CHAPTER 4

PREPARATION AND CHARACTERIZATION OF LIPOSOMES

Liposomes are microscopic vesicles (Bangham et al. 1965), which consist of an aqueous core surrounded by one or more lipid bilayers They are classified on the basis of their size and lamellarity as Multilamellar vesicles (MLVs), comprise five or more concentric aqueous and lipid layers with wide range of sizes (100-1000 nm), Small Unilamellar Vesicles (SUVs), comprise a single bilayers surrounding a aqueous compartment, in the size range of 25-100 nm and Large Unilamellar Vesicles (LUVs), have a large aqueous compartment surrounded by a single bilayers The size of LUVs commonly ranges from 100-1000 nm. Liposomes may also be referred to in terms of their method of manufacture, e g reverse phase evaporation vesicles (REVs) (Szoka and Papahadjopoulos, 1978), dehydration rehydration vesicles (DRVs) (Kirby and Gregoriadis, 1984)) and multivesicular liposomes (MVLs) (Kim et al., 1983)

Many methods have been developed to prepare liposomes There are at least fourteen major reported methods (Ostro, 1987) The seven most commonly employed method are lipid film hydration also referred as thin layer evaporation method (Bangham et al , 1965), reverse phase evaporation technique (Szoka and Papahadjopoulos, 1978), rehydration-dehydration technique (Shew and Deamer, 1985), ethanol injection method (Batzri and Korn, 1975), ether infusion method (Deamer et al., 1976), French press technique (Barenholz et al , 1979) and detergent dialysis technique (Kagawa and Racker, 1971)

The liposomes are characterized for their shape, lamellarity, size and size distribution, quantity of entrapped solute and for their chemical composition The most precise method to determine size of the liposomes is electron microscopy. Unfortunately, is very time consuming and costly Laser light scattering method is very simple and rapid but having disadvantage of measuring an average property of the bulk of the liposomes The approximate idea of size range can be obtained by gel exclusion chromatographic techniques and optical microscopy. Considering the cost of equipments like electron microscope and size analyzer based on laser light scattering, optical microscopy at higher magnification can be performed during optimization of the liposomes and the size of final liposomal batches can be confirmed by laser light scattering size analyzer. The percent drug entrapment (PDE) is determined after separation of free drug from the entrapped drug. Method used for this purpose may be

dialysis (using dialysis sack), ultra centrifugation, mini column centrifugation using hydrated gel (Sephadex G-50) and protamine aggregation, using solution of protamine sulphate in the concentration of 10 mg/ml (New, 1990) The lamellarity of liposomes is determined by freeze fracture electron microscopy and by ^{31}P-NMR The chemical composition of liposomes can be determined by analyzing the components of liposomes after their separation using reported analytical methods (Stewart 1980)

Liposomes of LN and LEU were prepared by TFH and REV methods Prepared liposomes were characterized for shape, size and size distribution, lamellarity, and PDE and for the contents of phosphatidylcholine and cholesterol Optimization was carried our by selection of various formulations and process variables.

4.1 REAGENTS

(i) Water: Double distilled water

(ii) Acetate Buffer pH 5 2. Prepared as per the procedure given in the Indian Pharmacopoeia

(iii) PBS· Prepared as per the procedure given in the Indian Pharmacopoeia.

(iv) Methanolic PBS Accurately measured 800 ml of PBS was transferred to a clean, dry 1000 ml volumetric flask and volume was made to 1000 ml with Methanol

4.2 PREPARATION OF LEVONORGESTREL LIPOSOMES

The liposomes of LN were prepared by two methods viz, TFH (Bangham et al, 1965) and REV (Szoka and Papahadjopoulos, 1978) methods

4.2.1 Thin Film Hydration method

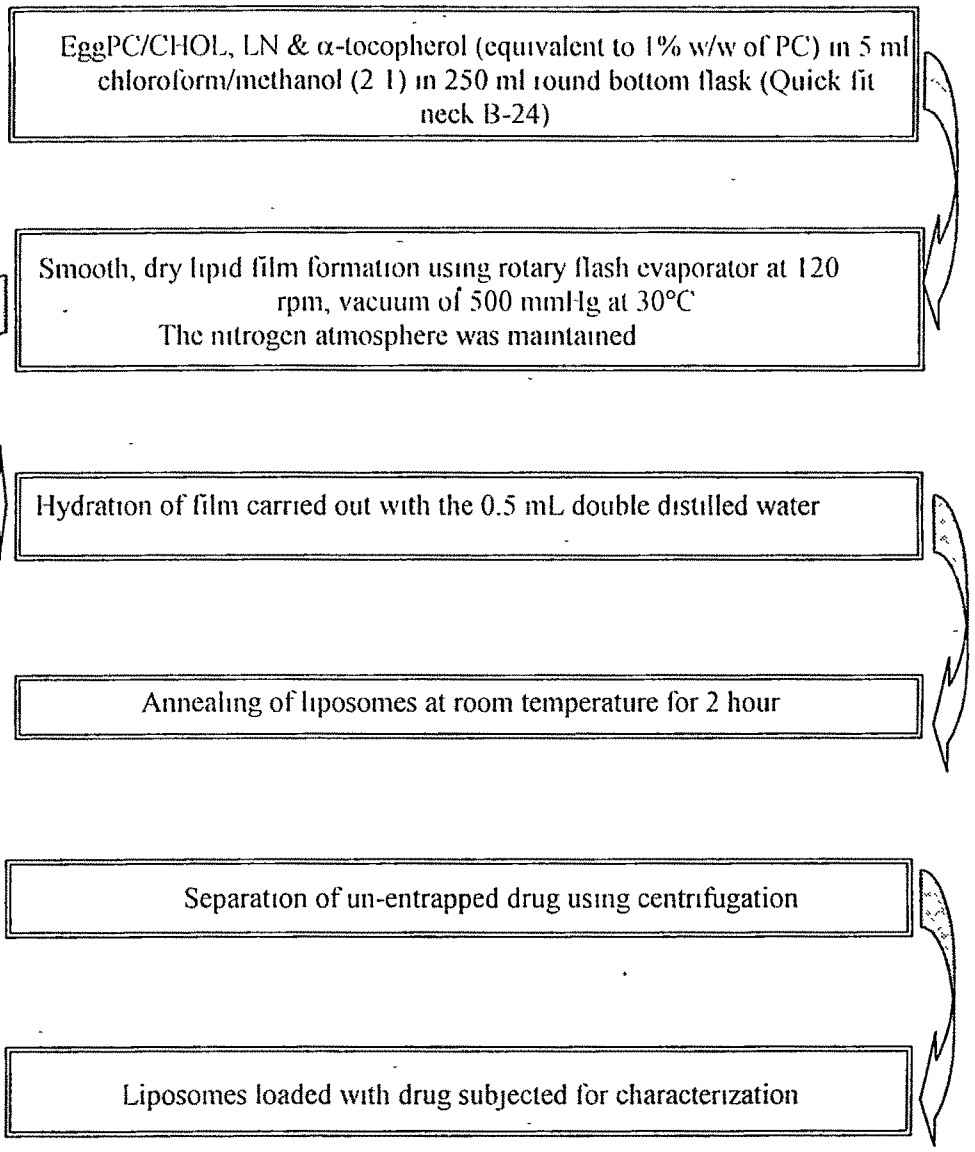
PC, CHOL and α-tocopherol (equivalent to 1% w/w of PC) were dissolved in solvent mixture in 250 ml round bottom flask (Quick fit neck B-24) Flask was rotated in a rotary flash evaporator at 120 rpm under vacuum of 500 mm of Hg at 30°C The nitrogen atmosphere was maintained for 20 minutes to form smooth, uniform and dried film Hydration of film was carried out by double distilled water under nitrogen atmosphere at room temperature for 45 minutes Annealing of liposomes at room temperature was carried out for 2 hour The complete procedure is shown in Flow chart A

The formulation technique was optimized with regards to Drug.PC ratio, PC CHOL ratio, selection of organic phase, selection of aqueous phase, vacuum, hydration medium, hydration time, speed of rotation of flask, size reduction of liposomes so as to get optimum drug entrapment and stability Each batch was prepared for three times on three different days and stored in refrigerator The optimized variables, organic phase, vacuum, hydration medium, hydration time, speed of rotation of flask are recorded in Table 4 1.

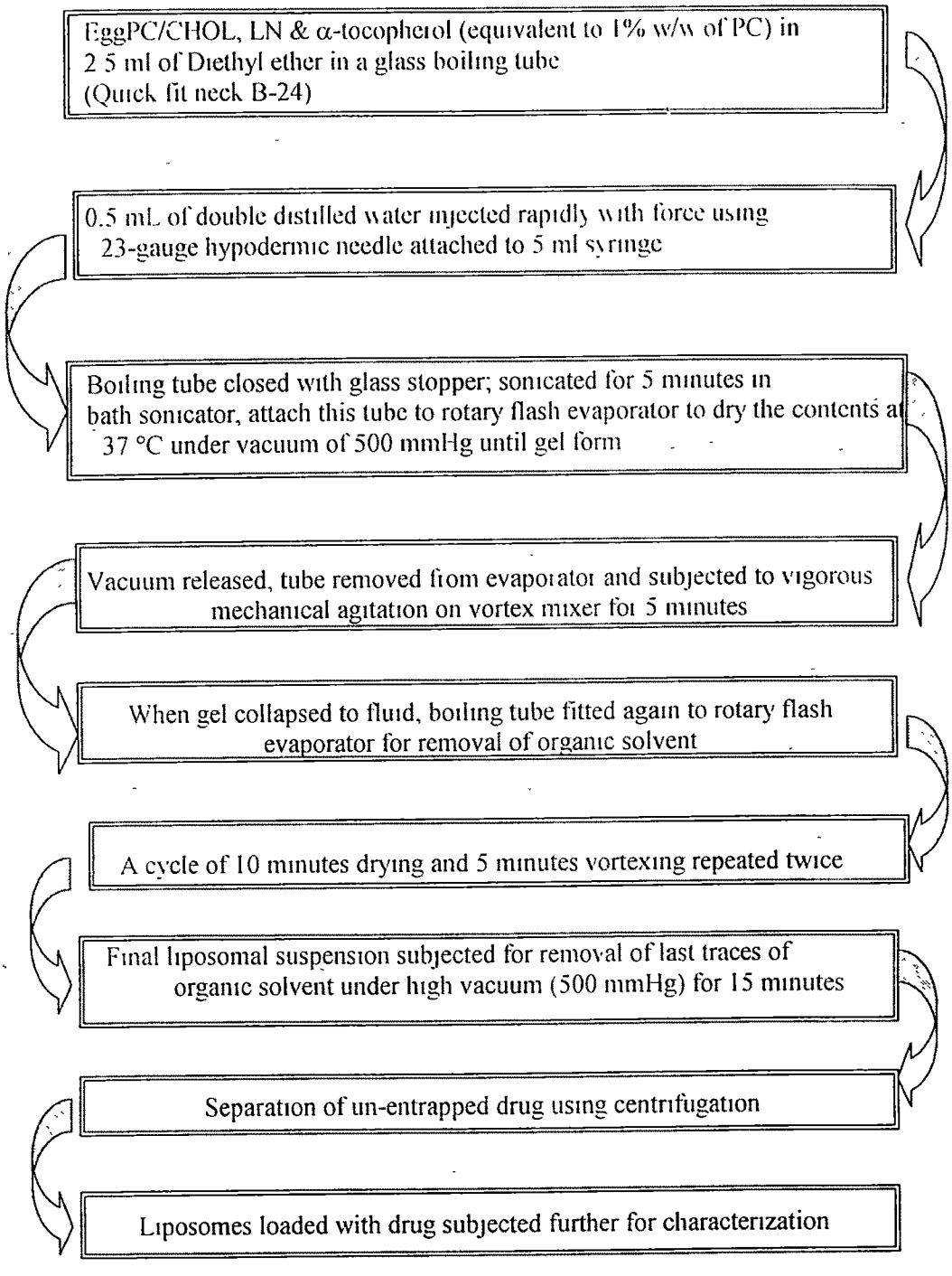
4.2.2 Reverse Phase Evaporation Method (REV Method):

PC, CHOL and α-tocopherol (equivalent to 1% w/w of PC) were dissolved in diethyl ether in a glass boiling tube (Quick fit neck B-24). Double distilled water was injected rapidly through 23-gauge hypodermic needle from a 5 ml syringe. The tube was closed with glass stopper and sonicated for 5 minutes in a bath sonicator. It was then attached directly to a rotary flash evaporator to dry the contents at 37°C with 500 mm Hg of vacuum until a gel was formed Vacuum was released and the tube was removed from the evaporator and subjected to vigorous mechanical agitation on vortex mixer for 5 minutes. When the gel collapsed to fluid, it was again fitted to rotary flash evaporator for the removal of organic solvent. A cycle of 10 minutes drying and 5 minutes vortexing was further repeated twice Final liposomal suspension was subjected for complete removal of last traces of organic solvent in a rotary flash evaporator at 500 mm Hg of vacuum for 15 minutes. Annealing of liposomes at room temperature was carried out for 2 hour after sonication. The complete procedure is shown in Flow chart B

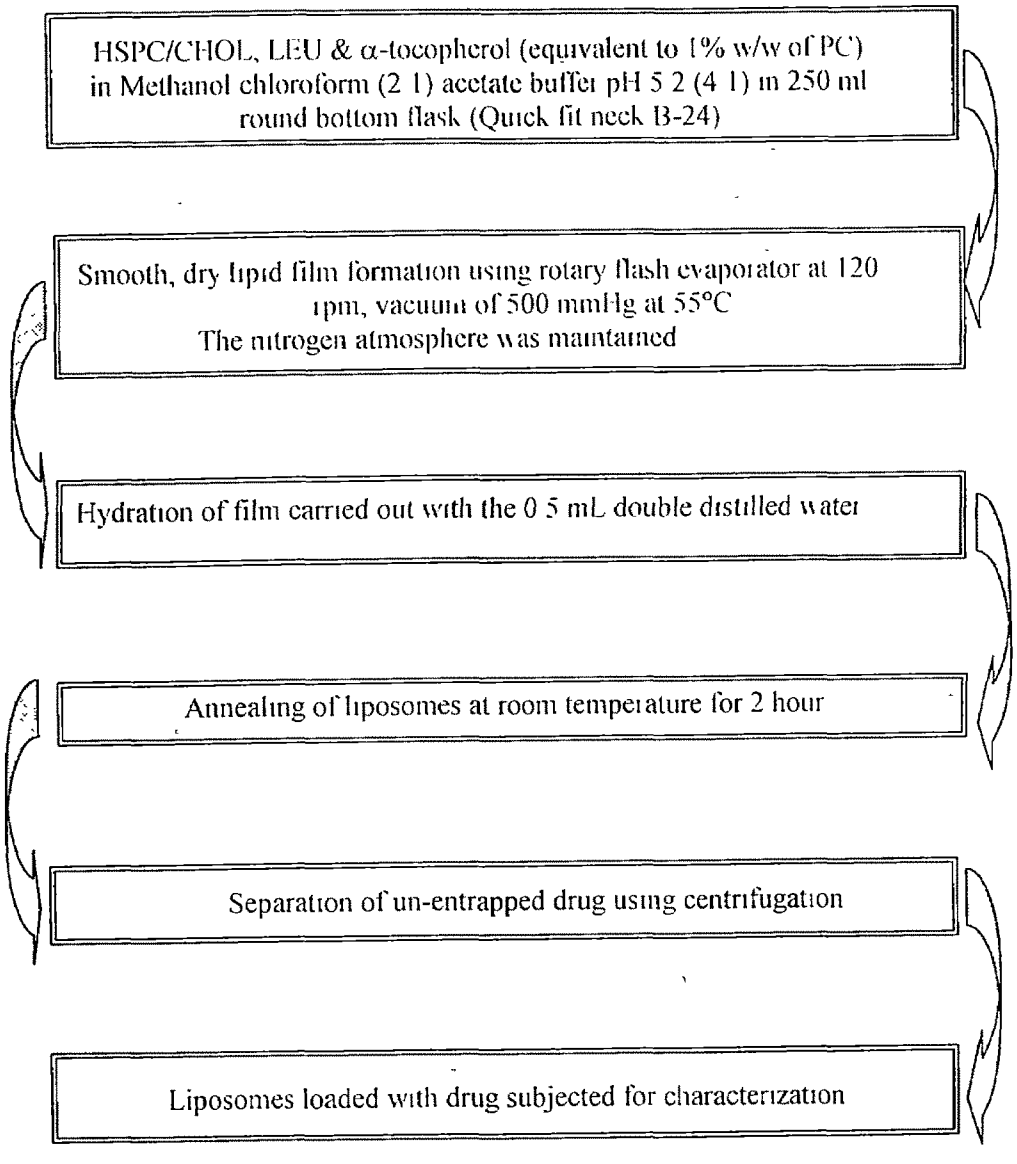
Flow Chart- A: Formulation technique for the preparation of LN liposomes by TFH method



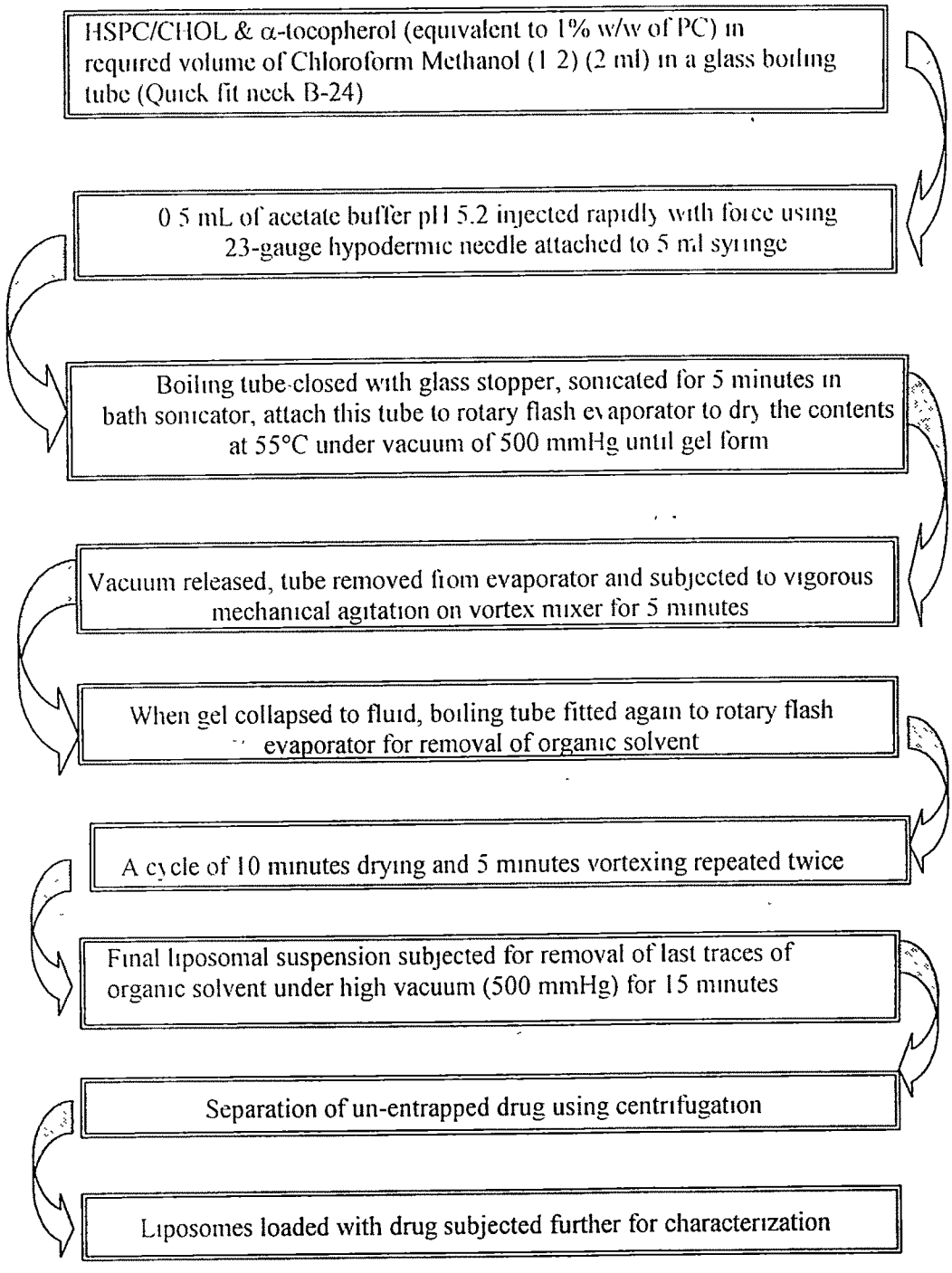
Flow Chart – B: Formulation technique for the preparation of LN liposomes by REV method



Flow Chart- C: Formulation technique for the preparation of LEU liposomes by TFH method



Flow Chart – D: Formulation technique for the preparation of LEU liposomes by REV method.



The formulation technique was optimized with regards to Drug PC ratio, PC CHOL ratio, hydration medium, organic to water phase ratio, annealing time, speed of rotation of flask so as to get optimum drug entrapment and stability Each batch was prepared for three times on three different days and stored in refrigerator Size reduction of LLN was carried out using sonication in a 10 ml borosil beaker under ice bath, by probe sonicator 10 minutes sonication cycles were carried out and numbers of sonication cycles were optimized with regard to required vesicle size

The prepared liposome batches prepared by both the methods were evaluated for PDE, PC and CHOL contents by the procedure discussed in Chapter 3 (section 3 4 3 and 3.3 respectively). The results of PDE along with compositions of liposomes are recorded in Table 4 2. Vesicle size of the optimized batches were also determined and results obtained are recorded in Table 4 2

4.2.3 Freeze thaw cycle for LN Liposomes

Freeze-thaw (F-T) cycles were carried out for liposomes prepared by both the methods describe above The prepared liposomal batches were frozen and the frozen suspension was thawed at room temperature for 15 min and the liposomes were again evaluated for vesicle size and PDE The results of PDE along with number of F-T cycles are recorded in Table 4.2 Freezing temperature and time were optimized with regards to PDE and results obtained are recorded in Table 4 4 and Figure 4 3

4.3 CHARACTERIZATION OF LEVONORGESTREL LIPOSOMES

Liposomes loaded with drug prepared in this work were characterized with respect to size, shape, lamellarity, PDE in addition to PC and CHOL content in the liposomes.

4.3.1 Determination of vesicle Size:

The vesicle size determination of the prepared liposomes carried out by light scattering was based on laser diffraction using Malvern. MasterSizer SM 2000k

(Malvern Instruments Inc , UK) The apparatus consisted of a He-Ne laser (5 mw) and a sample holding cell of 50 ml capacity Liposomes were diluted with sufficient amount of diffusion medium so that 50 ml volumes give obscuration between 10-20% as per the manufacturer's recommendation The samples were stirred using a blade stirrer at 1000 rpm to keep the sample in suspension

The instrument settings used were as follows Temperature 25°C, viscosity 0 01 poise, refractive index 1·33, scattering angle 90°, run time 30 sec, range 0-1000 µm

4.3.2 Determination of vesicular shape & lamellarity:

All the batches of the liposomes after sufficient dilution were viewed under Olympus (BX 40F4, Japan) with the provision of dark background and attachment of polarizing lens, to study their shape and lamellarity The photomicrographs of representative batches were taken to confirm the results (Figure 4 11)

4.3.3 Analysis of liposomes for PDE and PC & CHOL contents:

PDE in liposomes is expressed as the percent of the drug actually entrapped in the liposomes out of the added drug. This was determined by the method described in Chapter 3 (section 3.4 3) PC and CHOL contents of the liposomes were also determined using the method described in Chapter 3 (section 3.3)

4.4 PREPARATION OF LEUPROLIDE ACETATE LIPOSOMES

The liposomes of LEU were prepared by two methods viz; TFH and REV methods The method of sequential analysis was adopted as there are many variables that were thought to have significant effect on PDE of liposomes

4.4.1 Thin Film Hydration method:

The method used was as described in section 4 2.1 under the heading of Thin Film Hydration method. The formulation and process variables selected were shown in Flow Chart C and Table 4.6.

4.4.2 Reverse Phase Evaporation method:

The method used was as described in section 4.2.1 under the heading of Reverse Phase Evaporation method. The formulation and process variables selected were shown in Flow Chart D and Table 4.6.

The formulation technique was optimized with regards to Drug:PC ratio, PC:CHOL ratio, organic phase, organic to water phase ratio, so as to get optimum drug entrapment and stability. Each batch was prepared for three times on three different days and stored in refrigerator. Size reduction of prepared LEU liposomes were carried out using two methods, first by sonication under ice bath, by probe sonicator for 10 minutes and second, by subjecting liposomes to extrusion. The extrusions of prepared liposomes were carried out by passing through 2 µm polycarbonate membrane for different number of extrusions.

For preparation of either negatively or positively charged liposomes, DCP or SA 5 % of the total lipid quantity (molar ratio) was incorporated along with the HSPC and CHOL.

The prepared liposome batches prepared by both the methods were evaluated for PDE, PC and CHOL contents by the procedure discussed in Chapter 3 (section 3.5.3 and 3.3 respectively). The results of PDE along with compositions of liposomes are recorded in Table 4.6.

4.5 CHARACTERIZATION OF LEUPROLIDE ACETATE LIPOSOMES

Liposomes loaded with drug prepared in this work were characterized with respect to size, shape, lamellarity, PDE in addition to PC and CHOL content in the liposomes.

4.5.1 Determination of vesicle Size:

The vesicle size determination of the prepared liposomes carried out by the method described in this chapter earlier (Section 4.3.1).

4.5.2 Determination of vesicular shape & lamellarity:

The shape and lamellarity of prepared liposomal batches were determined by the method described in this chapter earlier (Section 4.3.2). The photomicrographs of representative batches were taken to confirm the results.

4.5.3 Analysis of liposomes for PDE and PC & CHOL contents:

PDE in liposomes is expressed as the percent of the drug actually entrapped in the liposomes out of the added drug. This was determined by the method described in Chapter 3 (section 3.5.3). PC and CHOL contents of the liposomes were also determined using the method described in Chapter 3 (section 3.3).

4.6 STATISTICAL ANALYSIS

In the optimization of liposome preparation, the effect of one variable was studied at a time keeping other variables same and experiment was repeated. Statistical analysis using ANOVA technique was applied for comparing the data of each set of experiment with others. The differences were considered significant at $p<0.05$. The reproducibility of optimized liposomal batches, in terms of PDE and vesicle size was checked by repeating the experiment for three times on three different days. The mean PDE and standard error mean was calculated to confirm reproducibility. The mean diameters (μm) obtained before and after Freeze-thaw cycles for LN liposomes were compared using Student's t-test and the differences larger than the value at $p<0.05$ were considered significant.

4.7 RESULTS AND DISCUSSION

TFH method and REV method were used to prepare liposomes of LN and LEU Process and formulation variables of these methods were altered to maximize the liposomal encapsulation of the drug

4.7.1 Levonorgestrel Liposomes

4.7.11 Thin Film Hydration method

TFH method involved co-evaporation of the lipid and drug from the solvent system in a round bottom flask The hydration and entrapping process is most efficient when the film of dry lipid is thin (Bangham et al., 1965) The film formation is depending on proper combination of solvent system, selection of temperature, vacuum and rotation speed. Efforts were made to establish combination of these variables to give liposomes with maximum PDE (Table 4 1)

Polarity of the solvent system initially and changes in polarity during solvents stripping due to different volatility of solvents have great impact on film formation Different solvents and/or solvent systems, viz, diethyl ether, chloroform, methanol. ethyl acetate and their mixtures, were tried The problems observed with inappropriate solvent/solvent systems were non-uniform film formation, precipitation of one or the other components at different time during evaporation of solvents, improper hydration of film once formed or combination of these problems The best solvent system was found to be Chloroform:Methanol, 1 2 used for preparation of liposomes by TFH method. The temperature above the glass transition temperature (Tg) of lipid (30°C) was found optimum The low speed of rotation produced thick, non-uniform film. The speed of rotary flask was increased from 90 rpm to 120 rpm and 120 rpm was found to be adequate in increasing sufficient surface area for evaporation (Payne et al., 1986) to give thin, uniform and completely dry film at optimized temperature The effect of vacuum on film formation was also established and 500 mmHg was found to be optimum and was used for further work PC & CHOL contents were determined and found to be in the range of 90-100% in all the prepared batches

Table 4.1: Selection of Process Variables for Preparation of LN Liposomes

INDEPENDENT VARIABLES	LEVELS		
	1	2	3
A Chloroform Methanol	2:1	1 1	**1:2**
B Vacuum	400	**500**	600
C Speed of rotation	80	**120**	160
D Temperature	20	**30**	40

The formulation components, viz , drug lipid ratio, Egg PC CHOL ratio and volume of hydration medium (distilled water), were optimized with regards to PDI Liposomes batches were prepared by altering any one variable and were evaluated for PDE The results are recorded in Table 4 2 and shown graphically in Figure 4 1

Formulation variables:

Initially, influence of drug lipid ratio on PDE was evaluated When drug PC ratio was increased from 1 2 to 1 5 (Batch 01 to 04, Figure 4 1), 15 fold increase in PDE was observed It may be due to the reason that definite amount of PC is necessary for proper emulsification to obtain optimum PDE (LN PC ratio, 1 4) When CHOL was incorporated keeping drug PC ratio 1 4 (Batch 05, Figure 4 2), marginal increase in PDE was observed (74 36±2.69 to 76 27±3 15) at drug:PC CHOL ratio 1.4 1 The increase in PDE may be because of increase in hydrophobicity of the lipid membrane Further increase in cholesterol concentration resulted in significant decrease in PDE (Batch 06, Figure 4 2) Increasing the CHOL beyond optimum concentration, it competes with the hydrophobic drug for incorporation into the liposomal membrane (Joshi and Misra, 2001)

Process variables

Distilled water and phosphate buffer, pH 7 0 was used and compared as a hydration medium No significant difference ($p>0.05$) in PDE was observed between the two and hence, distilled water was used as a hydration medium The volume of hydration medium was changed from 0 5ml to 1 5ml, but the effect on PDE was found to be non significant ($p>0.05$). The drug being hydrophobic in nature, no change in PDE was noticed with increase in the volume of hydration medium Annealing time was altered and significant difference ($p<0.05$) was observed upon changing the time (Table 4.3). Annealing time was found to be optimum at 2 hrs. Further increase in hydration time did not result in to significant ($p>0.05$) change in PDE Hence, annealing time of 2 hrs was used for further experiments.

Influence of F-T cycles on PDE of liposomes were also evaluated and PDE before and after exposure of liposomes to F-T cycles are recorded in Table 4 4 and shown graphically Figure 4 3. Significant improvement in PDE was observed after exposure of liposomes to F-T cycles Significant variation ($p<0.05$) in PDE were observed before exposure of liposomes to F-T cycles and these variations reduced to insignificant level after exposure of LN liposomes to freeze thaw cycles Batch 05 were subjected to vesicle size determination and the data obtained are recorded in Table 4 4 Non-significant ($p>0.05$) variation in vesicle size after 1-T cycle was observed It may be due to the freezing of aqueous phase, proximity between lipid molecules and hydrophobic drug increases Hydrophobic interactions between lipid molecules of liposomal membrane and hydrophobic drug, LN, maximize and the drug substitutes the hydrophobic component, cholesterol, in liposomal bilayer membrane resulting into increase in PDE of liposomes The molecular proximity of lipid and drug molecules for molecular interactions also increases due to the drug solubilization in aqueous phase in presence of PC at higher temperatures and separation of lipophilic molecules once the temperature is decreased to -20°C The rate of freezing was found to have significant effect ($p<0.005$) on PDE (Table 4 4 and Figure 4 3) Ice crystal size is controlled by the rate of freezing - the faster the rate - the more nucleation is promoted, and the greater number of crystals of smaller size that will result When freezing temperature was kept to -20°C, the rate of freezing is slower and hence formation of large ice crystals may break the membrane structure and hence resulted in less PDE However, at the higher freezing rate (-40°C) smaller size crystals results which has lesser tendencies to disrupt the membrane structure and hence resulted into high PDE Optimization of PDE was achieved only after the second freeze-thaw cycle

4.7.12 Reverse Phase Evaporation (REV) method:

REV method involves emulsification of organic and aqueous phase containing lipid and drug followed by evaporation of organic phase under vacuum The removal stage results in either formation of the liposomal suspension, or a viscous gel that on addition of an aqueous buffer forms the liposomal suspension The emulsification of the two-phase system is an important process that affects the final formation of

liposomes and their characteristics (Pidgeon et al . 1987, Szoka and Papahadjopoulos, 1980, Pidgeon et al , 1986) The ratio of aqueous to organic phase is most important variable in REV method for proper emulsification and formation of uniformly distributed fine aqueous globules of aqueous phase surrounded by layers of lipid (Knight 1981). The resulting emulsion must be reasonably stable (Szoka and Papahadjopoulos, 1978) Diethyl ether was used as the organic phase Distilled water was selected as aqueous phase as in case of TFH method Initially, efforts were made to optimize aqueous to organic phase ratio and was found optimum at 1.5 (2 5 ml.0.5 ml) The volume of organic phase and aqueous phase was observed to have significant effect on PDE This may be due to the total amount of lipid which formed the interfacial film around the droplets, decides the resulting emulsion stability (Tabandeh and Ghasemi, 2001).

Process variables, viz; size of hypodermic needle for injecting aqueous drug solution into lipid solution (23 gauge), vortexing time before evaporation (5 minutes), initial vacuum until gel was formed (500 mm of Hg), vortexing time (5 minutes) to collapse the gel to fluid, two cycles of 10 minutes drying and 5 minutes vortexing and vacuum (500 mm of Hg) for removal of last traces of organic solvent for 15 minutes were fixed to prepare each batch.

The LN liposome batches were evaluated for PDE and results obtained are recorded in Table 4.2 and shown graphically in Figure 4 1 Increase in PC concentration (LN.PC; 1 02 to 1.04) resulted in 10 fold increase in PDE. Incorporation of CHOL (Drug PC·CHOL: 1 04.01) resulted in marginal increase in PDE (83.32±2 87 to 88 78±3.00). Further increase in CHOL concentration was resulted in significant decrease in PDE as in case of TFH method (Table 4.2 and Figure 4.2). The results obtained were similar to that obtained for TFH method However, significantly higher PDE values were observed in all the batches prepared by REV method compared to that of TFH method (Table 4 2). The high PDE of MLV vesicles prepared from water/organic solvent emulsions depend on maintaining a core during the process of liposome formation (Pidgeon et al., 1987)

Table 4.2: Influence of Freeze-thaw cycles on Percent Drug Entrapment of Levonorgestrel Liposomes

Batch	Molar ratio (Drug PC Chol)		TFH Method PDE ± SEM	REV Method PDE ± SEM
01	1:2 0	B	5 765±2 25	8 254±2 00
		A1	11 54±1 21	15 67±0 74
		A2	15 13±0 58	15 85±0 41
		A3	15 25±0 42	15 98±0 38
02	1 3 0	B	16 25±2 35	21 08±2 52
		A1	35 43±2 13	43 87±0.11
		A2	42 69±0 97	44 38±0 79
		A3	43.847±0.37	44 254±0 35
03	1:4 0	B	71.23±2 45	78 650±2 69
		A1	88 32±1 18	96 98±1 09
		A2	96 10±0 65	97 32±0.45
		A3	96 02±0 31	97 56±0.29
04	1:5 0	B	74 36±2.69	83 32±2 87
		A1	91 31±2 01	95 61±0.98
		A2	95 86±1 08	96 56±0 25
		A3	95 98±0 28	96 69±0 26
05	1 4 1	B	76.275±3 15/15.0±0 2*	88 789±3 00/12 6±0.2*
		A1	89 54±2.54	97 98±1.36
		A2	96 87±1 17	99 01±0.44
		A3	97.98±0.29	98.99±0 21
06	1:3 2	B	35 014±2 11	40 519±2 31
		A1	49 23±1.16	55 36±1.21
		A2	57.01±0.74	56 45±0.47
		A3	58 321±0.21	56 551±0.26

*D[4,3], B=Before Freeze thaw Cycle, A1= After 1st Freeze thaw cycle

A2= After 2nd Freeze thaw cycle, A3= After 3rd Freeze thaw cycle,

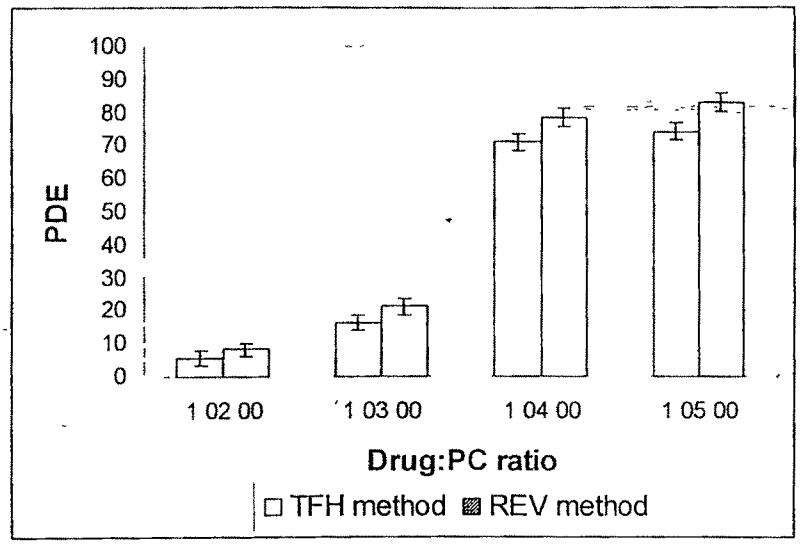


Fig. 4.1: Effect of Drug:PC ratio on PDE of LN liposomes prepared by TFH and REV methods

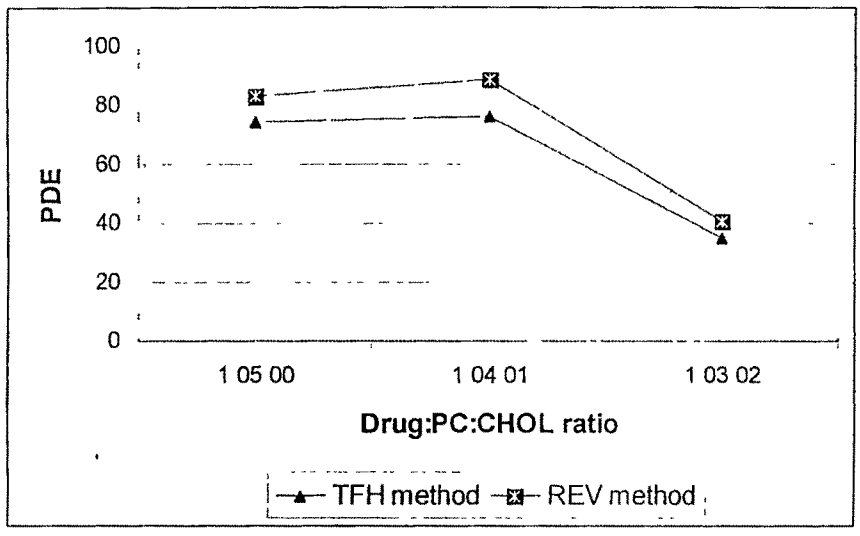


Fig. 4.2: Effect of PC:CHOL ratio on PDE of LN liposomes prepared by TFH and REV methods

Table 4.3: Effect of Hydration time on PDE of liposomes prepared by TFH and REV method

Time of Hydration	TFH method	REV method
2	68 14±3 17	88 789±3.00
4	76 27±3 15	88 715±2 86
8	76 29±3 21	--
12	--	--

Table 4.4: Effect of time and temperature of freezing on PDE evaluated in Batch LLN5

Time/Temperature	TFH method	REV method
Before	76 27±7 73	88 79±7 35
30 min/-20°C	76 32±7 45	87 98±6 98
60 min/-20°C	78 65±2 13	88 67±0 98
120min/-20°C	79.54±1 21	89 98±0 32
180 min/-20°C	79 24±1 12	97 01±0.29
30min/-40°C	77 96±6 83	89 13±6 42
60min/-40°C	88 13±1 05	96 61±0.78
120min/-40°C	**89.49±0.98/15.1±0.2***	**97.89±0.31/ 12.8±0.2***

*** D,[4,3]**

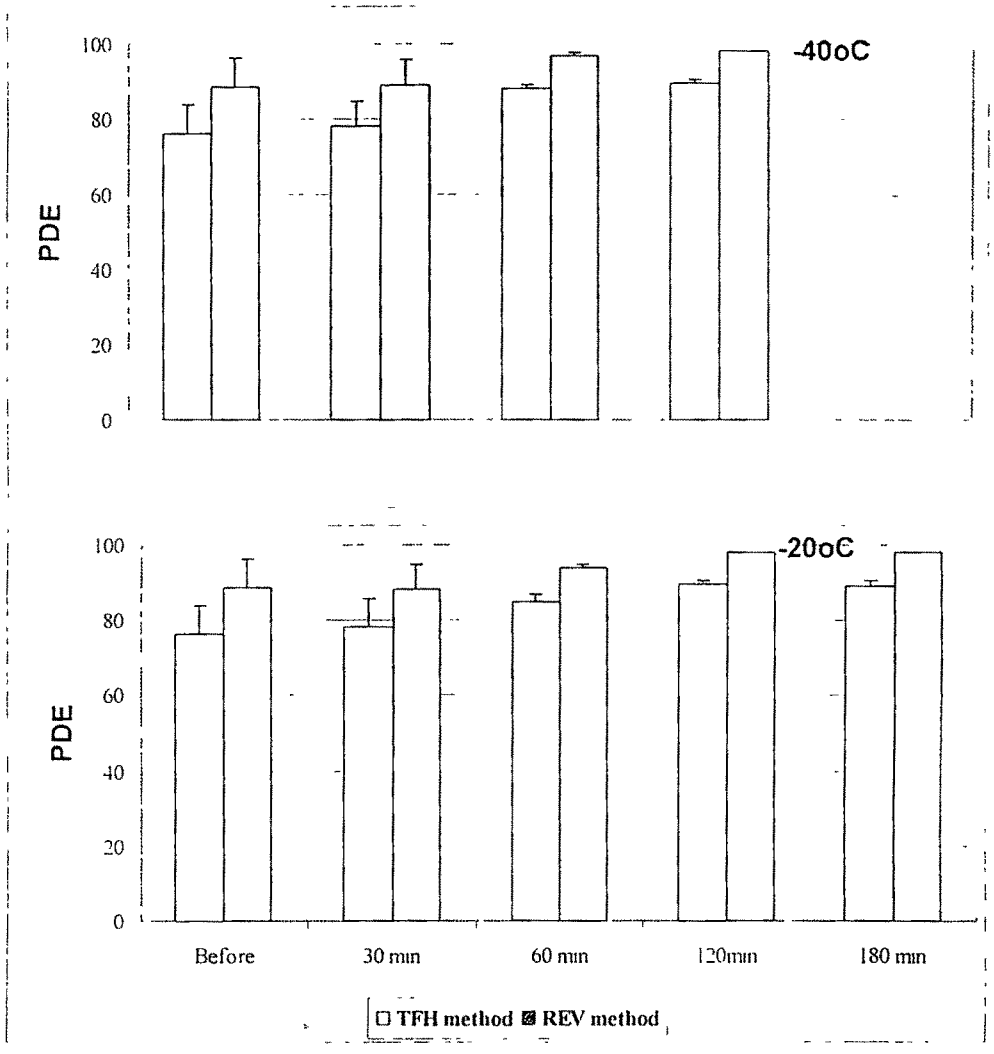


Fig. 4.3 Effect of temperature and time of freezing on PDE of LN liposomes

Table 4.5 Effect of Post Sonication Freeze-thaw cycle on LLN, Batch 05

Sonication Time	PDE before Freeze-thaw cycle/ Liposome size (D[4,3])	PDE after Freeze-thaw cycle/Liposome size (D[4,3])
0 min	--	98 3 ± 0 211/12 8 ± 0 2
10 min	84 14 ± 1 26/8 4 ± 0 14	98 27 ± 0 20/8 5 ± 0.03
20 min	72 06 ± 1 15/2 5 ± 0 11	98 3 ± 0 21/2 5 ± 0 01
30 min	37.34 ± 1 51/2 1 ± 0 11	88 29 ± 0 35/2 2 ± 0 11

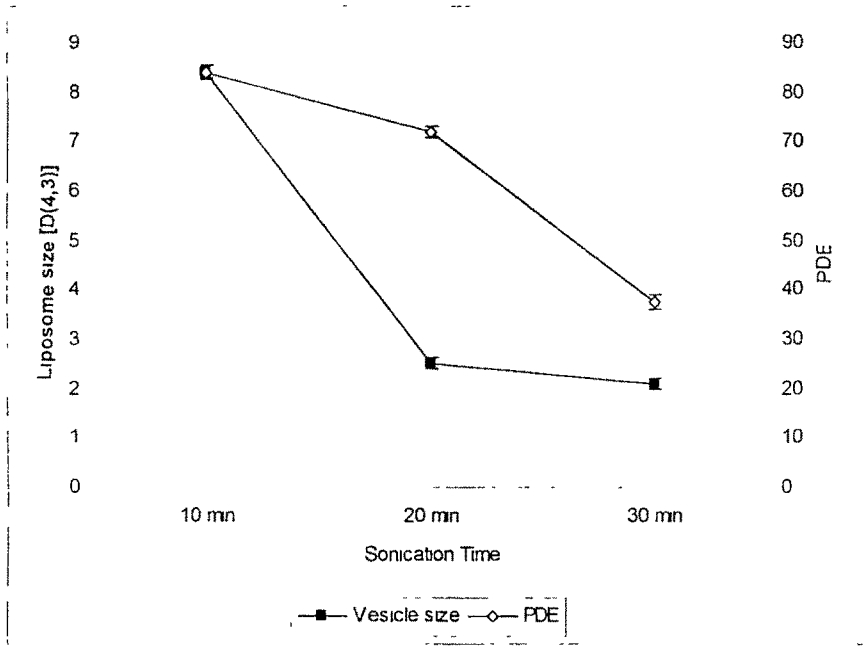


Figure 4.4: Effect of Sonication Time on PDE and Vesicle size of LLN, Batch 05

Optimization of PDE in liposomes prepared by REV method was achieved by the first Freeze-thaw cycle (Table 4 2) Time and temperature of Freezing was also optimized to get the maximum PDE and freezing at -40°C for 2 hours were the optimum conditions (Table 4 4) REV method was used to prepare liposomes for further studies

4.7.13 Size Reduction of Optimized Liposomal batch (Batch 05 (LLN)):

Size reduction of LLN was carried out using sonication in a 10 ml borosil beaker under ice bath, by probe sonicator Twenty minutes sonication time was found to be optimum with regard to required size distribution (below 5 μm) Post sonication F-T cycles helps in regaining the equilibrium with the leaked drug (Table 4.5 and Figure 4 4) The further increase in sonication time to 30 minutes resulted into higher drug leakage and further stabilization with Freeze-thaw cycle is poor (Table 4 5) Sonication brings about size reduction by breaking large liposomes to smaller ones, and in doing so, leakage of small quantities of drug from the liposomes occurs Hence, further decrease in the size by increasing sonication time to 30 minutes from 20 minutes was ruled out Sonication also narrowed down the size distribution, which determines the drug deposition and release rate in lung

Hence, the results of this investigation demonstrate that the exposure of liposomes to F-T cycles may help in optimizing PDE of LN (lipophilic drug) Also, it can contribute to the reproducibility of the technique used for preparation of liposomes and also reduction in significantly affecting variables (method of preparation, time of hydration and volume of hydration) Hence, it can be used as in preparation of LLN at commercial scales without increasing the cost of production significantly

4.7.2 LEUPROLIDE ACETATE LIPOSOMES (LLEU)

LEU being a hydrophilic in nature tends to entrap inside the aqueous compartments of the liposomes. It was also thought that LEU being a peptide in nature also has tendency for protein-lipid interactions and hence can also either incorporated or associated with the phospholipid bilayers As the volume of aqueous compartments

were remain more or less similar in liposomes having similar size and similar structure (multilamellar), the efforts were made to maximize the PDE by favouring lipid-protein interactions The method of sequential analysis was adopted, as there are many variables that were thought to have significant effect on PDL of liposomes

Initially, liposomes were prepared using EPC The resulting liposomes were observed to be unstable with regards to drug retention Also, the PDE obtained were also not satisfactory Hence, further efforts were made using HSPC HSPC & CHOL contents were determined and found to be in the range of 90-100% in all the prepared batches

4.7.21 Reverse Phase Evaporation Method

LLEU were initially prepared by the REV method and efforts were made to get maximum PDE by varying various formulation and process parameters The process was carried out above the Tg of HSPC (55° C) and acetate buffer pH 5.2 was selected as hydration medium because the drug stability is highest at this pH (Ref) Process variables viz; size of hypodermic needle for injecting aqueous drug solution into lipid solution (23 gauge), vortexing time before evaporation (5 minutes), initial vacuum until gel was formed (500 mm of Hg), vortexing time (5 minutes) to collapse the gel to fluid, two cycles of 10 minutes drying and 5 minutes vortexing and vacuum (500 mm of Hg) for removal of last traces of organic solvent for 15 minutes were fixed to prepare each batch Prepared liposomal batches were analyzed for PDE and results obtained are recorded in Table 4.6.

Each parameter was studied at three different levels and optimized batches were further studied by doing minor changes in optimized parameter as and when necessary When total lipid was increased from 100µmol to 200µmol, PDE was improved from 23 15% to 29 54%. Further increase in lipid quantity to 300µmol was resulted into insignificant improvement in PDE ($p>0.05$) (Figure 4.5). Only six to seven percent improvement in PDE was observed even after doubling the lipid quantity (100µmol to 200µmol) and no significant improvement in PDE on further doubling the lipids (200µmol to 300µmol). These results indicated that poor correlation occurs between lipid quantity and PDE; other factors might be more

responsible for PDE Therefore it was thought worthwhile to change the HSPC CHOL ratio to modify the lipophilicity of the liposomal bilayer It was found that 100µmol 50µmol HSPC.CHOL ratio yield higher PDE (37 25%) compare to other ratios (100µmol 100µmol and 100µmol 150µmol) The ratio was further optimized on minor scale and above ratio (100µmol 50µmol) was taken as an optimized ratio for further studies on the basis of highest PDE obtained (Table 4 6 and Figure 4 6)

Once the HSPC CHOL ratio was optimized, third major contributing variable, i e , solvent system was altered to see it's effect on PDE Both process variables like choice of organic solvent and ratio of organic phase to aqueous phase were optimized to have proper orientation of vesicles and high PDE Different solvents like diethyl ether, chloroform, and methanol were tried either alone or in combination Maximum PDE was observed in case of methanol chloroform (2 1) Ratio of organic aqueous phase was optimised and maximum PDE was observed at 3 0 5 (ml) organic to aqueous phase ratio (Table 4 6 and Figure 4 7-4 8) It may be due to the synergistic effect of this combination of organic solvent with aqueous phase to form stable emulsion, which is pre-requisite for reverse phase evaporation cycle (Betageri et al, 1993). This system also have benefit of being miscible and can solubilize both the drug and lipids, larger surface contact between lipids and LEU was possible and hence higher PDE was obtained

The complete removal of aqueous phase may help in diffusion of LEU into lipids as the proximity occurs between the drug and the lipid molecules This is reflected in PDE when aqueous phase was evaporated to dryness and fresh aqueous medium was then added to get the final liposomal suspension (PDE, 91.4% ± 1 5%) (Table 4.6).

The 5% of total lipids of either SA (negative) or DCP (positive) was incorporated to see the effect of charge on the PDE in liposomes The PDE in liposomes observed were 96 5% ± 1.2% and 84 27% ± 1 11% for negative and positive charge liposomes respectively (Table 4 6) The positively charged liposomes were found to be unstable in nature. The high PDE of negatively charged liposomes (LLEUn) and instability of positively charged liposomes may be because of the positively charged LEU LEU

has three ionizable sites, the imidazolyl nitrogen of His (pKa 6 0), the phenolic hydroxyl of Tyr (pKa 10 0), and the guanidine nitrogen of Arg (pKa 13 0) Since the guanidine nitrogen is extremely basic, LEU exists in the protonated form (Akwete et al , 1993)

4.7.22 Thin Film Hydration Method:

The optimized LLEU by REV method (Table 4 6) were subjected to TFH method to see the effect of method of preparation on PDE of liposomes There is no significant difference ($p>0.05$) between the PDE values of liposomes prepared by either of the method The results indicated that once the proper selection of formulation and process variables made, the method of preparation does not affect the PDE of liposomes significantly Further preparations of liposomal batches were continued with REV method

4.7.23 Size reduction of Leuprolide acetate Liposomes:

Size reduction of both neutral liposomes (LLEU) and negatively charged liposomes (LLEUn) were also tried using sonication, however, it resulted in high drug leakage and hence, further efforts for size reduction were made using extrusion The prepared liposomes were extruded by passing through 2 µm polycarbonate membranes to a reproducible mean liposomal size below 5µm (Martonen et al, 1993) PDE and liposomal size were determined after each extrusion and results obtained are recorded in Table 4 7 and shown graphically in Figure 4.9 and 4 10 Minimum of three times extrusion was necessary to obtain mean liposome size below 5µm for both LLEU and LLEUn Five to six percent drug leakage observed in both the liposomes was unavoidable. The extrusions were carried out at 55°C as the HSPC liposomes remains in fluid state at this temperature

Table 4.6: Set parameters in Methodology selected for preparation of LEU liposomes

FACTOR	VARIABLES			
A	50µmol 50µmol (23 15±1 15)	100µmol 100µmol (29 54±1 02)	150µmol 150µmol (29 98±0 96)	
B	100µmol·50µmol (37.25±0 99)	100µmol 100µmol (29 54±1 02)	100µmol 150µmol (13 21±1 15)	
C	100µmol 40µmol (35 26±1.08)	100µmol 50µmol (37 25±0 99)	100µmol 60µmol (32 03±0 87)	
D	Diethyl Ether/water,5 1 (37.25±0 99)	Chloroform/water (5 1) (49 96±1 16)	Methanol/water (5 1) (46 29±1 10)	
E	Methanol chloroform (1 2) (56 37±1 00)	Methanol chloroform (1 1) (63 87±1 30)	Methanol chloroform (2 1) (70 32±1 41)	
F	Methanol chloroform water (3 1) (73 23±1 26)	Methanol chloroform .water (4·1) (79 26±1 31)	Methanol chloroform water (5 1) (70 32±1 41)	
G	Yes (90.15±1 13)		No (79 26±1 31)	
H	TFH (91.12±0 96)	REV (90 15±1 13)	TFH (63 21±1 23)	REV (79.26±1 31)
I	+ (84.27±1.11)	0 (90 15±1 13)	- (96 5±1.20)	

A= Lipid quantity (Major), Solvent ratio (Diethyl ether water, 5 1)
B= Lipid: cholesterol ratio (Major), Solvent ratio (Diethyl ether· water, 5·1)
C= Lipid cholesterol ratio (Minor), Solvent ratio (Diethyl ether water. 5.1)
D= Solvent
E= Solvent ratio
F= Solvent water ratio (Minor)
G= Solvent evaporated to dryness, again hydrated with water
H= Method of preparation, In case of TFH method, LEU added during the film formation
I= Inclusion of charged lipids (+, positively charged, -, negatively charged)

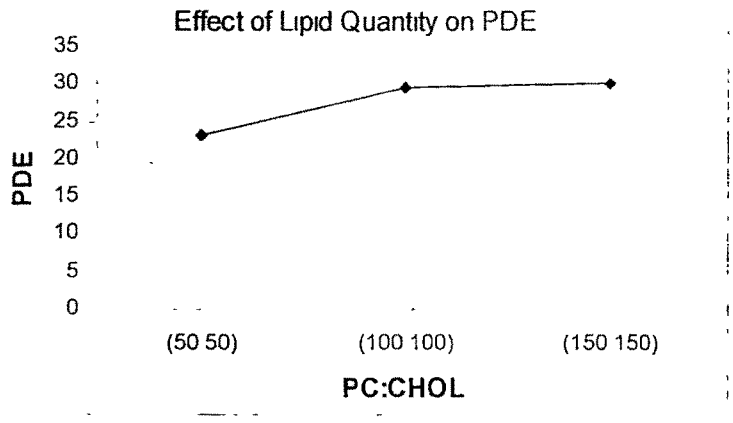


Figure 4.5: Effect of lipid quantity on PDE of LLEU

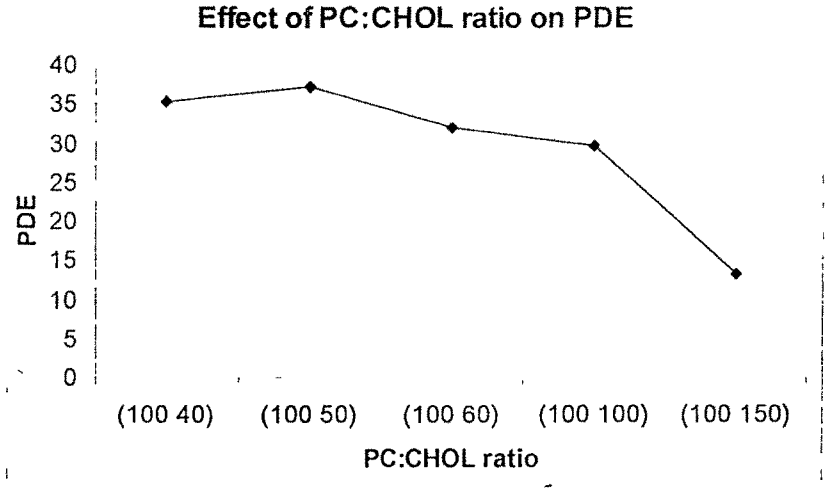


Figure 4.6: Effect of HSPC:CHOL ratio on PDE of LLEU

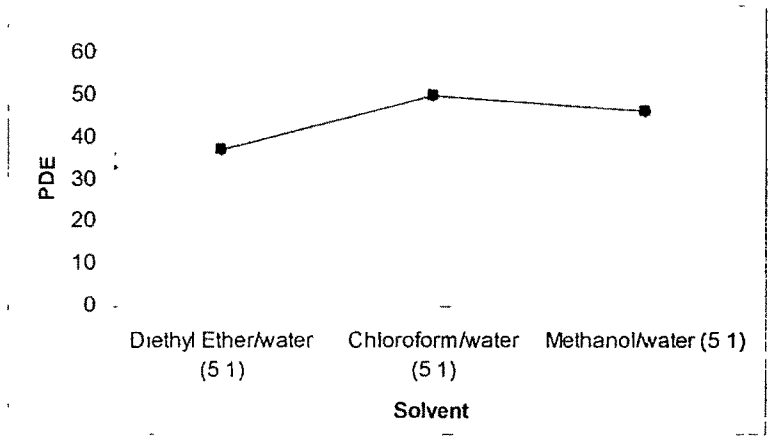


Figure 4.7: Effect of solvent system on PDE of LLEU

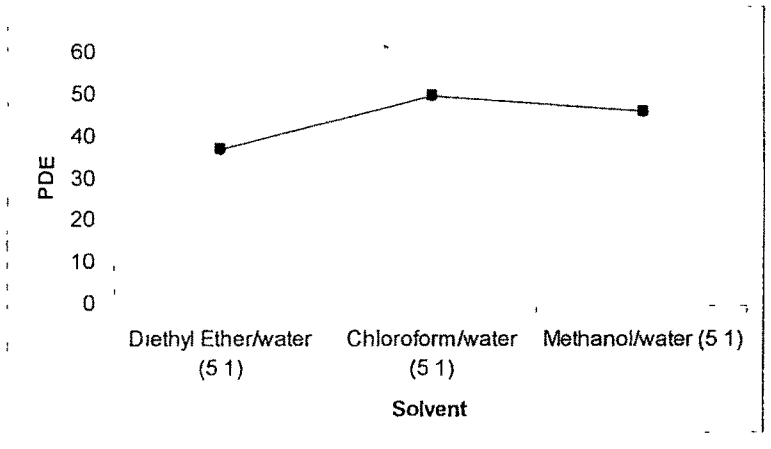


Figure 4.8: Effect of methanol:chloroform ratio on PDE of LLEU

Table 4.7 Effect of Extrusions on Prepared Liposomes of LEU

Extrusion	Neutral Liposomes PDE/ Size (D[4,3])	Negative Liposomes PDE/ Size (D[4,3])
Zero	91 4 ± 1 5/11 5 ± 0 01	96 5 ± 1 3/10 71± 0 01
First	87 21±1 87/6 04 ±0 15	93 11±1 08/7 63±0 09
Second	86.74±1 23/4 98 ±0 11	92.90±1 21/6 08±0 14
Third	85 41±1.34/3 42±0 12	91 14±1 09/4 14±0 10

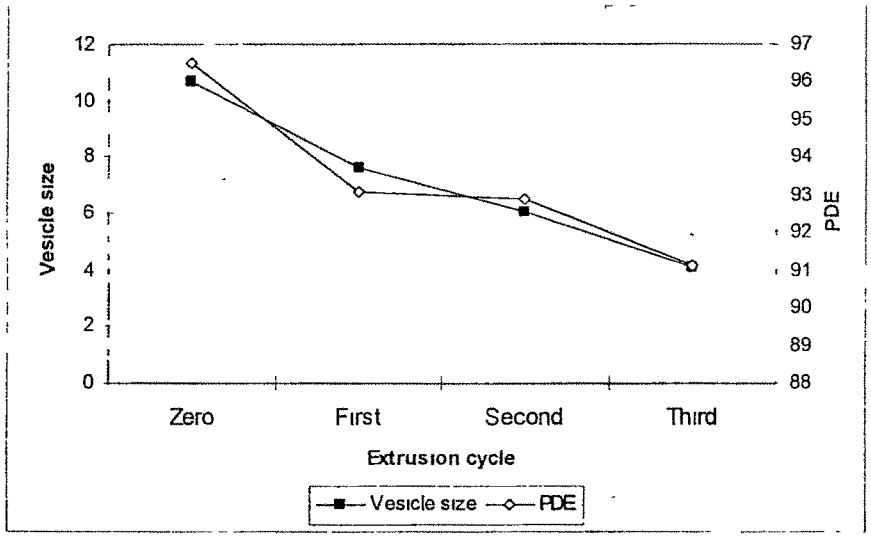


Figure 4.9. Effect of Extrusion cycle on PDE and Vesicle size of LLEU

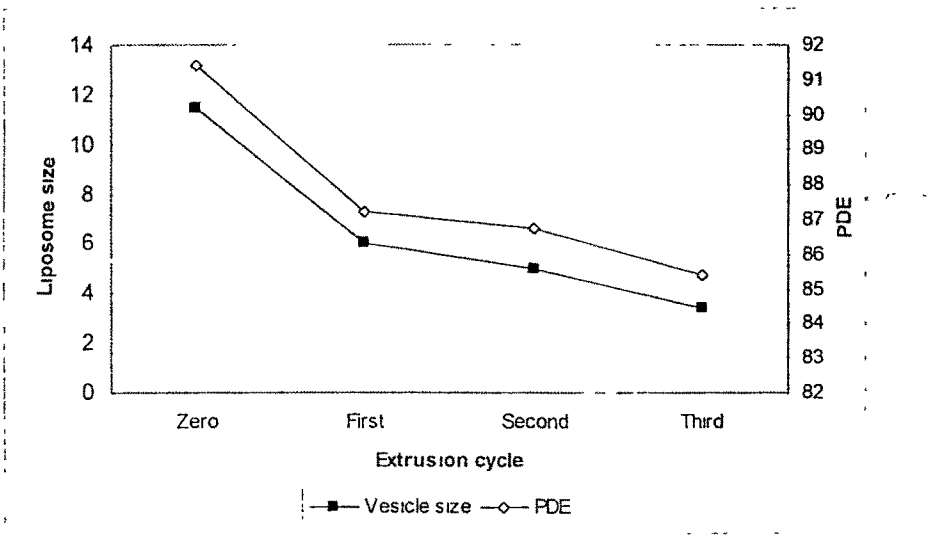
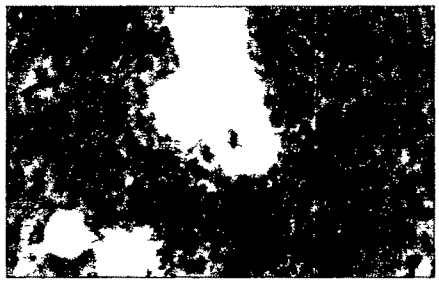
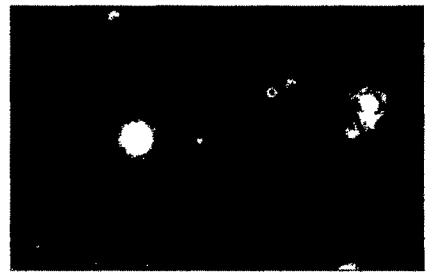


Figure 4.10. Effect of Extrusion cycle on PDE and Vesicle size of LLEUn

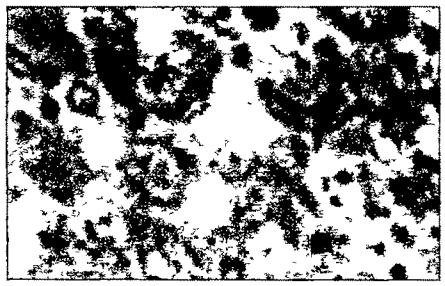


Before F-T cycle

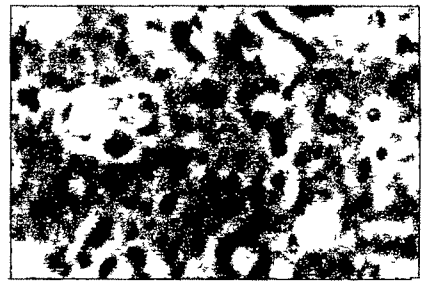


After F-T cycle

Photographs of LLN, Batch 05



LLEU



LLEUn

Photographs of LLEU AND LLEUn

Figure 4.11. Photomicrographs of the optimized liposomal batches

4.7.3 COMPARISONS

When LN liposomes prepared by two different methods compared, REV method showed higher PDE at all the drug:PC CHOL ratio compared to TFH method The high entrapment found for MLV vesicles prepared from water/organic solvent emulsions (REV method) depend on maintaining a core during the process of liposome formation (Pidgeon et al., 1987) Even during the Freeze-thaw cycles, equilibrium with respect to PDE attained faster (after 1st cycle) for REV method compared to that of TFH method (after 3rd cycle) The difference in number of freeze thaw cycles required to establish equilibrium on prepared liposomes for both the methods, may be due to the structural differences observed for liposomes produced by both the methods as TFH method resulted into multilamellar and REV method resulted into oligolamellar liposomes

In case of LEU liposomes, liposomes were initially prepared by REV method TFH method was applied once the PDE in liposomes optimized to see the effect of method of preparation with the optimized process and formulation variables obtained by the REV method When thin film was prepared using organic solvents of REV method and hydrated with the same quantity of LEU containing aqueous phase, significantly less ($p<0.05$) PDE was observed compared to REV method However, when the solvent system including aqueous phase containing LEU of REV method was used during thin film preparation and hydration was carried out with the same quantity of fresh aqueous phase, no significant difference in PDE ($p>0.05$) was observed The reason might be at these conditions, the lipid protein interactions get optimized, selection of other variables plays crucial role and not the method of preparation

When compositions of LN and LEU liposomes were compared, it was observed that Egg PC was suitable for carrying out LN liposome preparations However, LEU liposomes when prepared with Egg PC were observed to be unstable with regards to drug retention. Also, the PDE obtained were also not satisfactory Hence, HSPC was used. HSPC being a saturated PC have higher Tg compared to Egg PC (unsaturated), helps in prevention of leakage of hydraophilic drug like LEU which entrapped inside

aqueous compartments However, LN being a hydrophobic in nature entrapped inside the lipid bilayers, Egg PC is found to be suitable

Also, when PC CHOL ratio of LN and LEU liposomes were compared, 4 1 ratio and 3 1 (15 5) ratio were found to be optimum respectively Increasing the cholesterol beyond a certain concentration can compete with the hydrophobic drug like LN in entrapment inside the membrane (Joshi and Misra, 2001) CHOL also imparts rigidity to liposomal membrane might be the reason preventing the leakage of hydrophilic drug like LEU

When the quantity of Drug PC CHOL were compared, 1 5 1 ratio was optimum for LN liposomes while 1·15·5 found to be optimum for LEU The ratio of drug lipids is depend on the molecular size (molecular weight) and nature (lypophilicity) of the drug The higher quantity of lipids required for optimizing LEU liposomes showed that entrapement of hydrophilic drug is more difficult to that of hydrophobic drug like LN The higher moleculat wt and therefore molecular size of a peptide drug LEU may also responsible for higher lipid quantity to encapsulate the drug

When size reduction of LN and LEU liposomes were carried out, sonication was found to be suitable for LN liposomes, however this method leads to higher drug leakage in case of LEU liposomes, therefore, size reduction of LEU liposomes by breaking large liposomes to smaller ones, and in doing so, leakage of small quantities of drug from the liposomes occurs In case of LN liposomes post-sonication freeze-thaw cycle re-equilibrate the leaked drug to liposomes.

Table 4.8: Analytical profile of Final Liposomal batches of LN and LEU

	LN LIPOSOMES (LLN)	**LEU LIPOSOMES (NEUTRAL) (LLEU)**	**LEU LIPOSOMES NEGATIVE (LLEUN)**
Composition	Drug·PC CHOL (1 4:1)	Drug HSPC CHOL (1 15 5)	Drug HSPC.CHOL DCP (1 15 5 1)
Method of Preparation	REV method followed by F-T cycles	REV method	REV method
Solvent system	Diethyl ether.water (5·1)	Methanol chloroform Phosphate buffer pH 5 2 (2 1 0 5)	Methanol·chlorofor m Phosphate buffer pH 5 2 (2·1 0 5)
PDE/D[4,3] before size reduction	97.89±0 31/ 12.8±0.2	91 4 ± 1 5/11 5 ± 0 01	96 5 ± 1 3/10 71± 0 01
PDE/D[4,3] after size reduction	98.3 ± 0.21/2.5 ± 0.01	85 41±1 34/3 42±0 12	91 14±1 09/4.14±0 10

4.7.4 Conclusions

Liposomes of LN and LEU were prepared and optimized with regard to percent drug entrapment by changing various process and formulation parameters. The compositions of optimized liposomal batches along with their PDE and mean vesicle size values are shown in Table 4.8. Prepared liposomes prior to size reduction were suitable for nasal delivery as their vesicle size distribution was in range of 10-20 µm that is required for nasal drug delivery. However, further processing for size reduction of liposomes was required for lung deposition in case of pulmonary drug delivery and hence liposomes after size reduction to 3-5 µm were used for preparation of DPI formulations.

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