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A Preliminary Pharmacokinetic Study of Liposomal Leuprolide Dry Powder Inhaler: A Technical Note

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INTRODUCTION

Many biologically active peptides have been discovered recently and have attracted attention as new drugs. Because of transport and enzymatic barriers, clinical dosage forms of these peptides have been primarily parenteral forms. Development of sustained release forms of these peptide drugs is also being actively researched[1-3] and the pulmonary route would seem to be a promising alternative for delivering them, because many drugs that are poorly absorbed from other mucosal sites are well absorbed from the lungs.[4] This route of administration offers several advantages over the conventional gastrointestinal pathway, including large surface area, extensive vasculature, easily permeable membrane, and low intracellular and extracellular enzymatic activity.[5-8] Recent clinical and preclinical reports reveal that delivery of peptide drugs such as leuprolide acetate and insulin is feasible through the pulmonary route.[9-12] However, the bioavailability of the drugs having relatively high molecular weight is still poor through the pulmonary route compared with the parenteral route.

Recent research is focused on the delivery of systemically acting drugs via the pulmonary route.[13] Dry powder inhalations are a promising application form for peptides and proteins for systemic delivery because they overcome the drawbacks of oral and invasive delivery forms, as enzymatic degradation in the GI tract; low oral bioavailability; the need for intramuscular (IM), subcutaneous (SC), or intravascular (IV) injection; and so forth.[4]

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.[14,15]

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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.[16-18] Liposomes are also known to sustain the release of the entrapped drug(s) and to decrease the mucociliary clearance of the drug(s) because of their surface viscosity. Therefore, more effective and sustained systemic absorption of a drug would be attained by administering the drug containing liposomes in the respiratory tract.

Leuprolide acetate, the peptide drug used in this study, is a potent agonist of luteinizing hormone-releasing hormone (LHRH) currently used for the treatment of prostatic cancer, endometriosis, and precocious puberty. Also, unlike steroid hormones (for contraception), gonadotropin-releasing hormones exert 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, and liver and gallbladder involvement and hence can be a better alternative for contraception. Although clinical application of these peptides is highly promising, their potential may be restricted by difficulties involved in self-medication. Chronic treatment with leuprolide has the disadvantage of requiring long-term, daily injections, as it is a very water-soluble nonapeptide with a 3-hour biological half-life following parenteral administration. Lupron (Abbott Laboratories, Casa Grande, AZ) and Eligard (Atrix Laboratories, Fort Collins, CO) are 2 depot Leucinostatin (LEU) formulations available in the market for 1, 2, and 4 months. Viadur (Bayer Healthcare) is also available in the market as a 1-year sustained release implant. However, pulmonary delivery provides better patient compliance, self-medication, and avoids the complications related to injection procedure. It also provides immediate discontinuation of therapy in case of any side effects. Hence, the aim of this investigation was to develop liposomal formulations for pulmonary administration of LEU with a view to enhance bioavailability of the drug from the pulmonary route.

MATERIALS AND METHODS

LEU was obtained from Abbott Laboratories. Hydrogenated soya phosphatidylchloine (HSPC), was a gift sample from Lipoid Gmbh, Ludwigshafen, Germany. Cholesterol, dicetyl

phosphate (DCP), and stearylamine (SA) purchased from S. D. Fine Chemicals, Mumbai, India. All other solvents and chemicals used were of analytical grade unless otherwise specified.

Preparation of LEU Formulations

LEU Solution (LEU S)

An amount of 500 µg of drug was weighed accurately and transferred to a 10-mL volumetric flask. Acetate buffer pH 5.2 (5 mL) was added and volume was made up to the mark to get a final concentration of 50 µg/mL with acetate buffer pH 5.2.

LEU Physical Mixture (LEU PM)

An amount of 500 µg of drug along with liposomal constituents, namely HSPC, cholesterol (CHOL), and DCP were weighed accurately and transferred to a 10-mL volumetric flask. Acetate buffer pH 5.2 was added and volume was made up to the mark. The proportion of the HSPC and CHOL were the same as used during liposome preparation.

LEU Liposomes

Liposomes of LEU (LLEU) were prepared by reverse phase evaporation (REV) method.[19] The liposomes were prepared in a glass tube (Quick fit Neck B-24, Durga Scientific Pvt Ltd, Vadodara, India) from drug, HSPC, and CHOL at a (2:4:1) molar ratio mixture, which was dissolved in solvent mixture composed of (2:4:1) chlorform:methanol: acetate buffer pH 5.2. The tube was closed with a glass stopper and vortexed for 5 minutes. It was then attached directly to a rotary evaporator (Superfit Continental Pvt Ltd, Mumbai, India) to dry the contents at 55°C under vacuum (400 mm Hg) until a gel was formed. Vacuum was released and the tube was removed from the evaporator and subjected to vigorous mechanical agitation on a vortex mixture for 5 minutes. When the gel collapsed to fluid, it was again fitted to a rotary flash evaporator for the removal of organic solvent. A cycle of 10 minutes drying and 5 minutes vortexing was again repeated twice. The prepared liposomes were extruded by passing through a 2-µm polycarbonate membrane using a syringe filter.

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

In the preparation of dry powder inhaler (DPI), the liposomal dispersion was diluted with sufficient hydrating medium to obtain a lipid:sugar (sucrose) ratio of 1:6 in the case of neutral (LLEU) and 1:5 in the case of negatively charged (LLEUn) liposomes. The dispersion was frozen at –40°C overnight and dried under negative displacement pressure (model DW1 0–60E; Heto Drywinner, Birkerod, Denmark) for 24 hours. The porous cake thus formed was sized successively through #200 and #240 sieves.[13] An equivalent proportion of sorbolac calculated to have a final strength of 250 µg entrapped drug per 10 mg formulation was dispersed into it. Capsules (size 2) were filled with individually weighed powder (10 mg) containing 250 µg 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 aluminum foils and stored in a refrigerator (2 to 8°C) until further use. A fraction of the powder was rehydrated with triple-distilled water with gentle, occasional agitation. The rehydrated liposomal dispersion was separated from the leaked drug by centrifugation and analyzed for percentage drug entrapment and percentage free drug in the DPI formulation.

Characterization

Measurement of percent drug entrapment (PDE) of liposomes was carried out by separating unentrapped drug from rehydrated liposomal suspension by centrifugation at 15000 rpm for 30 minutes and analyzed with a spectrophotometric method at 240 nm using 0.1 N sodium hydroxide.[20] Beer's law is obeyed in the concentration ranges of 2 to 10 µg with a correlation coefficient of 0.999. Results of PDE obtained are recorded in Table 1.

The mean vesicle size of rehydrated liposomes was determined by a laser light scattering technique using Mastersizer (Malvern Instruments, London, UK). The particle size of the formulations was described by the volume mean diameter (D [4,3]). The polydispersity of the powder was expressed by the span. Span = [D(v,90) – D(v,10)]/D(v,50),

Table 1. Analytical Profile of LEU Formulations*

	LLEU-DPI	LLEUn-DPI
Drug entrapped in liposomes (%)	66.08 ± 1.6	72.08 ± 2.1
Size (µm) D [4,3]	3.5 ± 0.1	4.3 ± 0.1
Span	1.91 ± 0.01	1.87 ± 0.01

*Data are the mean ± SEM (n = 3).
D [4,3], volume mean diameter.

where D(v,90), D(v,10) and D(v,50) are the equivalent volume diameters at 90%, 10%, and 50% cumulative volume, respectively. The results are given in Table 1.

Animals and Treatment

Adult albino rats of either sex weighing 200 ± 20 g were housed in polypropylene cages with free access to pelletized chow and tap water. The animals were exposed to alternate cycles of 12 hours light and darkness. The temperature was maintained at approximately 26°C to 28°C. Animal experiments were approved by Social Justice and Empowerment Committee, Ministry of Government of India, New Delhi, India.

Methodology

Pulmonary Administration

The method of Enna and Schanker[21] 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 hours after instillation. Animals were anesthetized using urethane intraperitoneal. Anesthetized 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 0.1 mL (LEU S and LEU PM as such and in case of DPI, after rehydration with sufficient amount of acetate buffer pH 5.2) containing 5 µg LEU were slowly instilled over a 1-minute 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. The animal was 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.

Subcutaneous Administration

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

Blood was sampled from the tail vein at different time points for first 3 days of treatment.

Serum were separated and stored at –20°C until hormone radioimmunoassay. After that the rats were killed following ethical procedure.

Hormone Assay

Serum lutuneizing hormone (LH) was measured by radioimmunoassay (RIA) as described in the instructions provided with the kit (Monobind, Costa Mesa, CA). The sensitivity of the assay was 0.2 mIU/mL for LH. Each sample was assayed in duplicate. The intra-assay coefficient of variation in each assay was 2.0% for LH.

Pharmacokinetic Study

Serum LH levels were measured to evaluate pharmacokinetics following administration of different LEU formulations. The serum LH concentrations at each sampling time point were plotted against time in hours. Maximum serum concentration (Cmax), time in hours to achieve Cmax (Tmax) and LH serum half-life ($t_{1/2}$) were determined from LH serum concentration-time curve from best fit curve using major and minor gridlines with ±0.2 unit accuracy. The area under the serum level curve was calculated by the trapezoidal rule. The pharmacokinetic data are recorded in Table 2.

Statistical Analysis

Data were expressed in mean ± SEM. Statistical analysis was performed by analysis of variance (ANOVA) and Student t test and differences at $P < .05$ were considered significant.

Table 2. Pharmacokinetic Parameters (LH) of LEU Formulations Following Subcutaneous (SC) and Intratracheal (IT) Administration

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

Results and Discussion

Neutral and positively and negatively charged liposomes containing LEU were prepared using REV technique. Incorporation of positive charge with SA into liposomes resulted in poor percent drug entrapment (PDE) and stability. Hence, work on positively charged liposomes was not pursued further. PDE of 91.4% ± 1.5% and 96.5% ± 1.3% were achieved with neutral and negatively charged liposomes respectively. The high PDE of neutral and negatively charged liposomes and instability of positively charged liposomes may be because the LEU has 3 ionizable sites: the imidazolyl nitrogen of His ($pK_a \approx 6.0$), the phenolic hydroxyl of Tyr ($pK_a \approx 10.0$), and the guanidine nitrogen of Arg ($pK_a \approx 13.0$). Since the guanidine nitrogen is extremely basic, LEU exists in the protonated form.[20] Hence, investigations of percent drug retention and contraceptive efficacy studies were performed only on neutral and negatively charged liposomes containing LEU. Dry powder of formed liposomes was prepared by lyophilization using sucrose as a cryoprotectant after separation of unentrapped drug. The percent drug retained after lyophilization was 66.1% ± 1.6% (for neutral liposomes) and 72.1% ± 2.1% (negatively charged liposomes) at 1:5 and 1:6 lipid: sucrose, molar ratios respectively.

In a group of animals, control formulation was administered and serum LH level was monitored. LH levels were found to be approximately 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 hour to 3 hours and then decreased gradually to the pretreatment level within 24 hours. The highest Cmax value of 263 mIU/mL was obtained after SC 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 were obtained after intratracheal instillation for LEU S, LEU PM, LLEU-DPI, and LLEUn-DPI formulations respectively. However, when relative bioavailability of intratracheally administered formulations were compared, LLEU-DPI and LLEUn-DPI formulations showed significantly higher bioavailability of 44.3% ± 1.6% and 48.2% ± 1.1% for LLEU-DPI and LLEUn-DPI formulations respectively compared with 13.0% ± 1.5% and 17.4% ± 1.4% for LEU and LEU PM respectively.

The results supported the role of liposomes in enhancement of drug permeation through alveolar epithelium by altering physicochemical properties of the drug (renders the drug hydrophobic). Liposomal encapsulation of LEU also acted as a biodegradable reservoir, which prolonged pulmonary residence time of LEU. Liposomes may have also decreased the mucociliary clearance due to their surface viscosity. Pronounced effect of apolipoproteins of the lung surfactants have been reported on liposomes.[22] Apolipoprotein SP-A has been shown to cause aggregation, whereas SP-B and SP-C have been shown to cause extensive leakage of liposome contents (lysis) and some degree of lipid mixing (fusion). This may be one reason for only 50% relative bioavailability with developed liposomal formulations compared with presently available parenteral route (SC).

Summary and Conclusions

The developed liposomal DPI of LEU (LLEUn-DPI) demonstrated approximately 50% bioavailability compared with SC route. The studies justify the role of the pulmonary route as a promising alternative to the presently available SC route. The components of liposomal vesicles may be suitably changed to achieve higher bioavailability. Pulmonary delivery of LEU is expected to help in improving patient compliance, self-administration and avoiding the complications related to injection procedure. The developed LEU-DPI may be employed for both male and female contraception and treatment of prostate cancer in men and early puberty in children. In women it may be used for ovarian, endometrial, pancreas, and breast cancer; endometriosis; Uterine Leiomyoma; and anemia due to uterine fibroid tumors. However, the role of LEU-DPI in clinical practice can only be justified only after in vivo studies on 2 species of animals followed by extensive clinical trials.

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