# Percutaneous Absorption Enhancement of Leuprolide

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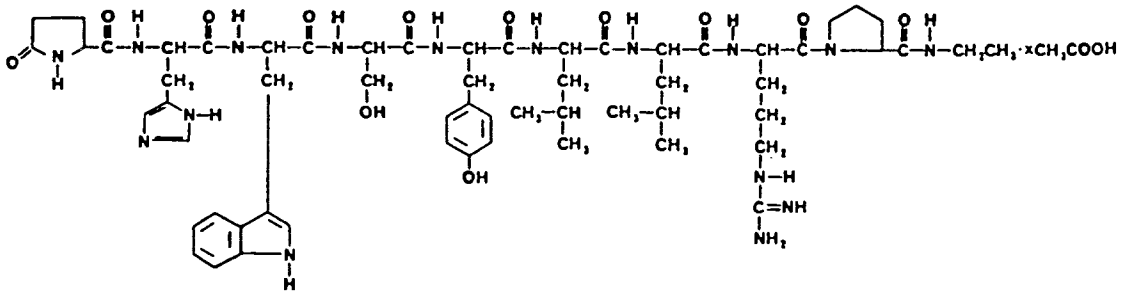
Chemical enhancers and vehicles were tested for their ability to improve the percutaneous absorption of leuprolide, a nonapeptide (luteinizing hormone releasing hormone analogue; MW 1209.4). *In vitro* permeabilities in nude mouse, snake, and cadaver skin were evaluated in either Franz diffusion cells or a Bronaugh flow-through system using an HPLC assay. Skin irritation caused by the formulations was evaluated in the rabbit. The chemical enhancer systems investigated strongly enhanced skin penetration of leuprolide. Maximum permeability enhancement of leuprolide acetate can be achieved with a nonirritating formulation containing ethanol, menthol, camphor, methyl salicylate, urea, and hydrogel. The *in vitro* permeability in nude mouse skin was 10 or 100 times higher than that obtained in cadaver skin, depending on the type of enhancer that was used in the formulation. Snake skin was at least 10 times less permeable than cadaver skin in this study. However, the effects of chemical enhancers on skin permeability were highly dependent on the skin model. Further, the *in vitro* permeability of leuprolide in the base form was 10 times higher than in the acetate form with the enhancers.

KEY WORDS: leuprolide; percutaneous absorption; human skin; snake skin; mouse skin; menthol; camphor; methyl salicylate; urea; skin irritation.

## INTRODUCTION

Leuprolide acetate is a nonapeptide chemically defined as 5-Oxo-Pro-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-ethylamide acetate, with the following chemical structure.



$C_{59}H_{84}N_{16}O_{12} \cdot xCH_3COOH$, MW 1209.41 as free base

Leuprolide base has three ionizable sites: imidazolyl nitrogen of histidine, phenol hydroxyl of tyrosine, and guanidine nitrogen of arginine. As a result of the synthetic procedure, leuprolide is associated with at least 1 mol of acetic acid, and therefore this peptide exists as an acetate salt.

Leuprolide acetate is a potent luteinizing hormone releasing hormone (LHRH) agonist which, when introduced to the portal circulation, induces the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the anterior pituitary (1). The oral bioavailability of leuprolide is low (2). Currently, this drug is administered by parenteral routes: 3.75- and 7.5-mg monthly depot intramuscular injections (Lupron Depot) and 1-mg daily subcutaneous injection (Lupron).

Various nonparenteral routes have been considered as alternative methods for administration of leuprolide acetate or LHRH analogue, such as aerosol inhalation (3), nasal spray (4), and iontophoresis (5,6). The present work focuses on passive transdermal delivery. In order to deliver this peptide to the systemic circulation through the skin, enhancement of the percutaneous absorption of this compound is required. The poor membrane permeability of leuprolide is due to its large molecular size and hydrophilic nature; however, we have shown that chemical enhancer systems can dramatically increase the percutaneous permeability of leuprolide acetate.

The ideal way to determine the potential of transdermal delivery of a drug in humans is to do the study in humans. Since the cost and complexity of studies in humans are considerable, laboratory animals have been used to screen formulations before clinical studies, but it is of concern (7) whether results from animal skin can be extrapolated to human skin. Previously, the variability of percutaneous drug absorption of drug compounds in different animal species has been reported (8). We report that chemical enhancer systems increased the permeability of leuprolide to a different extent in human cadaver, nude mouse, and snake skin.

Skin irritation following topical drug application remains a major concern in dosage form design (9,10). Primary skin irritation is an immediate irreversible nonimmunologic localized inflammatory response. It is usually characterized by nonspecific effects such as erythema, edema, and necrosis (11). In this work, skin irritation caused by the formulations tested has been examined in rabbits.

## MATERIALS AND METHODS

### Chemicals and Formulations

*Formulation.* The following chemicals were used to prepare formulations for *in vitro* diffusion studies: leuprolide

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acetate (Abbott Laboratories), 10–40 mg/ml; ethyl alcohol, 200 proof, USP, 20–80% (in vehicle); hydroxypropylcellulose (Hercules Incorporated), 2% (in vehicle); menthol, reagent grade, 0.5–1.0%; camphor, reagent grade, 0.5–1.0%; methyl salicylate, reagent grade, 0.5–2.0%; urea, reagent grade, 0 or 10%; and purified water, USP, 80–20% (in vehicle).

The vehicle was a homogeneous gel solution containing 2% Klucel EF and different proportions of ethyl alcohol and water according to the formulation. Other chemicals and leuprolide were weighed and added into the vehicle, and the volume was adjusted with the vehicle. Formulations were prepared within 24 hr prior to study and were kept at −5°C. The potency of leuprolide acetate in formulations was determined by HPLC assay (12).

*Preparation of Leuprolide Base.* A solution of 1 g of leuprolide acetate in 15 ml of methanol was loaded onto an anionic exchange resin (Bio-Rad AG-1 × 2; 200- to 400-mesh) column in the $OH^-$ form, which was prepared by passing a solution of 0.5 *N* methanolic sodium hydroxide to 31.4 g of $Cl^-$ form resin. The free base was eluted from the column with methanol. The methanol solution was evaporated on a rotary evaporator and the residue was further dried under vacuum for 3 hr. The purity of free base was measured by HPLC as 96.5%

### *In Vitro* Diffusion Study

*Procedure. In vitro* diffusion experiments were done using a Franz diffusion apparatus (Vanguard International, Inc., Neptune, NJ) and a Bronaugh flow-through system (13). The exposed skin surface area was 4.52 $cm^2$ in Franz cells and 0.32 $cm^2$ in the Bronaugh system. The dose of formulation per cell was 0.4 ml for the Franz apparatus and 0.13 ml for the Bronaugh flow-through system. The receptor fluid, 40% polyethylene glycol (PEG) 400 and 60% 0.1 *M* HEPES buffer (*N*-2-hydroxyethylpiperazine *N'*-2-ethanesulfonic acid; Sigma Chemical Co.), was initially chosen for reducing peptide binding to the glass. However, in comparison to the 100% HEPES buffer, the receptor fluid containing 40% PEG 400 was able to preserve skin integrity for a longer period (up to 48–72 hr) during *in vitro* experiments. The donor compartment was sealed with parafilm after application of formulation. The water jacket was maintained at 37°C throughout the test period. Samples were taken from the receptor compartment at predetermined intervals up to 36 hr after application of formulation on the skin. Leuprolide acetate was measured by HPLC (12).

The purity of permeant compounds and the possibility of chemical or enzymatic degradation of the compounds during experiments were checked by analysis of the donor chamber and the receiver chamber solutions at different times. In all formulations, and in the receptor fluid, leuprolide was stable during diffusion experiments. Metabolism of leuprolide was not observed during transport across skins.

The cumulative amounts of leuprolide acetate in receptor fluid, divided by skin area, were plotted as a function of time. The steady-state flux (slope) was calculated by linear regression. The flux value was divided by the leuprolide concentration in the formulation to give the permeability *P* value.

*Nude Mouse Skin Preparation.* Skins were obtained from 6- to 8-week-old hairless female mice (Charles River Laboratories). The freshly excised skins (from an abdominal site) were mounted on diffusion cells and then preconditioned for 40 min. The surface of the skin on the donor side was fully covered with formulation when the experiment started.

*Human Cadaver Skin Preparation.* Skin was obtained from the National Disease Research Interchange (Philadelphia PA). Skin pieces were from the thigh area and were dermatomed to a thickness of 200–300 μm and frozen at −70°C. Skins were thawed and hydrated in saline solution overnight (about 15 hr). The fully hydrated skins were mounted on diffusion cells (in the flow-through system) and preconditioned for 40 min before application of formulations. Skin in the assembled diffusion cells was rapidly screened for barrier integrity by applying [$^3$H]water to the surface of the skin (13).

*Snake Skin Preparation.* The dorsal portion of shed snake skin was a gift from the University of Kansas. The dried skin was placed in deionized water for 10–15 min and cut into pieces large enough to fit the diffusion cell. Dirt particles attached to the snake skin were removed by gentle cleaning. The pieces of skin were dried and stored in a freezer. Just prior to use, the pieces of skin were soaked in the receptor fluid (40% PEG and 60% HEPES buffer) for 2 hr to allow for full hydration. The hydrated pieces of skin were mounted on diffusion cells (in the flow-through system) and preconditioned for 40 min before application of formulation.

### Partitioning Studies

Partitioning studies were conducted in Type I glass culture tubes provided with Teflon-lined caps. Octanol-saturated water was used to prepare a solution of leuprolide acetate or base at a concentration of 500 μg/ml. Ten milliliters each of the solution and 10 ml of water-saturated octanol were pipetted into the tubes. These were tightly screw-capped and placed in a shaker bath (at 30°C) and agitated for 24 hr. After equilibrium the tubes were centrifuged at 2000 rpm for 10 min. The octanol and aqueous phases were both analyzed by HPLC for leuprolide. The octanol–water distribution coefficient, *Kp*, was calculated from the ratio of leuprolide concentrations in the nonaqueous and aqueous phases.

### Skin Irritation Test in Rabbits

Female albino rabbits weighing 2–3 kg were used to evaluate the formulation effect on skin irritation. Each individual formulation was tested in four rabbits. A fixed volume (0.4 ml) of formulation was filled into Hill Top Chamber patches (surface area, 2.6 $cm^2$ in each patch). The patch was applied to the rabbit for 24 hr and then the skin surface was visually examined for redness, discoloration, swelling, and necrosis. Twenty-four hours before the test, rabbits were shaved at the abdominal site using a clipper. It is assumed that 24 hr would allow skin to recover from the shaving process. After patch application, all rabbits were housed in stainless-steel cages equipped with feeders and automatic water dispensers.

## RESULTS AND DISCUSSION

The initial *in vitro* transport studies were conducted using a static diffusion system before the continuous-flow system was available in the laboratory. A comparison study was done using hairless mouse skins to establish equivalence of these two systems. No significant difference in permeation results from these two systems was observed. Both systems can provide sink conditions for the diffusing chemical (leuprolide), considering the high solubility of leuprolide acetate in the receptor fluid.

### Permeability in Nude Mouse Skin

*Effect of Ethyl Alcohol.* The effect of ethyl alcohol concentration (at volume fraction 20–80%) on skin permeability is shown in Fig. 1. The results showed that permeability increased with an increasing content of ethyl alcohol in the formulation. Skin penetration enhanced by ethanol has been reported by many investigators with various permeants (14,15). The permeability of leuprolide acetate from a totally aqueous solution was below the HPLC assay limit ($P < 1 \times 10^{-6}$ cm/hr) through nude mouse skin. As shown in Table I, the permeability ($2 \times 10^{-6}$ cm/hr) of leuprolide acetate from the formulation containing ethanol (at the 80% level) alone as an enhancer through nude mouse skin was barely measurable. In order to obtain flux values high enough to be measured by HPLC, all formulations shown in Fig. 1 contained 1% menthol as a second permeability enhancer besides ethanol. However, this study demonstrated that a higher ethanol concentration is more favorable for percutaneous penetration of leuprolide.

*Effect of Chemical Enhancers.* The permeability of leuprolide acetate through nude mouse skin was dramatically enhanced by menthol, camphor, methyl salicylate, lauric acid, or decanoic acid (Table I). All formulations tested were prepared in a common vehicle containing ethyl alcohol and water (4/1, v/v) as well as 2% hydroxypropyl cellulose. A small amount of this low-viscosity hydrogel (hydroxypropyl cellulose) can minimize solvent evaporation during the experiments, resulting in lower standard deviations (coefficient of variation, 10–30 vs 50–100%). Menthol has shown superior permeability enhancement over the other four chemicals tested. There was moderate skin irritation observed in rabbits treated with formulations containing 2% menthol or lauric acid. The observations from skin irritation tests indicated that the combination of a high level of ethyl alcohol and chemical enhancer was the main cause of irritation in skin. When the level of ethyl alcohol in the vehicle decreased, the skin could generally tolerate the other chemical enhancers better and at a higher concentration. For example, skin irritation studies have shown that with a solvent containing 80% ethyl alcohol and 20% water, a safe formulation cannot exceed a 1% concentration of menthol.

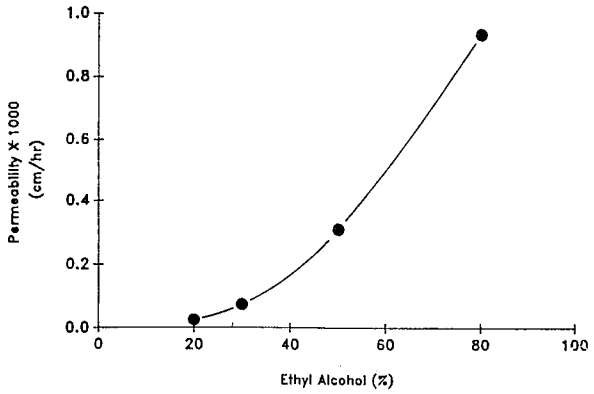


Fig. 1. The effect of ethyl alcohol concentration in vehicle on permeability of leuprolide acetate through nude mouse skin. Formulation: 10 mg/ml leuprolide acetate, 1% menthol, 2% Klucel EF, and water.

Table I. Effect of Chemical Enhancer on Permeability of Leuprolide Acetate Through Nude Mouse Skin ($n$ = 6): Static Diffusion System[a]

| Enhancer | Permeability × 1000 [cm/hr; mean (SD)] |
|---|---|
| None | 0.002 (0.001) |
| Menthol | |
| 0.5% | 0.182 (0.094) |
| 1% | 0.942 (0.321) |
| 2%[b] | 3.044 (0.810) |
| Camphor, 2% | 0.132 (0.059) |
| Methyl salicylate, 2% | 0.130 (0.078) |
| Lauric acid, 2%[b] | 1.350 (0.515) |
| Decanoic acid, 2% | 0.766 (0.291) |

[a] All formulations were prepared in a solvent containing ethyl alcohol/water (v/v, 4/1) and 2% Klucel. Drug concentration, 40 mg/ml.
[b] These formulations caused erythema in rabbits.

The addition of camphor and methyl salicylate in the 1% menthol formulation (as shown in Table II) was found to enhance further the permeability of leuprolide, but not to cause any skin irritation. Table II compares the permeability of leuprolide acetate in five formulations which involved three chemical enhancers, menthol, camphor, and methyl salicylate. The permeability obtained from the three-enhancer system (Formulation C) was higher than those obtained from the formulations containing only menthol (Formulation A) or menthol and camphor (Formulation B). Increasing the concentration of methyl salicylate to 2% and camphor to 1% in Formulation E only slightly increased *in vitro* permeability. No skin irritation was noticed from any of these formulations. Very slight variation in permeability data

Table II. Effect of Menthol, Camphor, and Methyl Salicylate on Permeability of Leuprolide Acetate Through Nude Mouse Skin ($n$ = 6): Static Diffusion System[a]

| | Menthol (%) | Camphor (%) | Methyl salicylate (%) | Permeability × 1000 (cm/hr) |
|---|---|---|---|---|
| A | 1 | — | — | 1.090 (0.435) |
| B | 1 | 0.5 | — | 1.201 (0.056) |
| C | 1 | 0.5 | 0.5 | 2.876 (0.787) |
| D | 1 | 0.5 | 2.0 | 2.985 (0.754) |
| E | 1 | 1.0 | 2.0 | 3.971 (0.537) |

[a] All formulations were prepared in a solvent mixture of ethyl alcohol/water (v/v, 4/1) and 2% Klucel. Drug concentration, 40 mg/ml.

Table III. Effect of Urea on the Permeability of Leuprolide Acetate Through Human Cadaver, Nude Mouse, and Snake Skins ($n$ = 6): Flow-Through Diffusion System[a]

| Skin | Urea (%) | Menthol (%) | Camphor (%) | Methyl salicylate (%) | Permeability × 1000 [cm/hr; mean (SD)] |
|---|---|---|---|---|---|
| Cadaver | 0 | 0 | 0 | 0 | 0.000 (0.000) |
| | 0 | 1 | 1 | 2 | 0.029 (0.038) |
| | 10[b] | 1 | 1 | 2 | 0.220 (0.115) |
| | 10 | 0 | 0 | 0 | 0.000 (0.000) |
| Mouse | 0 | 0 | 0 | 0 | 0.020 (0.016) |
| | 0 | 1 | 1 | 2 | 2.649 (1.331) |
| | 10[b] | 1 | 1 | 2 | 2.510 (1.395) |
| | 10 | 0 | 0 | 0 | 0.000 (0.000) |
| Snake | 0 | 1 | 1 | 2 | 0.000 (0.000) |
| | 10[b] | 1 | 1 | 2 | 0.017 (0.003) |

[a] Solvent: ethyl alcohol/water (v/v, 4/1) and 2% Klucel. Drug concentration, 40 mg/ml leuprolide acetate.

[b] Figure 2 plots the cumulative amount of leuprolide permeated through human, mouse, and snake skins over time from this formulation.

from the same formulation (Formulation E) was observed in two independent studies (data shown in Tables II and III). The variability from one lot of mouse skin to another lot was acceptable.

### Comparative Permeability in Human Cadaver Skin, Snake Skin, and Hairless Mouse Skin

Table III compares formulation effects on permeability of leuprolide acetate in human, mouse, and snake skin. Formulations were prepared in a vehicle containing ethanol/water (4/1, v/v) and 2% Klucel. This study was done with a flow-through diffusion system. The permeabilities through mouse skin and human skin were enhanced by menthol, camphor, and methyl salicylate (as Formulation E in Table II). With this formulation, human skin was 10-fold less permeable than mouse skin, but much more permeable than snake skin. The results obtained from this study agree with the general observations: The skins of common laboratory animals are more permeable than the skin of humans (7) for various compounds.

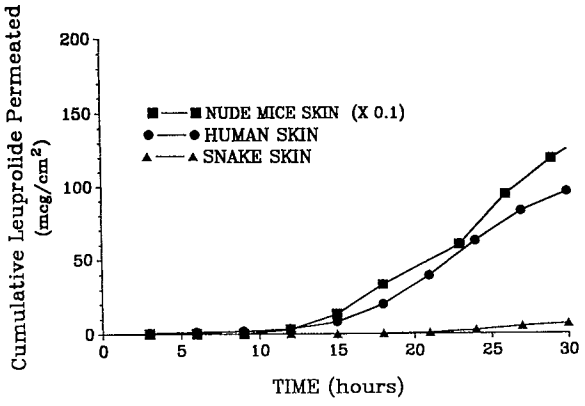


Fig. 2. Cumulative amount of leuprolide permeated through human skin, mouse skin, and snake skin over time, from the formulation shown in Table III.

*Effect of Urea.* The effect of urea on enhancing permeability in human skin, snake skin, and hairless mouse skin is also shown in Table III. The addition of 10% urea in the formulation further increased the permeability of leuprolide acetate up to 10-fold in human cadaver skin and snake skin. However, a similar penetration enhancement effect of urea on nude mouse skin was not observed. Therefore shed snake skin may be a better animal model than mouse skin to study the effect of urea on penetration enhancement to leuprolide. The permeability of snake skin has been reported to resemble more closely that of human skin (16).

A formulation containing 10% urea alone in vehicle containing ethyl alcohol/water (4/1, v/v) without other agents did not enhance the permeability of leuprolide acetate in human cadaver skin or mouse skin. Urea alone is ineffective for penetration enhancement of leuprolide. With the optimized formulation (containing 1% menthol, 1% camphor, 2% methyl salicylate, and 10% urea in ethanol/water vehicle), nude mouse skin was 10-fold more permeable than human skin ($P$, $2.5 \times 10^{-3}$ vs $2.2 \times 10^{-4}$ cm/hr), and snake skin was about 13-fold less permeable than human skin ($P$, $1.7 \times 10^{-5}$ vs $2.2 \times 10^{-4}$ cm/hr). Figure 2 plots cumulative amount of leuprolide permeated through human, mouse, and snake skins over 30 hr from the same formulation. Ethanol in the formulation had penetrated and been replaced by water, which back-diffused from the receptor compartment after 30 hr. The

Table IV. Comparison of Partition Coefficients and Permeability of Leuprolide Acetate to Base Through Human Cadaver Skin ($n$ = 6): Flow-Through Diffusion System[a]

| Leuprolide form | Octanol/water $K$ | Permeability × 1000 [cm/hr; mean (SD)] |
|---|---|---|
| Acetate | 0.022 | 0.220 (0.115) |
| Base | 0.119 | 2.119 (0.338) |

[a] Formulation: 1% menthol, 1% camphor, 2% methyl salicylate, and 10% urea. Solvent, ethyl alcohol/water (v/v, 4/1) and 2% Klucel. Drug concentration, 40 mg/ml.

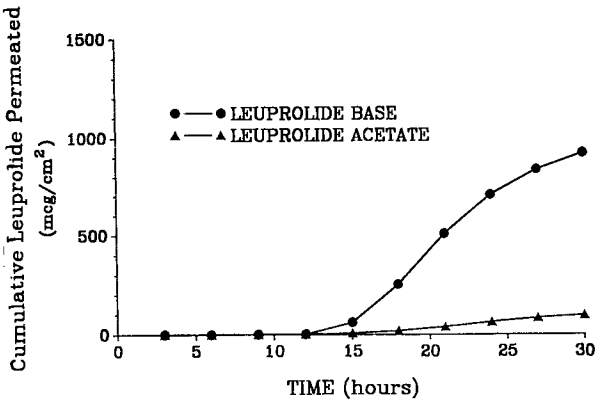


Fig. 3. Cumulative amount of leuprolide permeated through human skin over time from leuprolide acetate and base. The formulation is shown in Table IV.

permeability coefficients from this study were determined during the time period of 15 to 30 hr.

Urea is a mild keratolytic agent used in the treatment of ichthyosis and other hyperkeratotic skin conditions. As a 10% cream it increases the water holding capacity of the stratum corneum by 100% and has little effect on the epidermal water barrier (17). The moisturizing and keratolytic effects of urea increase the activity and bioavailability of hydrocortisone from Alphaderm cream (18). Urea analogues in propylene glycol enhance the permeability of 5-fluorouracil sixfold by increasing the diffusivity of the stratum corneum in human skin (19).

The role of urea in the present formulation for further enhancing the permeability of leuprolide in human and snake skin could be through a hydration or/and keratolytic effect. Hydration of skin is believed to be a mechanism to enhance the rate of drug transport (20).

### Permeability of Leuprolide Base vs Acetate

Leuprolide acetate was converted to the more lipophilic form, leuprolide base, according to the methods described under Materials and Methods. A comparison of the partition coefficient and permeability of leuprolide acetate and base is shown in Table IV. The flow-through system was used for the diffusion studies. The octanol-to-water partition coefficient of the base is fivefold higher than that of the acetate salt. Plots of cumulative amount of leuprolide from the acetate salt and base form permeated through human skins over 30 hr are shown in Fig. 3. The permeability coefficients from this study were determined during the time period of 15 to 27 hr. Figure 3 and Table IV show that on converting the acetate form to the base form, the permeability of leuprolide in cadaver skin increased 10-fold. The improved lipophilicity may contribute to the superior permeability of leuprolide base over acetate.

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