Abstract
Human growth hormone (hGH) is one of the most important large protein drugs, which is secreted by the pituitary gland. Deficiency in this hormone causes stunted growth and mental retardation. Recently, replacement therapies including hGH have become one of the most popular anti-aging treatments especially in cosmocauticals. In this study, the transdermal route as an alternative route for in vitro hGH hormone delivery was investigated. hGH was formulated using various cyclodextrins (CDs). The enhancing effect of CDs on hGH skin permeation was investigated in vitro using Franz diffusion cells. The results suggest the enhancing effect of CDs on in vitro transdermal delivery hGH.

Key words: hGH, Transdermal delivery, Chemical enhancers, Skin, Cyclodextrins.

Introduction
Transdermal delivery of therapeutic agents has been used successfully for decades. Transdermal systems for hormone replacement therapy, smoking, cessation and pain management are well accepted. However, there have been challenges in extending the use of the technology to the delivery of peptides, proteins and other macromolecules (4). In view of the above mentioned factors, this research aimed to develop the transdermal delivery of hGH. Hence, this drug was chosen for delivery via the transdermal route.

Transport of macromolecules into the skin is slow due to the resistance of the outer most layer of the skin, known as the stratum corneum (SC) (7). A variety of methods such as physical, biochemical and chemical have been studied in attempts to overcome this barrier. One of the most promising and the most extensively studied techniques is the use of chemical enhancers (8). Many studies have investigated the mechanisms of the action of chemical enhancers and the following have been suggested as possible explanations for activity:

1. Interaction with intercellular lipids of the SC resulting in disorganization of the highly ordered structures thus enhancing paracellular diffusion through the SC.
2. Interaction with intracellular proteins of the corneocyte to increase transcellular permeation.
3. Increasing partitioning of the drug into the SC.

Enhancing effect of cyclodextrins
Several excipients are able to promote the transport of an active substance across the skin barrier. (1). Potential substances used for this purpose need to have both features, i.e., drug penetration-promoting effects and a low or no skin irritating potential. In fact pharmaceutical scientists are searching penetration enhancement techniques for transdermal penetration of drugs with no side effects. Complex of drugs with cyclodextrins has been used to enhance aqueous solubility and drug stability. Cyclodextrins of pharmaceutical relevance contain 6, 7 or 8 dextrose molecules (α-β-8 cyclodextrin) bound in a 1,4 configuration to form rings of various diameters, with a hydrophilic exterior and lipophilic core in which appropriately sized organic molecules can form non-covalent inclusion complexes resulting in increased aqueous solubility and chemical stability (6). Derivatives of α-cyclodextrin with increased water solubility (e.g., hydroxypropyl-β-cyclodextrin (hp-β-CD)) are most commonly used in pharmaceutical formulation.

Theoretically, cyclodextrin can enhance drug bioavailability by stabilizing drug molecules at the biomembrane surface. In general, drug stabilization associated with cyclodextrin complexation plays only a very minor role when it comes to drug delivery through biological membranes since it is their solubilizing effect that is usually related to improved drug delivery. Based on improvement in the quality of life that replacement therapy produces in patients with hGH-deficiency and based on clinical similarities between aging and hGH deficiency, researchers are now exploring possible additional therapeutic applications for hGH and using supplemental hGH in physiological doses to slow the normal catabolic changes of aging. In the 1990s, the FDA approved hGH therapy for adults who are deficient in the hormone, which includes most people who are over 40 years of age (5).

More readily available biosynthetic (recombinant) forms of hGH have helped to extend its clinical applications to adult patients who are not pathologically deficient in hGH. Because of the large size of the molecule and its labile structure, there is no acceptable delivery system for the human growth hormone other than by subcutaneous injection. There are no hGH products that bear labeling approval from the FDA for delivery by oral or transdermal routes. This absence of more appropriate dosage forms is the result of the fact that enzymatic degradation and poor absorption (across the skin) because of its large molecular weight presents access of the molecule to systemic circulation by non-parenteral administration. The objective of this paper was to contribute new experimental data in order to analyze and compare the effects of chemical enhancers on transdermal absorption of hGH. Chemical enhancers employed were α-β and hp-β cyclodextrin.

Materials and Methods

rhGH (4 IU/mg) was obtained from Novo Nordisk pharma Co (Tokyo, Japan). ELISA kits for determination of hGH concentration in the samples were purchased from PADTAN ELM (Iran). α-cyclodextrin, β- cyclodextrin and hp-β-CD were obtained from the Sigma Chemical Company. Male rats were obtained from the animal house of the Pasteur Institute (Tehran, Iran). All other solvents and reagents used in this study were procured from Merck and prepared with purified water.

Preparation of drugs: The Chemical penetration enhancers (CPEs) used in the permeation studies of the drug were α-CD, β-CD and hp-βCD. The hGH solution was prepared in the presence of CDs at weight ratios of 1:2 (hGH: CDs) and 1:4 (hGH: hp-βCD). For preparation of the 1:2 and 1:4 ratios, aqueous CD solutions were made by

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dissolving 2.6 mg of CDs and 5.2 mg of hP-βCD in distilled water (2.6 ml). Then 1.3 mg of hGH was added to each aqueous CD solution and mixed with a magnetic stirrer at 300 rpm for 60s. A control solution of hGH in the absence of the enhancers was prepared at a concentration of 0.5 mg/ml. A 1.3 mg sample of hGH was dissolved in 2.6 ml of distilled water, then mixed with a magnetic stirrer at 300 rpm for 60 s. A 1 ml sample of the prepared solution was placed in the donor compartment of the skin equipped diffusion cells.

**In vitro permeation studies:** The drug diffusion studies were performed using the Franz diffusion cells (Fig.1). The total volume of the cell in the receptor compartment was 30 ml. The rat skin was placed between the donor and receptor compartments. The compartments were then clamped together. Drug (1 ml) was applied uniformly. The receptor compartment was filled with 30 ml of phosphate buffer (0.05 M, pH 7.5). The cell was placed in a water bath maintained at 37 ± 0.5°C. The receptor compartment was filled with 30 ml of degassed phosphate buffer solution (pH 7.2) under constant stirring with a magnetic stirrer. Samples (1 ml) were drawn from the receptor compartment periodically every 2, 4, 6, 8 and 24 h and then replaced by the same volume of receptor solution. The samples were analyzed for hGH content by using sandwich ELISA technique. The results were plotted, as the cumulative amount released (Q) versus time (T).

The parameters of the in vitro skin permeation study were calculated by plotting the cumulative drug amount permeated through the skin versus time. The slope of the linear portion of the permeation curve provided the flux value (μg cm⁻²h⁻¹) at steady state. The lag time (Tlag) was determined by extrapolating the linear portion of the curve to the X-axis. The cumulative drug amount in the receptor compartment after 24 h was defined as Q24 (μg cm⁻²). Enhancement ratio (ER) for flux was calculated using the following equation:

\[
ER = \frac{\text{Flux for skin treated with enhancer}}{\text{Flux for control (skin without enhancer treatment)}}
\]

**Preparation of rat abdominal skin:** Because of the similarity between male rat and human skins, male rat skin (150-200g) was used as a biological model for in vitro skin penetration studies. A rat was killed by chloroform and hairs of the abdominal region were excised. Skin from the outer surface of the abdominal region was carefully dissected. Subcutaneous fat was carefully removed with a scalpel and rinsed with normal saline and stored at -20°C until further use.

**Determination of hGH concentration by ELISA:** The concentration of hGH in the receptor phase (an indicator of transdermal delivery) was analyzed by using the sandwich ELISA technique. The hGH quantitative test kit based on the solid phase enzyme immunoassay (EIA) used two mouse monoclonal antibodies directed against distinct antigenic determinants on the hGH.
molecules. The GH present in the standards and samples was bound to the anti-GH antibodies, resulting in the development of a blue color. The intensity of the color produced was proportional to the amount of GH in the sample. The color intensity was determined in a microtiter plate by a spectrophotometer at 450 nm. Standard curves were constructed for each assay by plotting the absorbance value against the concentration of each standard. The GH concentrations of the samples were then obtained from the standard curve.

**Stability analysis:** Stability studies of hGH in the enhancer solutions were performed under defined collection and processing conditions at room temperature (RT). The hGH solutions were prepared in the presence of CDs at weight ratios of 1:2 (hGH: CDs). The control solution of hGH in the absence of any enhancer was prepared at a concentration of 0.5 mg/ml. Samples were obtained periodically at 2, 4, 6, 8, 24 h and one week (168 h) post-incubation. Samples were then analyzed for hGH stability with 13% (w/v) SDS-PAGE and the ELISA assay.

**Data and statistical analysis:** The amounts of hGH that permeated through the excised rat skin were plotted as a function of time. Statistical analysis of the data was performed using one-way analysis of variance (ANOVA, $\alpha=0.05$) the least significant different test (LSD) was followed if the ANOVA indicated that a difference existed. LogP value of the model drugs was determined using the ACD program. All the skin permeation experiments were repeated three times. The mean values and corresponding standard deviations are presented in the figures and tables. The student's $t$-test was performed to find any significant difference in the permeation between or among hGH containing CPEs and free enhancer formulation (control). A value of $p < 0.05$ was considered statistically significant.

**Results and Discussion**

The skin permeation profiles of hGH in the control group and complexed with various CDs and comparison of the three CDs’ effectiveness are shown in Fig. 2.

![Fig. 2. Permeation profiles of hGH in the free state (control group) and complexed with CDs (+: hP-βCD, ♦: α-CD, ▲: β-CD, □: control group).](image)

The skin penetration of hGH without CDs was negligible because of its hydrophilic nature and also high molecular weight (20 KDa). As shown, CDs can enhance the penetration of hGH through the skin more effectively (Fig. 2). Among the various CDs, β-CD showed the lowest enhancing effects on hGH transdermal delivery. Probably, this is due to the limited aqueous solubility of β-CD in which the interaction of lipophilics with these CDs is poor, thus causing precipitation of the solid CD complexes from water and other aqueous system (9). Among the applied CDs, hp-βCD showed the highest enhancing effects on hGH transdermal delivery. In contrast to βCD, hp-βCD increases the solubility of the complexes...
by approximately 10-100 times. By dividing the amount of drug released together with enhancer by the amount of released drug without enhancer, the percentage of enhanced drug penetration is achieved at different time courses (Fig. 3).

Different enhancers by interaction with intracellular lipids cause decomposition and increased fluidity of them. The results indicate that maximum permeation is related to hpCD and equals 15501 ng ml cm⁻² thus having the most effect on growth hormone permeation. Also, studying the stability of the growth hormone in the presence of hp-βCD by the ELISA test (table 1) and SDS-PAGE (Figs 4-a, b), indicated that the amount of the growth hormone remained constant after one week and was thus stable during this period of time.

Fig. 3. Percent of enhanced for various CDS. ■: βCD, ◆: α-CD, ●: hp-β-CD, constant after one week and was thus stable during this period of time.

Fig. 4. a) SDS-PAGE for hGH (Nurditropin) standard. Lane 1: The low molecular size marker. Lanes 2-8: The samples were obtained after 2, 4, 6, 8, 24 h and one week. b) SDS-PAGE for hpβCD. Lane 1: The low molecular size marker. Lanes 2-8: The samples were obtained after 2, 4, 6, 8, 24 h and one week.
Table 1. Studying the stability of the growth hormone

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Control (ng/ml)</th>
<th>hpβCD (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>10.12</td>
<td>10.67</td>
</tr>
<tr>
<td>4</td>
<td>10.08</td>
<td>10.57</td>
</tr>
<tr>
<td>6</td>
<td>10.24</td>
<td>10.31</td>
</tr>
<tr>
<td>8</td>
<td>9.98</td>
<td>9.57</td>
</tr>
<tr>
<td>24</td>
<td>10.39</td>
<td>10.2</td>
</tr>
<tr>
<td>168</td>
<td>10.24</td>
<td>9.88</td>
</tr>
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Among the three applied enhancers in this study, hp-βCD is the cheapest cyclodextrin, which can be obtained from a variety of sources. So the following studies were performed on the effect of the hp-βCD.

Effect of hp-βCD concentration on the skin permeation of hGH: The permeation multiplier effects of hp-βCD were studied at concentrations of 2 and 4 than growth hormone. As seen in fig. 5, the amount of the growth hormone permeation in the four fold concentration than twofold has indicated some deal increase to 6 h after sampling. A probable justification for this observation is the increase in the amount of the drug contact with hpβ molecules that cause an increase in the levels of the drug complexed with hp-βCD.

According to former reports, existence of some interaction between the available hydrophobic side chains of the amino acids and hydrophilic CDs have been proved, examples of which are buserelin, insulin and α-chymotrypsin (2). In this regard cyclodextrins can be used to solubilize and stabilize various biomedically important peptides and proteins including growth hormones (3) by complexing with hp-βCD to become entrapped. The outer surface of the hp-βCD is very hydrophilic and interacts well with water to carry the guest into solution. It was found that large water soluble molecules with side chains capable of forming a complex react with cyclodextrins in aqueous solutions, resulting in modified solubility and stability (10). The objective of this paper was to contribute new experimental data in order to find any significant differences in the permeation the hGH containing CPE and free enhancer formulation (control). A value of P < 0.05 was considered statistically significant.

In conclusion cyclodextrins have significant potentials as drug enhancers in penetration of proteins and peptides. Therefore, growth in the number of cyclodextrin based commercial products can be expected in the future. Thus, it can be concluded that CDs (especially hp-βCD) are very suitable delivery systems for penetration of hGH.

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