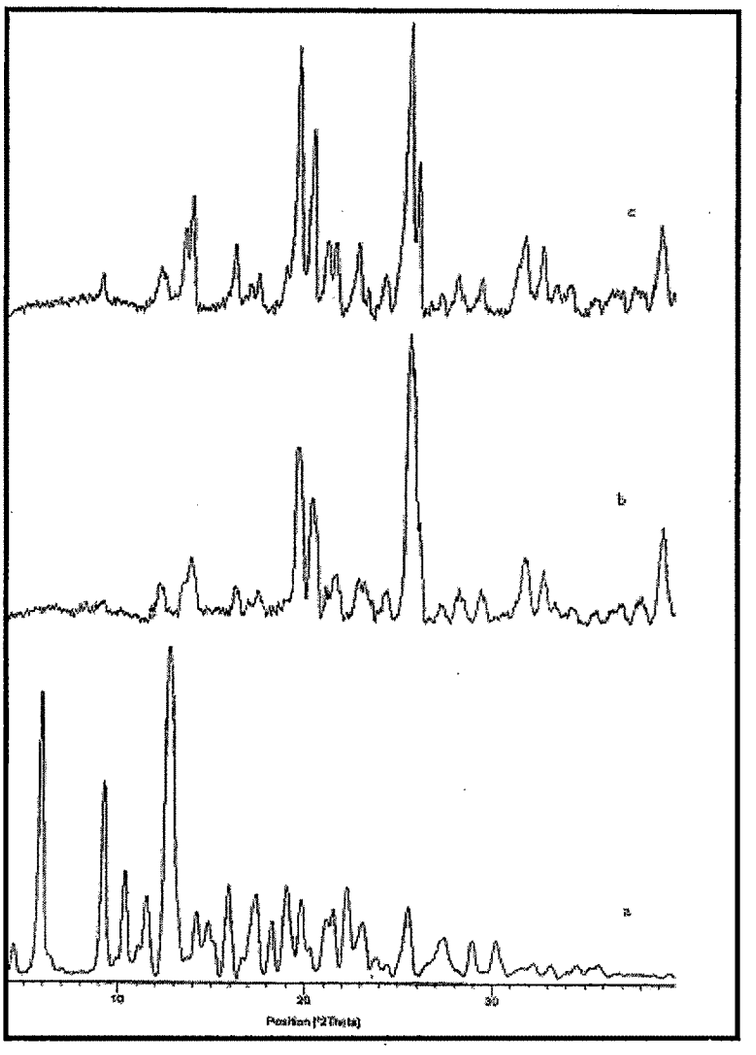
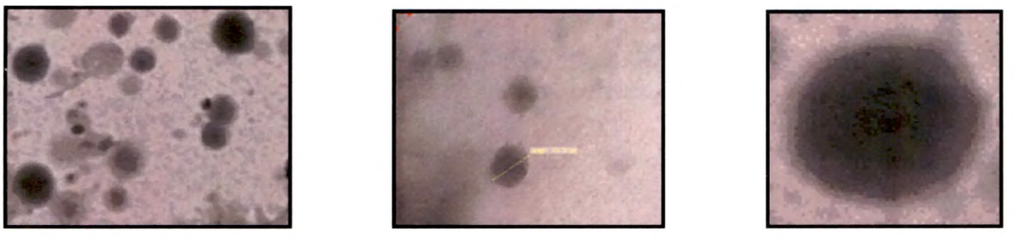


Figure 5.2.4. X-Ray Diffraction pattern of (a) Paclitaxel, (b) Placebo pH sensitive liposome and (c) Paclitaxel pH sensitive liposomes.

v) **Transmission Electron Microscopy (TEM):**

Transition Electron Microscopic (TEM) studies were done for liposomal dispersion using Transition Electron Microscope (Philips, Morgagni 268). One drop of the liposomal dispersion (fresh sample or reconstituted) was incubated on 200-mesh carbon coated copper grid after staining with 2.5% uranyl acetate for 30 seconds and dried. The copper grid was fixed into sample holder and placed in vacuum chamber of the Transition

Electron Microscope and observed under low vacuum (10^{-3} torr). The aim is to study the particle shape, size and surface characteristics in the liquid dispersion.



(a) (b) (c)

Figure 5.2.5. Transmission Electron Microscopy of Paclitaxel loaded pH sensitive liposomes at different magnifications.

vi) **pH induced liposomal aggregation and serum stability:**

Liposomal aggregation in response to reduced buffer pH was measured by increase in particle size and also measuring the change in zeta potential values. The Paclitaxel liposomal suspension was diluted with specific volume of sodium acetate buffer of various pH values and incubated at 37°C for 1 hour. After specified time, aliquots of the sample were withdrawn and the mean particle size and zeta potential was determined using Malvern particle size analyzer (Shi et. al., 2002).

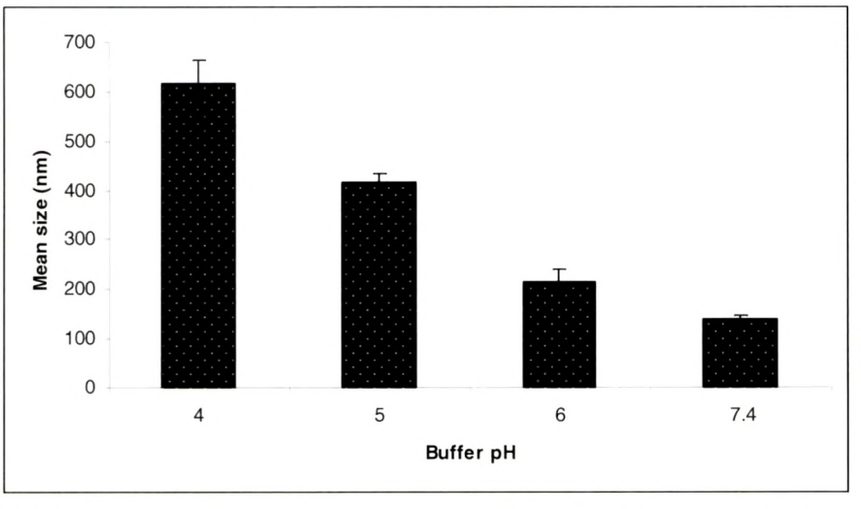


Figure 5.2.6. Change in size distribution pattern of pH sensitive liposomes of Paclitaxel in response to various pH values incubated at 37 °C for 1 hour.

Serum stability of the pH sensitive Paclitaxel liposomal preparation was also determined by the same method, using 80 % serum instead of buffers with varied pH, measuring the change in particle size and zeta potential values, incubated at 37°C for 48 hours. The size and zeta potential of the withdrawn samples were determined as above.

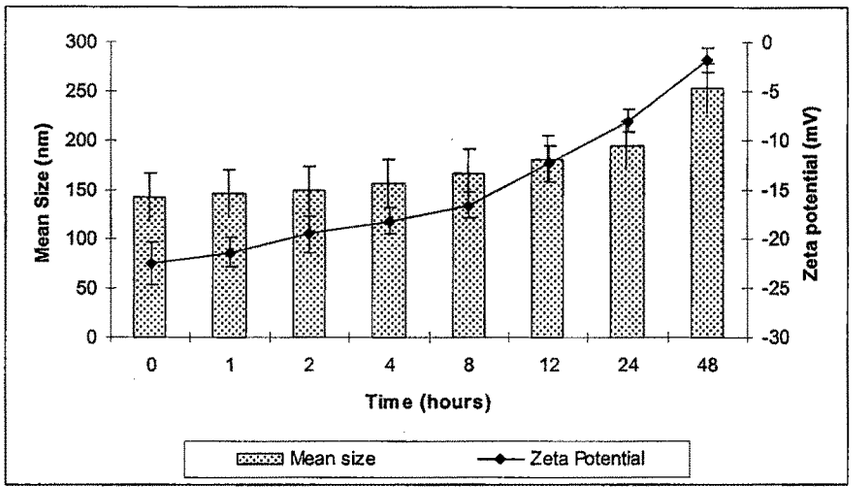


Figure 5.2.7. Size distribution pattern and Zeta potential values of pH sensitive liposomes of Paclitaxel at various time points in serum at 37 °C upto 48 hours.

vii) ***In-vitro* drug release study:**

In-vitro release of plain Paclitaxel, conventional liposomes of Paclitaxel and Paclitaxel loaded pH-sensitive liposomes were studied by dialysis method in Phosphate buffered saline, pH 7.4 & pH 5.0 as dissolution media at 37°C. Briefly, 2 ml of liposomal suspension or plain drug solution of known concentration was taken in dialysis bag and suspended in the buffer with particular pH. At various time intervals aliquot samples were withdrawn and drug content was analysed.

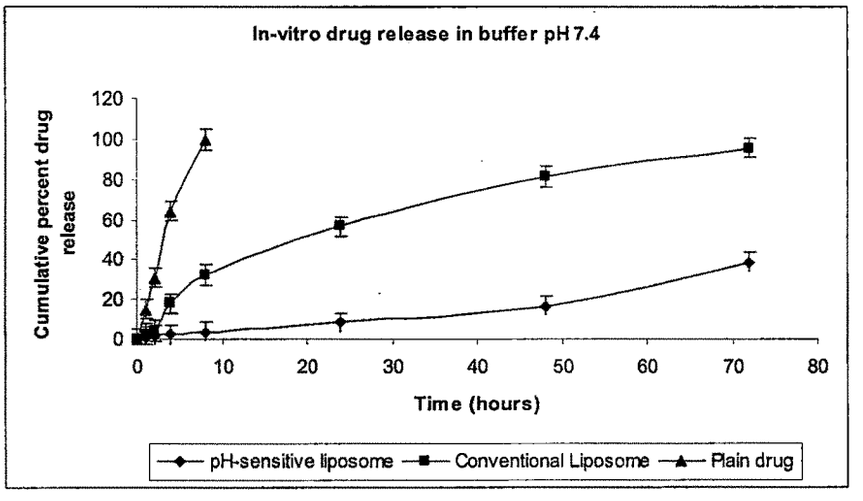


Figure 5.2.8. ***In-vitro*** **release of plain drug Paclitaxel, conventional liposomes of Paclitaxel and pH sensitive liposomes of Paclitaxel in buffer pH 7.4**

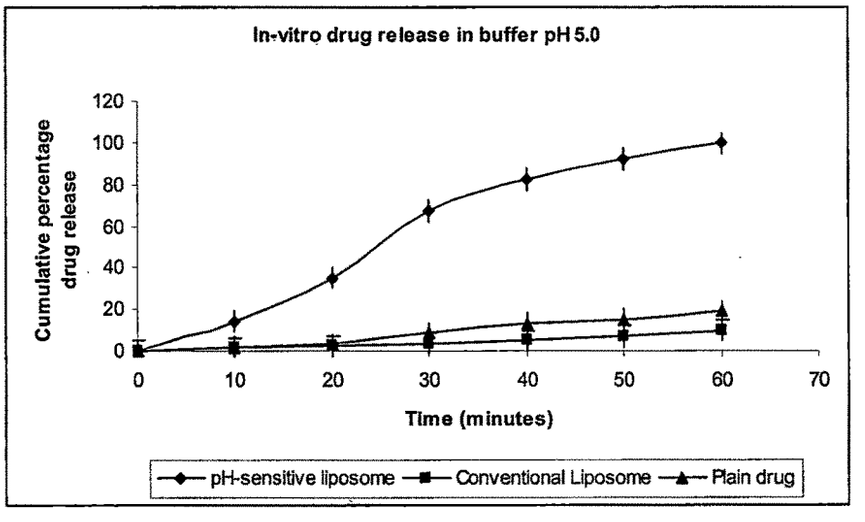


Figure 5.2.9. ***In-vitro*** **release of plain drug Paclitaxel, conventional liposomes of Paclitaxel and pH sensitive liposomes of Paclitaxel in buffer pH 5.0.**

viii) **Stability Study:**

The stability of the sterically stabilized pH sensitive liposomal dispersion and lyophilized liposomal formulation containing Paclitaxel was investigated. Two temperatures were selected viz. refrigerator (2-8°C) and room temperature (30 ± 2°C). The liposomal dispersions were placed in colourless USP type-I glass vials stoppered with 20 mm grey

bromobutyl stoppers. The vials were purged with nitrogen before stoppering and sealing using 20 mm aluminium seals.

At predetermined intervals of time, samples were removed and studied for parameters such as change in size and size distribution, zeta potential, assay and percentage drug retention/drug leaching. Changes in the size, size distribution and zeta potential of the liposomal formulations were determined by particle size analyzer. While the assay was performed spectrophotometrically by the developed method and percent drug leaching was estimated by centrifuging the formulation at 2000 rpm and analyzing the supernatant containing liposomal dispersion for the drug content.

Table 5.2.1. Effect of storage on particle size of Paclitaxel loaded pH sensitive liposomal formulations

Formulations	Particle size (nm)									
	At (2-8) °C					At (30 ± 2) °C/65% RH				
	Time in months					Time in months				
	Initial	1	2	3	6	Initial	1	2	3	6
PSPT	139	146	151	158	168	139	153	168	175	189
PSPT-Lyophilized	148	151	156	157	164	148	157	163	167	170

Table 5.2.2. Effect of storage on uniformity value of Paclitaxel loaded pH sensitive liposomal formulations.

Formulations	Poly Dispersity Index (Uniformity Value)									
	At (2-8) °C					At (30 ± 2) °C/65% RH				
	Time in months					Time in months				
	Initial	1	2	3	6	Initial	1	2	3	6
PSPT	0.089	0.101	0.122	0.135	0.166	0.089	0.137	0.164	0.178	0.196
PSPT-Lyophilized	0.107	0.111	0.112	0.125	0.146	0.107	0.117	0.124	0.138	0.169

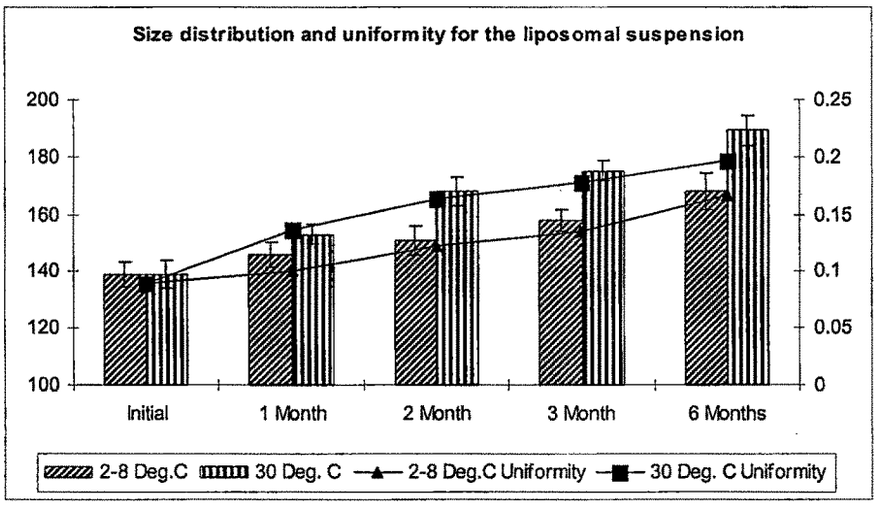


Figure 5.2.10. Mean particle size and uniformity value for Paclitaxel pH Sensitive Liposomal suspension stored at different temperatures.

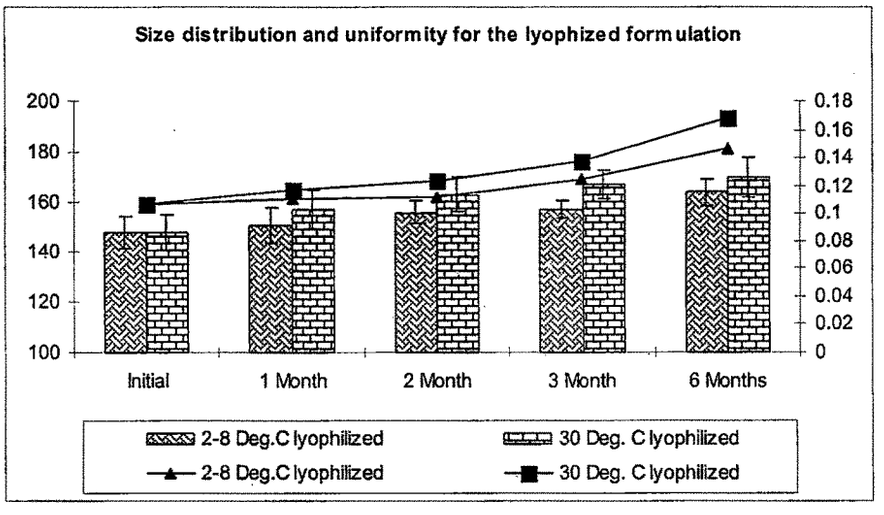


Figure 5.2.11. Mean particle size and uniformity value for lyophilized Paclitaxel pH Sensitive liposomal formulation stored at different temperatures.

Table 5.2.3. Effect of storage on Zeta potential and uniformity value of Paclitaxel loaded pH sensitive liposomal formulations

Formulations	Zeta potential (mV)									
	At (2-8) °C					At (30 ± 2) °C/65% RH				
	Time in months					Time in months				
	Initial	1	2	3	6	Initial	1	2	3	6
PSPT	-22.5	-22.3	-22.1	-21.4	-20.8	-22.5	-21.6	-21.1	-20.1	-18.1
PSPT-Lyophilized	-21.9	-21.8	-21.8	-21.5	-21.2	-21.9	-21.8	-21.6	-21.4	-21.0

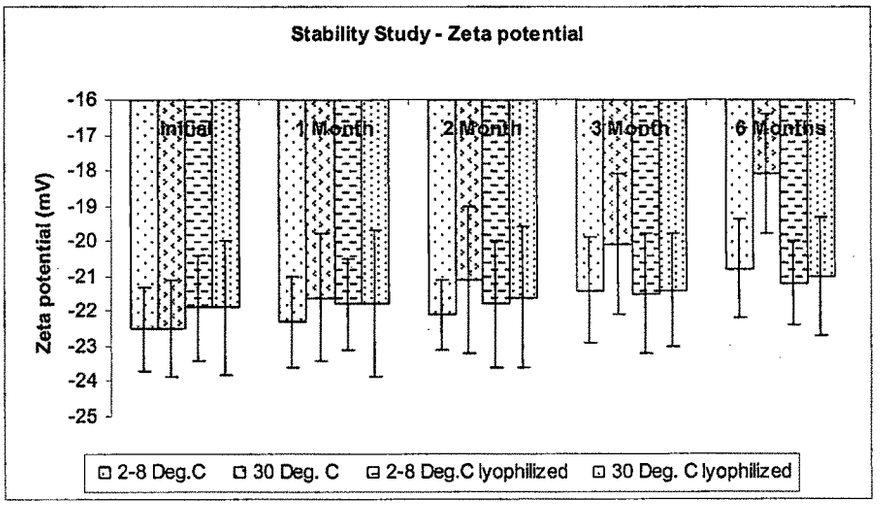


Figure 5.2.12. Zeta potential values for Paclitaxel pH sensitive liposomal suspension and lyophilized formulations stored at different temperatures.

Table 5.2.4. Effect of storage on Assay of Paclitaxel loaded pH sensitive liposomal formulations.

Formulations	Assay (%)									
	At (2-8) °C					At (30 ± 2) °C/65% RH				
	Time in months					Time in months				
	Initial	1	2	3	6	Initial	1	2	3	6
PSPT	98.94	98.54	97.73	97.22	96.82	98.94	98.08	95.85	92.18	90.63
PSPT-Lyophilized	97.46	97.21	97.08	97.02	96.85	97.46	97.15	97.01	96.86	96.74

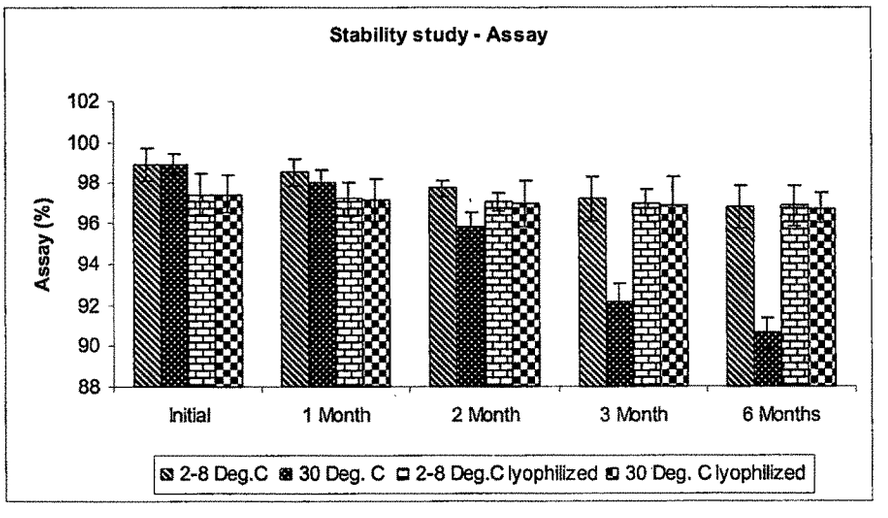


Figure 5.2.13. Assay for Paclitaxel pH sensitive liposomal suspension and lyophilized formulations stored at different temperatures.

Table 5.2.5. Effect of storage on drug retention in Paclitaxel loaded pH sensitive liposomal formulations.

Formulations	Drug retention (%)									
	At (2-8) °C					At (30 ± 2) °C/65% RH				
	Time in months					Time in months				
	Initial	1	2	3	6	Initial	1	2	3	6
PSPT	98.56	97.42	96.18	95.12	93.06	98.56	97.02	95.04	91.53	88.68
PSPT-Lyophilized	97.01	96.67	96.24	96.08	95.89	97.01	96.58	96.21	96.01	95.76

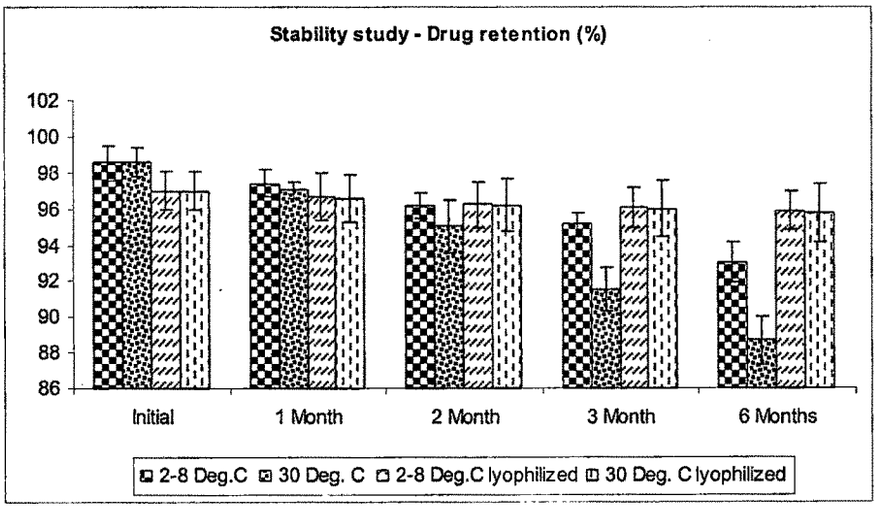


Figure 5.2.14. Percentage drug retention for Paclitaxel pH sensitive liposomal suspension and lyophilized formulations stored at different temperatures.

5.2.2 Characterization of pH sensitive Liposomes of Irinotecan Hydrochloride:

Characterization of the prepared pH sensitive, serum stable, long circulating liposomes for its physicochemical characteristics such as

i) **Particle size:**

This was performed using Nano-ZS particle size analyzer (Malvern Instruments, UK). The sample after appropriate dilution was filled in glass cuvette with round aperture and the readings were taken by maintaining the temperature at 25° C.

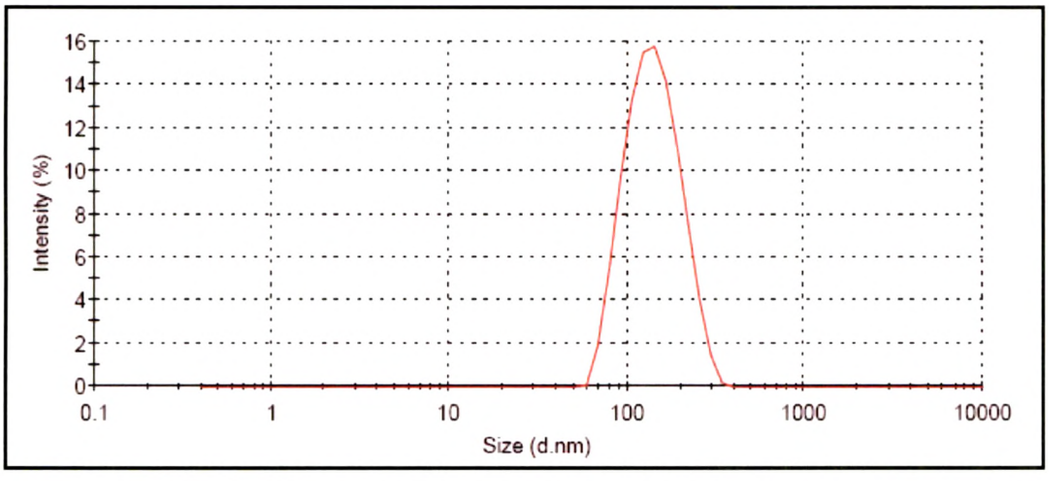


Figure 5.2.15. Particle size distribution of Irinotecan loaded pH sensitive liposomes.

ii) **Zeta potential:**

This was performed using Nano-ZS particle size analyzer (Malvern Instruments, UK). The sample was filled in unique disposable folded capillary cell and the readings were taken by maintaining the sample at 25° C temperature. Zeta potentials were calculated from the mean electrophoretic mobility by applying the Smoluchowski equation. The results are the means of 3 determinations ± standard deviation.

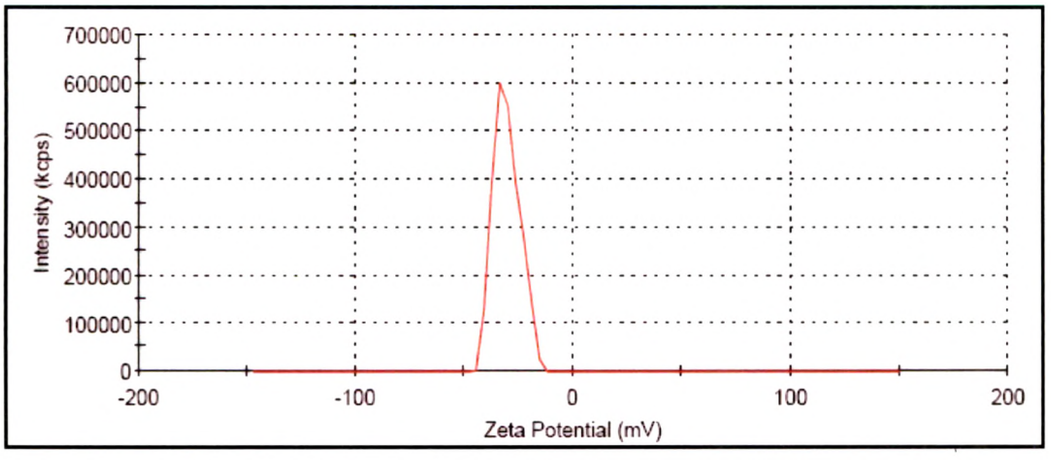


Figure 5.2.16. Graph depicting zeta potential of Irinotecan loaded pH sensitive liposomes.

iii) **Entrapment efficiency of Irinotecan Hydrochloride in liposomes:**

The entrapment efficiency (EE) is defined as the ratio of the amount of the Irinotecan Hydrochloride encapsulated in liposome to that of the total Irinotecan Hydrochloride in liposomal dispersion. The amount of Irinotecan Hydrochloride encapsulated in liposomes was measured following the reported method (Shieh et. al., 1997: Yang et. al., 2007). Briefly, aliquot (0.1mL) of liposomal dispersion diluted to 1.0mL by PBS (pH 7.4) immediately after preparation was centrifuged at 25,000 rpm for 30 min. After removing the supernatant by aspiration, the precipitate (*i.e.*, liposome pellet) was washed twice with PBS (pH 7.4). The liposome pellet was dissolved in organic solvents mixture of methanol & chloroform (9:1parts). The concentration of Irinotecan HCl was determined by spectrophotometrically at 361 nm after appropriate dilution with the mixed solvent that destroyed the liposome pellet. An aliquot (0.1mL each) of the liposome suspension was also dissolved with the same mixed solvent to determine the total amount of Irinotecan in the liposome suspension, after which the EE was calculated from the following equation:

$$\text{EE}\ (\%) = \frac{\text{Amount of Irinotecan HCl in liposome pellet } (\mu g)}{\text{Amount of Irinotecan HCl in liposomal dispersion } (\mu g)} \times 100$$

Supernatant was also analysed for free drug in it.

$$\text{EE (\%)} = \frac{\text{Drug}_{\text{Total}} - \text{Drug}_{\text{Supernatant}}}{\text{Drug}_{\text{Total}}} \times 100$$

A fluorescence spectrophotometric drug estimation method was also developed for Irinotecan HCl. The excitation and emission wavelengths found to be 374 nm and 435nm respectively.

An aliquot (50 mg each) of the freeze-dried liposome powder was dissolved with the same mixed solvent (4 mL) to determine the content of Irinotecan Hydrochloride in the freeze-dried liposome powder using the following equation after appropriate dilution with the same mixed solvent:

$$\text{Content} = \frac{\text{Amount of Irinotecan HCl in freeze dried liposome (µg)}}{\text{Amount of Freeze dried liposome (mg)}}$$

The EE and Irinotecan Hydrochloride content were determined from three separately prepared liposome suspensions, and were expressed as the mean ± standard deviation.

iv) **Solid-state analysis (DSC and XRD):**

Differential Scanning Calorimetry (DSC) studies were conducted for optimum batches having minimum particle size and maximum entrapment efficiency using Diamond Differential Scanning Calorimeter (Mettler, Star SW 8.10). These included studies on pure drug, bulk lipids, and lyophilized liposomal preparations. The analysis was performed at a rate 10 °C/ min from 35.0°C to 350 °C temperature range under inert nitrogen atmosphere at flow rate of 50 ml/min. The main objective of these studies is to determine the melting behaviour of lipids in the liposomal dispersions and possible various polymorphic modifications that the lipid may assume during the process of liposomal preparation.

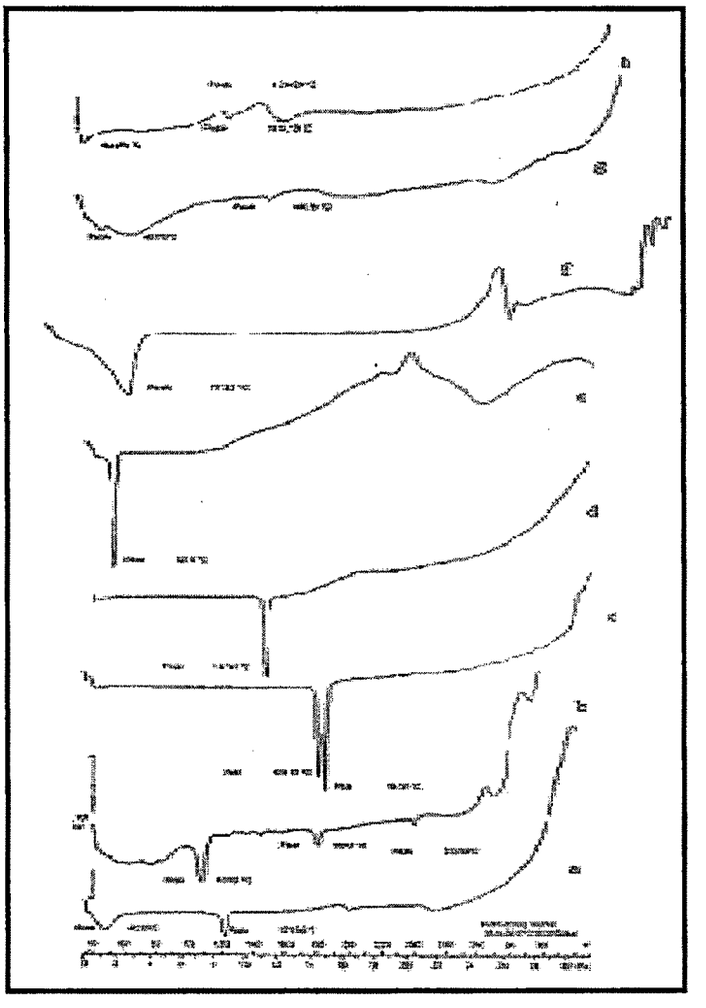


Figure 5.2.17. Differential Scanning Calorimetry thermograms of (a) DOPE, (b) HSPC, (c) CHEMS, (d) Cholesterol, (e) mPEG-DSPE, (f) Irinotecan Hydrochloride, (g) Placebo liposomes and (h) pH sensitive Irinotecan liposomes.

X-ray Diffraction studies (XRD): Powder X-ray diffraction patterns were obtained using an X-ray diffractometer (Philips PW 1710) with Cu Kα radiation generated at 30 mA and 40 kV. The source of X-ray was copper anode with a wavelength of 1.54060 A.

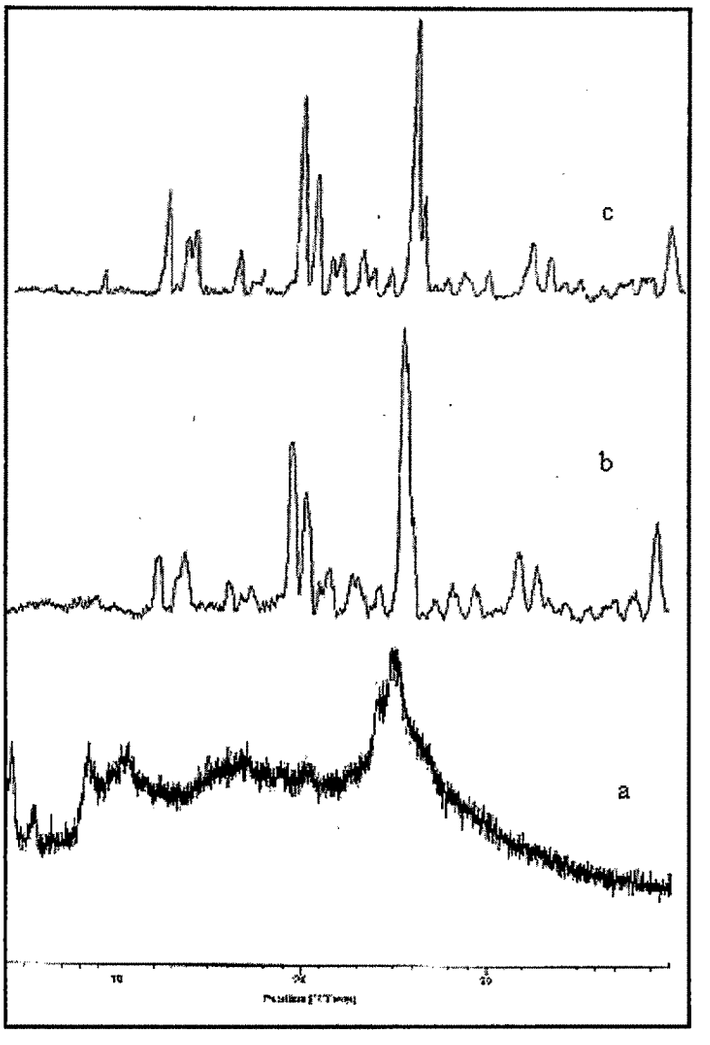
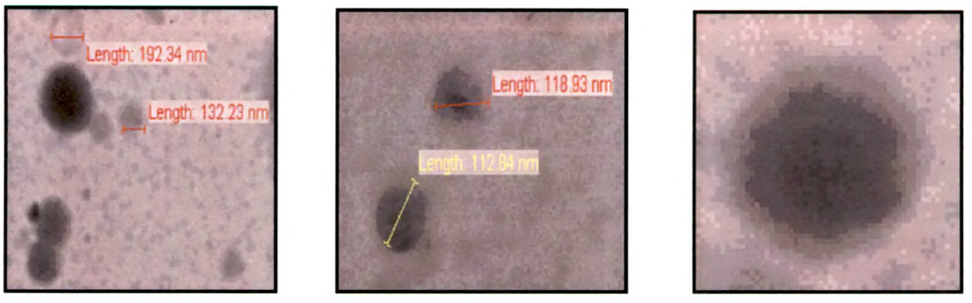


Figure 5.2.18. X-Ray Diffraction pattern of (a) Irinotecan Hydrochloride, (b) Placebo pH sensitive liposome and (c) Irinotecan pH sensitive liposomes.

v) Transmission Electron Microscopy (TEM):

Transition Electron Microscopic (TEM) studies were done for liposomal dispersion using Transition Electron Microscope (Philips, Morgagni 268). One drop of the liposomal dispersion (fresh sample or reconstituted) was incubated on 200-mesh carbon coated copper grid. The copper grid was fixed into sample holder and placed in vacuum chamber of the Transition Electron Microscope and observed under low vacuum (10^{-3} torr). The aim is to study the particle shape, size and surface characteristics in the liquid dispersion.



(a) (b) (c)

Figure 5.2.19 Transmission Electron Microscopy of Irinotecan Hydrochloride loaded pH sensitive liposomes at different magnifications.

vi) **pH induced liposomal aggregation and serum stability:**

Liposomal aggregation in response to reduced buffer pH was measured by increase in particle size. The Irinotecan encapsulated liposomal suspension was diluted with specific volume of sodium acetate buffer of various pH values and incubated at 37°C for 1 hour. At specified time point, aliquots of the sample were withdrawn and the mean particle size was determined using Malvern particle size analyzer (Shi et. al., 2002).

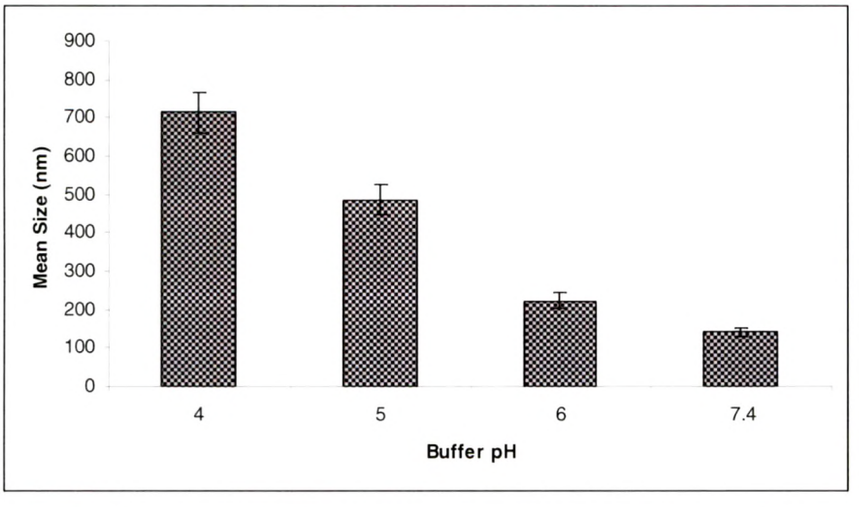


Figure 5.2.20. Change in size distribution pattern of pH sensitive liposomes of Irinotecan in response to various pH values, incubated at 37 °C for 1 hour.

Serum stability of the liposomal preparation was also determined by the same method, using 80 % serum instead of buffers with varied pH, measuring the change in particle size and zeta potential values, incubated at 37°C for 48 hours. The size and zeta potential of the withdrawn samples were determined as above.

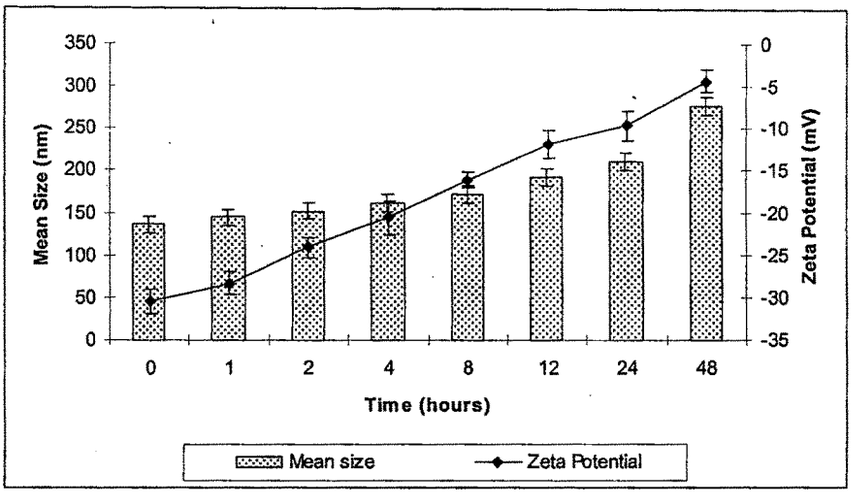


Figure 5.2.21. Size distribution pattern and Zeta potential values of pH sensitive liposomes of Irinotecan at various time points in serum at 37 °C upto 48 hours.

vii) ***In-vitro*** **drug release study:**

In-vitro release of plain Irinotecan, conventional Irinotecan liposomes and Irinotecan loaded pH-sensitive liposomes were studied by dialysis method in Phosphate buffered saline, pH 7.4 and pH 5.0 as dissolution media at 37°C. Briefly, 2 ml of liposomal suspension or plain drug solution of known concentration was taken in dialysis bag and suspended in the buffer with particular pH. At various time intervals aliquot samples were withdrawn and drug content was analysed.

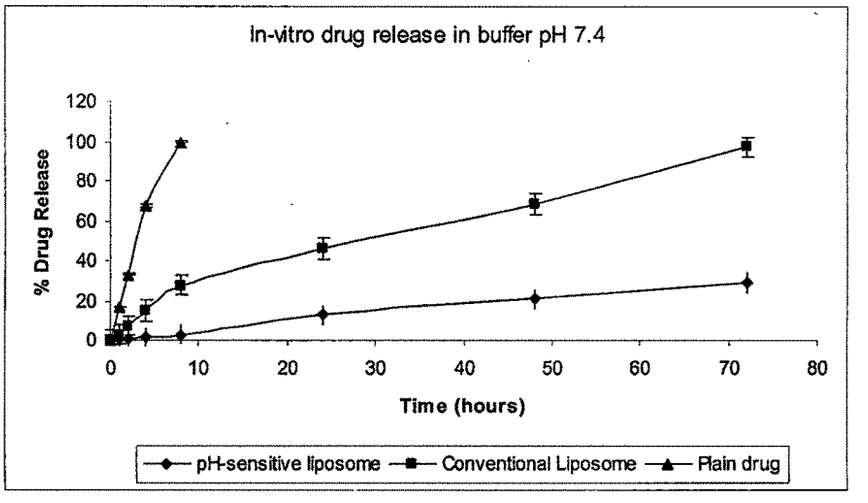


Figure 5.2.22. ***In-vitro*** **release of plain drug Irinotecan, conventional liposomes of irinotecan and pH sensitive liposomes of Irinotecan in buffer pH 7.4**

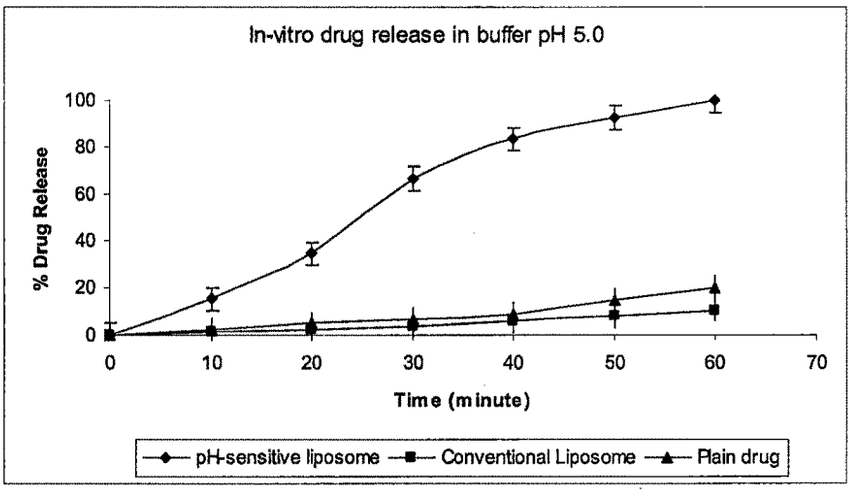


Figure 5.2.23. ***In-vitro*** **release of plain drug Irinotecan, conventional liposomes of irinotecan and pH sensitive liposomes of Irinotecan in buffer pH 5.0.**

vii) **Stability Study:**

The stability of the sterically stabilized pH sensitive liposomal suspension and lyophilized liposomal formulations containing Irinotecan HCl was investigated. Two temperatures were selected viz. refrigerator (2-8°C) and room temperature (30 ± 2°C). The liposomal dispersions were placed in amber coloured USP type-I glass vials

stoppered with 20 mm grey bromobutyl stoppers. The vials were purged with nitrogen before stoppering and sealing using 20 mm aluminium seals.

At predetermined intervals of time, samples were removed and studied for parameters such as change in size and size distribution, zeta potential, assay and drug retention/percent drug leaching. Changes in the size, size distribution and zeta potential of the liposomal formulations were determined using particle size analyzer. The assay was performed spectrophotometrically by the developed method while the percent drug leached from the liposomes was determined by centrifuging the liposomal suspension and analyzing the supernatant for the drug content.

Table 5.2.6. Effect of storage temperature on particle size of Irinotecan loaded pH sensitive liposomal formulations.

Formulations	Particle size (nm)									
	At (2-8) °C					At (30 ± 2) °C/65% RH				
	Time in months					Time in months				
	Initial	1	2	3	6	Initial	1	2	3	6
PSIH	132	143	156	161	174	132	154	170	185	199
PSIH-Lyophilized	154	156	160	162	165	154	158	168	169	174

Table 5.2.7. Effect of storage on uniformity value of Irinotecan loaded pH sensitive liposomal formulations

Formulations	Poly Dispersity Index (Uniformity Value)									
	At (2-8) °C					At (30 ± 2) °C/65% RH				
	Time in months					Time in months				
	Initial	1	2	3	6	Initial	1	2	3	6
PSIH	0.067	0.112	0.114	0.139	0.178	0.067	0.118	0.164	0.182	0.203
PSIH-Lyophilized	0.096	0.108	0.122	0.125	0.149	0.096	0.118	0.129	0.144	0.163

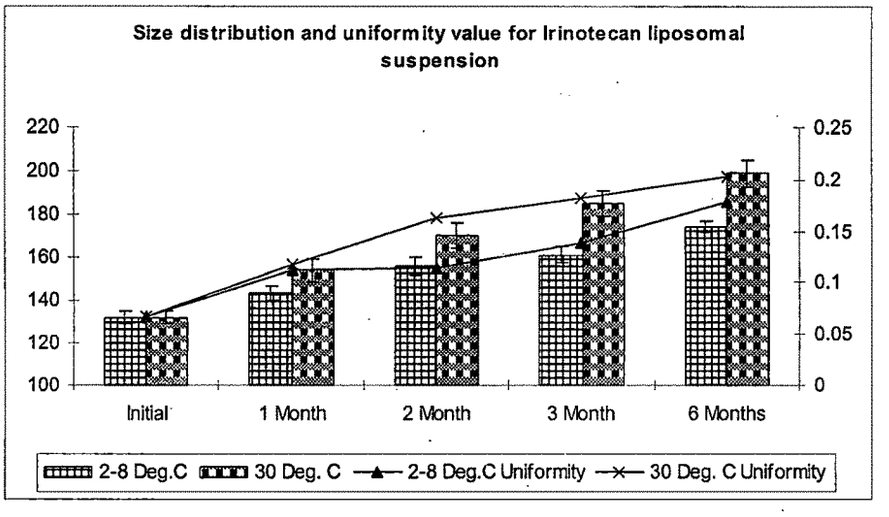


Figure 5.2.24. Mean particle size and uniformity value for Irinotecan liposomal suspension stored at different temperatures and dark condition.

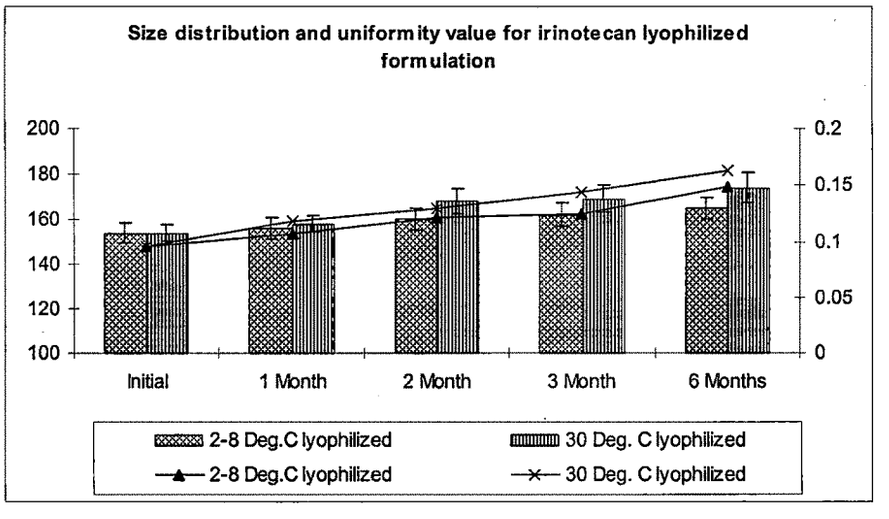


Figure 5.2.25. Mean particle size and uniformity value for lyophilized Irinotecan liposomal formulation stored at different temperatures and dark condition.

Table 5.2.8. Effect of storage temperature on Zeta potential of Irinotecan loaded pH sensitive liposomal formulations

Formulations	Zeta potential(mV)									
	At (2-8) °C					At (30 ± 2) °C/65% RH				
	Time in months					Time in months				
	Initial	1	2	3	6	Initial	1	2	3	6
PSIH	-30.4	-28.3	-27.1	-25.4	-25.3	-30.4	-27.6	-24.3	-22.9	-20.2
PSIH-Lyophilized	-28.9	-28.4	-28.4	-28.2	-28.1	-28.9	-28.3	-27.5	-27.2	-27.1

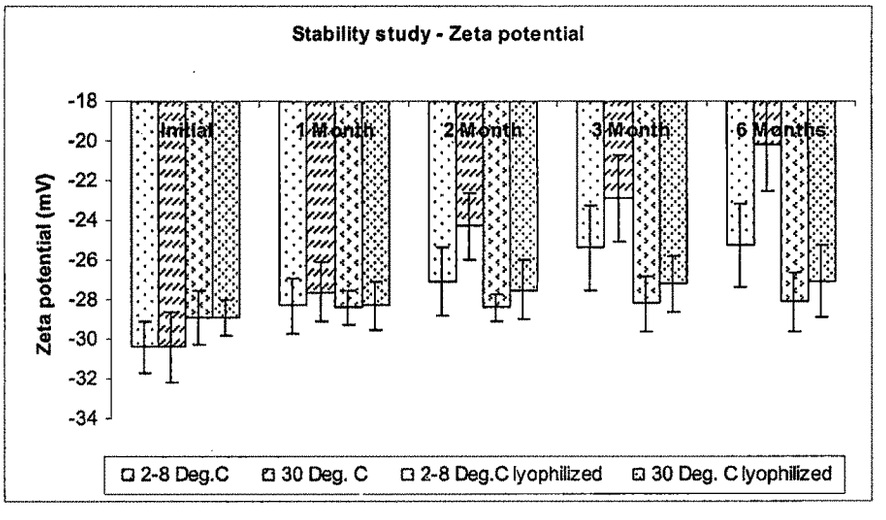


Figure 5.2.26. Zeta potential values for Irinotecan liposomal suspension and lyophilized formulations stored at different temperatures and dark condition.

Table 5.2.9. Effect of storage temperature on Assay of Irinotecan pH sensitive liposomal formulations.

Formulations	Assay (%)									
	At 2-8 °C					At (30 ± 2) °C/65% RH				
	Time in months					Time in months				
	Initial	1	2	3	6	Initial	1	2	3	6
PSIH	99.84	98.46	97.21	96.57	95.12	99.84	98.01	95.24	91.11	86.14
PSIH-Lyophilized	98.65	97.45	97.06	97.02	96.85	98.65	97.27	97.16	96.98	96.71

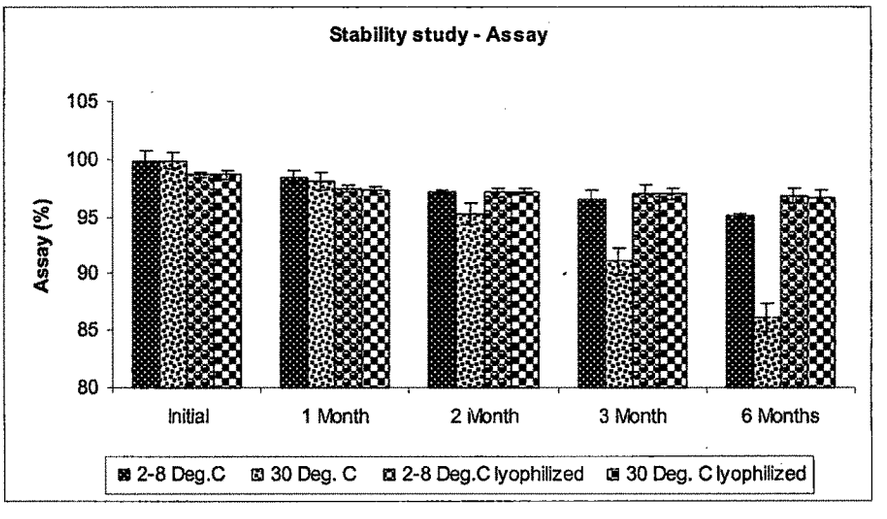


Figure 5.2.27. Assay of Irinotecan liposomal suspension and lyophilized formulations stored at different temperatures and dark condition.

Table 5.2.10. Effect of storage temperature on Drug retention of Irinotecan pH sensitive liposomal formulations.

Formulations	Drug retention (%)									
	At 2-8 °C					At (30 ± 2) °C/65% RH				
	Time in months					Time in months				
	Initial	1	2	3	6	Initial	1	2	3	6
PSIH	99.12	97.56	96.21	95.02	91.17	99.12	96.13	94.28	90.09	84.12
PSIH-Lyophilized	98.45	96.38	96.46	96.12	96.01	98.45	96.37	96.16	95.78	95.06

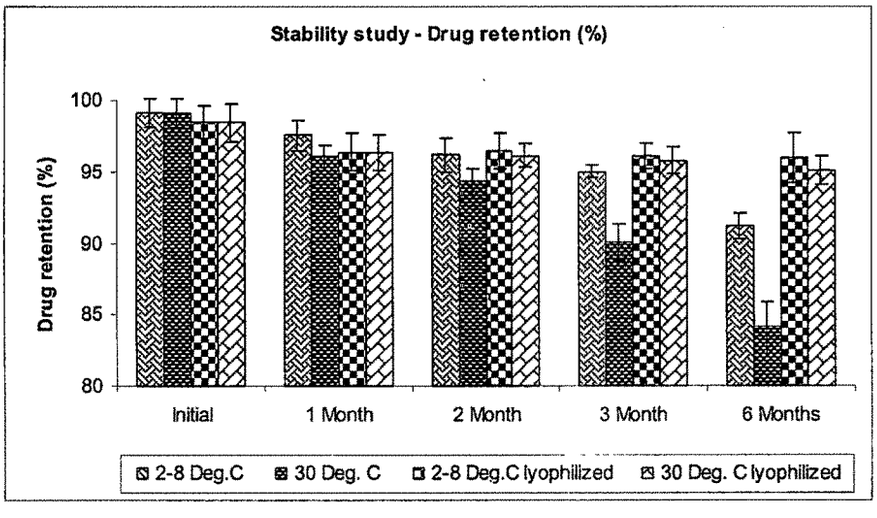


Figure 5.2.28. Percentage drug retention of Irinotecan liposomal suspension and lyophilized formulations stored at different temperatures and dark condition.

5.2.3 RESULTS AND DISCUSSION

The sterically stabilized, serum stable, pH sensitive liposomes were characterized by following parameters.

Size and Zeta potential:

The mean particle size of the prepared pH sensitive liposomal formulations after extrusion was obtained by using Particle size analyzer, Nano ZS (Malvern Instruments, UK). The initial particle size of 139 ± 2.3 nm was measured for pH sensitive liposomal suspension containing Paclitaxel (Figure 5.2.1) with uniformity value (PDI) of 0.089 and zeta potential of -22.5 ± 2.1 mV (Figure 5.2.2). The lyophilized formulation was reconstituted with water for injection and the size was measured. It was found to be 148 ± 6.4nm with uniformity value of 0.107 and zeta potential of -21.9 ± 2.5 mV.

In case of Irinotecan pH sensitive liposomal suspension the initial particle size was found to be 132 ± 3.2 nm with uniformity value of 0.067 (Figure 5.2.15) and zeta potential of -30.4 ± 1.3 mV (Figure 5.2.16). The lyophilized formulation was reconstituted with water for injection and size was found to be 154 ± 7.2 nm with uniformity of 0.0.096 and zeta potential of -28.9 ± 2.1 mV.

The results of the size analysis and zeta potential measurement supports the size and charge required for the liposomes to be long circulatory and tumor targeting which is also an important determining factor in *in-vivo* biodistribution.

Drug entrapment:

The unentrapped Paclitaxel was separated by centrifugation at 1,000 rpm for 10 minutes at 4 °C which will settle it from the liposomal dispersion (Yang et. al, 2007). Then the liposomal pellet was separated by centrifugation at 25,000 rpm for 30 minutes. The unentrapped Paclitaxel and encapsulated Paclitaxel were determined by methods explained earlier in Analytical development section. Entrapment of about 87.13 ± 1.49 % was obtained for Paclitaxel. For separation of unentrapped Irinotecan, the liposomal

suspension was centrifuged at 25,000 rpm for 30 minutes and the supernatant was analysed for unentrapped drug in it. The liposomal pellet was also analysed simultaneously to determine the encapsulated Irinotecan by methods explained earlier in Analytical development section. Entrapment of about 75.18 ± 1.62 % was obtained for Irinotecan.

Differential Scanning Calorimetry (DSC):

DSC analysis was performed for the pure drugs (Paclitaxel and Irinotecan), bulk lipids (DOPE, HSPC, CHEMS, Cholesterol and $mPEG_{2000}$-DSPE), and lyophilized liposomal preparations of both Paclitaxel and Irinotecan along with the placeboes respectively (Figure 5.2.3 and Figure 5.2.17). The DSC curve of DOPE showed a glass transition temperature (*Tg*) of 46.59 °C. The DSC curve of plain Paclitaxel showed a melting endotherm for the drug at 221.55 °C. This melting peak was absent in the DSC curve of the Paclitaxel loaded pH sensitive liposomal formulation. This indicates the presence of Paclitaxel in the amorphous form after entrapment into the DOPE containing liposomes. The thermogram also showed an endotherm at 47.91 °C (*Tg* of DOPE).

The DSC curve of Irinotecan showed endotherms at 77.32 °C and 271.3 °C. These melting peaks were absent in the DSC curve of the Irinotecan loaded pH sensitive liposomal formulation (Figure 5.2.17). This indicates the presence of Irinotecan in the amorphous form after entrapment into the DOPE containing liposomes. The thermogram also showed endotherms at 42.26 °C (*Tg* of DOPE) and at 114.13 °C (melting peak of HSPC 107.05 °C). DSC curve of placebo liposome showed endotherms at 48.73 °C (*Tg* of DOPE) and at 146.79 °C (melting peak of cholesterol).

X-Ray Diffraction studies (XRD):

Crystal diffraction software tools are widely used to simulate Powder X-Ray Diffraction (PXRD) patterns as reference standards for individual crystal forms such as polymorph, solvates and salts. The small differences between observed and simulated PXRD patterns, such as the appearance of the new peak(s), additional shoulders and shifts in the peak position or abnormal intensity distribution can indicate the presence of different forms

(e.g. polymorphs or solvates). Comparisons of the XRD patterns were performed by considering relative intensities of the diffracted peaks and inter planar spacing 'd'. The relative intensity is defined by the ratio of the peak intensity of a particular diffraction angle to the intensity of the standard peak. The diffraction peak with the strongest maxima is usually considered as standard peak. XRD pattern of plain Paclitaxel showed 40 peaks where as XRD patterns of Paclitaxel pH sensitive liposomes and placebo preparation showed 26 peaks and 24 peaks respectively. XRD studies of plain Paclitaxel revealed the crystalline nature of the drug, due to the presence of characteristic peaks (Figure 5.2.4). XRD studies of Paclitaxel loaded pH sensitive liposomes revealed the amorphous nature of the encapsulated drug in the formulation due to the absence of the characteristic peaks that were observed in the XRD of the plain drug. XRD of the placebo formulation also lacks the principle peaks which are present in plain drug. The XRD pattern of placebo and Paclitaxel pH sensitive liposomes shows almost similar peaks at different 2θ angles. The XRD pattern of Paclitaxel shows principle peak at angle 12.6072° 2θ. There was reduction in the intensity of all the peaks in the liposomal formulation. This result indicates that Paclitaxel is encapsulated inside the liposomes in the amorphous form.

X-Ray diffraction studies of plain Irinotecan revealed that the drug is in the amorphous state with characteristic broad, diffused diffraction pattern (Figure 5.2.18). The amorphous materials exhibit only short-range order where as crystalline materials exhibits long-range order. X-Ray diffraction studies of Irinotecan loaded pH sensitive liposomes revealed the amorphous nature of the encapsulated drug in the formulation due to the absence of the characteristic drug peaks in it. XRD of the placebo formulation also lacks the peaks which are present in plain drug. The XRD pattern of placebo and Irinotecan pH sensitive liposomes shows almost similar peaks at different 2θ angles. There was reduction in the intensity of all the peaks in the liposomal formulation. This result indicates that Irinotecan is encapsulated inside the liposomes in the amorphous form.

Transmission electron Microscopy (TEM):

TEM is one of the techniques that are used to characterize the liposomes by particle size, vesicle shape and lamellarity. The vesicle size was well below 200 nm and the unilamellar vesicles were spherical in shape in both Paclitaxel (Figure 5.2.5) and Irinotecan pH sensitive liposomal formulations (Figure 5.2.19). Majority of the liposomal vesicles were in the size range of 100 – 150 nm. The surface of the pH sensitive liposomes of Paclitaxel and Irinotecan does not show any visual difference.

pH induced flocculation and Serum stability:

pH induced flocculation study in buffers with varied pH demonstrated the flocculation and quick release of both Paclitaxel and Irinotecan at lower pH (i.e acidic range) where as it is more stable in neutral buffers. The change in the size of the liposomes at different buffer pH demonstrates the stability of the liposomal system. As the pH becomes more and more acidic the size increased drastically specifying the flocculation of liposomes in both the cases. In case of Paclitaxel pH sensitive liposomes the mean particle size was 139 ± 9.1 nm at buffer pH 7.4 where as it increased to 617 ± 48.5 nm at pH 4.0 (Figure 5.2.6). In case of Irinotecan pH sensitive liposomes the mean particle size was 142 ± 10.6 nm at buffer pH 7.4 and it increased to 714 ± 52.4 nm at buffer pH 4.0 (Figure 5.2.20).

Serum stability of the pH sensitive liposomal preparations were also assessed in the same method, using 80 % serum instead of buffers, measuring the change in particle size and zeta potential values, incubated at 37°C for 48 hours. The change in size and zeta potential of the withdrawn samples were determined as above. In case of Paclitaxel pH sensitive liposomes a gradual increase in mean size was observed over the time period of 48 hours (Figure 5.2.7). The initial mean size of 142 ± 2.1 nm increased to 254 ± 2.7nm at the end of 48 hours where as in case of Irinotecan pH sensitive liposomes it was 136 ±1.2 nm increased to 278 ± 2.2nm (Figure 5.2.21). Simultaneously change in zeta potential was also measured for both the formulations. For Paclitaxel pH sensitive liposomes the initial zeta potential value of -22.5 ± 2.1 mV increased to -1.8 ± 1.2 mV at the end of 48 hours where as in case of Irinotecan pH sensitive liposomes it was 136 ±1.2

nm increased to 278 ± 2.2nm. On storage in serum, the colloidal system started to aggregate due to the adherence of proteins in serum, there by increase in size and zeta potential values.

***In-vitro* release study:**

In-vitro release of Paclitaxel solution, conventional liposomes of Paclitaxel, Paclitaxel loaded pH-sensitive liposomes were carried up to 72 hours in phosphate buffered saline, pH 7.4 and for 1 hour in pH 5.0 as dissolution media.

Paclitaxel in the solution form showed 99.94 ± 0.18 % release in 8 hours in PBS pH 7.4 where as conventional liposome of Paclitaxel released 97.46 ± 1.22 % and Paclitaxel pH sensitive liposomes released 29.35 ± 0.54 % of drug in 72 hours of release study under the same experimental conditions (Figure 5.2.8). In buffer pH 5.0 the Paclitaxel pH sensitive liposomes showed 99.73 ± 0.92 % of drug release within 60 minutes where as conventional liposome of Paclitaxel showed 10.67± 0.23 % and Paclitaxel solution showed 19.91 ± 0.58 % of drug release under similar experimental conditions (Figure 5.2.9).

In the same way Irinotecan in solution form, conventional Irinotecan liposomes and pH sensitive liposomes of Irinotecan were also assessed for release pattern as that was carried for Paclitaxel formulations. Irinotecan in the solution form showed 99.89 ± 0.84 % release in 8 hours in PBS pH 7.4 where as conventional liposome of Irinotecan released 95.78 ± 1.43 % and Irinotecan pH sensitive liposomes released 38.86 ± 0.48 % of drug in 72 hours of release study under the same experimental conditions (Figure 5.2.22). In buffer pH 5.0 the Irinotecan pH sensitive liposomes showed 99.65 ± 0.38% of drug release within 60 minutes where as conventional liposome of Irinotecan showed 9.98 ± 0.22 % and Irinotecan solution showed 18.95 ± 0.28 % of drug release under similar experimental conditions (Figure 5.2.23).

The *in-vitro* release studies revealed that the long circulation property of Paclitaxel and Irinotecan loaded pH-sensitive liposomes in PBS pH 7.4 compared to both the Plain drug

solutions, conventional Paclitaxel and Irinotecan liposomes respectively, where as complete destabilization at cytosolic acidic pH of 5.0.

Different dissolution models were applied to drug release data of pH sensitive liposomal formulations in order to evaluate release mechanisms and kinetics. A criterion for selecting the most appropriate model was based on linearity (coefficient of correlation). The drug release data fit well to zero order kinetics but fairly good fit into the Higuchi and Peppas expression (in PBS pH 7.4) from pH sensitive Paclitaxel liposomes where as it demonstrated Peppas and Higuchi expression in acidic buffer pH 5.0. In case of Irinotecan pH sensitive liposomes the drug release data fit well to Peppas but fairly good fit to zero order expression (in PBS 7.4) whereas Peppas and Higuchi expression in acidic buffer pH 5.0.

Stability studies:

Effect of temperature on particle size and uniformity value:

The particle size distribution of the prepared Paclitaxel pH sensitive liposomal suspension and the lyophilized liposomal products stored at refrigerated temperature (2-8°C) with ambient humidity and at (30 ± 2°C)/65% R.H. were determined at regular time intervals. The particle size was found to increase slowly with the increasing time duration of storage, as a result of particle fusion or agglomeration. The particle size increase was more in case of liposomal suspensions stored at (30 ± 2°C)/65% R.H than that stored at 2-8 °C. The mean particle diameter of the Paclitaxel pH sensitive liposomal suspension stored at 2-8 °C increased to 168 nm (uniformity value 0.166) from the initial diameter of 139 nm (uniformity value 0.089), where as the sample stored at (30 ± 2°C) increased to 189nm (uniformity value 0.196) after 6 months (Figure 5.2.10, 5.2.11 and Table 5.2.1, 5.2.2).

The mean particle diameter of lyophilized samples of pH sensitive liposome containing Paclitaxel stored at 2-8 °C increased to 164 nm (uniformity value 0.146) from the initial diameter of 148 nm (uniformity value 0.107), where as the sample stored at (30 ± 2°C)

increased to 170nm (uniformity value 0.169) after 6 months (Figure 5.2.10, 5.2.11 and Table 5.2.1, 5.2.2).

The mean particle diameter of the Irinotecan pH sensitive liposomal suspension stored at 2-8 °C increased to 174 nm (uniformity value 0.178) from the initial diameter of 132 nm (uniformity value 0.067), where as the sample stored at (30 ± 2°C) increased to 199nm (uniformity value 0.203) after 6 months (Figure 5.2.24, 5.2.25 and Table 5.2.6, 5.2.7).

The mean particle diameter of lyophilized samples of pH sensitive liposome containing Irinotecan stored at 2-8 °C increased to 165 nm (uniformity value 0.149) from the initial diameter of 154 nm (uniformity value 0.096), where as the sample stored at (30 ± 2°C) increased to 174nm (uniformity value 0.163) after 6 months(Figure 5.2.24, 5.2.25 and Table 5.2.6, 5.2.7).

The above results indicate that the liposomal suspensions stored at higher temperature lacks suitable stability profiles, which intern indicates the need for lyophilization (freeze drying) of the liposomal suspension formulations for long time storage maintaining the integrity of the preparation.

Effect of temperature on zeta potential:

The zeta potential of the Paclitaxel pH sensitive liposomal suspension stored at 2-8 °C increased to -20.8 mV from the initial value of -22.5 mV, where as the sample stored at (30 ± 2°C) increased to -18.1mV after 6 months (Figure 5.2.12 and Table 5.2.3).

The zeta potential of lyophilized pH sensitive liposomes containing Paclitaxel stored at 2-8 °C increased to -21.2 mV from the initial value of -21.9 mV, where as the sample stored at (30 ± 2°C) increased to -21.0 mV after 6 months (Figure 5.2.12 and Table 5.2.3).

The zeta potential of the Irinotecan pH sensitive liposomal suspension stored at 2-8 °C increased to -25.3 mV from the initial value of -30.4 mV, where as the sample stored at (30 ± 2°C) increased to -20.2 mV after 6 months (Figure 5.2.26 and Table 5.2.8).

The zeta potential of lyophilized pH sensitive liposomes containing Irinotecan stored at 2-8 °C increased to -28.1 mV from the initial value of -28.9 mV, where as the sample stored at (30 ± 2°C) increased to -27.1 mV after 6 months(Figure 5.2.26 and Table 5.2.8).

A system is considered stable if the electrostatic repulsion dominates the attractive van der Waals forces. When the kinetic energy of the particle is high enough to overcome the barrier of electrostatic repulsion, they undergo collision. Increase in temperature usually leads to increase in kinetic energy of the system, which in combination with a reduction in zeta potential leads to the aggregation of the particles (Freitas and Muller, 1998). The lyophilized formulations found to be more stable in different temperature conditions than the liposomal suspensions, in case of both the drugs.

Effect of temperature on Assay:

The initial assay of the Paclitaxel pH sensitive liposomal suspension stored at 2-8 °C found to be 98.94 % decreased to 96.82 %, where as the same batch sample stored at (30 ± 2°C) decreased to 90.63 % after 6 months (Figure 5.2.13 and Table 5.2.4).

The initial assay of the lyophilized Paclitaxel pH sensitive liposomes stored at 2-8 °C found to be 97.46 % decreased to 96.85 %, where as the same batch sample stored at (30 ± 2°C) decreased to 96.74 % after 6 months (Figure 5.2.13 and Table 5.2.4).

The same trend was also observed in case of Irinotecan formulations. The initial assay of the Irinotecan pH sensitive liposomal suspension stored at 2-8 °C found to be 99.84 % decreased to 95.12 %, where as the same batch sample stored at (30 ± 2°C) decreased to 86.14 % after 6 months (Figure 5.2.27 and Table 5.2.9).

The initial assay of the lyophilized Irinotecan pH sensitive liposomes stored at 2-8 °C found to be 98.65 % decreased to 96.85 %, where as the same batch sample stored at (30 ± 2°C) decreased to 96.71 % after 6 months(Figure 5.2.27 and Table 5.2.9).

The assay failed in case of Irinotecan pH sensitive liposomal suspension stored at (30 ± 2°C) and it was on the border line in case of Paclitaxel pH sensitive liposomal suspension after 6 months of storage. But the lyophilized formulations showed stability in different temperature conditions in case of both the drugs.

Effect of temperature on drug retention:

Drug retention is one of the major criteria for stability assessment of liposomal preparations. The drug retention of the Paclitaxel pH sensitive liposomal suspension stored at 2-8 °C was found to be 93.06 % from the initial value of 98.56 %, where as the sample stored at (30 ± 2°C) decreased to 88.68 % after 6 months (Figure 5.2.14 and Table 5.2.5).

The drug retention of the lyophilized Paclitaxel pH sensitive liposomes stored at 2-8 °C was found to be 95.89 % from the initial value of 97.01 %, where as the sample stored at (30 ± 2°C) decreased to 95.76 % after 6 months (Figure 5.2.14 and Table 5.2.5).

The drug retention of the Irinotecan pH sensitive liposomal suspension stored at 2-8 °C was found to be 91.17 % from the initial value of 99.12 %, where as the sample stored at (30 ± 2°C) decreased to 84.12 % after 6 months (Figure 5.2.28 and Table 5.2.10).

The drug retention of the lyophilized Irinotecan pH sensitive liposomes stored at 2-8 °C was found to be 96.01 % from the initial value of 98.45 %, where as the sample stored at (30 ± 2°C) decreased to 95.06 % after 6 months (Figure 5.2.28 and Table 5.2.10).

The percentage drug retention in liposomes decreased drastically in case of pH sensitive liposomes of Irinotecan and Paclitaxel stored at (30 ± 2°C). But in case of lyophilized formulations the drug retention was quite good and proved to be highly stable during storage. Sucrose was used as cryoprotectant while lyophilizing the liposomal samples gave better retention ability to the carrier system.

5.2.4 Conclusion:

Conventional and pH sensitive liposomes containing Paclitaxel and Irinotecan were prepared using film hydration method with good encapsulation efficiency. Liposomal suspension was lyophilized with sucrose as cryoprotectant in order to increase the stability of the product. Characterizations of the liposomal formulations were done and stability studies of the samples conducted. Stability studies of the formulated pH sensitive liposomes for both the drugs indicated that the liposomal suspensions were not stable enough to be commercialized whereas freeze dried products showed excellent stability in different temperatures selected for the study.

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