CHAPTER 6

PREPARATION, CHARACTERIZATION AND EVALUATION OF FORMULATIONS FOR PULMONARY DELIVERY

Recent research is focused on the delivery of systemically acting drugs via the pulmonary route (Ganderton, 1999) Especially dry powder inhalations are a promising application form for peptides and proteins for systemic delivery as they overcome the drawbacks of oral and invasive delivery forms, as enzymatic degradation in the GI-tract, low oral bioavailability, the need for i m , s c or i v injection, etc (Wall, 1995)

The administration of liposome-encapsulated drugs by aerosols seems to be a feasible way of targeting these delivery systems to the lung The tolerability and safety of liposome aerosols has been previously tested in animals as well as in human volunteers, no untoward effects have been recognized (Waldrep et al , 1997, Saari et al , 1999)

The requirement for viable alternatives to ozone-depleting meter dose inhalers, coupled with the opportunity for dehydrating liposomes to powder form, make dry powder aerosol of liposomal drug an attractive choice for modulated inhalation drug delivery It has been proposed that sugars preserve membrane structure (cryoprotection) by hydrogen bonding to the phospholipid head group and effectively replacing the bound water (Crowe and Clegg, 1973) Evidence in support of this hypothesis has been provided by differential scanning calorimetry and infrared spectroscopic studies (Crowe et al , 1984) Sugars when added to the liposome dispersion form a glassy matrix during freezing This prevents fusion of the vesicles and provides protection against ice formation (Edwoud et al., 1997) The stability of various liposomal formulations encapsulating drugs and/or forming complexes with plasmid DNA has been evaluated (Eastman et al., 1997, Schwarz et al., 1996). Several formulation and operating conditions play a crucial role in the stability profile of such formulations, thus, the conditions for efficient liposomal aerosolization are to be well monitored and the stability of formulations is to be evaluated (Niven et al., 1992; Niven and Shreier, 1990).

Flow and dispersion characteristics of the developed liposomal DPI formulation are critically important in development of DPI products. To deliver the liposomes to the lung, size below 5 μm is necessary. However, strong adhesive and cohesive properties

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of such fine liposomes lead to their unreliable filling into inhalation device (e.g. capsule) because of their poor flowing properties They tend to adhere and remain in the capsule and inhalation device during emission process, resulting in lower and unreliable dosing To dissolve these problems, coarse carrier particle (30.0 -90 0 μm in diameter) system such as lactose particle loading fine liposomes has been originally developed for DPIs by Bell et al , 1971 The carrier of choice for DPI products is currently lactose monohydrate (Lahrib et al , 1999) Nearly all DPI products already on the market or approaching the market are relying on lactose as a carrier material, with few exceptions present that contain glucose (Steckel, 2003) The advantages of lactose monohydrate are its well-investigated toxicity profile, its broad availability and the relatively low price In addition, lactose crystals do have a smooth surface, a regular shape and show good flowability

This chapter focuses on the pharmaceutical development of liposomal drug formulations for DPI and the evaluation, optimization, and control of flow and dispersion characteristics of the formulations developed. At present, in vitro test systems have not been developed which can accurately predict the rate of drug release from liposomes in vivo. Therefore an in vitro diffusion technique is proposed, validated and utilized for drug diffusion studies from potential DPI formulations The in vitro studies were followed by in vivo studies in rats. The optimized liposomal batches LLN and LLEU were used to develop DPI formulations.

6.1 REAGENTS

(i) Water Double distilled water.

(ii) Acetate buffer (ionic strength, 0.261), pH 5.2: It was prepared as per the procedure given in the Indian Pharmacopoeia

(iii) Phosphate buffer saline, pH 7.4 (PBS): As described in Indian Pharmacopoeia

(iv) 20% methanolic PBS: Accurately measured 800 ml of pH 7.4 Phosphate buffer saline was transferred to a clean, dry 1000 ml volumetric flask and to it was added 200 ml of Methanol.

6.2 PREPARATION OF DRY POWDER INHALER FORMULATIONS

The prepared liposomal batches, LLN, LLEU and LLEUn were centrifuged and formed pellets were further diluted with the required quantity of hydration medium containing sugar to obtain different lipid sugar mass ratio The resulting suspension was frozen at –40°C overnight and dried under negative displacement pressure for 24 h The porous cake obtained were mixed either with Sorbolac 400 or Pharmatose 325 M and sieved successively through #200 and #240 sieves (Joshi and Misra, 2001b) Capsules (size '2') were filled with individually weighed powder (10 mg) containing 250 µg drug and packed under nitrogen atmosphere in high-density polyethylene (HDPE) bottles containing silica bags as desiccant The bottle with desiccant was sealed with polyvinyl chloride-coated aluminum foils and stored in a refrigerator (2–8°C) until further use.

Lyophilization was optimized with regards to selection of cryoprotectant (sugar), phase of cryoprotectant addition, Lipid to cryoprotectant ratio, and phase of diluent addition so as to get maximum percent drug remain entrapped Each batch was prepared for three times on three different days and stored in refrigerator.

Compositions of various batches prepared and percent drug remained entrapped are recorded in Table 6.1-6.3 Percentage efficiency of lyophilization were also calculated for the batches prepared and recorded in Table 6.1-6 3.

6.3 CHARACTERIZATION OF DRY POWDER INHALER FORMULATIONS

The prepared DPI formulations were characterized with respect to various physical and chemical parameters as described below:

6.3.1 ANALYSIS OF LIPOSOMAL DPI FORMULATIONS FOR PDE

PDE in liposomal DPI formulations were determined by the method described in Chapter 3 (section 3.4.6 and 3.5.6).

6.3.2 DETERMINATION OF SHAPE AND LAMELLARITY:

A fraction of the powder was rehydrated with triple-distilled water with gentle, occasional agitation. All the batches were then viewed under Olympus microscope with the provision of dark background and attachment of polarizing lens, to study their shape and lamellarity at 1000X magnification

6.3.3 VESICLE SIZE DETERMINATION

The vesicle size of the rehydrated liposomes of optimised DPI formulations were determined by laser diffraction spectroscopy using Mastersizer (Malvern Instruments Ltd , UK) operating at a beam length of 2.40 mm and range of lens at 300 mm The dehydrated freeze-dried cake was rehydrated with equivalent proportion of water (i e. one fourth of the cake needed to be diluted upto 1ml with triple distilled water) for 30 minutes and the obtained dispersion was centrifuged to remove lactose and subjected to particle size determination These dispersions were very concentrated to be analyzed by laser diffraction, so were further diluted with the hydrating medium to a factor of 1,000 The mean liposomal size with their respective size range is summarized in Table 6.4.

6.3.4 DETERMINATION OF PARTICLE SHAPE

Morphology of the prepared DPIs was studied by Scanning Electron Microscopy (SEM) Photomicrograph of SEM are as shown in Figure 6 1.

6.3.5 RESIDUAL WATER CONTENT

The residual water content of the freeze-dried cakes of DPI formulations (1g) was determined by K-F method Commercially available pyridine free reagent was standardized with known quantity of water (250mg) and used. Before adding sample, 40 ml of methanol was added into the titration vessel and titrated with the reagent to an audio-visual endpoint to consume any moisture that may be present. The water

content determination was carried out three times and the results are recorded in Table 6 4

6.3.6 POWDER FLOW PROPERTIES

6.3.61 Angle of Repose

To determine the angle of repose of liposomal DPI formulations, a pile of the sample was carefully built up by dropping the material through a funnel till the formed pile touches the tip of the funnel, 1 cm above the flat surface. The angle of repose (Table 6 4) was calculated by inverting tangentially the ratio of height and radius of the formed pile

6.3.62 Tapped Density

Tapped density was determined by mechanically tapping a measuring cylinder containing 2 g of powder sample. After observing the initial volume, the cylinder was mechanically tapped, and volume reading was taken until little to no change in volume is observed. The plateau condition was obtained after 500 taps for all samples The obtained values are recorded in Table 6 4.

6.3.63 Compressibility Index:

The compressibility index values recorded in Table 6 4 were obtained by tapping the powder for 500 tap (taps sufficient to obtain plateu condition), similar to the process described in the previous section and calculated using the formula.

$$\text{Carr's compressibility index} = \frac{\text{Tapped density} - \text{Fluff density}}{\text{Tapped density}} \times 100$$

6.3.64 Dispersibility

The dispersibility was determined using a miniature assembly to that described by Carr, R L., 1965 Liposomal DPI formulation (5gm) was dropped 'en masse' through a cylinder (length 6 5 inch and internal diameter 2 inch) held 2 inch above a watch glass (Diameter 1 inch) The dropping point was 3 inch above the cylinder, from a funnel tip Dispersibility was calculated as the relative proportion of material lost to the material dropped. Six individual batches were evaluated for dispersibility and the results are recorded in Table 6 4.

6.3.7 Fine Particle Fraction

The twin impinger (Apparatus A) official in British Pharmacopoeia was used to obtain the FPF values The volumes of the capturing solvents (methanol) in the upper stage (stage 1) and lower stage (stage 2) of the impinger were 7 and 30 ml; respectively. Fluidization of the formulation was achieved by the aid of rotary vacuum pump The pump was previously set with a flow control valve to generate a physiologically relevant airflow rate of 60 L/min measured with a Rotameter A trap of sulphuric acid was placed between the impinger and the vacuum pump to protect it from the vaporizing solvent. Capsule was placed in Rotahaler (Cipla, India) and attached to mouthpiece adapter of the impinger Rotahaler was twisted to release the contents of the capsule. The actuation time of the impinger was kept 5 seconds The methanol in the lower portion of the device was evaporated to dryness and the contents were analysed for the drug by the individual testing procedure described in Chapter 3 The device (Rotahaler) was rinsed with methanol and analyzed to determine the fraction remained in the device (DF) for calculating the effective Index. The results of FPF and DF are recorded in Table 6 4

6.4 DRUG RETENTION STUDIES

The prepared liposomal DPI formulations LLN-DPI, LLEU-DPI and LLEUn-DPI formulations were subjected to drug retention studies for a period of 6 months. Prepared batches were sealed in HDPE bottles containing silica bags as desiccant and

stored at refrigeration temperature (2-8°C), room temperature (25±2°C) and at accelerated temperature (40±2°C) The percent drug retained in liposomes was determined at specific time intervals using the method described in Chapter 3 (Section 3 4.7 and 3 5.7) The results of this study are recorded in Table 6 6-6 7 and shown graphically in Figure 6 2-6.3

The increase in vesicle size of liposomes was determined from changes in vesicle diameter for liposomes at refrigeration temperature (5°C ± 3°C) and room temperature (25°C ± 2°C) after 6 months storage period as described in Section 6 5.2 The results are recorded in Table 6 6-6 7

6.5 IN VITRO DIFFUSION STUDIES

6.5.1 EXPERIMENTAL SETUP

6.5.11 Preparation of the membrane:

Dialysis membrane (250-9U, molecular weight cut off 12000 Dalton; Sigma, Hyderabad, India), 200µm in thickness, pH 5 8 to 8, breaking strength 2.75 kg f/cm and porosity 0.45 µm was used as a membrane for in vitro diffusion studies because of simplicity, homogeneity and uniformity This membrane was pretreated with ethanol (95%) followed by hydration in PBS for 24 hr prior to permeation runs.

6.5.12 Design of diffusion Cell:

For the present study a vertical type of membrane diffusion system was designed (Figure 5.1) The system consists of a hollow glass tube open at both ends with inner diameter of 18 mm and length of 6 cm. The diffusion membrane was tied to one end of the tube with a nylon string, serving the purpose of a donor compartment. The tube was immersed in 50 ml diffusion medium (20% methanolic PBS for LN formulations and PBS for LEU formulations), maintained at 37 ± 0 5°C under continuous stirring at

a rate of 50 rpm, in a way that the membrane just flushes to the surface of the diffusion fluid

6.5.13 Validation of Diffusion Cell

The hydrodynamic characteristics of the diffusion cell were established using the benzoic acid disc method (Chein and Valia, 1984, Shahiwala, 1999)

6.5.2 METHOD

Diffusion studies were carried out for plain drug and liposomal DPI formulations of LN and LEU Plain drug suspension/solution and liposomal DPI formulations containing 1 mg drug were transferred to the donor compartment. One ml of sample was withdrawn from the receptor compartment at definite time intervals and equivalent amount of fresh medium was replaced to the receptor compartment. The estimation of the drugs in sample was carried out using the procedure described in Chapter 3 (Section 3.4 8 and 3.5.8) All diffusion runs and sample analysis were carried out in triplicate on three consecutive days and mean values along with standard error of mean are recorded in Table 6.8 and Table 6 11 and shown graphically as Q vs t (hours) in Figure 6.4 and Figure 6.6 Regression coefficients by different release kinetic models were calculated and recorded in Table 6.9 and Table 6 12 The mean flux values, J (µg/min), and diffusion coefficients were also calculated for all the formulations and recorded in Table 6 10 and Table 6.13 and shown graphically in Figure 6.5 and Figure 6 7

6.6 IN VIVO STUDIES

6.6.1 LN FORMULATIONS

6.6.11 Animals

White albino female rats weighing 200± 50 g were used. All the animals used for the study were of proven fertility record All animals were housed in polypropylene cages with free access to palletized chow and tap water. The animals were exposed to

alternate cycles of 12 h light and darkness Animal experiments were approved by Social Justice and Empowerment Committee, Ministry of Government of India, New Delhi, India

6.6.12 Methodology

Rats were divided into 4 groups of 6 animals each Three groups were administered intratracheally with LN suspension, LN + PM and LLN-DPI (rehydrated with distilled water) formulations respectively and one group administered orally with LN suspension

Oral administration

For oral administration. 10-µg drug suspension (LN) was instilled through mouth using 28gauge long blunt needle

Pulmonary administration

The method of Enna and Schanker, 1972; for measurement of absorption rates of instilled compounds from the lungs of anesthetized rats was modified to allow measurements in conscious animals for periods of up to 72 hr after instillation Animals were anesthetized using urethane i p. Anaesthetized animals were placed in supine position on a 45° slanted support, and a small middle incision was made over the trachea The trachea was exposed by blunt dissection of the sternohyoideus muscle A small hole was made in the trachea between the fifth and the sixth tracheal rings using a 20-gauge needle A short (10- to 15-cm) length of PE50 tubing was inserted into the hole and advanced to the bifurcation of the trachea. Formulations of LN 0.1mL were slowly instilled over a 1-min period using a 1 mL syringe attached to the PE50 tubing Following instillation, the tubing was withdrawn and a small drop of cyanoacrylate adhesive was placed over the hole to seal the opening The skin was clothed with 3-0 Dexon sutures. Animals were removed from anesthesia and allowed to recover under a heating lamp. After recovery, animals were housed in individual plastic cages with access to food and water for the remainder of the study

200µl blood samples were withdrawn from tail vein at specific time points and estimation of the drugs in sample was determined using the procedure described in Chapter 3 (Section 3 6 9) The data of drug plasma concentration are shown in Figure 6 8 Various pharmacokinetic parameters, (Tmax, Cmax, and $t_{1/2}$) were calculated from the Figure and recorded in Table 6 14 AUC and bioavailability (F*) were also calculated and are recorded in Table 6 14 Each set of result represents the mean values of six experimentral determinations along with its standard error mean

6.6.2 LEU FORMULATIONS:

6.6.21 Animal:

White albino rats of either sex (equal in numbers) weighing 170± 20 g (120~140 days of age) were used Male and female rats were caged separately during the study unless otherwise required All the animals used for the study were of proven fertility record. All animals were housed in polypropylene cages with free access to palletized chow and tap water The animals were exposed to alternate cycles of 12 h light and darkness Animal experiments were approved by Social Justice and Empowerment Committee, Ministry of Government of India, New Delhi. India

6.6.22 Methodology

Rats of either sex were divided into 6 groups of 6 animals each One group was kept as control Control group was treated with the composition containing HSPC, CHOL and CS of the concentration used in formulations. Four groups of animals were treated with different DPI formulations containing LEU (LEU solution, LEU+PM and LLEU-DPI and LLEUn-DPI (rehydrated with Phosphate buffer pH 5.2). One group of animals were treated with LEU solution subcutaneously (s c).

Subcutaneous administration

For subcutaneous administration, 5-µg drug solution (LEU) was injected into the nape of the neck.

Pulmonary administration

The method described earlier in this chapter (section 6.8.12), under heading of Pulmonary administration was used for intratracheal instillation of LEU formulations

Blood was sampled from tail vein at different time points. Heparinized plasma was prepared and stored at -20°C, till the serum LH concentrations determined by as described in Chapter 3 (Section 3.5.9). The data of serum LH concentration are shown in Figure 6.9. Various pharmacokinetic parameters, (T_{max}, C_{max}, and $t_{1/2}$) were calculated from the Figure and recorded in Table 6.15. AUC and bioavailability (F^*) were also calculated and are recorded in Table 6.15. Each set of result represents the mean values of six experimentral determinations along with its standard error mean.

6.7 STATISTICAL ANALYSIS

(i) Optimization:

In the optimization of cryo-protection, the effect of one variable was studied at a time keeping other variables same and each experiment was repeated three times. Statistical analysis using ANOVA technique was applied for comparing the data of each set of experiments with others. The differences were considered significant at $P < 0.05$. Percent drug remained entrapped is the percentage of the drug initially added, determined after dehydration and rehydration cycle. Percentage efficiency of lyophilization was calculated using following formula:

$$\text{Percentage efficiency of lyophilization} = \frac{\text{Percent drug remained entrapped} \times 100}{\text{Percent drug entrapped}}$$

The mean particle size, drug entrapment efficiency and coefficient of variation were calculated to confirm reproducibility. The mean diameters (μm) obtained for LLEU-DPI and LLEUn-DPI were compared using student's t-test and the differences were considered significant at $P < 0.01$.

The particle size of the formulations was described by the volume mean diameter (D [4,3]) Effective index is the geometric mean of the emission fraction and FPF, represented by the equation

$$EI = \sqrt{(100 - DF) \times FPF}$$

Where, DF is the device fraction

(ii) Drug Retention Studies

For drug retention studies, the data of percent drug retained in liposomal DPI at 3 different storage conditions were compared using ANOVA and the differences were considered significant at $P<0.05$.

The vesicle size of liposomal DPIs (LLN-DPI, LLEU-DPI and LLEUn-DPI) was measured immediately after preparation and after 3 month and 6 months storage at refrigeration temperature (2-8°C), room temperature (25±2°C) and accelerated temperature (40±2°C) The mean vesicle diameter obtained immediately after preparation and after 3 months storage was statistically evaluated using student's t-test and the differences were considered significant at 5% level

(iii) Diffusion Studies

Six batches were evaluated for in vitro diffusion and the results are expressed as mean± SEM.

(a) Percent Drug Diffused (Shah et al., 1993).

The percent drug diffused across artificial membrane at each sampling points was determined by the formula given below

$$\text{Pecent Drug Diffused (R)} = \frac{CrVr}{CdVd} \times 100$$

Cr = Concentration of drug in receptor compartment

Vr = Volume of the receptor compartment

Cd = Initial concentration of drug in donor compartment

Vd = Initial volume of donor compartment

(a) Kinetics of Release

The order of drug release was determined by performing regression over the mean values of percent drug diffusion vs t and percent drug diffusion vs Root t.

(b) Mean Steady state flux,

The flux across the membrane was calculated using the following formula

$J = V (dc/dt)$

Where J = flux of the drug across the membrane

V = Volume of receptor compartment

(dc/dt) = Rate of change of concentration

Mean steady state flux is the mean of individual flux values at all sampling points.

(c) Diffusion Coefficient

The diffusion coefficient of the drug at every sampling point was calculated using the following equation:

$R= 200 \text{ Sq rt } Dt/\pi h^2$

Where, R = Percent drug diffused

h = thickness of the membrane (0 02 cm)

t = time (sec)

D = diffusion coefficient (cm^2/sec)

The diffusion coefficient used for the discussion is the mean of the value (D) obtained at each sampling point

The mean flux values (n=3), J, and mean percent drug diffused (n=3), Q, obtained for LLN-DPI were compared with that of LN by applying student's t-test and the differences were considered significant at 5% level. Similarly, the mean flux values (n=3), J, and mean percent drug released (n=3), Q, obtained for LLEU-DPI and LLEUn-DPI were compared with that of LEU by applying ANOVA and the differences were considered significant at $P < 0\ 05$

(iv) In vivo studies

Each experiment was repeated six times and the results obtained are expressed as mean± SEM

The drug plasma concentrations at each sampling time point were plotted against time in h Maximum plasma concentration (Cmax), time in h to achieve Cmax (Tmax) and drug plasma half-life ($t_{1/2}$) were determined from drug plasma concentration-time curve from best fit curve using major and minor gridlines with ±0.2 unit accuracy. The area under the plasma level curve was calculated by the trapezoidal rule Data were compared using ANOVA and difference at $p<0.05$ were considered significant

The relative percent pulmonary bioavailability/bioactivity (F*) with respect to orally/subcutaneously administered LN was calculated by,

$$F^* = \frac{\text{AUC Intratracheal Route} \times \text{Subcutaneous Dose}}{\text{AUC Subcutaneous Route} \times \text{Intratracheal Dose}} \times 100$$

6.8 RESULTS AND DISCUSSION

Optimized liposomal batches of LN and LEU with the mean diameter D[4,3] less than 5 μm obtained as shown in Chapter IV were used for further development of Dry Powder Inhaler (DPI) formulations.

Lyophilization and optimization of cryoprotectant in the preparation of DPI formulations:

Liposomal pellet after centrifuging liposomal dispersion was again suspended in distilled-water containing either lactose or maltose or trehalose or sucrose or dextrose in mass ratio of lipid:sugar (1.1) The amount of LN and LEU retained in liposomes following lyophilization and rehydration was determined The results are recorded in Table 6.1-6 3. Data revealed that trehalose and sucrose gave the highest percent drug retained in liposomes (89 4±1.9% and 88.3±2 3% respectively for LLN-DPI, 37.4±1 9% and 38.3±2.3% respectively for LLEU-DPI and 39 15±1 9% and 36.99±2.3% respectively for LLEUn-DPI)

This is in agreement with the finding that effectiveness of number of sugars in maintaining structural and functional properties of microsomal membranes at low mean liposomal size, sucrose and trehalose were found to be the most effective cryoprotectant (Cullis et al, 1985). Sucrose was selected as optimized cryoprotectant for preparation of lyophilized liposomal LN and LEU for further experiments due to its easy availability and low cost

In case, when sucrose was added either outside or inside, 88 3±2 3% or 76.6±2 6%, 38.3±2.3% or 35 6±2.6% and 36.99±2 3% or 31.41±2.6% drug retention were observed for LLN-DPI, LLEU-DPI and LLEUn-DPI respectively, while vesicles retained 94.3±2.1%, 48.3±2.1% and 55.09±2.1% drug with sucrose on both the sides of the bilayers for LLN-DPI, LLEU-DPI and LLEUn-DPI respectively. When sucrose as cryoprotectant was provided only on the outside or the inside of the vesicles, there was increased leakage of liposomal drugs were observed which might be due to difference in osmotic pressure across liposomal membrane (Table 6.1-6 3) (Cullis et al, 1985; Crowe et al, 1988)

The batches of liposomes were lyophilized in the presence of varying concentrations of sucrose The amount of initially entrapped LN and LEU retained by the lyophilized liposomes upon rehydration was found to be dependent on cryoprotectant concentration (Table 6 1-6 3). While changing the mass ratio of lipid sucrose, the percent of drug retained was found to be maximizing at 1 1 (97 03±1 9%), 1·5 (66.08±1 6%) and 1.6 (72.08±2 1%) for LLN-DPI, LLEU-DPI and LLEUn-DPI formulations Further increase in sucrose concentration, there were no significant change in percent drug retained in rehydrated liposomes This may be due to drying process of liposomes, the liposomes are believed to be constricted and coated on the surface of internally crystallized sugar This stabilization by coating is in synergism with the hydration of polar head groups with hydroxyl group of sucrose, which replaces the lyophilizing water molecule If the sucrose concentration is less than optimum, the internally crystallized sugar does not allow adequate surface for the adherence of constricted bilayers. Thus an optimal lipid to sugar mass ratio is required to have a better retention of drug in the porous cake formed during lyophilization. It seems that sufficient dilution of liposomes in the sugar solution is required to have the polar head groups on its surface completely saturated with sugar, as well as to protect it from the deleterious effect of icing of sugar These findings are in congruence with the findings of Crowe and Crowe, 1988

The effects of diluent's addition on percent drug remained entrapped were also carried out and it was found that diluent's addition after lyophilization leads to better PDE in all the formulations Either 10 mg Sorbolac 400 or Pharmatose 325 M was added to the lyophilized cake. The resulting mixture was mixed properly and sieved gently to reduce to a respirable size and also to deaggregate, the powders were sieved from 200# and 240# sieves and resulting powder was filled in capsule size "2". The formed powder after lyophilization is generally very hygroscopic and so the product was stored under nitrogen atmosphere, sealed with PVC coated aluminium foil.

Characterization

The mean vesicle size of liposomes before dehydration and after rehydration was determined by a laser light scattering technique using Mastersizer (Malvern

Instruments, London, UK) The vesicle size of liposomes after rehydrating the formulations was described by the volume mean diameter (D [4,3]) The results obtained are given in Table 6.4 No significant change in the vesicle size was observed following dehydration and rehydration This infers that freeze-drying according to this protocol (formulation variable) provided sufficient cryoprotection to the liposomes

All the batches of liposomal DPI prepared were rehydrated and viewed under Olympus (BX 40F4, Japan) with the provision of dark background and attachment of polarizing lens, to study their shape and lamellarity. Liposomes were found to be multilamellar and spherical in shape

Evaluation and control of flow and dispersion (deaggregation) characteristics of the formulation are of critical importance in the development of DPI products Inter-particle forces that influence flow and dispersion properties are particularly dominant in micronize or microcrystalline powders required for inhalation therapy (< 5μm) (Gonda, 1992; Hickey, 1996) It has been demonstrated that powder adhesion, mediated in part by Van der Waal forces, is directly related to particles < 10 μm (Hamaker, 1937).

It is apparent that predictions of powder rheology based on the potential interplay of a number of physicochemical properties are extremely complicated Instead flow and dispersion properties are generally characterized such as angle of repose, dispersibility, moisture content and fine particle fraction. These properties were determined and are recorded in Table 6 4 It is important to identify and control the critical parameters, both fundamental and derived, to ensure optimum and consistent product performance. The angle of repose has been used in several branches of science to characterize the flow properties of solids Nelson was the first to use angle of repose measurements to determine the flow properties of pharmaceutical material.

Table 6.1 Selection and optimization of cryoprotectant for efficient lyophilization of LN liposomes

Batch No.	Variable studied	Percent drug entrapped* Mean ±SEM	Percent drug remained entrapped* Mean ±SEM	Percentage efficiency of lyophilization* Mean ±SEM
SELECTION OF CRYOPROTECTANT				
LLN-DPI1	Maltose	98 30 ± 0 21	74 8 ±1 9	76 09± 0.89
LLN-DPI2	Trehalose	98 30 ± 0 21	89.4 ± 1 9	90.95±0 78
LLN-DPI3	Dextrose	98 30 ± 0.21	77 8 ± 2 2	79.15±0.99
LLN-DPI4	Lactose	98.30 ± 0 21	67 6 ± 2 2	68 77±0.65
LLN-DPI5	Sucrose	98 30 ± 0 21	88 3 ± 2 3	89.83±0 75
PHASE OF CRYOPROTECTANT ADDITION				
LLN-DPI5	External	98.30 ± 0 21	88 3 ± 2 3	89.83±0.91
LLN-DPI6	Internal	98.30 ± 0 21	76 6 ± 2.6	77 92±0 90
LLN-DPI7	Both	98 30 ± 0 21	94 3 ± 2 1	95.93±1.01
MASS RATIO OF CRYOPROTECTANT (Lipid Sucrose; molar ratio)				
LLN-DPI7	1 . 0.5	98 30 ± 0.21	94 3 ± 2 1	95.93±1 01
LLN-DPI8	1 1	98 30 ± 0 21	98 17 ± 0 18	98.71±0 99
LLN-DPI9	1 · 2	98 30 ± 0 21	97 18 ± 1 7	98 86±0.80
PHASE OF DILUENT ADDITION				
LLN-DPI8	Post lyophilization	98 30 ± 0 21	98 17 ± 0 18	99 23±0.11
LLN-DPI10	Pre lyophilization	98.30 ± 0.21	97 54 ± 0.33	99.87±0.24

*n = 3

Table 6.2 Selection and optimisation of cryoprotectant for efficient lyophilization of LLEU liposomes

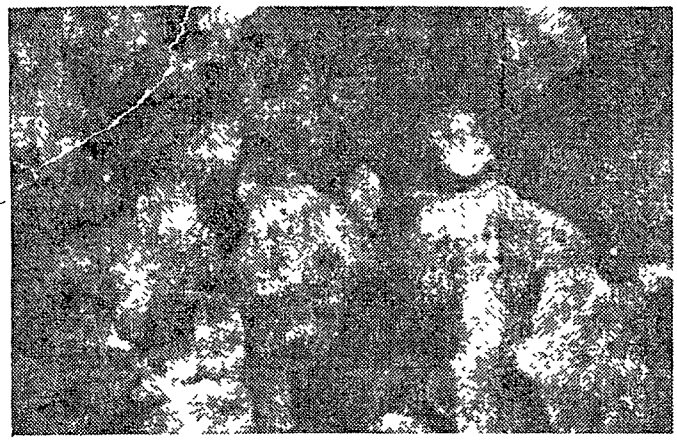
Batch No	Variable studied	Percent drug entrapped* Mean ±SEM	Percent drug remained entrapped* Mean ±SEM	Percentage efficiency of lyophilization* Mean ±SEM
SELECTION OF CRYOPROTECTANT				
LLEU-DPI1	Maltose	85 41±1 34	19 35± 1 9	22 66±0 98
LLEU-DPI2	Trehalose	85 41±1 34	37 4 ± 1 9	43.79±0 99
LLEU-DPI3	Dextrose	85.41±1.34	31.8 ± 2 2	37.23±1 05
LLEU-DPI4	Lactose	85 41±1 34	21.6 ± 2.2	25.29±1 00
LLEU-DPI5	Sucrose	85 41±1 34	38.3 ± 2.3	44.84±0 95
PHASE OF CRYOPROTECTANT ADDITION				
LLEU-DPI5	External	85 41±1 34	38 3 ± 2 3	44.84±0 95
LLEU-DPI6	Internal	85 41±1 34	35 6 ± 2 6	41.68±0 87
LLEU-DPI7	Both	85.41±1 34	48 3 ± 2.1	56 55±0 90
MASS RATIO OF CRYOPROTECTANT (Lipid Sucrose, molar ratio)				
LLEU-DPI7	1 1	85.41±1.34	48 3±2 1	56.55±0 90
LLEU-DPI8	1 2	85.41±1.34	50.36 ±1.9	58.96±0 83
LLEU-DPI9	1 3	85 41±1.34	55 18 ± 2.1	64 61±0.87
LLEU-DPI10	1 4	85.41±1 34	59 71 ±1.8	69.91±0 79
LLEU-DPI11	1 5	85 41±1 34	66 08±1 6	77.37±0.77
LLEU-DPI12	1 6	85.41±1.34	66.98±1 6	73.49±0.76
PHASE OF DILUENT ADDITION				
LLEU-DPI12	Post lyophilization	85.41±1.34	66.98 ± 1.6	99.23±0 74
LLEU-DPI13	Pre lyophilization	85.41±1 34	61.17 ± 1.7	71.62±0.56

*n = 3

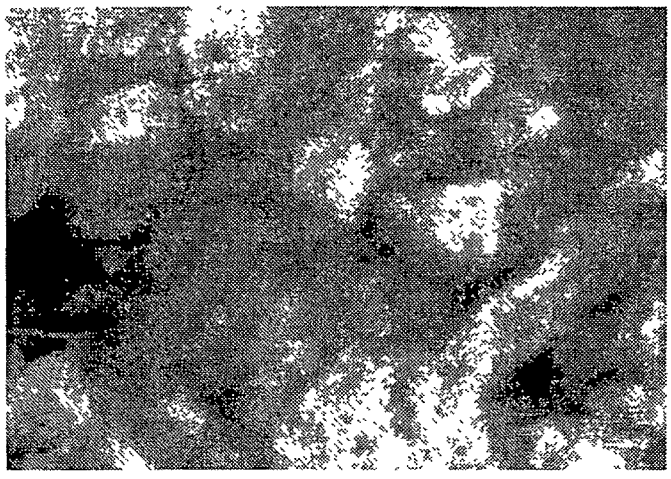
Table 6.3. Selection and optimisation of cryoprotectant for efficient lyophilization of LLEUn liposomes

Batch No	Variable studied	Percent drug entrapped* Mean ±SEM	Percent drug remained entrapped * Mean ±SEM	Percentage efficiency of lyophilization* Mean ±SEM
SELECTION OF CRYOPROTECTANT				
LLEUn-DPI1	Maltose	91 14±1 09	23 08 ±1 9	25 32±1.06
LLEUn-DPI2	Trehalose	91 14±1.09	39.15 ± 1.9	42.96±1.11
LLEUn-DPI3	Dextrose	91 14±1.09	26 87 ± 2.2	29 48±0.98
LLEUn-DPI4	Lactose	91 14±1 09	19 24 ± 2.2	21 11±0 89
LLEUn-DPI5	Sucrose	91.14±1.09	36 99 ± 2 3	40.59±0 90
PHASE OF CRYOPROTECTANT ADDITION				
LLEUn-DPI5	External	91 14±1 09	36 99 ± 2.3	40.59±0 90
LLEUn-DPI6	Internal	91 14±1 09	31 41 ± 2.6	34 46±1 11
LLEUn-DPI7	Both	91 14±1 09	55 09 ± 2.1	60 44±1.06
MASS RATIO OF CRYOPROTECTANT (Lipid Sucrose, molar ratio)				
LLEUn-DPI7	1 : 1	91 14±1 09	55 09 ± 2.1	60 45±1.06
LLEUn-DPI8	1 2	91.14±1 09	57.33 ± 2.4	62 90±0.97
LLEUn-DPI9	1 . 3	91 14±1 09	61.08 ±1 9	67.02±0.83
LLEUn-DPI10	1 4	91.14±1 09	62 35 ±1.9	68.41±0 88
LLEUn-DPI11	1 : 5	91 14±1 09	66.10±2.0	72.53±0.95
LLEUn-DPI12	1 : 6	91 14±1.09	72.08±2.1	79.09±0 98
LLEUn-DPI13	1 : 7	91.14±1.09	72 24±1.4	79.26±1.00
PHASE OF DILUENT ADDITION				
LLEUn-DPI12	Post lyophilization	91 14±1.09	72.08 ± 2.1	79.26±1.23
LLEUn-DPI14	Pre lyophilization	91.14±1.09	61.03 ± 1.5	66.96±1.07

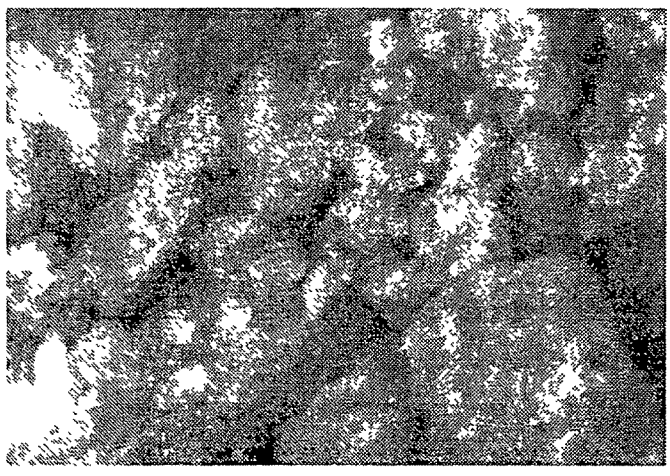
*n = 3



SEM of LLN-DPI



SEM of LLEU-DPI



SEM of LLEUn-DPI

Figure 6.1. SEM of LLN-DPI, LLEU-DPI and LLEUn-DPI at 2000X magnification

The flowability and floodability expressed by angle of repose (26-32) and dispersibility (20-23) falls in the category of good and floodable (Carr, 1965) This suggests that there is no significant interparticulate interactions among the liposomal DPI formulations and thereby assures optimal dispersion in stream of air, upon inhalation

The Compressibility Index is a measure of the propensity of a powder to be compressed. As such, they are measures of the relative importance of interparticulate interactions In a free-flowing powder, such interactions are generally less significant, and the bulk and tapped densities will be closer in value For poorer flowing materials, there are frequently greater interparticle interactions, and a greater difference between the bulk and tapped densities will be observed These differences are reflected in the Compressibility Index The Compressibility index for all the formulations were below 30% suggests low interparticulate interactions

The tap density of formulations falls in the range of 0 33 to 0.37 g/cc. The tap density below 0 4 g/cc and a mean size below 5 μm together will yield an aerodynamic diameter of the particles between approximately one and three microns. Moisture content determination is important for liposomal structural and drug stability on storage and deaggregation upon inhalation The lyophilized formulations are found to have moisture content below 1.5 percent (before diluent addition) (Table 6 4). It confirms low aggregation tendency

In DPI formulations, certain particles may aggregated to form free flowing spheres, reducing the surface free energy of the micronized powder The aerosol performance of the agglomerated system is dictated predominately by the interparticulate forces acting between the particles Optimal inhalation performance requires that the dispersive forces, generated within the device upon patient inspiration, exceed the strength of the interparticulate forces acting between the particles. The in vitro aerosol behavior of the liposomal DPI formulations was therefore investigated in terms of respirable fine particle fraction (FPF). From the two devices described in British Pharmacopoeia Commission (1993) for particle characterization of fine particle fraction (FPF). glass liquid impinger (apparatus A) was used. Ideal formulation of

DPIs should provide small device fraction (effective emission from the device), and large FPF when inhaled The results obtained from the studies suggest the fraction of the DPI likely to deposit in the trachea to bronchioles The FPF value (Table 6 4) for the optimized formulations were in the range of 22-38, suggestive of substantial deposition of the developed DPI formulations in lung

From the results, it is evident that by diluting the liposomal drug with carrier will give increase in FPF of liposomal drug Sorbolac and Pharmatose were used as diluents to study their effect on powder flow properties and fine particle fraction for LEU products We hypothesize that it may be due to association of liposomal drugs to the lactose particles possess high energy adhesion sites (HA) which are able to bind strongly to the liposomal drug particles while low energy adhesion sites (LA) which allow the formation of more reversible bonds with it and thus gives efficient detachment of liposomal drug from the carrier molecules as this type of observation is seen in case of plain DPI formulations (Staniforth, 1996). The better FPF for Pharmatose 325M product compared to that of Sorbolac 400 product is may be due to the particle size distribution of these materials (Table 6 5) The finer lactose particles will occupy HA sites leaving LA sites for attachment of liposomal drug and thus gave higher FPF in case of Pharmatose 325M From SEM photomicrographs also it is evident that liposomal structures were found to be adhered to the lactose particles and gave required FPF when delivered as dry powder inhaler (Figure 6.1). These formulations were characterized and evaluated for stability under various storage conditions followed by *in vitro* diffusion studies and *in vivo* studies

Drug retention studies of LN liposomal DPI formulation:

The drug retention studies were carried out at refrigerated temperature (2-8°C), at controlled room temperature (25±2°C) and at accelerated temperature (40±2°C) for the LLN-DPI stored in HDPE bottles containing silica bags as desiccant. LLN-DPI batches were evaluated for PDE in liposomes and the results are recorded in Table 6.6 and shown graphically in Figure 6.2.

Table 6.4: Characterization of potential batches of liposomal DPI Formulations

Variable Studied	Potential liposomal batches*					
	LLN-DPI with Sorbolac	LLN-DPI with Pharmatose	LLEU-DPI with Sorbolac	LLEU-DPI with Pharmatose	LLEUn-DPI with Sorbolac	LLEUn-DPI with Pharmatose
D[4,3] before dehydration (μm)	3 3±0 1	3 3±0 1	3 5±0 1	3 5±0 2	4 5±0 2	4 5±0 2
D [4,3] after rehydration (μm)	3 2±0 1	3 2±0 1	3 5±0 1	3 5±0 1	4 3±0 1	4 3±0 1
Angle of Repose (θ)	31 4±0 8	29.5±0 9	29 6±0 5	27 8±0 8	29 4±0 9	26 7±0 8
Tapped Density	0 37± 0 03	0 36±0 05	0 34±0 04	0 33±0 03	0 33±0 04	0 33±0 03
Compressibility Index	26 34±1 14	21 08±1 09	19 51±1 07	13 26±0 98	18 97±0 97	13 47±0 98
Dispersibility	20 8±1 0	21 9±1 0	21 4±0 6	22 15±0 9	21 6±0 9	22 8±1 0
Moisture content (after lyophilization %)	1 1±2 0	1 1±1 8	0 9±2 4	1 0±0 8	1 1±1 5	1 0±1 3
Respirable Fraction (FPF)	22 5±2 2	30 15±2 1	29 8±2 2	35 36±2 0	31 2±2 4	37.8±2 1
Device Fraction (DF)	26 38±2 0	24 22±1 9	32 65±2.1	23 24±2 0	26 24±2.0	22 10±1 9
Effective Index	40 7 ± 1 9	47 8 ± 2.0	44.8 ± 1 6	52.1 ± 1 9	47 9±1 8	54.2±1 7
Control Ashthalin (Cipla Ltd , India) FPF = 27 1 ± 2.0, EI = 48.6 ± 1.7						

*n = 3

Table 6.5: Comparison of Bulk density and particle size data for Pharmatose 325M and Sorbolac 400

Pharmatose 325M	Sorbolac
B.D = 0 67 g/cc Particle Size = 5-10% < 32 μ NLT 70% 63 μ 100%- less than 100 μ	B D = 0 37 g/cc Particle Size –NLT 97% = 32 μ 100% = 63 μ

LLN-DPI showed more than 7 percent drug leakage at 40±2°C after 3 months storage period and hence drug retention studies were discontinued for product stored at this temperature LLN-DPI was found to be stable over 6 months stability period for the drug retention (less than 5%) at refrigerated conditions and room temperature The size of liposomes was also determined immediately, after 3 months and 6 months storage period and no significant difference ($p<0.05$) were observed at refrigerated and room temperature storage conditions

Drug retention studies of LEU liposomal DPI formulation:

The drug retention studies were carried out at refrigerated temperature (2-8°C), at controlled room temperature (25±2°C) and at accelerated temperature (40±2°C) for the LLEU-DPI and LLEUn-DPI stored in HDPE bottles containing silica bags as desiccant. Products were evaluated for PDE in liposomes and the results are recorded in Table 6 7 and shown graphically in Figure 6.3

LLEU-DPI and LLEUn-DPI products showed 9-10% percent drug leakage at 40±2°C after 6 months storage period. Both the products were found to be stable over 6 months stability period for the drug retention (less than 5%) at refrigerated conditions and room temperature. The size of liposomes was also determined immediately, after 3 months and 6 months storage period and no significant difference ($p<0.05$) were observed at refrigerated and room temperature storage conditions When percent drug retention data for both the products were compared, LLEUn-DPI formulation showed higher percent drug retention at all the sampling time points This might be due to the attractive forces between negatively charged liposomal membrane and positively charged LEU restricts the drug movement from the liposomes

As obvious, when compared between the batches stored at lower temperature (refrigerator) compared with those stored at higher temperatures (25±2°C, 40±2°C) batches at lower temperatures showed higher preservation.

IN VITRO DRUG DIFFUSION STUDIES

LN FORMULATIONS

Comparative diffusion studies were carried out between plain LN formulation and LLN-DPI formulation for a period of 24 h to 72 h using dialysis membrane in self designed and validated diffusion cell As the drug was soluble in 20% methanolic PBS, sink conditions were maintained when 50 ml of the medium used as a diffusion medium and hence zero-order flux conditions not violated The results obtained are recorded in Table 6 8 and shown graphically in Figure 6 4

The percent drug release is plotted against time (hr) in Figure 6 4 The non linearity of the graph suggests that the release pattern does not follow zero order kinetics An attempt was made to understand the diffusion kinetics, kinetic parameters were calculated and are shown in Table 6 9 The drug diffusion data from both the formulations fit a Higuchi's equation as the highest correlation (R^2 = 0 96-0.98) obtained suggesting that the release obeys Higuchi's diffusion controlled model.

Mean flux values of both the formulations were calculated and recorded in Table 6.10 and shown graphically in Figure 6 5 The diffusion co-efficient of both the formulations also calculated and recorded in Table 6.10 and its graphical presentation is given in Figure 6 5. The mean flux value of the LN formulation was found to be two to three times higher than those of LLN-DPI formulation, indicating that liposomal formulation prolong the drug diffusion Similarly the diffusion coefficient of the LN formulation is much higher to that of the LLN-DPI formulation confirming a prolong drug diffusion following liposomal encapsulation of drug

Table 6.6: Percent drug retention (PDR) in LLN formulation at different storage conditions

Time in months	PDR±SEM* at 2-8°C	PDR±SEM* at 25±2°C	PDR±SEM* at 40±2°C
0 5	99.9±0 20	99 3±0 27	98 7±0 56
1	99 7±0.18	98 5 ±0 52	96 8±0 80
2	99.1±0.26	97 7±0 59	95 3±0.99
3	98 4±0 27	96 6±0 68	91 7±1 87
6	97 5± 0 44	95 9±0 77	83 7±2 31

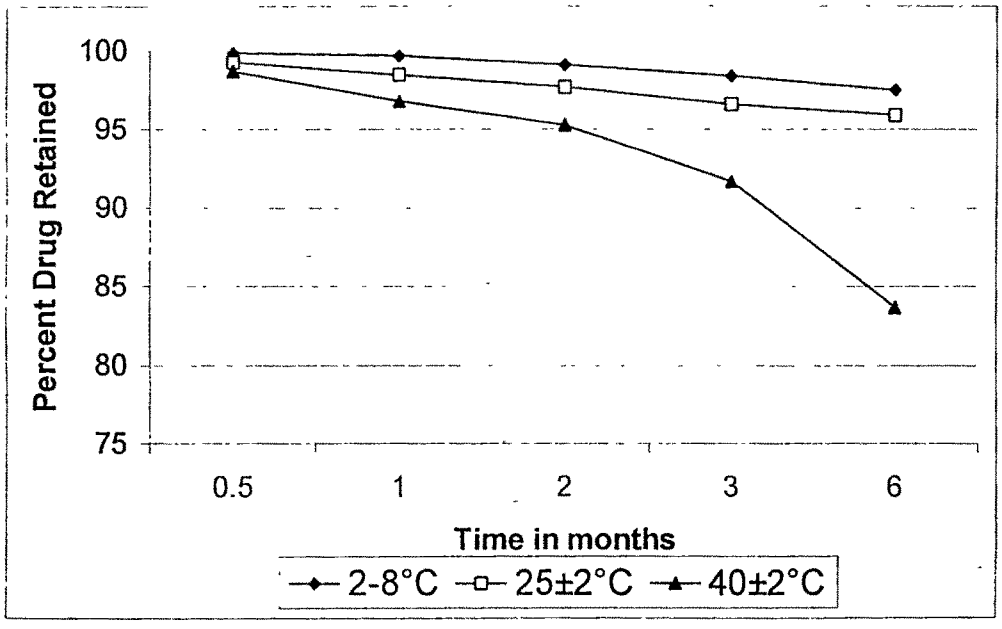


Figure 6.2 Drug Retention Studies of LLN-DPI formulation

Table 6.7: Percent drug retention in LLEU formulations at different storage conditions PDR in liposomal formulations of LEU at different storage conditions

	LLEU-DPI*-			LLEUn-DPI*		
Time in months	PDR±SEM at 2-8°C	PDR±SEM at 25±2°C	PDR±SEM at 40±2°C	PDR±SEM at 2-8°C	PDR±SEM at 25±2°C	PDR±SEM at 40±2°C
0.5	99 9±0 5	99 6±0 65	99.±0 79	00 0±0 2	99 8±0 55	99 3±0 65
	99 9±0 3	99.2±0.69	98 6±0 77	99 9±0 5	99.7±0 5	99 0±0 50
2	99 8±0 4	98.7±0 6	97 ±0 98	99 9±0 7	99 4±0 49	97 5±0 74
3	99 2±0 8	98 ±0 77	95.8±	99.7±0	99 2±0 54	96.±0 85
6	98 8±0 30 /3 53±0 2	96 5±0.68 /3 5±0 2 2	9 2± 23	99 4±0.9 /4.6±0 2	98.5±0.36 /4 63±0 3 4	9.5±0.9

*(n=3)

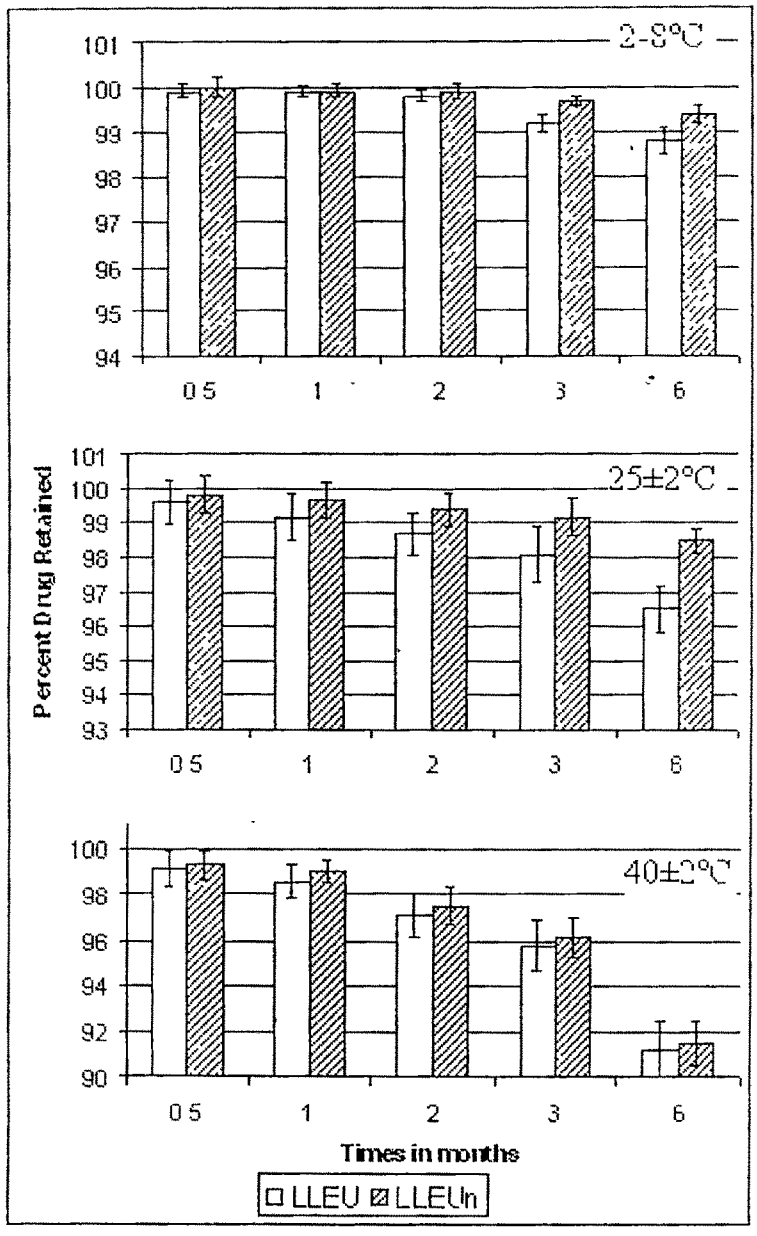


Figure 6.3: Drug Retention Studies of LLEU formulations

Table 6.8: In vitro diffusion studies of LN formulations

Time	LN	LLN-DPI
01	19 07 ± 0 69	6 7 ± 0 84
02	43 21± 1 17	28 ± 0 99
04	68.71 ± 1 56	44 8 ± 1 67
06	75 36 ± 1 20	46 7 ± 1 09
08	96 99 ± 1 77	59 09 ± 1 35
10	--	68 6 ± 1 64
12	--	75 2 ± 1 21
24	--	100 01 ± 2 15

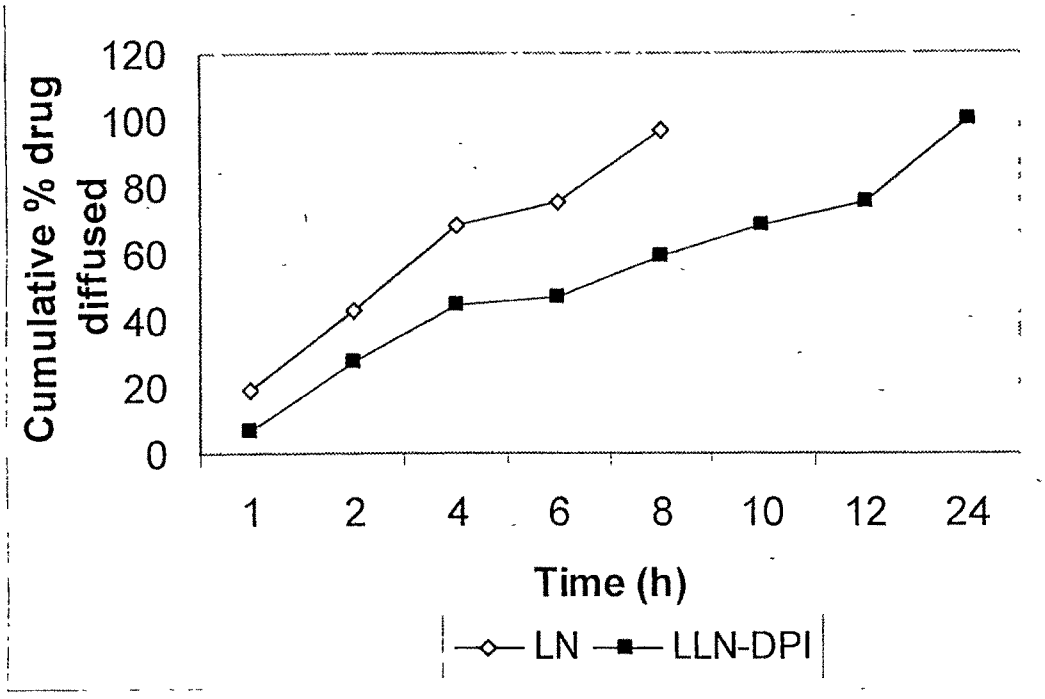


Figure 6.4: Cumulative % LN diffused during In vitro diffusion studies

Table 6.9 : Regression Coefficients of LN Formulations by different models

Formulations	Zero-order equation	Higuchi's equation	First-order equation
	R^2	R^2	R^2
LN	0 9378	0 97L3	0 8079
LLN-DPI	0 8683	0.9693	0 537L

Table 6.10: Mean Flux and Diffusion Coefficient values of LN formulations

	LN	LLN-DPI
Mean Flux	3 51	1.68
Diffusion Coefficient	8 03E-09	3 36E-09

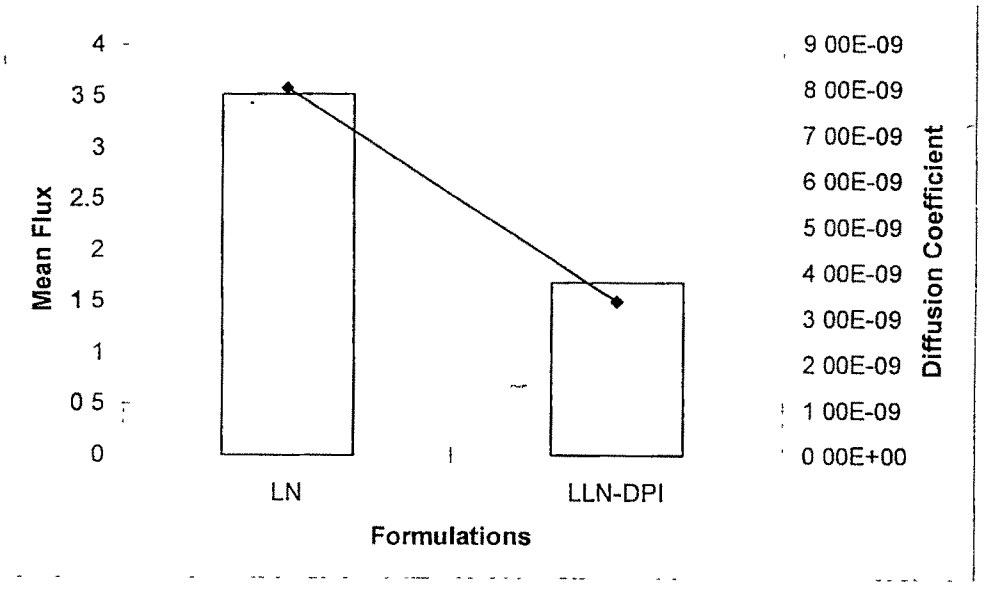


Figure 6.5: Mean Flux and Diffusion Coefficient values of LN formulations

Table 6.11: In vitro diffusion studies of LEU formulations

Time	LEU	LLEU-DPI	LLEUn-DPI
01	17 6 ± 1 08	4 21±0 71	--
02	44 0± 1 15	14.4±0 96	9.84±0 81
04	64 72± 99	34±0 97	24.52±1 02
06	77 3±1 24	45 7±1 11	32 09±1 16
08	94 0±1 30	52±1 23	41.88±0 97
10	--	57.7±1 45	47 2±0 99
12	--	67 3±1.30	58 71±1 21
24	--	95 2±1.72	84 9±1 26
36	--	--	94 07±1 00

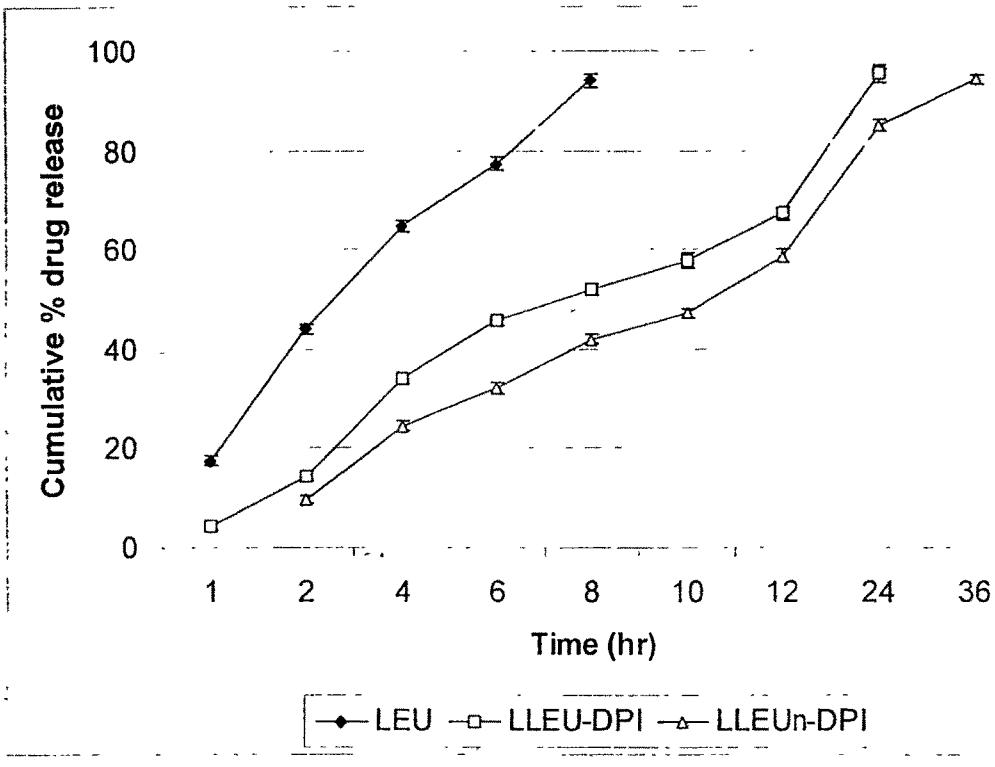


Figure 6.6: Cumulative % LEU diffused during In vitro diffusion studies

Table 6.12: Regression Coefficients of LEU Formulations by different models

Formulations	Zero-order equation	Higuchi's equation	First-order equation
	R^2	R^2	R^2
LEU	0.9421	0.9818	0 g916
LLEU-DPI	0.9013	0.9888	0.5g0g
LLEUn-DPI	0 9051	0.9g0g	0 508

Table 6.13: Mean Flux and Diffusion Coefficient values of LEU formulations

	LEU	LLEU-DPI	LLEUn-DPI
Mean Flux	3.45	1 35	1.11
Diffusion Coefficient	7.72E-09	2 3806E-09	2.0812E-09

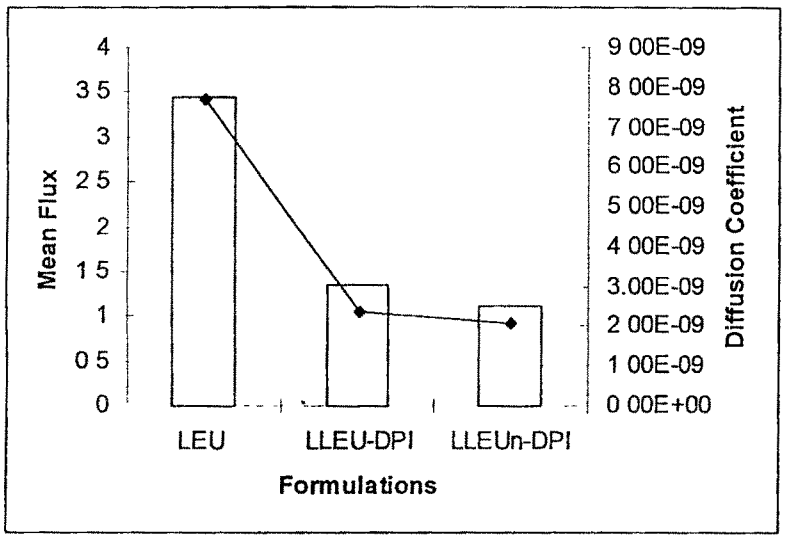


Figure 6.7: Mean Flux and Diffusion Coefficient values of LEU formulations

LEU FORMULATIONS

Comparative diffusion studies were carried out between various LEU formulations (LEU, LLEU-DPI and LLEUn-DPI) for a period up to 36 hours using dialysis membrane in self designed and validated diffusion cell As the drug was soluble in PBS, sink conditions were maintained when 50 ml of the medium used as the diffusion medium and hence zero-order flux conditions not violated The results obtained are recorded in Table 6 11 and shown graphically in Figure 6 6

Figure 6.6 clearly shows that encapsulating LEU in liposomes substantially slowed down its release from release assay. The non-linearity of the graph suggests that the release pattern does not follow zero order kinetics However, when correlation coefficients for different kinetic models were compared (Table 6.12), highest correlation (R^2 = 0.97-0.99) by Higuchi's equation obtained suggesting that the release obeys Higuchi's diffusion controlled model

Mean flux values of various LEU formulations were calculated and recorded in Table 6.13 and shown graphically in Figure 6.7 The diffusion co-efficient of different LEU-DPI formulations were also calculated and recorded in Table 6 13 and its graphical presentation is given in Figure 6 7 The mean flux values of the LLEU-DPI and LLEUn-DPI formulations are found to be three to four times higher than those of liposomal formulations, indicating that liposomal formulations are potentially sustaining the drug release.

On comparing the individual formulations it was found that the flux values were depend on the charge of liposomes as shown in Figure 6.7 The reduction in mean flux and diffusion coefficient values of negatively charged liposomes (LLEUn-DPI) were may be due to the attractive forces between negatively charged lipid (DCP) and positively charged LEU.

Assuming that the permeability of artificial membrane remains constant, the diffusion coefficient is ultimately governed by the concentration of free drug in donor

compartment, which intern depends upon the rate of drug diffusion from liposomes Thus there are two rate controlling barriers acting on the drug diffusion to the receptor compartment, one is the liposomal membrane and other is the artificial membrane The artificial membrane acts as a physical barrier preventing liposomes to enter into the sampling port and is not regulating the drug diffusion to receptor compartment

IN VIVO STUDIES

LN Formulations

LLN-DPI (after rehydration with water), LN PM or LN suspension containing 10-µg LN were administered intratracheally in three different group of rats Similarly, 10-µg of LN suspension was administered orally Blood samples were collected at specific time points and plasma LN concentrations were estimated by spectrofluorometric method The data of drug plasma concentration are shown in Figure 6 8 Various pharmacokinetic parameters. (Tmax. Cmax, and $t_{1/2}$) were calculated from the Figure 6 8 and recorded in Table 6 14 AUC and bioavailability (F*) were also calculated and are recorded in Table 6 14.

The AUC following oral and intratracheal administration of formulations were found to be significantly different ($p<0$ 05) However, no significant difference ($p>0.05$) was observed in AUC after intratracheal administration of these formulations The F* values after pulmonary administration were 97 6%, 109.88%, and 98 55% for LN, LN PM and LLN-DPI formulations, respectively The Tmax for these formulations were found to be 6.0, 6 8, and 7 0 hours for pulmonary administration with the Cmax of 4.40ng/ml. 4.42ng/ml, and 4.20ng/mL, respectively, followed by a plateau up to 48 hours, while for oral administration of LN suspension, Tmax and Cmax were 2.1 hours and 14 4 ng/mL, respectively The rate and extent of lung uptake depend on drug physicochemical properties such as degree of ionization and lipophilicity (Anderson et al, 1974, Suhara et al., 1998; Roerig et al., 1989; Jorfeldt et al., 1979; Dollery and Junod, 1976) Pulmonary delivery of all 3 formulations resulted in similar pharmacokinetic behavior because of the similarity in lipophilicity and size of the drug and liposomes.

Table 6.14: Pharmacokinetics of different formulations of LN following oral and intratracheal administration in rats

Formulation	AUC (ng-h/mL)	F*	Tmax (hours)	Cmax (ng/mL)	T1/2 (hours)
LN (oral)	261.41 ± 12.36	–	2.1 ± 0.2	14.4± 0.6	16.9 ± 0.2
LN (i.t.)	255.16 ± 9.87	97.6 ± 1.2	6.0 ± 0.2	4.4± 0.4	61.2 ± 0.2
LN PM (i.t.)	257.63 ± 10.15	98.6 ± 1.4	7.0 ± 0.2	4.2± 0.5	61.4 ± 0.2
LLN-DPI (i.t.)	287.24 ± 11.29	109.9 ± 1.4	6.8 ± 0.2	4.4± 0.6	64.4 ± 0.2

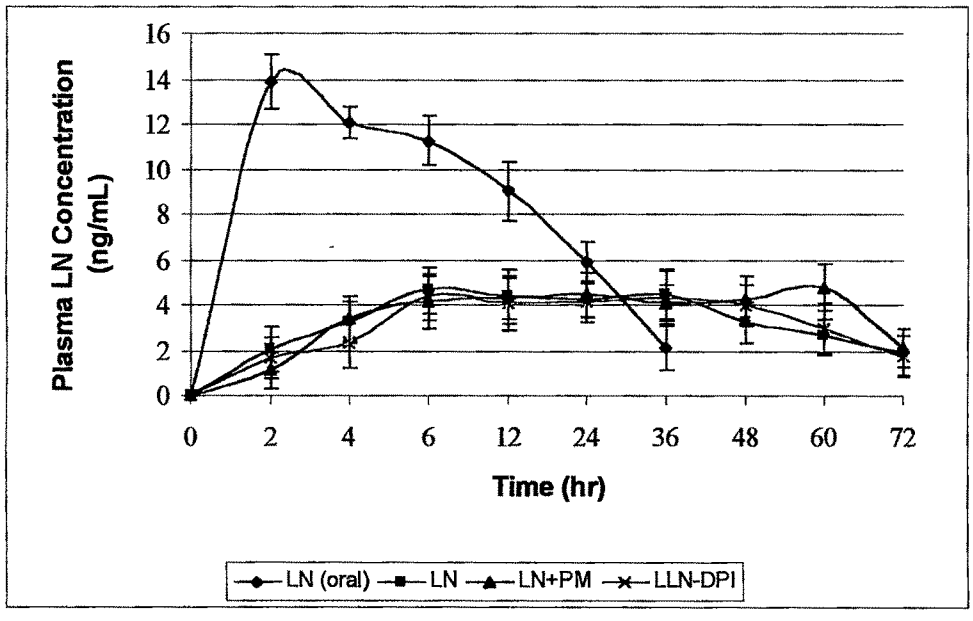


Figure 6.8. Plasma LN concentration profile after intratracheal and oral administration of LN formulations

Table 6.15: Pharmacodynamic parameters of LEU formulations following subcutaneous (s.c.) and intratracheal (i.t.) administration

Formulation	AUC (ng-h/mL)	F* (%)	Tmax (hours)	Cmax (ng/mL)	$T_{1/2}$ (hours)
LEU (s.c.)	720.5 ± 78.21	--	1.2 ± 0.2	263 ± 0.2	3.1 ± 0.2
LEU (i.t.)	78.5 ± 8.14	12.98 ± 1.5	1.0 ± 0.4	27 ± 0.4	2.3 ± 0.4
LEU PM (i.t.)	79 ± 11.04	17.35 ± 1.4	1.0 ± 0.5	27 ± 0.5	2.6 ± 0.3
LLEU-DPI (i.t.)	153.5 ± 8.11	44.27 ± 1.6	2.0 ± 0.3	47 ± 0.3	4.2 ± 0.5
LLEUn-DPI (i.t.)	200.5 ± 7.36	48.23 ± 1.1	2.0 ± 0.4	59 ± 0.4	4.5 ± 0.4

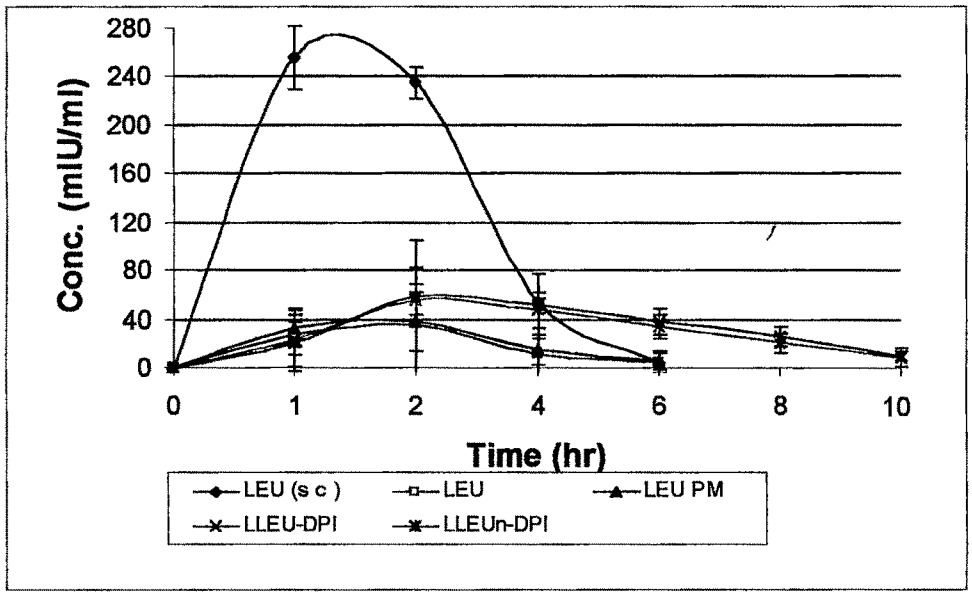


Figure 6.9: LH release profile after intratracheal and subcutaneous administration of LEU formulations

Following oral drug delivery, Cmax of 14 4 ng/mL was followed by decline in plasma concentration with t1/2 of 16 9 hours. In contrast, pulmonary delivery gave effective plasma drug concentration for the period of 56 to 60 hours with the zero-order release kinetics following Cmax of 4 40, 4 42, and 4 20 ng/mL for LN, LN PM, and LLN-DPI formulations, respectively Solubilization and diffusion of the drug and drug from liposomes into alveolar fluid before absorption into systemic circulation through transcellular uptake may also responsible for prolonged and zero-order absorption of LN (up to 60 ± 2 hours) It has also been reported that liposomally encapsulated drug remains in the lung for a prolonged period of time (Juliano and McCullogh, 1980) Slow and prolonged absorption of the drug after pulmonary delivery significantly reduces Cmax and is also expected to reduce dose-dependent progestronic side effects associated with orally administered LN

LN, an orally active progestronic derivative, is associated with various side effects possibly due to the initial very high plasma concentration (Cmax) achieved, which is significantly higher than the therapeutic window of the drug (active therapeutic window, 4-6 ng/mL) Pulmonary dosage forms, however, give an extended release of the drug over a long period of time without resulting in initial higher plasma concentrations. This may further reduce the frequency of dose administration While more work is needed to extrapolate these findings to better contraceptive efficacy by pulmonary route, the present study clearly indicates the important role of this route as an alternative to oral administration with regards to sustainability, and slow zero-order release kinetics may help in reduction of various side effects of oral contraceptives

LEU FORMULATIONS

The plasma bioactivities of the LEU were observed after intratracheal and s c. administration of various formulations. Drug dose of 5 µg was administered by both the route. Blood samples were collected at specific time intervals and serum LH concentrations were estimated by specific radioimmunoassay and plotted in Figure 6 9 Various pharmacokinetic parameters, (Tmax, Cmax, and $t_{1/2}$) were calculated from the Figure 6.9 and recorded in Table 6.15. AUC and bioavailability (F*) were also calculated and are recorded in Table 6 15

In a group of animals, control formulation was administered and serum LH level was monitored LH levels were found to be very low (around 2 mIU/ml) In LEU treated animals, regardless of the route of administration and formulations of LEU, serum LH concentrations transiently rose to peak at 1 h-3 h then decreased gradually to the pretreatment level within 24 h The highest Cmax value of 263 mIU/mL was obtained after s.c. administration Lower Cmax values of 27 ± 0 4 mIU/ml, 27 ± 0 5 mIU/ml, 47 ± 0.3 mIU/ml, and 59 ± 0 4 mIU/ml respectively for LEU, LEU PM, LLEU and LLEUn formulations were obtained after intratracheal instillation However, when relative bioactivity of intratracheally administered formulations were compared LLEU and LLEUn formulations showed significantly higher bioactivity, i e ,44 27 ± 1 6 % and 48.23 ± 1.1 % for LLEU and LLEUn formulations respectively compared to LEU and LEU PM (12 98 ± 1 5 % and 17 35 ± 1.4 % respectively Almost 50% relative bioactivity compared to presently available parenteral route (s c) achieved with developed liposomal formulations This confirms the role of liposomes in enhancement of drug permeation through alveolar epithelium by altering physicochemical properties of the drug (renders the drug hydrophobic). Liposomes are also serving as a biodegradable pulmonary reservoir with prolonged pulmonary residence times. They may also decrease the mucociliary clearance due to their surface viscosity

The developed liposomal DPI of LEU demonstrated almost 50% bioactivity was achieved through pulmonary route compared to subcutaneous route infers that pulmonary route can be an alternative to presently available subcutaneous route by just doubling the dose Double dose can be justified by patient compliance, self medication and avoiding the complications related to injection procedure The developed formulations of LEU with improved bioactivity can also be useful for treatment of prostate cancer in men, early puberty in children and for ovarian, endometrial, pancreas, and breast cancer, endometriosis, Uterine Leiomyoma, anemia due to uterine fibroid tumors in women. Before findings of this investigation can be commercially realized, the detailed pharmacokinetic and pharmacodynamic studies in one species of animal and clinical investigations with special emphasis on side effects are to be accomplished for success in market.

Different portions of the bronchopulmonary tree possess different characteristics, it is possible that drug release from liposomes is affected by the distribution of formulation achieved during administration and later altered by mucociliary transport and other mechanisms Animal studies to date have utilized instillation of liquid formulations in order to obtain accurate dosimetry (Shek and Jurima-Romet,1990. Juliano and McCullogh, 1980) Such results are dependent upon the spreading of the instilled dose within the lung and then interpretation may be complicated by the presence of components capable of affecting the spreading process The distribution and absorption of inhaled aerosols in the lungs and airways are different from those of instilled liquids, (Brown and Schanker,1983, Schanker et al . 1986, Brain et al., 1976) and it is possible that release kinetics of drug from instilled formulations in animals and from inhaled aerosols may be significantly different In addition, the size and aerodynamic behavior of the powder through human airways may result in a significantly different distribution and rehydration of aerosolized liposomes compared with rodent test systems, which may affect observed release kinetics, duration, onset. and intensity of effect.

Comparisions:

Cryoprotectant effects of different sugars on developed liposomal formulations of both the drugs were also evaluated. Data revealed that trehalose and sucrose gave the highest percent drug retained in liposomes, 89 4 ± 1.9 percent and 88 3 ± 2 3 percent respectively for LLN, 37 4 ± 1.9 percent and 38 3 ± 2 3 percent respectively for LLEU and 39.15 ± 1 9 percent and 36.99 ± 2 3 percent respectively for LLEUn. When further optimized with sucrose by varying concentration, sequence of addition and effect of diluent (Pharmatose or Sorbolac) addition LLN showed significantly higher perseverance during all the stages compared to that of LLEU and LLEUn formulations. This is may be due to the difference in hydrophobicity of the drugs.

The FPF value for the optimized formulations LN16 and LEU25 (22.5 ± 2.2 and 29.8 ± 1.9 respectively), significantly higher ($p<0.05$) values were observe for LEU25 compared to LN16 may be due the difference in Tg of lipid used in preparation of LN

and LEU liposomes Egg PC has a lower Tg, aggregation tendency due to their sticking tendency at room temperature may resulted into lower FPF

When drug retention study data for LN and LEU formulations were compared, LEU formulations showed higher PDR at all the sampling time points at all the storage conditions. This may be due to the Tg of the phospholipids used for the preparation of these liposomes The higher Tg of HSPC used in the preparation of LEU liposomes compared lower Tg of PC resulted into higher stability of LEU liposomes.

In vitro drug diffusion studies were also carried out for LN and LEU formulations Different diffusion medium were used as LN and LEU being lipophilic and hydrophilic in nature hydro-alcoholic medium was used for LN and PBS was used for LEU to maintain the flux and sink conditions.

In vivo studies were carried out in rats for LN and LEU formulations. Developed formulations were given intratracheally in comparison to their presently available route of administration (oral for LN and s c for LEU) Pulmonary delivery of all LN formulations (LN formulation, LN physical mixture and LLN formulation) resulted in similar pharmacokinetic behavior because of the similarity in lipophilicity and size of the drug and liposomes Plain LN formulation showed 100% relative bioavailability compared to that of orally administered drug However, LEU formulation only showed 13% relative bioactivity to that of s.c administered LEU This may be due the higher partition coefficient of the LN compared to that of LEU formulation

Conclusions

Stable liposomal DPI formulations of the drugs for both the routes of administration were developed and optimized with regard to percent drug retained after lyophilization. In vivo studies (pharmacokinetics) in rats were carried out followed by in vitro diffusion studies to create in vitro testing procedures. Slow and prolonged absorption of the drug after pulmonary delivery significantly reduces Cmax and is also expected to reduce dose-dependent progestronic side effects associated with orally administered LN However, liposomal encapsulation of LN did not result into

any improvement in terms of bioavailability/duration of action over plain drug formulation after pulmonary administration Similar lipophilicity of the drug and the liposomally encapsulated drug may be responsible for similar pharmacokinetic/pharmacodynamic behavior of the formulations of plain and liposomally encapsulated LN formulations The developed liposomal DPI of LEU demonstrated almost 50% bioactivity was achieved through pulmonary route compared to subcutaneous route infers that pulmonary route can be an alternative to presently available subcutaneous route by just doubling the dose. Double dose can be justified by patient compliance, self medication and avoiding the complications related to injection procedure

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