Chapter 7

SUMMARY & CONCLUSION

Finding an effective, safe, acceptable, reversible and preferably long-acting, hormonal contraceptive methods are research priority for WHO and other health organizations Although there are currently no systemic methods of contraception for use by men, the development of a male-use equivalent of oral, injectable and implantable female steroid hormone methods of contraception has been the subject of research for the past 30 years or more A number of studies in animals and men have shown that the administration of androgens alone, androgen and progestin combinations, and combinations of androgens with gonadotrophin-releasing hormone receptor ligands can suppress gonadotrophin secretion and thereby reduce spermatogenesis to render men infertile

Also, in case of female contraception, all oral contraceptive drugs interference with the production and action of endogenously synthesized steroid hormones Also induction of hepatic enzymes by oral contraceptives may interfere with potency and duration of other medications such as anticoagulants, antibiotics, or anticonvulsant drugs In addition, orally administered steroids interfere to different degrees with hepatic protein synthesis of procoagulatory and fibrinolytic proteins and fatty liver as a consequence of long-term treatment It is also likely that factors originating from or due to hepatic metabolism of exogenous steroids play a role in hypertension and dyslipidaemia, side effects frequently observed with oral contraceptive treatment. Therefore there is a need of development of female contraceptives other than presently available oral contraception without compromise of safety and user compliance

New materials and new technologies have stimulated pharmaceutical researchers to identify and use alternatives to the classical oral and injectable routes One of the routes currently being studied is the nasal way. Today, nasal drug delivery is receiving much attention from the pharmaceutical industries. However, the mucociliary clearance under normal conditions rapidly clears the applied material since there is a little time of contact between the drug and the mucosa. Therefore, employing nasal delivery for prolonged release required the development of particular strategies in order to keep the substance on the mucosa for a long time without altering the functionality of the nose. Prolonging the contact of the drug with the absorptive surfaces by means of an appropriate delivery system can increase the bioavailability of intranasally administered drug. Weakly cross linked polyacrylates as

carbomers (which are FDA approved) by Ca^{2+} complexation are able to trigger the reversible opening of the tight-junction between the cells and to allow the paracellular transport of peptides. Chitosans have been shown to have similar properties to reversibly open the tight junctions This mechanism is thought to occur by ionic charge transfer between the positive charge of chitosan molecule and the negative charges (sulfate and sialine groups) of the glycocalix.

The pulmonary route is also being used for the effective delivery of drugs into the systemic circulation For a long time, the lung has been used for the administration of drugs for the treatment of local conditions However, more recently, spurred on by the advent of novel delivery devices, there is a growing interest in the use of the lung for the systemic delivery of challenging molecules, such as peptides and proteins, as well as analgesic agents and even vaccines The larger surface area of the lung is well known, although, interestingly, the permeability of the lung tissue in itself is not that different from other mucosal surfaces, it is the large area that provides for the rapid absorption The challenging aspect still remains unanswered are the mode of delivery for liposomally encapsulated drug to lungs Metered dose inhalers (MDI) are currently being reformulated as a result of the ban being implemented throughout the world by the United Nations on the use of chloro-fluoro carbons To meet this challenge, one such alternative is the development of new and improved "Dry Powder Inhaler (DPI)" system that will allow inhalants administration of all drugs presently delivered with MDIs.

Liposomes are attracting considerable interest in the area of drug delivery because of their biocompatible, biodegradable and relatively nontoxic nature Liposomes are also known to sustain the release of the entrapped drug(s) and to decrease the mucociliary clearance of the drug(s) due to their surface viscosity. Therefore, more effective and sustained systemic absorption of a drug would be attained by administering the drug containing liposomes in respiratory tract As a drug delivery system, liposomes can significantly alter the pharmacokinetics and pharmacodynamics of entrapped drugs, for example, by enhancing drug uptake, delaying rapid drug clearance, and reducing drug toxicity.

LN has been used for many years both alone (in low doses) in the POP and in combination with estrogen in COC preparations. There have been many fewer studies on the safety of long-term use of the POP than of the COC, but the existing data are

largely reassuring Unlike steroid hormones, gonadotropin-releasing hormone exerts specific action on the pituitary gonadotrophs and the human reproductive tract This specificity reduces the likelihood of secondary adverse effects such as gynecomastia, thromboembolism, edema, liver and gallbladder involvement Although clinical application of these peptides is highly promising, their potential may be restricted by difficulties involved in self-medication

The aim of these investigations was to develop nasal and pulmonary drug delivery systems for effective contraception both in males and females A second generation steroidal contraceptive and a progesterone derivative, LN, and a peptide based potent contraceptive and a gonadotropin releasing hormone agonist, LEU, were selected as drugs for incorporation into nasal and pulmonary drug delivery systems Objectives of these investigations were to develop effective drug delivery systems of selected drugs for nasal and pulmonary administration In development of pharmaceutically rational drug-delivery systems for nasal and pulmonary administration, liposomal encapsulation and/or mucoadhesion of the delivery systems were the techniques used for maximizing the therapeutic index, reducing the dose/ frequency of dosing, and systemic side-effects and, thereby, reducing the cost of therapy

EXPERIMENTAL

The drug content and the excipients of liposomes were analyzed by the reported analytical methods with suitable modification whenever necessary to meet the requirement of this investigation. The methods were standardized for the estimation of drugs (LN and LEU) under study, PC and CHOL content.

Calibration curve of LN was prepared by spectrophotometric method where LN gives yellow color with INH reagent. The method was found to be sensitive between 0-10 µg/ml. Calibration curve of LEU was prepared by spectrophotometric method where LEU gives λmax at 240 nm IN 0.1N NaOH and the method was found to be sensitive between 0-10 µg/ml The analyses of LN and LEU in plasma were carried out by spectroflorimetric method and radioimmuno assay respectively. The methods were found to be sensitive between 200-1000 pg/ml and 5-100 mIU/ml respectively. The ability of PC to form a red colored complex with ferrothiocyanate in organic solutions was used to estimate PC. The method was found to be sensitive between 100-700 µg/3 ml concentration of PC in chloroform. Complexation of CHOL with ferric

chloride and sulphuric acid was the basis of the colorimetric method used for estimation of CHOL The method was found to be sensitive between 10-100 µg/ml of CHOL in glacial acetic acid Absorbance of the standard solutions were measured at the absorption maxima and plotted graphically to get calibration curves Regression analysis of the data proved the linearity of the plots in the concentration range used The interference of formulation components were checked by measuring the absorbance/peak area of the maximum concentrations at the corresponding wavelength of drug

Liposomes encapsulated LN and LEU were prepared by TFH and REV methods techniques TFH method involves co-precipitation of lipid and drug to a thin film, by solvent stripping followed by hydration to get liposome dispersion, while REV method involves emulsification of organic and aqueous phase containing lipid and drug followed by evaporation of organic phase under vacuum The optimization of various formulation and process variables namely, drug lipid ratio, PC CHOL ratio, sonication time/extrusion cycles, solvent mixture, hydration, vacuum and method of purification were studied during the preparation of liposomal dispersion for both the methods

For TFH method, optimized process variables i e., solvent system; Chloroform.Methanol (1 2), temperature; 30°C, speed of rotary flask,120 rpm, vacuum: 500 mmHg and 0 5 ml distilled water as hydration medium and hydration time of 2 hours were used during the preparation of liposomes. LN:PC CHOL ratios were altered and PDE was studied after separation of free drug by ultracentrifugation The PDE in liposomal batches were further optimized by subjecting them to three F-T cycles at freezing temperature of -40°C for 2 hours followed by thawing at room temperature for 15 min The prepared liposomes were characterized with respect to size, shape and lamellarity and drug entrapment efficiency. Significant improvement in PDE was observed after exposure of liposomes to F-T cycles Significant variations 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 Similarly, no significant ($p>0.05$) change in vesicle size after freeze-thaw cycle was observed. Optimized LN:PC:CHOL ratio (1:4:1) shows PDE value of 76 275±3 15 and 97 98±0.29, D[4,3]; 15.1±0.2 before and 15.4±0.1 after freeze thaw cycles.

For REV method, 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 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 Efforts were made to optimize aqueous to organic phase ratio and was found optimum at Diethyl ether water, 1.5 (2.5 ml·0 5 ml). LN:PC.CHOL ratios were altered and PDE was studied after separation of free drug by ultracentrifugation The PDE in liposomal batches were further optimized by subjecting them to F-T cycles at freezing temperature of -40 ℃ for 2 hours followed by thawing at room temperature for 15 min. The prepared liposomes were characterized with respect to size, shape and lamellarity and drug entrapment efficiency. Significantly higher PDE values were observed in all the batches prepared by REV method compared to that of TFH method Significant improvement in PDE was observed after exposure of liposomes to F-T cycles Significant variations 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 Similarly, no significant ($p>0.05$) change in vesicle size after freeze-thaw cycle was observed Optimized LN·PC.CHOL ratio (1 4.1) shows PDE value of 88.789±3.00, 99.01±0.44, and volume mean diameter (D[4,3]), 12.8±0 2, 12.8±0.2 before and after F-T cycles respectively Less number of F-T cycles were necessary to attain equilibrium for PDE for REV method when compared to TFH method and hence REV method was used to prepare further liposomal batches. Size reduction of LLN was carried out using sonication and twenty minutes sonication time was found to be optimum with regard to required size distribution (PDE; 72.06 ± 1.15, D[4,3]; 2.5 ± 0.11). Post sonication freeze thaw cycles helps in regaining the equilibrium with the leaked drug (PDE; 98.3 ± 0.21, D [4,3], 2.5 ± 0.01).

In case of LEU, efforts were made to maximize the PDE by favoring lipid-protein interactions. The method of sequential analysis was adopted to optimize liposomal encapsulation. LLEU were initially prepared by the REV method and efforts were

made to get maximum PDE by varying various formulation and process parameters 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 prepared liposomes were characterized with respect to size, shape and lamellarity and drug entrapment efficiency LEU.HSPC.CHOL ratio was 1:15·5 with optimum solvent ratio of Chlorform methanol water 2 4.1 The vacuum and temperature were kept 400 mm Hg and 55°C respectively. Maximum PDE was observed when solvent system was evaporated to dryness with a value of 91.4±1.5%, 96.5±1 3% for neutral and negative charged liposomes respectively for REV method Positively charged liposomes results in to low PDE and they were unstable in nature This may be due to the interaction/incompatibility with positively charge amino acids present on the LEU. The optimized LLEU by REV method 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 All the batches of the liposomes prepared were viewed under Olympus (BX 40F4, Japan) microscope with polarizing attachment to study their shape and lamellarity Polarizing microscopy confirmed the multilamellarity. identified by the presence of Maltese crosses. The vesicle size was determined by laser light scattering technique using Mastersizer (Malvern Instruments Ltd., UK) Prepared liposomes were having the mean vesicle size (11.5 ± 0.01 for neutral and 10.71± 0.01 for negative charged liposomes).

Size reduction of both neutral liposomes (LLEU) and negatively charged liposomes (LLEUn) were carried out by extrusion at 55°C through 2 µm polycarbonate membranes (Whatmann, USA) to a reproducible mean liposomal size below 5µm. Minimum of three times extrusion was necessary to obtain mean liposome size below 5µm for both LLEU and LLEUn (D [4,3], 3.42±0.12 and 4.14±0.10 respectively). Five to six percent drug leakages observed in both the liposomal batches were

unavoidable The extrusions were carried out at 55°C as the HSPC liposomes remains in fluid state at this temperature

Formulations for Nasal Administration:

Plain drug/liposomes encapsulated LN and LEU, their solution/suspension with and/or without CS/CP hydrogels were prepared, characterized and investigated for in vitro diffusion studies, and comparative pharmacokinetic and antifertility performance studies on rats Various parameters like pH, viscosity, mucoadhesion, in vitro diffusion studies and in vivo studies were evaluated Liposomal formulations were also subjected to stability studies

For preparation of LN formulations, LN was weighed accurately and dispersed in double distilled water and sonicated for approximately 1 hour to get particle size in range of 10-15 micron The resulting suspension was further diluted either with the equal volume of 1% CS solution (in 0.01% acetic acid) or 1% CP solution (in double distilled water) The resulting mixture was mixed well and stored in amber colored glass vial in refrigerator till use Optimized LN liposomal batches (without size reduction) were also used for further studies

For preparation of plain LEU formulation and LLEU+CS formulation, either 500 µg of drug in acetate buffer pH 5.2 or LEU liposomes containing 1mg of drug was diluted with equal volume of 1% chitosan solution (acetate buffer pH 5 2) respectively. The resulting solution or suspension was mixed well and stored at refrigerator till required Optimized LLEU and LLEUn liposomal batches (without size extrusion) were also used for further studies.

pH of the LN+CS and LN+CP suspensions were 3.8-3.9 and other LN formulations having pH of 6.5 to 7.5. All LEU formulations were having pH of 5.2. The pH of all the formulations makes them well tolerated Viscosities of various formulations were also determined by Oswald Viscometer and were found to be satisfactory. The mucoadhesion test was also performed in selected formulations. Liposomes (LLEU) and drug particles (LN) in CP and/or CS hydrogel exhibited good mucoadhesive properties in the *in vitro* wash-off test when compared to liposomes (LLN, LLEU and LLEUn) and drug particles (LN) alone. LN+CS showed better mucoadhesion property compared to formulation with LN+CP. This might be due to the strong interactions between the positively charged CS and negatively charged nasal mucosa When

mucoadhesion of LLEU was compared to that of LLN. higher mucoadhesion was observed This may be due to the ionic interactions between LEU induced positive charges in liposomes and negatively charged nasal mucosa

The drug retention studies were carried out at refrigerated temperature (2-8°C) and room temperature (25±2°C) for the LLN stored in sealed glass vials LLN batches were evaluated for PDE in liposomes. Drug retention studies at 25±2°C indicate that about 10% of drug was leaked after storage for the three months from LLN and therefore were discontinued afterwards at this condition LLN stored at refrigerated condition was found to be stable with regard to percent drug retention for 6 months stability period The size of liposomes was also determined immediately and after 3 and 6 months storage at refrigerated conditions The D [4,3] was increased insignificantly ($p>0.05$) after storage at refrigeration temperature up to 6 months

Similarly, liposomal batches of LEU (LLEU), negatively charged liposomes (LLEUn) and liposomal formulation with CS (LLEU+CS) were also subjected to drug retention studies at refrigerated temperature (2-8°C) and room temperature (25±2°C) stored in sealed glass vial. Stability studies at 25±2°C indicate that about 6-7% of drug was leaked after storage for the three months from LLEU and LLEUn. therefore stability studies were discontinued afterwards for this product at this condition However, LLEU and LLEUn batches were found to be stable over 6 months stability period with regard to percentage drug retained under refrigerated conditions (less than 5%) The D [4,3] was increased insignificantly ($p>0.05$) after storage at refrigeration temperature during the stability period of 6 months After incorporation of CS in LLEU formulations were found to result into significant ($p<0.05$) improvement with regards to percent drug retention. LLEU+CS formulations were found to be stable for 3 months at 25±2°C compared to two months stability of LEU formulations. The values of PDR were significantly higher at all sampling points at both the conditions for LLEU+CS formulations compared to LLEU formulations The increase in stability of liposomes after CS incorporation may be due to the repulsive forces between positively charged and positive charged LEU may prevent the drug leakage from liposomes The increase in viscosity also contributed to increase in liposomal stability.

As obvious, when compared between the batches stored at lower temperature (refrigerator) compared with the one stored at higher temperature (controlled room temperature), batches at lower temperatures showed higher preservation When

stability data for LN and LEU formulations were compared, LEU formulations showed higher PDR 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

Comparative diffusion studies were carried out between various LN formulations for a period upto 24 hr across dialysis membrane using validated vertical in vitro diffusion set-up The diffusion medium (20% methanolic PBS) was analyzed at specific time intervals upto 24 hrs for its drug content The mean cumulative amount of drug released and the mean flux values across the dialysis membrane were calculated at each sampling time points Regression coefficients by different release kinetic models suggest that drug release from formulations followed the Higuchi's diffusion model The mean flux values and diffusion coefficients of the LN formulation were found to be two to three times higher than those of liposomal formulations, indicating that liposomal formulations are potentially sustaining the drug release Lower mean diffusion flux and diffusion coefficients values for LN+CS and LN+CP compared to that of plain drug formulation of LN may be due to the increase in viscosity of the formulations Gel formation takes place at the contact points of CP containing formulation with the membrane diffusion medium pH of 7.4 contributing to further lowering of mean flux and diffusion coefficient values

Comparative diffusion studies were also carried out between various LEU formulations across dialysis membrane using validated diffusion cell and the results were compared The diffusion medium (PBS) was analyzed at specific time intervals upto 36 hours for its drug content The mean cumulative amount of drug released across the dialysis membrane was calculated at each sampling time points and the mean flux values were calculated. Regression coefficients by different release kinetic models suggested that the release follows the pattern delineated in Higuchi's diffusion controlled model. The mean flux and diffusion coefficient values of the LEU formulation were found to be two to three times higher than those of liposomal formulations, indicating that liposomal formulations are potentially sustaining the drug release. The further lowering in mean flux and diffusion coefficients value for LLEUn were may be due to the attractive forces between negatively charged lipid (DCP) and positively charged LEU. The increase in viscosity after addition of CS to

LLEU and also repulsive forces between positive charge of CS molecule and positively charged LEU may be responsible for slow diffusion of the drug from the liposomes compared to LLEU

***In vivo* studies**

Rat was used as an animal model as it is commonly used animal model for screening of anti-fertility agents

LN Formulations:

Size of the drug particles and liposomes in all the formulations was kept between 10-15 μm, as the particles with 10-20 μm are all deposited in the nasal cavity, whereas particles smaller than 1 μm pass with inspired air into the lungs LLN, LN PM or LN suspension containing 10-μg LN were administered intranasally in three different group of rats Similarly, one group of animals were treated with 10-μg of LN suspension was administered orally Blood samples were collected at specific time points and plasma LN concentrations were estimated. The drug plasma concentration at each sampling time point were plotted against time in hr Various pharmacokinetic parameters (C_{max}, Tmax and $t_{1/2}$) were determined from drug plasma concentration-time curve Plasma levels of LN after a single oral dose indicate that a considerably higher level of LN (C_{max} 14 4 ng/mL) occurs with T_{max} of 2.1 h as compared to LN suspension, LN PM and LLN formulations given intranasally (C_{max} 7.13 ng/mL, 6 1 ng/mL, 5 24 ng/mL) at T_{max} of 4 2 h, 4 6 h and 4.6 h respectively Levels of LN fall precipitously to levels-below 1 ng/mL in all the cases LN suspension, LN PM and LLN formulations were having significantly less bioavailability (25-32%) The mucociliary clearance under normal conditions rapidly clears the applied material since there is a little time of contact between the drug and the mucosa When the drug was formulated with mucoadhesive agents, CS (LN+CS) and CP (LN+CP), significant improvement in F* of drug were observed (101 70% and 99 42% respectively) Plasma half lives ($t_{1/2}$) were also significantly increased from 7.0 hr to 55.7 hr and 52.9 hr, having the T_{max} of 4 4 hr and 5.0 hr and C_{max} of 4.73 ng/mL and 4.70 ng/mL respectively for LN+CS and LN+CP formulations. Prolonging the contact time of the drug with the absorptive surfaces by means of appropriate mucoadhesive agent contributed to increase in the F* of intranasally administered drug The clearance of administered drug was delayed by using mucoadhesive polymers such as

CS and CP CS acts by opening tight junction between epithelial cells It may also enhance the absorption of drugs by being a useful bioadhesive and slowing mucociliary transport Carbopol hydrogel is a thin liquid at acidic pH but it gels at physiological pH and thus has great potential for nasal delivery of drugs When the $t_{1/2}$ value of orally administered formulation was compared to nasally administered mucoadhesive formulations, significant increases in $t_{1/2}$ were observed (16 9 h to 52.9 h-55.7 h) The results clearly indicate that the dosing interval can be changed to once in two days from daily oral administration without changing the dose. The reduction in the drug dose and maintenance of therapeutic concentration in blood plasma of the drug for at least up to the 48 h is expected to reduce the reported side effects in humans and probably the cost of the therapy due to lower dose Pharmacokinetic studies were followed by pharmacodynamic studies, where the animals were administered with different formulations intranasally for four weeks and allowed for mating during the treatment period Animals when treated nasally with LN suspension, LN+PM and LLN were failed to show contraceptive efficacy, may be due to short plasma half lives of the drug However, in case of LN+CS and LN+CP cent percent anti-fertility was observed even formulations were administered on alternate days. These results are in agreement with pharmacokinetics, which further confirms the contraceptive efficacy of proposed formulations for prolonged period of time.

To investigate the contraceptive activity, LEU solution, LEU PM, LLEU and LLEU containing 5-µg LEU were administered intranasally. Similarly, 5-µg LEU solution was administered through s.c route Blood samples were collected at specific time intervals and plasma LH concentrations were estimated by specific radioimmunoassay Various parameters for LH release in plasma were calculated from LH plasma concentration Vs time plot. In LEU treated animals, regardless of the route of administration and formulations of LEU, serum LH concentrations transiently rose to peak at 1 h-2.1 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 mIU/ml, 27 mIU/ml, 59 mIU/ml and 47 mIU/ml for LEU solution, LEU PM, LLEU, and LLEUn formulations were obtained respectively after intranasal administration. Due to the mucociliary clearance of nasal cavity, nasally delivered formulations clear rapidly from site of absorption resulting into little contact time between the drug and the nasal mucosa and poor drug absorption When relative

bioactivities of nasally administered formulations were compared, LLEU and LLEUn showed higher relative percent bioactivity (F* 27 83% and 21 3% respectively) compared to LEU solution and LEU PM (F* 10 89% and 10 96% respectively) The relatively higher bioactivity of liposomal formulations compared to plain formulations may be due to their action on nasal mucosa by incorporating phospholipids in the membrane and opening "new pore" in the paracellular tight junction The prevalence of the repellent forces between negatively charged liposomes and negatively charged nasal mucosa may be responsible for low bioactivity of negatively charged liposomes compared to plain liposomes To enhance the residence time of the formulation and to impart mucoadhesion CS was incorporated into the formulations LLEU was selected as it showed significantly higher ($p < 0\ 05$) F* compared to other formulations LEU solution was also selected to see the effect of CS on bioactivity of plain drug For both LEU solution and LLEU formulations with 0 5% CS, marked increase ($p< 0\ 05$) in F* were observed (10 89 to 49 13% for LEU+CS and 27 83 to 88 90% for LLEU+CS) Significantly higher $t_{1/2}$ of 8 8-9 0 h was also observed in both the cases Prolonging the contact time of the drug with the absorptive surfaces by means of CS was contributed to increase in the F* of intranasally administered formulations. CS also acts by opening tight junction between epithelial cells The F* determines ultimate fate of the formulation in the body while, lower Cmax followed by plateau for prolonged period of time for LLEU+CS formulation may decrease the chances of concentration related side effects of the drug Intranasal administration of LLEU+CS showing comparable bioactivity to that of LEU solution administered subcutaneously (s c) was used for further studies. In male rats, sperm count and fertility performance studies were carried out for LEU solution administered s c. and LLEU+CS formulation administered nasally. Complete azoospermia was achieved in case of LLEU+CS formulation administered intranasally and LEU solution subcutaneously after 26 days treatment. Duration of treatment was kept to 26 days to cover two seminiferous cycles (13.2 x 2 days) in rats Females were mated with treated males and no implantation sites were observed in case of intranasal administration of LLEU+CS formulation and s.c. administration of LEU solution due to the azoospermic potential of both the formulations. In case of female rats, cyclicity was observed to evaluate fertility performance and cessation of estrous cycles was observed from the first treatment cycle in female rats treated with LEU solution and LLEU+CS through s.c. and nasal routes. Animals were return to normal cyclicity after

the cessation of the treatment Preliminary experiments conducted on rats have demonstrated the use of nasal administration of LLEU+CS developed in this investigation in producing contraception by treating male and female rats The results of the developed formulation were found to comparable to available parenteral dosage of LEU in producing contraception on rats

Formulations for Pulmonary Administrations (DPIs):

For the preparation of DPI formulations, lyophilization cycles were 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 The dispersion was frozen at -70°C and dried under negative displacement pressure (Heto Drywinner model DW1 0-60E, Denmark), both for 24 hrs The porous cake obtained was mixed either with Sorbolac 400 or Pharmatose 325 M and were sieved successively through #200 and #240 sieves to disaggregate particles. Capsules (size '2') were filled with individually weighed powder (10 mg) either containing 250 µg LN or LEU 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 aluminium foils and stored in a refrigerator (2– 8°C) until further use

The ability of various sugar namely, lactose, maltose, sucrose and dextrose to preserve the permeability barrier in dried vesicles were compared The amount of drug retained by the vesicles following dehydration and rehydration were determined Sucrose was found to be more effective with respect to PDE of liposomes The samples were sequentially diluted to produce mixtures with varying lipid sucrose mass ratio While changing the mass ratio of lipid.sucrose, the percent 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, LLEU and LLEUn liposomes 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. The effects of diluent's addition on percent drug retained were also carried out and it was found that diluent's addition after lyophilization leads to better percent drug retained in all the formulations.

Liposomal powder blends were characterized for density, dispersibility and powder flow using appropriate derived properties including, but not limited to, angle of repose,

bulk density, tapped density, compressibility index, dispersibility, moisture content and FPF The flowability and floodability expressed by angle of repose (26-32) and dispersibility (20-23) falls in the category of good and floodable The tapped density of formulations falls in the range of 0 33 to 0 37 g/cc The tapped 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 The low compressibility index and low moisture content were observed which reflects the less inter-particulate interactions and powders are free flowing in nature The *in vitro* deposition pattern of various liposomal dry powder formulations were made from a unit dose dry powder inhaler device (Rotahaler®) was investigated using Twin Impinger The fine particle fraction (FPF) values (22 to 38) for optimized formulations are suggestive of substantial deposition of the powder in the lower airways

The drug retention studies were carried out at refrigerated temperature (2-8°C), at room temperature (25±2°C) and at accelerated temperature (40±2°C) for the developed liposomal DPI formulations in HDPE bottles containing silica bags as desiccant Products were evaluated for PDE in liposomes at each sampling point 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 were observed at refrigerated and room temperature storage conditions.

LLEU products were also subjected to drug retention studies for 6 months. 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 (25±2°C). The size of liposomes was also determined immediately, after 3 months and 6 months storage period and no significant difference were observed at refrigerated and room temperature (25±2°C) storage conditions. When percent drug retention data for both the products were compared LLEUn-DPI formulations 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 the one stored at higher temperature (controlled room temperature), batches at lower temperatures showed higher preservation Also, dry state restricts the movement of liposomes and thus the aggregation and the leakage of the drug from the liposomes When stability data for LN and LEU formulations were compared, LEU formulations showed higher percentage drug retention 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 LLEU compared to lower Tg of PC used in the preparation of LLN resulted into higher stability of LLEU

Comparative diffusion studies were carried out between LN and LLN-DPI formulations for a period up to 24 hrs across dialysis membrane using validated vertical in vitro diffusion set-up. The diffusion medium (20% methanolic PBS) was analyzed at specific time intervals for up to 24 hrs for its drug content The mean cumulative amount of drug released and the mean flux values across the dialysis membrane were calculated at each sampling time points Regression coefficients by different release kinetic models were indicated that the drug release from formulations followed the simplified Higuchi's diffusion model. 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

Comparative diffusion studies were also carried out between LEU, LLEU-DPI and LLEUn-DPI formulations across dialysis membrane using validated diffusion cell and the results were compared The diffusion medium (PBS) was analyzed at specific time intervals for 36 hours for its drug content. The mean cumulative amount of drug released across the dialysis membrane was calculated at each sampling time points. Regression coefficients by different release kinetic models were indicated that the release follows the pattern delineated in Higuchi's diffusion controlled model. The mean flux and diffusion coefficient values of the LEU formulation were found to be three to four times higher than those of liposomal DPI formulations, indicating that

liposomal formulations are potentially sustaining the drug release The further reduction in mean flux and diffusion coefficients value for negatively charged liposomes were may be due to the attractive forces between negatively charged lipid (DCP and positively charged amino acids present in LEU)

***In vivo* studies**

LN, LN PM and LLN-DPI formulations 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 The drug plasma concentration at each sampling time points were plotted against time in hr Various pharmacokinetic parameters (Cmax, Tmax and $t_{1/2}$) were determined from drug plasma concentration-time curve The area under the plasma level curve was calculated by the trapezoidal rule 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 intratracheal administration were 97 6%, 109 88%, and 98.55% for LN, LN PM and LLN-DPI formulations, respectively. Following oral drug delivery, Cmax of 14 4 ng/mL was followed by decline in plasma concentration with $t_{1/2}$ of 16 9 hrs. In contrast, pulmonary delivery gave effective plasma drug concentration for the period of 56 to 60 hrs 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. The rate and extent of lung uptake depend on drug physicochemical properties such as degree of ionization and lipophilicity. Pulmonary delivery of all 3 formulations resulted in similar pharmacokinetic behavior because of the similarity in lipophilicity and size of the drug and liposomes 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.

The serum bioactivities of the LEU were observed after intratracheal and s.c. administration of various formulations. A drug dose of 5µg was administered by both the route Blood samples were collected at specific time intervals and plasma LH concentrations were estimated by specific radioimmunoassay and various parameters for LH release in blood (Cmax, Tmax, $T_{1/2}$, and F*) were obtained from drug plasma concentration-time curve. 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

Conclusion

Nasal and pulmonary routes have been most effectively used for drug delivery to general circulation and considerable interest is centered on the use of liposomal and mucoadhesive systems in the delivery of drugs and other biologically active molecules. The ability of liposomes and mucoadhesives in sustained and improved partitioning of drug in overcoming biological barriers offers a number of advantages over conventional drug delivery. In this investigation, liposomal and mucoadhesive drug delivery systems for two drugs namely LN and LEU were developed and evaluated for their contraceptive efficacy performance after nasal and pulmonary administrations. Stable liposomal formulations of the drugs for both the routes of administration were developed and optimized with regard to percent drug entrapment by changing various process and formulation parameters. In vivo studies including pharmacokinetics/pharmacodynamics in rats were carried out followed by in vitro diffusion studies to create in vitro testing procedures.

Plain LN and Liposome encapsulated LN formulations showed similar pharmacokinetics, with significantly low bioavailability after intranasal administration may be due to the mucociliary clearance of both the formulations. LN formulation with mucoadhesive agents demonstrated 100% bioavailability and prolonged effective therapeutic concentration of LN for 48-54 hour compared to presently available its oral dosage form in experimental studies conducted in female rats Fertility performance studies in rats after nasal administration were also carried out and cent percent contraception was observed in female rats after alternate day intranasal administration of formulations with mucoadhesives compared to daily oral administration of the drug Results demonstrated superiority of pulmonary plain and liposomal LN delivery with regards to maintenance of effective therapeutic concentration in the plasma over a period of 56-60 h over oral administration of drug in equivalent dose 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 nasal/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 Maintenance of lower but effective LN plasma concentrations for extended period of time after administration of LN nasal and pulmonary formulations developed in this investigation are expected to reduce frequency of dosing of the oral route and likely to reduce systemic side effects associated with oral administration of the drug

In case of LEU, both male as well as female anti-fertility studies were carried out by determination of release of gonadotropin hormone, LH, in blood and results demonstrated improved bioactivity after liposomal encapsulation (28% and 21% F* for neutral and negatively charged liposomes respectively) compared to plain LEU (10% F*) after nasal administration. Two to three fold increase in bioactivity of LEU after liposomal encapsulation may be due to the action of liposomes on nasal mucosa by incorporating phospholipids in the membrane and opening "new pore" in the paracellular tight junction. The prevalence of the repellent forces between negatively charged liposomes and negatively charged nasal mucosa may be responsible for low

bioactivity of negatively charged liposomes compared to plain liposomes Both LEU solution and LLEU formulations with chitosan demonstrated marked increase in F* (10 89 to 49 13% for LEU+CS and 27 83 to 88 90% for LLEU+CS) Pharmacodynamic studies demonstrated complete azoospermia in male rats after 26 days of treatment and ceased estrous cycles from the first treatment cycle in female rats were achieved with nasal liposomal LEU formulation with CS The results of the developed formulation were found to comparable to available parenteral dosage of LEU in producing contraception on rats Significantly improved bioactivity after liposomal encapsulation of LEU was observed after intratracheal instillation compared to that of plain LEU formulation Also, negatively charged liposomes containing LEU showed higher bioactivity (48%) compared to neutral LEU liposomes (44%). 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