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Decrease of genital organ weights and plasma testosterone levels in rats following oral administration of leuprolide microemulsion

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Abstract

Studies were conducted to develop oral leuprolide microemulsions using oleic acid as an absorption enhancer and to evaluate its absorption and pharmacological responses in rats. Oral administration of leuprolide microemulsion at a dose of 3 mg/kg showed a greater in vivo exposure level (C_{max} and AUC) than its saline solution. When male rats were orally given a microemulsion formulation of leuprolide acetate at 0.25, 0.5, and 1 mg/day for 14 consecutive days, a significant decrease in testis, prostate and seminal vesicle weights was observed. In a 35-day study, the reduction of the male genital organ weights by once a day treatment (2 mg/rat, qd) was similar to that by twice a day treatment (1 mg/rat, bid) at the same dose level. From both 14- and 35-day studies, plasma testosterone levels were sharply increased at the beginning of the treatment, and then significantly decreased to below normal control level which was also maintained during the treatment. In female rats, similar reduction of uterus and ovary weights was obtained following oral administration of leuprolide microemulsion for 35 days. These antagonistic activities from oral leuprolide microemulsion were similar to a single subcutaneous injection of Lupron® depot (3.75 mg/rat), a commercial leuprolide product. The results indicated that leuprolide absorbed into systemic blood circulation from the oral microemulsion containing oleic acid reached the plasma level which can exert its pharmacological effects. Increasing oral absorption of leuprolide observed in this study could be mediated by improved membrane permeation from oleic acid and reduced enzymatic degradation from microemulsions. These findings suggest that systemic absorption of highly water-soluble protein or peptide drugs could be enhanced by oral microemulsions containing oleic acid.

Keywords: Leuprolide acetate; Oleic acid; Permeation enhancer; Peptide; Microemulsion; Testosterone; Absorption; Rat; formulation; Peptide delivery

1. Introduction

Leuprolide acetate is a synthetic nonapeptide and an analog of naturally occurring luteinizing hormone releasing hormone (LHRH) (Fujino et al., 1974), which is chemically defined as:

5-OxoPro-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-ProNHEt.

$\times CH_3COOH$

It stimulates pituitary gonadotropin secretion at acute doses, while inhibiting pituitary gonadotropin secretion and suppressing testicular and ovarian steroidogenesis (chemical castration) when administered chronically in therapeutic doses. These antagonistic effects on the pituitary and gonads are thus used in treatment of metastatic prostate cancer and endometriosis (Sharifi and Soloway, 1990; Okada et al., 1988). Leuprolide acetate is presented in a number of injectable dosage forms including Lupron® sterile solution for subcutaneous administration (1 and 5 mg/ml) and Lupron® depot controlled release formulation for intramuscular injection (3.75 and 7.5 mg). To obtain a convenient and reliable method for non-parenteral self-administration of leuprolide, numerous studies have been performed over the years including: oral, rectal, vaginal, sublingual, nasal, transdermal, and pulmonary route (Okada et al., 1982; Zheng et al., 1999; Zheng and FuLu, 1999; Adjei et al., 1992, 1993; FuLu et al., 1992). However, none of nonparenteral leuprolide product is commercially available due to very low bioavailability, cost of goods, manufacturability, and marketing concerns on patient compliance.

The oral dosage form for pharmaceutical products is for many reasons the most common route of administration in humans. Several reports in literature have indicated that water-in-oil emulsion and microemulsion formulations can enhance

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the oral bioavailability of drugs, including proteins and peptides (Ritschel, 1991; Constantinides et al., 1994, 1996). It is also reported that oleic acid and certain fatty acids could improve insulin enteral absorption from rat ileum and colon using emulsions containing oleic acid (Wang et al., 1994; Morishita et al., 1998). Oleic acid has been known to increase the percutaneous and transmucosal absorption rates (Lee et al., 1991; Niazy, 1991) and is commonly used as a permeation enhancer for transdermal delivery. However, less attention has been paid to oral absorption enhancement for protein or peptide drugs using microemulsions containing oleic acid. In the present study, we investigated the feasibility of using microemulsion preconcentrate, i.e., self-microemulsifying drug delivery system (SMEDDS) containing oleic acid as an alternative to daily or monthly injections of leuprolide acetate. A leuprolide microemulsion preconcentrate formulation was developed and assessed in rats by determining inhibitory effects on the genital organs and plasma testosterone profiles following chronic oral administration. The results from the leuprolide microemulsion formulation were compared with that from both untreated animals and animals treated by Lupron® depot. Also, plasma profiles of leuprolide acetate following single oral administration of both saline solution and microemulsion were determined.

2. Experimental

2.1. Materials

Leuprolide acetate was obtained from Abbott Labs (Lot 94-822-AL). Lupron® depot (leuprolide acetate, 3.75 mg) was from TAP Holdings, Inc. (Lot 14-126-S2). Other chemicals and reagents used in the investigation were: ethanol (McCormick Distilling Co., Lot I08416), oleic acid (Abbott Labs, Lot 17-289-AL), polysorbate (Abbott Labs, Lot 03-268-KA), and α-tocopherol (Vitamin E) (Sigma Chemical Co., Lot 44H0121).

2.2. Microemulsion preconcentrate preparation

Leuprolide acetate was formulated in a water-in-oil (w/o) microemulsion preconcentrate, and dispersed into an aqueous phase to form a water-in-oil-in-water (w/o/w) microemulsion prior to administration. Briefly, leuprolide acetate was weighed and dissolved in water, and then mixed with ethanol. Into the mixture, oleic acid and Vitamin E were added and mixed well. Finally, Tween 80 was added and mixed to form a w/o microemulsion preconcentrate. The preconcentrate is practically clear and yellowish, and stable at room temperature. The formulation composition is listed in Table 1. A placebo formulation was prepared using similar method without leuprolide acetate.

Table 1
Formulation composition of oral leuprolide microemulsion preconcentrate

Ingredients	Unit formula
Leuprolide acetate	5 mg/ml
Ethanol	10% (v/v)
Water	2% (v/v)
Oleic acid	38% (v/v)
Polysorbate (Tween 80)	40% (v/v)
Vitamin E	q.s.

2.3. Particle size measurement

Photon correlation spectrometer using laser light scattering technology (Malvern model 4700, Southborough, MA) was employed to determine particle size of leuprolide and placebo microemulsions. The microemulsion preconcentrate was diluted approximately 100-fold with water and the diluted samples were loaded onto 1 cm^2 cuvette. Light scattering was monitored at 90° angle and 25 °C. The data were expressed as a mean of triplicate determinations. Typically, the relative standard deviation of the assay was less than 5%.

2.4. Animal studies

Sprague–Dawley rats weighing between 300 and 320 g were purchased from Charles River Labs (Wilmington, MA). Prior to dosing to animals, the microemulsion preconcentrate was dispersed into appropriate amount of water and vortexed for 1 min to form a w/o/w microemulsion with a leuprolide concentration of 0.25–2 mg/ml. The resultant clear microemulsion (w/o/w) was then given to rats (1 ml/rat) through gavage into the stomach once a day (qd) or twice a day (bid). Lupron® depot (3.75 mg) was given as a single subcutaneous injection (1.5 ml/rat). During the study, the animals were given free access to food and water and maintained in a controlled temperature (21 ± 1 °C) and (12-h light/24-h dark) environment.

The following four experiments were executed: (I) Single dose study (3 mg/kg)—leuprolide saline solution (1 mg leuprolide in 1 ml of 0.9% NaCl) or microemulsion was given orally to conscious male rats, respectively. Blood samples were taken at predetermined time intervals and analyzed for plasma leuprolide level. (II) Effective dose study—the leuprolide microemulsion was given to male rats (three groups) for 14 days at doses of 0.25, 0.50, and 1.0 mg/rat/day (qd), respectively. (III) Gender effect study—the leuprolide microemulsion was given to female rats (two groups) for 35 days at doses of 1.0 and 2.0 mg/rat/day (qd), respectively. (IV) Dose regime—the oral leuprolide microemulsion was given to male rats (three groups) for 35 days at 1 mg/rat/day (qd), 2 mg/rat/day (qd), and 1 mg/rat, bid (a total of 2 mg/rat/day). Each experiment also included additional two groups of rats as controls: a untreated group which was given the placebo microemulsion and another group treated with a single dose of Lupron® depot, 3.75 mg/rat, by subcutaneous injection.

Pharmacological responses for studies II–IV were evaluated including animal weight changes in body and genital organ weights (testis, prostate, seminal vesicle, uterus, and ovary) at the end of the treatment, and also plasma testosterone concentrations of male rats at specified time intervals were determined. To assay testosterone levels in plasma, blood was collected periodically from the tail veins of the rats. On the first day, the rats were bled before the first dose, and then 15 min, 1 h, 2 h, 4 h, and 8 h after the dose was given. On subsequent days, the blood samples were taken 30 min before dosing.

2.5. Assay methods for plasma testosterone and leuprolide levels

Plasma testosterone concentrations were determined in duplicate by a radioimmunoassay (RIA) method. The reagent and protocol were supplied by Diagnostic Products Corporation (Los Angeles, CA). Fifty microliters of plasma was used for assay. The lower limit of detection was 0.2 ng/ml.

The plasma concentration of leuprolide acetate was determined using a RIA method described previously (Adjei et al., 1993). Briefly, leuprolide was labeled with ^{125}I via Chloramine-T oxidation and purified by ion-exchange chromatography. Approximately 1200 Ci/mmole of ^{125}I-[Tyr^5]-leuprolide was used as a tracer. An antibody capable of recognizing the tripeptide antigenic determinant ×-Leu-Arg-Pro-NHEt was utilized. The EC_{50} and limit of detection for the assay were approximately 150 and 10 pg/sample. A standard curve with sample concentrations within 5–25,000 pg/tube was used in the assay.

3. Results and discussion

3.1. Microemulsion preconcentrate and particle size

Microemulsion preconcentrate, also known as self-microemulsifying drug delivery system, is a mixture consisting of active pharmaceutical ingredient, oils, and surfactant. Upon dilution with aqueous medium, the preconcentrate spontaneously forms clear isotropic solution, e.g., microemulsion just by gentle agitation. For long-term physical stability reason, microemulsion preconcentrate is preferred over ready-to-use microemulsion and can be directly filled into soft or hard gelatin capsules for commercialization. As listed in Table 1, the leuprolide microemulsion preconcentrate employed Tween 80 as a surfactant and oleic acid as oil as well as an absorption enhancer. Due to the poor oil solubility, water was needed to dissolve leuprolide into the lipid system and ethanol, a cosolvent, was added to reduce surface tension by stabilizing film formation between the two phases (Lawrence and Rees, 2000). When the preconcentrate was diluted with water, a fine w/o/w microemulsion formulation was spontaneously formed. The particle sizes of the microemulsion immediately after their formation were determined. The mean particle size upon 100-fold dilution was 22.5 nm for leuprolide microemulsion and 19.8 nm for placebo microemulsion, respectively. Both visual observation and particle size measurement indicated that w/o/w the microemulsions formed were stable at room temperature for at least a week (data not shown). The microemulsion used in animal studies was prepared fresh and dosed to the animals within a day after dilution.

3.2. Oral absorption of leuprolide in conscious rats

Oral bioavailability of leuprolide is very low. A mixed micellar solution with bile salts and monoolein was found to have 0.05% oral bioavailability compared to an appropriate intravenously injected dose of leuprolide (Adjei et al., 1993). Fig. 1 shows the plasma profiles following oral administration of leuprolide saline solution and microemulsion in conscious rats at a dose of 3 mg/kg. Absorption of leuprolide from the saline solution was poor with the maximum plasma level (C_{max}) of 3.0 ± 2.3 ng/ml at 71.3 min (Fig. 1). However, C_{max} value of leuprolide following oral administration of the microemulsion formulation increased significantly to 31.8 ± 27.8 ng/ml at 10 min. The mean area under the leuprolide plasma level versus time curve (AUC) from the microemulsion was 41.3 ± 16.2 ng h/ml, which is significantly higher than that from the saline solution (5.7 ± 4.3 ng h/ml, $p < 0.01$). The results indicate that oral administration of leuprolide microemulsion containing oleic acid provided better absorption compared with the saline solution. Absorption improvement by emulsion/microemulsions has also been reported for other protein and peptide drugs (Ritschel, 1991; Constantinides et al., 1996). However, it is not well understood about the biochemical mechanism by which permeability of hydrophilic protein/peptide drugs crossing mucosal membranes from microemulsions has been enhanced. Possible mechanisms by microemulsions may include prevention of enzymatic degradation in the gastrointestinal tract and increase of membrane permeability due to oleic acid-induced structure and fluidity change. This hypothesis is supported by several in vitro studies that have shown oleic acid altered intercellular lipid fluidity and disrupted lipid bilayers (Walker and Hadgraft, 1991; Aungst, 1996) and that in vitro degradation of leuprolide in intestinal mucosa was significantly reduced with microemulsions (Zheng and FuLu, 1999).

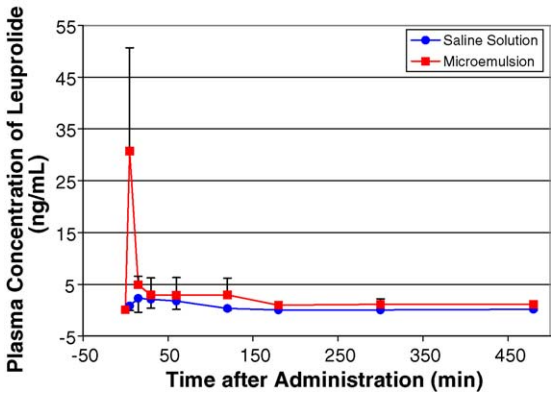

Fig. 1. Plasma concentrations of leuprolide acetate following single oral administration of leuprolide saline solution and microemulsion at a dose of 3 mg/kg in rats. Each data point represents the mean ± S.D. of five rats.

3.3. Effect on testis, prostate, and seminal vesicle of male rats

Leuprolide is a hormone analog and very potent. As a result, the amount of the drug existing in the blood to produce a pharmacological response can be very low. Usually, determination of the pharmacological response might be easier than assaying trace amounts of drug concentrations in the blood because analyses of very low drug concentrations in biological samples are often challenging and sometimes highly variable. Thus, the pharmacological effects become useful in this situation to evaluate potent drug formulations with low bioavailability, especially

for peptide and protein drugs. For example, the vaginal absorption of leuprolide was assessed by determining its ovulation-inducing activity in rats (Okada et al., 1982). When Lupron® depot (100 μg/kg/day) was subcutaneously injected into rats, the significant change in the genital organ weight was observed after 2 weeks of treatment (Okada et al., 1989). As seen in Fig. 1, plasma concentrations of leuprolide following oral administration of microemulsion were very low and also highly variable. Thus, pharmacological responses of leuprolide, such as changes in genital organ weight and plasma testosterone levels, could be another way to evaluate the absorption of leuprolide from the microemulsion.

Table 2 summarizes changes in genital organ weights in male rats after leuprolide microemulsion was orally administered once a day for 14 days at doses of 0.25, 0.5, and 1.0 mg/rat/day, respectively. The weight of testis was significantly lower by treatment at leuprolide doses of 0.25, 0.5, and 1.0 mg/rat/day compared with the placebo control group ($p < 0.05$, Table 2). The weights of seminal vesicle were reduced strikingly by treatment at doses of 0.5 and 1.0 mg/rat/day ($p < 0.05$). However, the weight of prostate was significantly decreased only by treatment at a dose of 1.0 mg/rat/day ($p < 0.05$). Thus, the degree of inhibition on the genital organ weight in rats increased dose-dependently. As seen in Table 2, all three genital organ weights were significantly reduced only at a dose of 1.0 mg/rat/day, indicating that an efficacious dose using leuprolide microemulsion could be greater than 1 mg/day/rat. A single injection of Lupron® depot at a dose of 3.75 mg/rat significantly reduced the weights of the genital organs (testis, prostate, and seminal vesicle) compared with untreated control ($p < 0.01$, Table 2). Interestingly, except the testis weight, no significant differences in the weights of the prostate and seminal vesicle were found between Lupron® depot and oral leuprolide microemulsion at doses of 0.5 and 1.0 mg/rat/day (Table 2). The results suggest that a higher dose of leuprolide microemulsion could lead to comparable pharmacological effects as observed in Lupron® depot.

The animal body weight increase was retarded in all groups after treatment of leuprolide acetate (Table 2). However, ANOVA testing among all groups did not show a significant difference ($p > 0.05$).

Fig. 2 shows plasma testosterone profiles in male rats following 14-day administration of leuprolide formulations or its placebo. When the placebo was given orally to rats (untreated control) for 14 days, the plasma testosterone levels were relatively constant with a mean of 2.34 ± 0.94 ng/ml (Fig. 2). After

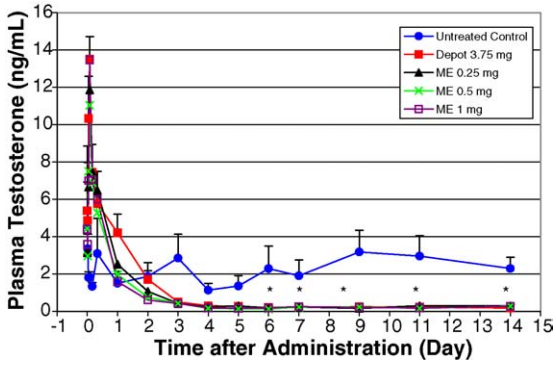

Fig. 2. Plasma testosterone levels in rats after oral administration of leuprolide for 14 days. (●) Untreated control group, placebo microemulsion; (■) Lupron® depot, a single injection of 3.75 mg/rat; (▲) Leuprolide microemulsion at a dose of 0.25 mg/rat, qd for 14 days; (×) Leuprolide microemulsion at a dose of 0.5 mg/rat, qd for 14 days; (□) Leuprolide microemulsion at a dose of 1 mg/rat, qd for 14 days. Each data point represents the mean ± S.D. of five rats. * $p < 0.05$ indicates significant differences compared to the untreated control by Student's *t*-test.

administration of oral microemulsion or injection of Lupron® depot, the plasma testosterone levels for all groups were elevated at the first day of treatment, and then were obviously reduced during 3–14 days of treatment (Fig. 2). The increase of plasma testosterone levels is consistent with the pharmacological activity of leuprolide (Johnson et al., 1976). From the treatment day 6, the plasma testosterone levels of treated groups were significantly lower than the untreated group ($p < 0.05$). However, there was no significant difference in the plasma testosterone levels between the Lupron® depot-treated group and microemulsion-treated groups at doses of 0.25, 0.5, and 1 mg. Therefore, the effect of plasma testosterone concentrations by daily administration of oral leuprolide microemulsion in rats appears comparable with a single injection of Lupron® depot during the 14-day study.

Oleic acid used as an absorption enhancer in the formulation is generally recognized as safe (GRAS) for food. It is also included in the FDA Inactive Ingredients Guide for inhalation, tablets and topical preparations (Weller, 1994). The effects of oleic acid on endocrine activity are widespread, especially in the hypothalamic–pituitary–adrenocortical axis (Widmaier, 1977). However, no in vivo evidence from literature suggests that oleic acid exert direct effects on genital organs and sex hormones in mammalian species. Thus, the pharmacological activities observed after oral administration of leuprolide microemulsion should be resulted from absorption of leuprolide in rats.

Table 2
Effect of dose levels on genital organs and body weight of male rats after oral administration of leuprolide microemulsion for 14 days ($n = 5$, mean ± S.D.)

Formula	Dose (per rat)	Testis	Organ weight (g) prostate	Seminal vesicle	Body weight increase (%)
Placebo control	Untreated	3.59 ± 0.30	0.44 ± 0.08	1.15 ± 0.28	9.5 ± 1.8
Lupron® depot	3.75 mg/35 days, s.c.	1.76 ± 0.27[a]	0.26 ± 0.03[a]	0.64 ± 0.16[a]	4.2 ± 5.0
Oral leuprolide microemulsion	0.25 mg, p.o., qd	2.35 ± 0.39[a,b]	0.36 ± 0.08	0.87 ± 0.10	5.8 ± 5.6
	0.5 mg, p.o., qd	2.21 ± 0.86[a,b]	0.32 ± 0.10	0.68 ± 0.13[a]	18.5 ± 7.5
	1.0 mg, p.o., qd	2.23 ± 0.34[a,b]	0.26 ± 0.09[a]	0.76 ± 0.18[a]	6.6 ± 8.1

[a] Student's *t*-test compared with the placebo control: $p < 0.05$ indicate significant differences at the 5% level.
[b] Student's *t*-test compared with Lupron® depot: $p < 0.05$ indicates significant differences at the 5% level.

Table 3
Effect of dose levels on genital organs and body weight of female rats after oral administration of leuprolide microemulsion for 35 days ($n = 5$, mean ± S.D.)

Formula	Dose (per rat)	Organ weight (g) uterus	Ovary	Body weight increase (%)
Placebo control	Untreated	0.56 ± 0.10	0.089 ± 0.032	25.9 ± 12.2
Lupron® depot	3.75 mg/35 days, s.c.	0.19 ± 0.02[a]	0.047 ± 0.012[a]	17.9 ± 8.6
Oral leuprolide microemulsion	1.0 mg, p.o., qd	0.17 ± 0.02[a,b]	0.043 ± 0.019[a,b]	22.5 ± 10.2
	2.0 mg, p.o., qd	0.20 ± 0.03[a,b]	0.041 ± 0.009[a,b]	20.6 ± 8.2

[a] Student's *t*-test compared with the placebo control: $p < 0.05$ indicate significant differences at the 5% level.
[b] Student's *t*-test compared with Lupron® depot: $p > 0.05$ indicates no significant differences at the 5% level.

3.4. Effect on uterus and ovary weigh of female rats

Leuprolide microemulsion was orally administered to two groups of female rats once a day for 35 days at doses of 1.0 and 2.0 mg/rat/day, respectively, and the uterus and ovary weights were determined at the end of the treatment. The weights of uterus and ovary were significantly reduced by treatments at both dose levels in comparison to untreated rats ($p < 0.05$, Table 3). After 35-day oral administration of leuprolide microemulsion, the weight of the uterus was 31% at the dose of 1 mg and 36% at the dose of 2 mg, when compared with untreated control rats. Also, the ovary weight following oral administration of leuprolide microemulsion for 35 days was 68% of the control rats at a dose of 1 mg and 64% of the control rats at a dose of 2 mg, respectively. Similar pharmacological responses in weights of uterus and ovary were observed when female rats were treated by a single injection of Lupron® depot at a dose of 3.75 mg/rat (Table 3). The reduction of the female genital weight by the leuprolide microemulsion is comparable to Lupron® depot injection. These results suggest that the genital organ weights can be reduced by oral leuprolide microemulsion formulation not only in male rats but also in female rats.

3.5. Effect of dose regime on pharmacological activity in rats

Lupron® depot is a controlled release dosage form and it is intramuscularly injected once a month in humans (Sharifi and Soloway, 1990). However, leuprolide microemulsion should be an immediate release formulation, and thus daily administration is needed for its pharmacological activity. From the 14-day studies, inhibition of rat genital growth by oral administration of leuprolide microemulsion once a day has been demonstrated. Further investigation on pharmacological activities of oral leuprolide microemulsion was conducted using a 35-day chronic study in male rats so that long-term effectiveness can be established. Pharmacological responses by once a day administration were also compared with twice a day administration. It was expected that the twice a day administration of leuprolide microemulsion could be more effective than the once a day dosing regime.

A single injection of Lupron® depot resulted in a significant decrease in weights of testis, prostate and seminal vesicle on the day 35 compared to the placebo control group (Table 4). After leuprolide microemulsion formulation was orally administered to male rats once a day (qd) for 35 days at doses of 1.0 mg/rat/day, the testis and prostate weights were significantly reduced in comparison to the untreated control ($p < 0.05$, Table 4). However, the growth of seminal vesicle was not significantly suppressed when compared to the control group ($p > 0.05$), which is different from the result from the 14-day study. When increasing the dose to 2 mg/rat/day (qd), a significant weight decrease in testis, prostate and seminal vesicle was obtained ($p < 0.05$, Table 4). Similar results of genital growth suppression were obtained when the leuprolide microemulsion was orally given to rats at a dose of 1 mg/rat, bid (a total of 2 mg/rat/day ($p < 0.05$, Table 4)). Statistically, no significant difference in the genital weight changes was observed between qd and bid treatment at the same dose level (Table 4). The data suggested that similar pharmacological activity has been obtained from either qd or bid at a same dose level.

Plasma testosterone levels after administration of leuprolide in rats are given in Table 5. The plasma testosterone concentrations increased greatly just after the first dose treatment with leuprolide microemulsion, and then decreased sharply to below the normal control level after treatments for 2 days. This pattern

Table 4
Effect of oral leuprolide microemulsion on genital organ weights of male rats after administration for 35 days ($n = 7$, mean ± S.D.)

Formula	Dose (per rat)	Organ weight (g)		
		Testis	Prostate	Seminal vesicle
Placebo control	Untreated	3.47 ± 0.22	0.509 ± 0.118	1.48 ± 0.17
Lupron® depot	3.75 mg/35 days, s.c.	1.80 ± 0.30[a]	0.225 ± 0.032[a]	0.26 ± 0.07[a]
Oral leuprolide microemulsion	1 mg, p.o., qd	2.74 ± 0.29[a]	0.464 ± 0.076[a]	1.12 ± 0.30
	2 mg, p.o., qd	2.51 ± 0.30[a]	0.437 ± 0.107[a]	0.87 ± 0.17[a]
	1 mg, p.o., bid (2 mg/day)	2.40 ± 0.38[a,b]	0.395 ± 0.095[a,b]	0.87 ± 0.28[a,b]

[a] *t*-Test compared with the placebo control group: $p < 0.05$ indicate significant differences at the 5% level.
[b] *t*-Test compared with once a day 2 mg group: $p > 0.05$ indicate no significant differences at the 5% level.

Table 5
Changes of rat plasma testosterone levels during oral administration of leuprolide microemulsion for 35 days ($n = 7$, mean ± S.D.)

Time (days)	Plasma testosterone levels (ng/ml)				
	Placebo control	Lupron® depot	Oral 1 mg qd	Oral 2 mg qd	Oral 1 mg bid
0	1.66 ± 0.66	2.46 ± 1.31	1.71 ± 1.02	0.95 ± 0.67	2.25 ± 0.80
0.01	–	5.82 ± 3.69	2.82 ± 1.32	2.62 ± 1.24	3.24 ± 0.90
0.04	–	–	5.28 ± 1.35	7.63 ± 2.58	7.34 ± 1.34
0.33	–	–	6.74 ± 1.53	5.53 ± 1.18	6.87 ± 1.82
1	2.95 ± 0.88	10.3 ± 3.77	1.26 ± 0.64	1.25 ± 0.41	1.66 ± 0.64
2	3.83 ± 3.27	4.32 ± 1.35	0.59 ± 0.21	0.46 ± 0.13	0.52 ± 0.15
5	2.25 ± 1.40	0.66 ± 0.27[a]	0.39 ± 0.16[a]	0.30 ± 0.08[a]	0.21 ± 0.13[a]
7	1.93 ± 0.70	0.66 ± 0.28[a]	0.40 ± 0.23[a]	0.32 ± 0.16[a]	0.35 ± 0.20[a]
11	3.21 ± 2.19	0.38 ± 0.16[a]	0.40 ± 0.21[a]	0.37 ± 0.14[a]	0.19 ± 0.13[a]
15	2.03 ± 0.83	0.28 ± 0.10[a]	0.36 ± 0.19[a]	0.38 ± 0.12[a]	0.12 ± 0.07[a]
18	1.54 ± 0.67	0.38 ± 0.28[a]	0.40 ± 0.15[a]	0.03 ± 0.06[a]	0.48 ± 0.46[a]
28	1.76 ± 0.55	0.26 ± 0.08[a]	0.24 ± 0.10[a]	0.26 ± 0.12[a]	0.06 ± 0.06[a]
35	3.60 ± 1.80	0.32 ± 0.17[a]	0.29 ± 0.22[a]	0.16 ± 0.08[a]	0.15 ± 0.02[a]

[a] *t*-Test compared with the placebo control group: $p < 0.05$ indicate significant differences at the 5% level.

following oral administration of leuprolide microemulsion was similar to that observed from the 14-day study. Reduced levels of plasma testosterone in rats were maintained over the 35-day experiment and were significantly different when compared with the untreated control group ($p < 0.05$). The similar profile was also observed in rats treated by Lupron® depot (Table 5). In this 35-day treatment study, decreases in plasma testosterone levels by oral leuprolide microemulsion were comparable to Lupron® depot, although the effects of the oral formulation on the genital organ weight losses were weaker (Table 4). Nevertheless, this data supports that absorbed leuprolide in rat systemic circulation when given by the microemulsion formulation has obvious pharmacological effects on steroidogenesis and reproductive organs. Thus, oral leuprolide microemulsion may be an alternative route to Lupron® depot injection as demonstrated in this work.

4. Conclusions

A microemulsion preconcentrate was developed in this study and oleic acid was used as an oral absorption enhancer. Upon dilution in water, the preconcentrate immediately formed a stable w/o/w microemulsion. Oral administration of the leuprolide microemulsion showed a better exposure level (C_{max} and AUC) than its saline solution. Leuprolide microemulsion also demonstrated reduction in the weights of rat genital organs in male rats and decreased plasma testosterone levels to the castrate level. Inhibition of genital growth was also observed in female rats by treatments with oral leuprolide microemulsion. The weights of the uterus and ovary following oral administration of leuprolide microemulsion for 35 days were significantly decreased in comparison to untreated rats. The pharmacological activities of oral leuprolide microemulsion in rat organ weights of prostate, seminal vesicle, uterus, and ovary were comparable with the commercial product Lupron® depot. These findings suggest that oral absorption of peptides, like leuprolide acetate, can be improved using microemulsion formulations with oleic acid as a permeation enhancer.

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