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Preliminary investigation of the nasal delivery of liposomal leuprorelin acetate for contraception in rats

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Abstract

The purpose of the study was to investigate the nasal route as a non-invasive alternative for delivery of leuprorelin acetate (leuprolide acetate, LEU) and to achieve an effective concentration of leuprorelin acetate in blood after nasal administration for contraception in rats. The plain drug solution, physical mixture (plain drug along with constituents of liposomes), or drug encapsulated in either neutral or charged liposomes containing 5 μg leuprorelin acetate were administered to rats through the nasal route. The plain drug solution was administered subcutaneously (s.c.). Simultaneous evaluation was performed on the influence of a mucoadhesive agent (chitosan) on nasal absorption of the plain drug and the liposome-encapsulated drug. Blood samples were taken at regular time intervals and subjected to luteinising hormone (LH) analysis using a specific immunoassay kit. The plasma luteinising hormone concentration vs time data of nasal and subcutaneous treatments were plotted and compared with that of subcutaneous administration. Relative percentage of bioavailability (*F*) for nasal treatments was calculated from plasma concentration vs time plots. Sperm count and fertility performance studies were carried out for selected formulations in rats. Neutral liposomes (LLEU) and negatively-charged liposomes (LLEUn) showed higher relative percentage of bioavailability (*F* 27.83 and 21.30%, respectively) as compared with the plain drug and the physical mixture (*F* 10.89 and 10.96%, respectively) after nasal administration. Hence, work on neutral liposomes was continued. *F* was further improved after incorporation of chitosan i.e. 10.89 to 49.13% for plain leuprorelin acetate and 27.83 to 88.90% for liposomal leuprorelin acetate formulations. Liposomal chitosan formulation administered nasally and leuprorelin acetate solution subcutaneously achieved complete azoospermia. No implantation sites were observed after the mating of female rats with treated males. It was observed that in the treated female rats, the estrous cycles ceased with the same formulations from the first treatment cycle. The findings of these investigations demonstrated that the bioavailability of the nasally-administered liposomal leuprorelin acetate with chitosan formulation was comparable with that of the subcutaneously administered drug. Complete contraception was obtained in male and female rats that had been treated with either the nasally administered liposomal leuprorelin acetate with chitosan or the subcutaneously administered drug.

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Introduction

Throughout history, family planning has been a shared responsibility, most methods requiring male involvement (Handelsman 2001). During the last century, convenient and highly reliable contraceptive methods were developed for women, yet not a single new male contraceptive method was introduced (Anderson & Baird 2002). The burden of contraceptive responsibility can be shifted and only be rebalanced by the availability of comparably attractive methods for men, allowing them to share more equitably the burden and benefits of effective family planning.

Finding an effective, safe, acceptable, reversible and preferably long-acting, hormonal contraceptive method for men is a research priority for WHO and other organizations. Although there are currently no systemic methods of contraception for use by men, the development of male-use contraception equivalent to 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 man 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 (Swerdloff et al 1989; Nieschlag et al 1992).

Regarding female contraception, all oral contraceptive drugs cause interference with the production and action of endogenously synthesized steroid hormones (Gruber et al 2002). Induction of hepatic enzymes by oral contraceptives may cause interference with potency and duration of other medications such as anticoagulants (Ellison et al 2000), 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 (Rosing et al 1999). 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 are frequently observed with oral contraceptive treatment. Therefore there is a need to develop female contraceptives other than the presently available oral contraception without compromising on safety and user compliance.

Unlike steroid hormones, gonadotrophin-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 gynaecomastia, thromboembolism, oedema, liver and gallbladder involvement. Although clinical application of these peptides is highly promising, their potential may be restricted by difficulties involved in self-medication, except by the nasal route (Berquist et al 1979). However, Akwete & Hsu (1993) noticed that nasal bioavailability of these peptides was as low as 2–5%.

Mucociliary clearance under normal conditions rapidly clears the applied material and so there is little time of contact between the drug and the mucosa. Prolonging contact of the drug with the absorptive surfaces by means of an appropriate delivery system could increase the bioavailability of an intranasally administered drug. In this study, various approaches have been made to enhance the persistence of the serum concentration of the peptide, which made use of liposomes and a mucoadhesive agent, chitosan. As a drug delivery system, liposomes can significantly alter the pharmacokinetics and pharmacodynamics of entrapped drugs. For example, by enhancing the drug uptake, drug clearance can be rapidly delayed, and drug toxicity can be reduced (Kimelberg & Mayhew 1978; Szoka & Papahadjopoulos 1981; Poznansky & Juliano 1984). Liposomes are known to sustain the release of the entrapped drug(s) when administered by different routes (Arrowsmith et al 1983; Knepp et al 1988; Akhtar & Juliano 1992). Therefore, administering the drug-containing liposomes via the nose would attain more effective and sustained systemic absorption of a drug.

To facilitate absorption, suitable penetration enhancers have to be developed which would only allow for paracellular absorption and which would be non-toxic. Chitosans have been shown to have similar properties of opening tight junctions. This mechanism is thought to occur by ionic charge transfer between the positive charge of the chitosan molecule and the negative charges of the glycocalix (Thanou et al 2000). Chitosan being a mucoadhesive agent could prolong the drug residence time within nasal mucosa. Chitosan-mediated tight junction disruption is caused by a translocation of tight junction proteins from the membrane to the cytoskeleton (Smith et al 2004; 2005).

Leuprorelin acetate (leuprolide acetate, LEU) is a highly potent analogue of luteinising hormone-releasing hormone (LHRH). However, chronic treatment with leuprorelin acetate has the disadvantage of requiring long-term, daily injections. For contraception, the objective was to enhance the bioavailability of leuprorelin acetate to be comparable with that of the presently available parenteral route, using liposomes and chitosan for the development of non-invasive drug delivery through the nasal route.

Materials and Methods

Materials

Leuprorelin acetate (leuprolide acetate) was obtained from Abbott Laboratories, USA. Hydrogenated soya phosphatidylchloine (HSPC) was a gift sample from Lipoid Gmbh, Germany. Cholesterol (CHOL) and dicetyl phosphate (DCP) were purchased from S. D. Fine Chemicals, India. Chitosan (150 000 Da, 75–85% deacetylated) and stearyl amine (SA) were purchased from BF Goodrich Company, USA and Sigma Chemicals, USA, respectively. All other solvents and chemicals used were of analytical grade unless otherwise specified.

Preparation of leuprorelin acetate formulations

Leuprorelin acetate solution (LEU)

Leuprorelin acetate (1 mg) was weighed accurately and transferred to a 10-mL volumetric flask. Acetate buffer pH 5.2 (5 mL) was added and the volume was made up to the mark to get a final concentration of 100 $\mu g\,mL^{-1}$.

Leuprorelin acetate physical mixture (LEU PM)

Leuprorelin acetate (1 mg) along with liposomal constituents HSPC and cholesterol were weighed accurately and transferred to a 10-mL volumetric flask. Acetate buffer pH 5.2 was added and the volume was made up to the mark. The proportion of the HSPC and cholesterol was the same as that used for liposome preparation.

Leuprorelin acetate liposomes (LLEU)

Liposomes of leuprorelin acetate were prepared by the reverse-phase evaporation method (Knight 1981). In a glass tube (Quick fit neck B-24, Durga Scientific Pvt Ltd, Vadodara, India), drug:HSPC:cholesterol in the molar ratio of 1:15:5 was dissolved in the solvent ratio of chloroform:methanol:acetate buffer pH 5.2, 2:4:1. The tube was closed with a glass stopper and vortexed for 5 min. The tube was then attached directly to a rotary evaporator to dry the contents at 55°C under vacuum (400 mmHg) 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 mixer for 5 min. When the gel collapsed to fluid, it was again fitted to the rotary flash evaporator for the removal of the organic solvent. The cycle of 10-min drying and 5-min vortexing was repeated twice. The final liposomal suspension

was subjected to complete removal of the last traces of organic solvent in a rotary flash evaporator at high vacuum for 15 min. Liposomal dispersion was further stabilized by 2-h hydration and then separated from unentrapped drug by centrifugation at 15000 rev min^{-1} for 30 min.

For the preparation of the negatively- or positively-charged liposomes, respectively, DCP or stearyl amine 5% of the total lipid quantity (molar ratio) was incorporated along with the HSPC and cholesterol.

Leuprorelin acetate solution with chitosan (LEU + CS)

Leuprorelin acetate (1 mg) was accurately weighed 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 with 1% chitosan solution in acetate buffer pH 5.2 (5 mL). The resulting solution was mixed well and stored in amber coloured glass vials in a refrigerator until use.

Leuprorelin acetate liposomes with chitosan (LLEU + CS)

Liposomes were centrifuged and diluted with fresh buffer to give a concentration of 1 mg leuprorelin acetate/5 mL LLEU. This was than diluted with an equal volume of chitosan solution. The resulting suspension was mixed well and stored in a refrigerator until required.

Characterization of formulations

Liposomes

Measurement of percentage drug entrapment (PDE) of liposomes was determined following solubilization of vesicles in 10% Triton X-100 in water, by a spectrophotometric method at 240 nm using 0.1 M sodium hydroxide (Akwete & Hsu 1993). Beer's law was obeyed in the concentration ranges 0–10 $\mu g\,mL^{-1}$ with a correlation coefficient of 0.999. The PDE and amount of leuprorelin acetate per mg of liposomes were calculated (Table 1).

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

Mucoadhesion

The mucoadhesive property of the developed formulations was evaluated by an in-vitro adhesion testing method known as the wash-off method (Lehr et al 1990). The mucoadhesiveness of developed formulations were evaluated on freshly excised bovine nasal mucosa (1 × 1 cm) mounted on glass slides (3 × 1 inch) with cyanoacrylate glue. The glass slides were connected with a suitable support. A sample (0.1 mL) of formulation was placed on nasal mucosa. The tissue was then placed in a desiccator to maintain it at >80% relative humidity at room temperature for 15 min. This allowed the formulation to interact with the nasal mucosa and prevented drying of the mucus. Tissues were examined under a microscope, and the numbers of particles/liposomes attached to the particular area were counted. The support was hung onto the arm of a USP tablet disintegrating test machine. When the disintegrating test machine was operated, the tissue specimen was given a slow, regular up-and-down movement in the simulating nasal fluid at 37°C contained in a 1-L vessel of the machine. The machine was stopped at 30 min, 1 and 2 h and the number of particles/liposomes still adhering to the same portion of the tissue was counted under a microscope. The adhesion number or percentage of mucoadhesion was calculated (Table 1).

In-vitro drug release studies

A vertical type of diffusion cell was designed and validated using the benzoic acid disc method (Chien & Valia 1984). This cell was used to undertake in-vitro drug release studies. Dialysis membrane (250-9U, molecular weight cut-off 12 000 Da; Sigma, Hyderabad, India), 200-μm thickness, pH 5.8–8, breaking strength 2.75 kg f cm^{-1} and porosity 0.45 μm was used as a membrane for in-vitro drug release studies because of simplicity, homogeneity and uniformity. This membrane was pretreated with ethanol (95%) followed by hydration in phosphate-buffered saline, pH 7.4 (PBS) for 24 h before drug release runs. The dialysis membrane was tied to one end of the tube with a nylon string, serving the purpose of a donor compartment.

Plain drug solution and liposomal formulations containing 500 μg leuprorelin acetate were transferred to the donor compartment. The tube was immersed in 20 mL release medium (PBS), maintained at 37 ± 0.5°C under continuous stirring at a rate of 50 rev min^{-1}, in such a way that the membrane was flush with the surface of the release medium.

Table 1 Analytical profile of leuprorelin acetate formulations

		LEU	LEU PM	LLEU	LLEUn	LEU + CS	LLEU + CS
mg liposomes per 1 mg LEU		–	–	11.84 ± 0.1 mg	11.66 ± 0.2 mg		11.97 ± 0.1 mg
PDE (%)		–	–	91.4 ± 1.5*	96.5 ± 1.3*	–	91.37 ± 1.4
Size (μm) D [4,3]		–	–	11.50 ± 0.01	10.71 ± 0.01	–	11.53 ± 0.01
Span		–	–	1.91 ± 0.01	1.87 ± 0.01	–	2.01 ± 0.01
Mucoadhesion (percentage of particles/	30 min	NP	NP	19 ± 1.7	–	NP	83 ± 1.8
liposomes adhering to tissue at different	60 min	NP	NP	–	–	NP	79 ± 1.9
time points)	120 min	NP	NP	–	–	NP	68 ± 2.0

Data are the mean ± s.e.m. (n=3). NP, not performed. D [4,3], volume mean diameter. *$P < 0.05$ by Mann-Whitney U-test. LEU, leuprorelin acetate solution; LEU PM, leuprorelin acetate liposomes (netural); LLEUn, leuprorelin acetate liposomes (negative); LEU + CS, leuprorelin acetate solution with 0.5% chitosan; LLEU+CS, leuprorelin acetate liposomes with 0.5% chitosan.

Samples were withdrawn at specific time intervals and analysed spectrophotometrically for leuprorelin acetate in samples. All experiments were carried out in triplicate and mean values ± s.e.m. are shown in Figure 1. The drug release data were subjected to different release kinetics to understand possible release mechanisms of drug from different formulations.

In-vivo studies

Animal selection

An equal number of white albino rats of both sexes (170 ± 20 g; 120–140 days old) were used. The male and female rats were housed separately in polypropylene cages during the study unless otherwise required. All the animals used for the study were of proven fertility record. The animals had free access to palletized chow and tap water, and were exposed to alternate cycles of 12-h light/darkness.

The Social Justice and Empowerment Committee, Ministry of Government of India, New Delhi, India, approved the animal experiments.

Methodology

Rats of both sexes were divided into eight groups of six animals each. One group served as the control. These animals were treated with the controlled formulation containing HSPC, cholesterol and chitosan at the concentration used in the other formulations. All the other groups were treated with different formulations containing leuprorelin acetate.

Nasal administration

At the time of administration, animals were held from the back in a horizontal position and 10 μL of a formulation (LEU, LEU PM, LLEU, LLEUn, LEU + CS, or LLEU + CS) containing 5 μg drug was placed along the nasal wall with a micropipette in one of the nostrils.

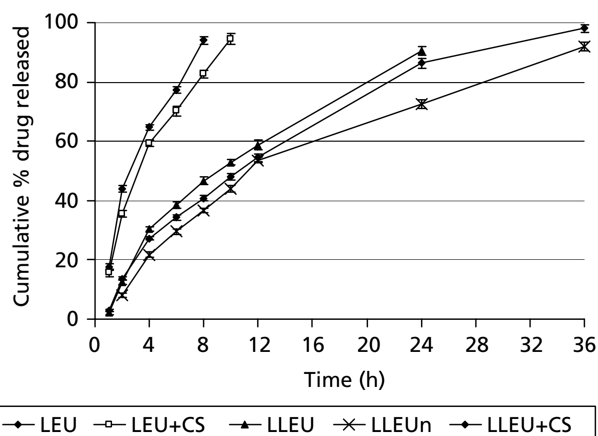

Figure 1 Cumulative % leuprorelin acetate released during in-vitro drug release studies. LEU, leuprorelin acetate solution; LLEU, leuprorelin acetate liposomes (neutral); LLEUn, leuprorelin acetate liposomes (negative); LEU + CS, leuprorelin acetate solution with 0.5% chitosan; LLEU + CS, leuprorelin acetate liposomes with 0.5% chitosan.

Subcutaneous administration

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

Blood was sampled from the tail vein at different time points. Serum was separated and stored at −20 °C until the serum luteinising hormone concentration was determined using hormone radioimmunoassay (described below).

Fertility performance of male rats

Fertility performance studies were carried out for selected leuprorelin acetate formulations (LEU (s.c.) and LLEU + CS intranasally containing 5 μg drug). The control group was kept untreated. The selected formulations were administered in male rats for 26 days. After the completion of the drug schedule the male rats (placebo/treated) were paired overnight with normal cyclic female rats (1:1) on their proestrous phase. The presence of spermatozoa in the vaginal smear following exposure was taken as an index of the first day of gestation. The male rats were killed by cardiac puncture, blood collected and plasma separated. Immediately after the animals were killed, sperm was collected from the left caudal epididymis for a sperm count. Mated normal female rats were autopsied on the tenth day of their gestation. The fertility test was considered positive when implantation sites were present.

Sperm count

The epididymal sperm count was by the method of Linder et al (1986). The epididymal sperm were collected by cutting the epididymis into small pieces and flushing the sperm in normal saline. The sperm collected were centrifuged at 2000 rev min^{-1} for 10 min. The pellet was resuspended in 2 mL normal saline. A 0.5-mL sample of sperm suspension was homogenized for a few seconds, centrifuged at 6000 rev min^{-1} for 10 min and again rehydrated with 5 mL normal saline. A sample of this solution was placed in a haemocytometer and motile sperm were counted using a microscope. To minimize error, the count was repeated at least five times for each rat.

Fertility performance of female rats

Two selected formulations (LEU (s.c.) and LLEU + CS intranasally containing 5 μg drug) were administered to female rats for two estrous cycles to see their effect on the normal cyclicity of the rats. An untreated group was kept as the control.

Hormone assay

Serum luteinising hormone was measured by radioimmunoassay (RIA) as described in the instructions provided with the kit (Monobind, USA). The sensitivity of the assay was 0.2 mIU mL^{-1} for luteinising hormone. Each sample was assayed in duplicate. The intra-assay coefficient of variation in each assay was 2.0% for luteinising hormone.

Statistical analysis

Data were expressed as mean ± s.e.m. The Mann–Whitney U-test was applied to see whether there was any significant difference between the drug entrapment and size of the

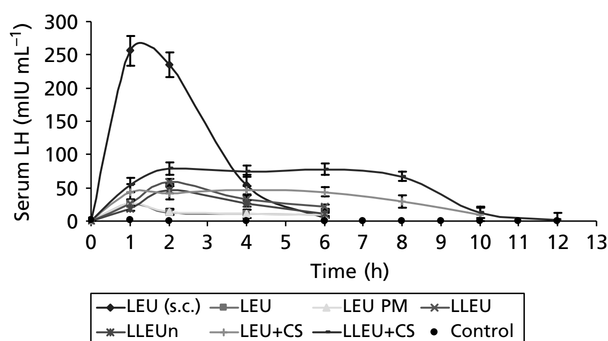

Figure 2 Plot of mean serum level of luteinising hormone (LH) vs time following subcutaneous and nasal administration of leuprorelin acetate. LEU (s.c.), leuprorelin acetate solution administered subcutaneously; LEU, leuprorelin acetate solution; LEU PM, leuprorelin acetate physical mixture; LLEU, leuprorelin acetate liposomes (neutral); LLEUn, leuprorelin acetate liposomes (negative); LEU + CS, leuprorelin acetate solution with 0.5% chitosan; LLEU + CS, leuprorelin acetate liposomes with 0.5% chitosan.

liposomal formulations. Comparison of all other data was carried out using analysis of variance and Student's *t*-test; differences at $P < 0.05$ were considered significant.

For the in-vivo studies, the serum luteinising hormone concentrations at each sampling time point were plotted against time (h) (Figure 1). Various pharmacokinetic parameters were calculated from the data in Figure 2 and were recorded in Table 3. Maximum plasma concentration (C_{max}), time in 1 h to achieve C_{max} (T_{max}) and drug plasma half-life (t½) were determined from the drug plasma concentration–time curve from best fit curve, using major and minor gridlines with ±0.2 unit accuracy. The area under the serum luteinising hormone level curve was calculated by the trapezoidal rule and recorded in Table 3. Data were compared using analysis of variance and a difference at $P < 0.05$ was considered significant. The bioavailability (F) was calculated by the following equation:

$$F = (\text{AUC nasal route} \times \text{subcutaneous dose}) / (\text{AUC subcutaneous route} \times \text{nasal dose}) \times 100 \quad (1)$$

Results

Incorporation of a positive charge with stearyl amine into liposomes resulted in poor percentage drug entrapment (PDE) and stability. Hence, work on positively-charged liposomes was not pursued. PDE values of 91.4 ± 1.5 and 96.5 ± 1.3 were achieved for neutral and negatively-charged liposomes, respectively (Table 1). When compared using the Mann–Whitney U-test, a significant difference ($P < 0.05$) between PDE and a non-significant difference ($P > 0.05$) in vesicle size were observed. Formulations of plain and liposomal drugs were prepared for intranasal application and were characterized for mucoadhesion (Table 1). Drug assay of all the formulations was found to be between 99% and 101%, pH approximately 5.2. Comparative drug release studies were carried out between various leuprorelin acetate formulations for up to 36 h. The results obtained are presented in Figure 1.

Table 2 Coefficient and exponent of leuprorelin acetate release from leuprorelin acetate formulations

Formulation	Equation coefficient (*a*)	Release exponent (*n*)
Leuprorelin acetate	0.2077 ± 0.002	0.7638 ± 0.018
LEU + CS	0.1819 ± 0.001	0.7521 ± 0.014
LLEU	0.0893 ± 0.002	0.7650 ± 0.020
LLEUn	0.0632 ± 0.001	0.7990 ± 0.017
LLEU + CS*	0.0556 ± 0.001	0.8984 ± 0.019

*$P < 0.05$ compared with LLEU. LEU + CS, leuprorelin acetate solution with 0.5% chitosan; LLEU, leuprorelin acetate liposomes (neutral); LLEUn, leuprorelin acetate liposomes (negative); LLEU + CS, leuprorelin acetate liposomes with 0.5% chitosan.

To examine the drug release kinetics and mechanism, the data were subjected to the following equation (Cardinal 1984):

$$Q(t) = a\, t^n \quad (2)$$

Where Q(t), *a, n* are the fraction of the drug released up to time t, equation coefficient, and release exponent expressing mechanism of release, respectively. When n approaches 0.5 a Fickian/diffusion-controlled release is implied, whereas $0.5 < n < 1.0$ is for non-Fickian release and $n = 1$ for zero-order release. Values of a and n for different formulations are recorded in Table 2. As observed, the mechanism of drug release from the various formulations was non-Fickian.

In-vivo pharmacokinetic studies were performed after intranasal and subcutaneous delivery of plain and liposomal formulations with and without chitosan to rats. In the control group, the control formulation (HSPC, cholesterol and chitosan at the concentration used in the other formulations but without drug) was administered and serum luteinising hormone level was monitored. Luteinising hormone levels were found to be very low (approximately 2 mIU mL^{-1}). In leuprorelin acetate-treated animals, regardless of the route of administration and formulation, serum luteinising hormone concentrations transiently rose to peak at 1–2.1 h and then decreased gradually to the pretreatment level within 24 h (Figure 2). The highest C_{max} value of 263 ± 1.8 mIU mL^{-1} was observed after subcutaneous administration. After intranasal administration (Table 3), a lower C_{max} of 27 ± 1.0, 27 ± 1.1, 59 ± 1.0 and 47 ± 1.2 mIU mL^{-1} were observed for leuprorelin acetate solution, LEU PM, LLEU and LLEUn formulations, respectively.

To enhance the residence time and mucoadhesion of the formulations, chitosan was incorporated in LLEU and leuprorelin acetate formulations. LLEU was selected as it showed significantly higher ($P < 0.05$) F as compared with the other formulations. Leuprorelin acetate solution was selected to see the effect of chitosan on bioavailability of plain drug. Leuprorelin acetate solution and LLEU formulation containing 0.5% chitosan solution showed a marked increase ($P < 0.05$) in F (i.e. 10.89 to 49.13% for LEU + CS and 27.83 to 88.90% for LLEU + CS) and significantly higher t½ of 7.2–8.4 h compared with these formulations without chitosan. Intranasally administered LLEU + CS formulation showed a relative F value of 88.90% compared with

Table 3 Pharmacodynamic parameters of leuprorelin acetate formulations following subcutaneous and nasal administration

Formulation	AUC (ng-h mL^{-1})	*F* (%)	T_{max} (h)	C_{max} (ng mL^{-1})	t ½ (h)
Leuprorelin acetate (s.c.)	720.5 ± 78.21	—	1.2 ± 0.2	263 ± 1.8	3.8 ± 0.2
Leuprorelin acetate*	78.5 ± 11.23	10.89 ± 1.3	1.0 ± 0.2	27 ± 1.0	2.8 ± 0.2
LEU PM*	79.0 ± 13.24	10.96 ± 1.1	1.0 ± 0.2	27 ± 1.1	3.0 ± 0.2
LLEU*	200.5 ± 18.75	27.83 ± 1.8	2.0 ± 0.2	59 ± 1.0	6.5 ± 0.2
LLEUn*	153.5 ± 16.03	21.30 ± 1.6	2.0 ± 0.2	47 ± 1.2	6.8 ± 0.2
LEU + CS*	354 ± 41.21	49.13 ± 2.5	1.2 ± 02	45 ± 1.4	7.2 ± 0.2
LLEU + CS*	640.5 ± 35.96	88.90 ± 2.1	2.1 ± 0.2	80 ± 1.2	8.4 ± 0.2

*$P < 0.05$ compared with leuprorelin acetate (s.c.). LEU PM, leuprorelin acetate physical mixture; LLEU, leuprorelin acetate liposomes (neutral); LLEUn, leuprorelin acetate liposomes (negative); LEU + CS, leuprorelin acetate solution with 0.5% chitosan; LLEU + CS, leuprorelin acetate liposomes with 0.5% chitosan.

subcutaneously administered leuprorelin acetate solution. Hence, the LLEU + CS formulation was subjected to fertility performance evaluation on male and female rats.

In male rats, sperm count and fertility performance studies were carried out for leuprorelin acetate solution administered subcutaneously and LLEU+CS formulation administered intranasally. Spermatozoa were collected from the left cauda epididymis and were counted under a microscope. The results showed that after 26 days of treatment complete azoospermia was achieved using the LLEU + CS formulation administered nasally and leuprorelin acetate solution subcutaneously. The duration of treatment was kept to 26 days to cover two seminiferous cycles (13.2×2 days) in rats. Females were mated with treated males and no implantation sites were observed with nasal administration of the LLEU + CS formulation or the subcutaneous administration of leuprorelin acetate solution. This was due to the azoospermic potential of both formulations.

For 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 leuprorelin acetate solution or LLEU + CS through subcutaneous and nasal routes, respectively. Animals returned to normal cyclicity after the cessation of the treatment.

Discussion

Issues that have been addressed while preparing proteins/peptides for delivery systems have included the large size, hydrophilicity, and physical and chemical lability of the drug molecule. These factors impact on the pharmacokinetics and pharmacodynamics of the drug in-vivo and must be considered when selecting a suitable formulation, storage, and delivery method. The nasal route is a non-invasive alternative route for the delivery of a number of macromolecules, including peptides, proteins and vaccines (Koushik & Kompella 2004). Intranasal albuterol was shown to offer an alternative to metered-dose inhalers for the treatment of acute bronchospasm and for prevention of exercise-induced asthma, especially for children and the elderly (Hussain et al 2004). Hence, liposomes and/or chitosan formulations containing leuprorelin acetate were prepared, characterized and subjected to pharmacokinetic and pharmacodynamic evaluation in rats.

The high PDE of neutral and negatively-charged liposomes and the instability of positively-charged liposomes might have been because leuprorelin acetate has three 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, leuprorelin acetate exists in the protonated form (Akwete & Hsu 1993). Hence, further investigations were carried out on only the neutral (LLEU) and negatively-charged liposomes (LLEUn) containing leuprorelin acetate. Assay values between 99 and 101% for all formulations assured the potency of the formulations delivered, while pH of formulations suggested that the formulations were non-irritant in the delivered volume. Increase in the viscosity of the formulations after addition of chitosan was expected to increase residence time in the nasal cavity and mucoadhesion. The LLEU + CS formulation exhibited maximum mucoadhesion compared with the other formulations. Ionic interactions between leuprorelin acetate induced positive charges in liposomes without chitosan and negatively-charged nasal mucosa might have been responsible for the mucoadhesive properties of the liposomes.

The results suggested that liposomal encapsulation substantially decreased drug release and a further delay in release was observed after incorporation of chitosan. When individual formulations were compared, a significant difference and delay in drug release ($P < 0.05$) was observed between plain drug solution and plain drug solution with chitosan. The increase in the viscosity might have been responsible for this effect. Initial analysis of drug release data of the formulations supported Higuchi's diffusion controlled release kinetics (Higuchi 1962). To understand the mechanism of diffusion-controlled release of leuprorelin acetate from leuprorelin acetate formulations, results were analysed according to equation 2 given by Cardinal (1984). High equation coefficient values (a) of leuprorelin acetate and LEU + CS suggested initial burst drug release (Ebube et al 1997). The release exponent (n) values gave an important clue to understanding the possible mechanism of drug release from the formulations. Values of n (0.7521–0.7990) indicated an anomalous mechanism of drug release (non-Fickian). The data recorded in Table 2 suggested that leuprorelin acetate incorporation in liposomes did not influence the n values or mechanism of drug release significantly ($P > 0.05$). However, when the drug-containing liposomes were

incorporated in chitosan solution (LLEU+CS), the n value increased significantly (0.8984 ± 0.019), suggesting a shift in drug release kinetics towards zero order (Korsmeyer et al 1983). To conclude, incorporation of leuprorelin acetate in liposomes and chitosan influenced drug release significantly and the possible leuprorelin acetate release mechanism from the LLEU + CS formulation was close to zero-order kinetics.

The in-vivo performance of a dosage form not only depends on the mechanisms occurring at the interface, but also on the properties of the total mucoadhesive complex: the dosage form, the mucosa and the interface between them (Edsman & Hagerstrom 2005). Due to the mucociliary clearance of the nasal cavity, nasally delivered formulations clear rapidly from the site of absorption resulting in little contact time between the drug and the nasal mucosa and, hence, poor drug absorption. When the relative bioavailability of nasally-administered formulations were compared, LLEU and LLEUn showed higher relative percentage bioavailability (F 27.83% and 21.3%, respectively) as compared with the leuprorelin acetate solution and LEU PM (F of 10.89% and 10.96%, respectively). Liposomal formulations showed significantly higher t½ (6.5–6.8 h; $P < 0.05$) as compared with the plain leuprorelin acetate formulations (2.3–2.6 h). The prevalence of the repellent forces between negatively-charged liposomes and negatively-charged nasal mucosa might have been responsible for the low bioavailability and t½ of LLEUn. An interaction between positively-charged leuprorelin acetate and negatively-charged liposomal constituents might have been a cause for low bioactivity as well. Liposomal encapsulation has been shown to sustain drug release (Arrowsmith et al 1983; Knepp et al 1988; Akhtar & Juliano 1992) and enhanced absorption after administration of liposomally encapsulated drug.

Chitosan has received substantial attention in novel bioadhesive drug delivery systems with the aim to improve the bioavailability of drugs by prolonging the residence time at the site of absorption (Lehr et al 1992; He et al 1998). Chitosan has been used as a stabilizing constituent of liposomes also (Henriksen et al 1994; Takeuchi et al 1996). Several studies have highlighted the potential use of chitosan (Aspeden et al 1997; Kotze et al 1998; Sandri et al 2004; Edsman & Hagerstrom 2005; Gavini et al 2005). Prolonging the contact time of the drug with the absorptive surfaces by means of chitosan contributed to the increase in the F value of intranasally administered formulations. Chitosan also acts by opening the tight junction between epithelial cells (Artursson et al 1994). The F value determined the ultimate fate of the formulation in the body, while lower C_{max} values followed by a plateau for a prolonged period of time for the LLEU + CS formulation might have decreased the chances of the concentration related side effects of the drug. The higher bioavailability and pharmacological activity observed with this formulation may be related to direct nose to brain drug transport, although it was not ascertained in this study. In recent years interest has been expressed in the use of the nasal route for delivery of drugs to the brain, exploiting the olfactory pathway. A wealth of studies has reported proof of nose-to-brain delivery of a range of different drugs in animal models. Recent studies in volunteers confirmed the likely existence of a direct pathway from nose to brain (Illum 2004).

Similarly, sperm count and fertility performance studies showed that treatment with the LLEU + CS formulation resulted in complete contraception in male and female rats. Continuous intranasal administration of LHRH agonists has been shown to inhibit ovulation in women and suppress gonadotrophin secretion enough to affect spermatogenesis in men (Nillius 1984). Inhibition of normal ovulation could be consistently achieved by daily intranasal super-agonist administration in women, in males, super-agonists of LHRH may be administered in combination with testosterone to induce oligospermia or azoospermia without causing impotence (Nillius 1984). However, no systematic studies have been conducted to demonstrate the possible role of these peptides in contraception for men or women. Hence, more studies are necessary to ascertain the role of these peptides in clinical practice.

Conclusions

Nasal administration of a liposomal leuprorelin acetate formulation with chitosan produced contraception in male and female rats. The results of the nasal administration of the developed formulation were comparable with leuprorelin acetate administered subcutaneously. When administered intranasally, a lower dose of the drug was needed and hence it would be expected to increase the therapeutic index and reduce toxicity. Potential clinical applications of these peptides in the therapy of chronic hormone-dependent diseases and in contraception are major new developments and studies focusing on new clinical uses must be undertaken, especially using slow delivery systems. However, its role in clinical practice can only be realised after extensive experiments on rats and one other species of animal followed by clinical trials.

References

Akhtar, S., Juliano, R. L. (1992) Liposome delivery of antisense oligonucleotides: adsorption and efflux characteristics of phosphorothioate oligodeoxy-nucleotides. *J. Control. Release* **22**: 47–56

Akwete, L. A., Hsu, L. (1993) Leuprolide and other LH-RH analogues. In: Wang, Y. J., Pearlman, R. (eds) *Stability and characterization of protein and peptide drugs: case histories*. Plenum Press, New York, pp 159–199

Anderson, R. A., Baird, D. T. (2002) Male contraception. *Endocr. Rev*. **23**: 735–762

Arrowsmith, M., Hadgraft, J., Kellaway, I. W. (1983) The in vitro release of steroids from liposomes. *Int. J. Pharm*. **14**: 191–208

Artursson, P., Lindmark, T., Davis, S. S., Illum, L. (1994) Effect of chitosan on the permeability of monolayers of intestinal epithelial cells (Caco-2). *Pharm. Res*. 11: 1358–1361

Aspeden, T. J., Mason, J. D. T., Jones, N., Lowe, J., Skaugrud, O., Illum, L. (1997) Chitosan as a nasal delivery system: the effect of chitosan on in vitro and in vivo mucociliary transport rates. *J. Pharm. Sci*. **86**: 509–513

Berquist, C., Nillius, S. J., Wide, L. (1979) Intranasal gonadotropin-releasing hormone agonist as a contraceptive agent. *Lancet*. **2**: 215–217

Cardinal, C. R. (1984) Drug release from matrix devices. In: Anderson, J. M., Kim, S. W. (eds) *Recent advances in drug delivery systems*. Plenum Press, New York, pp 229–248

Chien, Y. W., Valia, K. H. (1984) Development of a. dynamic skin permeation system for long term studies. *Drug Dev. Ind. Pharm*. **10**: 575–599

Ebube, N. K., Hikal, A., Wyandt, C. M., Beer, D. C., Miller L. G., Jones, A. B. (1997) Sustained release of acetaminophen from heterogeneous matrix tablets: influence of polymer ratio, polymer loading and co-active on drug release. *Pharm. Dev. Technol.* **2**: 161–170

Edsman, K., Hagerstrom, H. (2005) Pharmaceutical applications of mucoadhesion for the non-oral routes. *J. Pharm. Pharmacol*. **57**: 3–22

Ellison, J., Thomson, A. J., Greer, I. A., Walker, I. D. (2000) Drug points: apparent interaction between warfarin and levonorgestrel used for emergency contraception. *Br. Med. J*. **321**: 1382

Gavini, E., Rassu, G., Sanna, V., Cossu, M., Giunchedi, P. (2005) Mucoadhesive microspheres for nasal administration of an antiemetic drug, metoclopramide: in-vitro/ex-vivo studies. *J Pharm. Pharmacol*. **57**: 287–294

Gruber, C. J., Tschugguel, W., Schneeberger, C., Huber, J. C. (2002) Production and actions of estrogens. *N. Engl. J. Med.* **346**: 340–352

Handelsman, D. J. (2001) Male contraception. In: DeGroot, L. J. (ed.) *Endocrinology*. 4th edn, Saunders, Philadelphia, pp 2344–2349

He, P., Davis, S. S., Illum, L. (1998) In vitro evaluation of the mucoadhesive properties of chitosan microspheres. *Int. J. Pharm.* **166**: 75–88

Henriksen, I., Smistad, G., Karlsen, J. (1994) Interaction between liposomes and chitosan. *Int. J. Pharm*. **101**: 227–236

Higuchi, W. I. (1962) Analysis of data on the medicament release from ointments. *J. Pharm. Sci*. **51**: 802–804

Hinchcliffe, M., Jabbal-Gill, I., Smith, A. (2005) Effect of chitosan on the intranasal absorption of salmon calcitonin in sheep. *J. Pharm. Pharmacol*. **57**: 681–687

Hussain, A. A., Dakkuri, A., Lai, Y. L., Traboulsi, A., Hussain, M. A. (2004) Nasal administration of albuterol: an alternative route of delivery. *J. Pharm. Pharmacol*. **56**: 1211–1215

Illum, L. (2004) Is nose-to-brain transport of drugs in man a reality? *J. Pharm. Pharmacol*. **56**: 3–17

Kimelberg, H. K., Mayhew, E. G. (1978) Properties and biological effects of liposomes and their uses in pharmacology and toxicology. *Crit. Rev. Toxicol*. **96**: 25–79

Knepp, M., Hinz, R. S., Szoka, F. C., Guy, R. H. (1988) Controlled drug release from a novel liposomal delivery system. I. Investigation of transdermal potential. *J. Control. Release* **5**: 211–221

Knight, C. G. (1981) *Liposomes: from physical structure to therapeutic application*. Elsevier/North-Holland Biomedical Press, New York.

Korsmeyer, R. W., Gurney, R., Doelker, E., Buri, P., Peppas, N. A. (1983) Mechanisms of solute release from porous hydrophilic polymers. *J. Pharm. Sci*. **15**: 25–35

Kotze, A., Lussen, H., de Boer, A., Verhoef, J., Junginger, H.(1998) Chitosan for enhanced intestinal permeability: prospects for derivatives soluble in neutral and basic environments. *Eur. J. Pharm. Sci*. **7**: 145–151

Koushik, K. N., Kompella, U. B. (2004) Transport of deslorelin, an LHRH agonist, is vectorial and exhibits regional variation in excised bovine nasal tissue. *J. Pharm. Pharmacol*. **56**: 861–868

Lehr, C. M., Bowstra, J. A., Tukker, J. J., Junginger, H. E. (1990) Intestinal transit of bioadhesive microspheres in an in situ loop in rat. *J. Control. Release* **13**: 51–62

Lehr, C. M., Bouwstra, J. A., Kok, W., De Boer, A. G., Tukker, J. J., Verhoef, J. C., Breimer, D. D., Junginger, H. E. (1992) Effects of the mucoadhesive polymer polycarbophil on the intestinal absorption of a peptide drug in the rat. *J. Pharm. Pharmacol*. **44**: 402–407

Linder, R. E., Strader, L. F., McElroy, W. K. (1986) Measurement of epididymal sperm motility as a test variable in the rat. *Bull. Environ. Contam. Toxicol*. **36**: 317–324

Nieschlag, E., Behre, H. M., Weinbauer, G. F. (1992) In: Nieschlag, E., Halbernicht, U. F. (eds) *Spermatogenesis – fertilization – contraception: molecular, cellular and endocrine events in male reproduction*. Springer, Heidelberg, pp 477–501

Nillius, S. J. (1984) Luteinising hormone releasing hormone analogues for contraception. *Clin. Obstet. Gynaecol*. **11**: 551–572

Poznansky, M. J., Juliano, R. L. (1984) Biological approaches to the controlled delivery of drugs: a critical review. *Pharmacol Rev*. **36**: 277–336

Rosing, J., Middeldorp, S., Curvers, J., Thomassen, M. C., L. G., Nicolaes, G. A., Meijers, J. C., Bouma, B. N., Buller, H. R., Prins, M. H., Tans, G. (1999) Low-dose oral contraceptives and acquired resistance to activated protein C: a randomised cross-over study. *Lancet* **354**: 2036–2040

Sandri, G., Rossi, S., Ferrari, F., Bonferoni, M. C., Zerrouk, N., Caramella, C. (2004) Mucoadhesive and penetration enhancement properties of three grades of hyaluronic acid using porcine buccal and vaginal tissue, Caco-2 cell lines, and rat jejunum. *J. Pharm. Pharmacol*. **56**: 1083–1090

Smith, J., Wood, E., Dornish, M. (2004) Effect of chitosan on epithelial cell tight junctions. *Pharm. Res*. **21**: 43–49

Smith, J. M., Dornish, M., Wood, E. J. (2005) Involvement of protein kinase C in chitosan glutamate-mediated tight junction disruption. *Biomaterials* **26**: 3269–3276

Swerdloff, R. S., Wang, C., Bhasin, S. (1989) In: Burger, H. G., de Kretser, D. M. (eds) *The testis*. 2nd edn, Raven Press, New York, pp 547–568

Szoka, F., Papahadjopoulos, D. (1981) Liposomes: preparation and characterization. In: Knight, C. G. (ed.) *Liposomes: from physical structure to therapeutic applications*. Amsterdam: Elsevier, North Holland, pp 51–82

Takeuchi, H., Yamamoto, H., Niwa, T., Hino, T., Kawashima, Y. (1996) Enteral absorption of insulin in rats from mucoadhesive chitosan-coated liposomes. *Pharm. Res*. **13**: 896–901

Thanou, M., Florea, B. I., Langemeyer, M. W., Verhoef, J. C., Junginger, H. E. (2000) N-trimethylated chitosan chloride (TMC) improves the intestinal permeation of the peptide drug buserelin in vitro (Caco-2 cells) and in vivo (rats). *Pharm. Res*. **17**: 27–31

Yang, T., Mustafa, F., Ahsan, F. (2004) Alkanoylsucroses in nasal delivery of low molecular weight heparins: in-vivo absorption and reversibility studies in rats. *J. Pharm. Pharmacol*. **56**: 3–60