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Research Article

Transdermal Co-Delivery of Urea and Recombinant Human Growth Hormone

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Background: Urea with super-hydrating and moisturizing properties is mainly used as an adjunctive treatment of diseases associated with dry skin. In this regard, the recombinant human growth hormone (rhGH) with rejuvenating properties is used as a base material in beauty creams. Although urea easily passes through the skin, the epidermal skin barrier restricts the passage of hGH due to its size.
Objective: in this research, in order to solve this problem, hydroxy propyl-beta cyclodextrin (HP-β-CD) is used as a soluble chemical enhancer.
Material and Methods: UV and circular dichroism spectroscopy were used for the investigation of structural modification. The permeation process was studied *in vitro* on rat skin using vertical Franz diffusion cells. Enzyme-linked immunosorbent assay were used for rhGH activity assessment and evaluation of transdermal delivery.
Results: First, due to the denaturing effects of urea on proteins its concentration was optimized to maintain biological structure and protein activity. UV spectroscopy and CD data proved that the secondary structure of rhGH is preserved in the presence of urea (0.5-2 M) and HP-β-CD, which elevates urea and rhGH permeation. Maximum permeability was observed at 120 min after sampling (1424.35 ng.ml.cm^{-2}), which was much higher than the control. Using a higher concentration of urea in the formulation will significantly decrease the level of rhGH delivery.
Conclusion: According to results, this strategy can be considered as a successful method for enhanced Co-delivery of rhGH and urea.

Keywords: Co-delivery, Cyclodextrins, hGH, Transdermal delivery, Urea

Abbreviations:
rhGH, Recombinant Human Growth Hormone; HP-β-CD, Hydroxypropyl Beta-Cyclodextrin; CD, Circular Dichroism

1. Background

The interest of individuals to rejuvenate the skin is increasing every day. Nowadays, various techniques are applied for skin rejuvenation, including creams containing stem cells to modern plastic surgery procedures. With the advent of science and technology, many different methods have arisen, which are often performed by injection. For example, Botox injection is one of the most commonly used methods for beauty in the whole world. By injecting poison into the muscles and paralyzing them for at least 4 to 6 months, the formation of muscle movements is blocked (1). However, the administration of the drug in injection forms for long-term leads to different problems, including swelling of the injection area. Oral medications use is limited due to digestive enzymes' effects, which decrease protein activity when proteins and peptides are used for treatment (2, 3).
Transdermal drug delivery (4) is a non-invasive method for the easy application of therapeutic agents that, in comparison with the injection methods. Benefits from prolonged release, lower dose, avoiding drug entry

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into the gastrointestinal tract, liver, and intestinal metabolism, and patient satisfaction (5). Despite the advantages, the main problem of this method is the low bioavailability of drugs due to difficulty in penetration through the natural barrier of the skin. The properties of the drug (6) and targeted location have a remarkable impact on the dermal penetration of chemicals (7). Each of these properties affects the speed and the amount of absorption and distribution of the drug (8).

Three mechanisms (polar, non-polar, and polar / non-polar) are suggested for drug penetration into the skin: (9). Enhancers as molecules that increase the penetration act by changing one of these mechanisms (10). Fatty acids (11, 12), chitosan, and its derivatives such as sodium carboxylates (13, 14) and terpenes (15, 16) are referred to as chemical enhancers. Different techniques such as ultrasound micro-needles, heating, iontophoresis, and electroporation have been used as physical enhancers for transdermal delivery. These type of enhancers influence the design of products and their development considerably (17, 18). Cyclodextrin is a group of chemical enhancers that have hydrophilic outer surfaces which infiltrate into the skin under normal conditions (19, 20). These molecules are also known as drug carriers due to their hydrophobic interior, which enables the loading of hydrophobic molecules/ drugs (21-23). Natural chemical enhancers such as α-β and γ CDs are made of 6, 7 and 8 glucopyranose units, respectively, and are part of the USFDA list of generally regarded as safe (GRAS) molecules. In addition to natural CDs, two of their important derivatives known as hydroxypropyl-β-CD (HP-β-CD) and sulfobutylether-β-CD (SBE-β-CD) have also been recognized as GRAS and added to the FDA guide that includes inactive ingredients approved for use in new pharmaceutical processes (24).

As mentioned at the beginning, today, products that have an anti-aging effect on the skin are trendy for manufacturers and consumers. Some of these products contain growth hormone, which is claimed to rejuvenate the skin. Normally, the growth hormone is secreted by the pituitary gland (8).

The main task of growth hormone after secretion is to stimulate the growth of various tissues of the body. It is involved in the enduring process of regulating metabolism and regenerating and repairing human tissues. hGH content is usually at the peak in adolescence with the rapid growth of the body and decreases by aging (25). The aging process happens as a result of a continuous drop in hGH levels. hGH has been used as a therapeutic for different diseases, including Prader–Willi syndrome, Turner syndrome, children suffering from idiopathic short stature , wasting disease associated with AIDS, chronic renal insufficiency, and accumulation of fat in adults suffering from lipodystrophy (26). hGH is a key player in the health and aging of our skin and so, it is ideal for a product with topical use and could improve the overall condition of the skin.

2. Objective

As expected, penetration of the growth hormone into the skin is negligible, which can be due to the large size of molecule and the impermeability of the horny layer. Hence, because of the protein's stability and large molecular size, designing appropriate drug carriers has become a difficult and challenging process. Recently, attempts have been carried out for the development of efficient transdermal delivery systems of proteins (27). Various approaches, such as radiofrequency ablation (28), microneedle (29), and self-dissolving micropile, are used for transdermal delivery of hGH (26). We have previously reported that cyclodextrins remarkably improve hGH transdermal delivery (30). The main idea of this study was to add a well-known natural substance with hydrating and skin-improving properties (such as urea) to the formulation.

Urea is a natural known for its skin softening effects and is used in different rejuvenating skin products (31). It allows the skin to stretch easily, increases the absorption of excess water into cells, and also prevents evaporation and loss of water from the surface, thereby increasing the moisture content of the upper layers of the skin. The efficiency and safety of urea have been reported for hydrating and treating skin diseases for longer than one century (32). Urea is naturally found in body tissues and is the reason for skin smoothness. Urea content in beauty creams easily penetrates deep into the horny layer and keeps the moisture for a long time. In fact, it has been shown that topical urea's hydrating effects can be due to the destruction of hydrogen bonds in the stratum corneum, reduction of keratin's structural strength in the epidermis, and rise in the number of sites that bind water (33). But there is a problem here; urea unfolds and denatures proteins such as hGH both directly and indirectly (34, 35). However,

HP-β-CD has been found to bind to hydrophobic areas on the surface of proteins, resulting in the formation of a hydrophilic layer that increases protein solubility. HP-β-CD can reduce aggregation of proteins and increase the permeability of skin through possible interaction with aromatic side-chains (36). In this study, transdermal Co-delivery of urea and hGH using HP-β-CD as permeation enhancers will be investigated, and the effectiveness of the biological activity of the protein will be reported.

3. Materials and Methods

The human growth hormone (hGH) was obtained from Novo Nordisk A/S. hGH ELIZA kit was purchased from PADTAN ELM (Iran): HP-β-CD from Acros organics. Urea and other chemicals from Aldrich chemical company, Inc. Rats used in animal studies were obtained from the Pasteur Research Institute (Iran).

3.1. hGH Structure Analysis

3.1.1. UV Spectroscopy

A 0.5 $mg.mL^{-1}$ sample of hGH was added to PBS and incubated with 0.5, 1, 2, and 4 M of urea. Samples were removed at different time intervals (0, 0.5, 1, 2, 3, and 6 h), and the stability of hGH was measured in the presence of urea and/or HP-β-CD by UV spectroscopy. The UV absorption spectrum of the samples was recorded at 190-300 nm.

3.1.2. Fluorescence Spectroscopy

3.1.3. Circular Dichroism Spectroscopy

The wavelength for fluorescence of tryptophan is used extensively to detect changes in proteins and deduce structure and dynamics in their immediate environment. It monitors tryptophan spectra as a function of protein conformation. All samples were excited at 280nm by using a Cary Eclipse fluorescence spectrometer (Agilent Technologies, Santa Clara, CA). The width of excitation slits were 2.5 mm and emission slits were 5mm. According to the previous studies, for a more rigorous interrogation of spectral changes, the barycentric mean λ_m can be used (37), where F (λ) denotes intensity of fluorescence at λ wavelength.

In order to measure transformations in the secondary structure of hGH induced by urea and/or HP-β-CD, the technique of far-UV circular dichroism spectroscopy was used. The 0.5 $mg.mL^{-1}$ hGH samples were added to 1 mm cuvettes, and spectra were obtained in the far UV region between 250 to 195 nm, at 35 °C using a Jasco J-810 spectrometer (Jasco Spectroscopic Co. Ltd., Japan).

3.2. Quantitative ELISA Assay of hGH

The effect of Urea on the quantity of hGH was analyzed using a sandwich ELISA kit. The biometric assay is based on the color intensity results from the attachment of hGH and anti-GH antibodies. Mixtures were incubated for 360 min and samples were removed at 0, 30, 60, 120, 240, and 360 min intervals so as to measure their color intensity at 450 nm. The samples were then dialyzed using a 10 kDa cut-off membrane (MWCO) for 4 hours against PBS buffer to remove the excess urea, and the quantity was measured again.

3.3.Transdermal Delivery Study

3.3.1.Preparation of Rats' Abdominal Skin

A male rat weighing approximately 175 g at the age of 3 weeks was anesthetized using chloroform. Hairs were removed, and the skin was dissected from the outer surface of the abdominal region. Subcutaneous fat was removed by a scalpel and then rinsed by normal saline. The prepared skin section was immediately used for the penetration experiments.

3.3.2. Permeation Study

The drug diffusion studies were performed using the Franz diffusion cells (1.2 cm^2). The total volume of the cell in the receptor compartment was 30 mL. The rat skin was positioned in between the donor and receptor compartments, which were then clamped together. One ml of samples (0.5 $mg.mL^{-1}$) was applied uniformly to the donor part. A 30 mL volume of 0.05 M phosphate buffer (at pH 7.4) was added to the receptor compartment under constant stirring with a magnetic stirrer. The cell was then kept at 37±0.5 °C in a water bath. Samples (1mL) were regularly drawn from the receptor compartment at 0.5, 1, 2, 4 and 6 h time intervals, and replenished with the same amount of buffer. Subsequently, they were analyzed for hGH content by using sandwich ELIZA technique. Cumulative released amount (Q) versus time were determined. Different formulations used in this study are listed in **Table 1**. According to optimum ratio obtained in our previous studies (38), in

Table 1 . Transdermal delivery of hGH at different formulations

Formulation	Material	Q				
		30 min	60 min	120 min	240 min	360 min
A	hGH	0	14±3	23±2	4±1	0
B	hGH+HP-β-CD+urea(2M)	250±12	1370±31	1426±49	1375±27	1380±38
C	hGH+HP-β-CD+urea(4M)	0	3±1	9±1	2±2	0
D	hGH+HP-β-CD	210±10	1250±15	1240±40	1331±43	1100±17
E	hGH+urea (2M)	0	0	0	0	0
F	hGH+urea (4M)	0	0	0	0	0

the experiments with HP-β-CD the ratios of 1:4 (hGH to HP-β-CD) was applied.

3.4. Gel Electrophoresis

To determine the molecular weight of protein SDS gel electrophoresis was carried out according to a previously reported method by Laemmli (39).

4. Results

4.1. Hgh Structure Analysis

4.1.1. UV Spectroscopy

To investigate the denaturating effect of urea on hGH, the UV spectrum of protein was recorded, before and after incubation with urea at different concentrations (0.5, 1, 2, 4 M). The results showed that the absorbance of hGH at 260 decreases at high urea concentration (4M), which may be due to the denaturalizing nature of urea (**Fig. 1A**). Based on the UV spectra, hypochromic effects are increased by increasing urea concentration. It seems that as the urea concentration increase, instability of tertiary structure of protein begins. Prove that this instability to some extent leads to loss of biological activity, needs to additional experiments.

4.1.2. Fluorescence Spectroscopy

Tryptophan is a vital intrinsic fluorescent amino acid that is used to assess the type of microenvironment surrounding tryptophan with a fluorescence excitation at

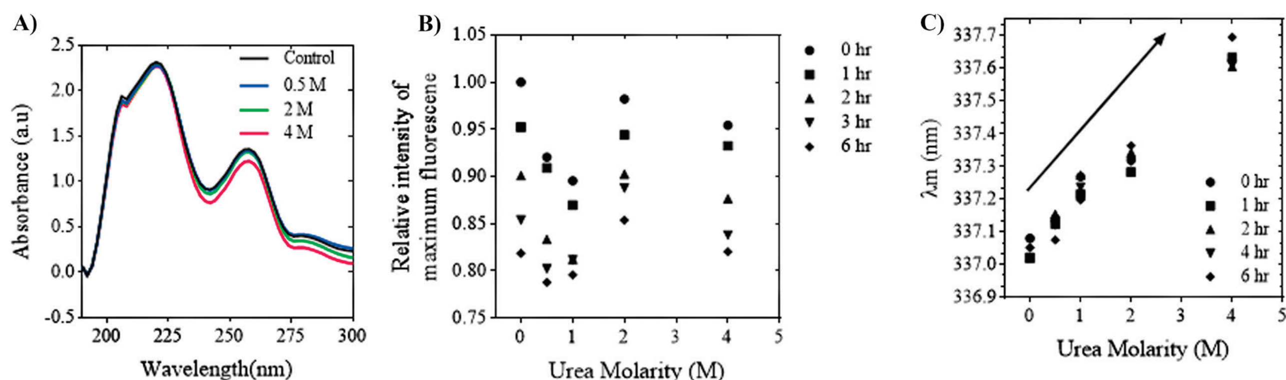

Figure 1. The effect of different concentration of urea (0-4 M) at different incubation times (0-6 hr) on the structure of hGH: **A)** UV spectroscopy of hGH in the presence of different concentration of urea. **B)** relative intensities of maximum tryptophan fluorescence and **C)** λ m changes in the presence of different concentration of urea obtained from the fluorescence spectra of hGH excited at 280 nm.

295nm. Intrinsic tryptophan fluorescence spectroscopy was used to explore the effect of urea on hGH structure. (**Fig. 1C**). The intrinsic tryptophan fluorescence study shows that urea has no significant effect on the relative intensity of maximum fluorescence of tryptophan, but the λ_m slightly changes by increasing the urea concentration, which shows a negligible change in the tertiary structure of hGH in the presence of Urea.

4.1.3. CD Spectroscopy

Protein's secondary structure can usually be investigated with the aid of circular dichroism (CD) spectroscopy. Effect of HP-β-CD on hGH with and without urea was studied using CD spectroscopy. The 1:4 ratio of HP-β-CD to hGH does not affect hGH's secondary structure of (**Fig. 2A**). The resulting CD spectra of hGH in the presence and absence of urea (**Fig. 2B**) showed no change in the secondary structure of hGH incubated with urea. As shown in **Figure 2B**, the magnitude of CD bands and the ratio of the ellipticity at 222 to 208 in the presence and absence of urea is the same and does not change over time. This observation confirms that urea in these concentrations (0-4M) does not affect the secondary structure of hGH.

4.2. Activity Study

Quantity of active hGH in the presence/absence of 2 and 4 molar urea was measured by an immunoenzymatic reaction. Also, influence of HP-β-CD on active hGH levels in the presence/absence of urea (2 and 4M) was investigated. (**Fig. 3A-E**). The Quantity of active hGH in the presence of 4M urea is decreased with a higher slope than 2M during the time. In both samples, the removal of urea caused a slight increase in quantity of active hGH as expected. This increase is less in the case of 4M urea (**Fig. 3A-B**). As shown in **Figure 3C**, HP-β-CD has no significant effect on the hGH activity. Not only HP-β-CD is capable of preserving the activity of hGH over time, but it can preserve the activity of hGH in the presence of urea (**Fig. 3D- E**). In all experiments (**Fig. 3A-E)** the quantity of active hGH reduction in 30 minutes was not significant in comparison to the native sample. The native hGH structure is preserved in the presence of HP-β-CD, with no significant reduction being observed until 360 minutes (**Fig. 3C**).

When, hGH was incubated with 2M urea, the significant reduction in quantity of protein observed during time. Similar effect was observed at 4M urea, leading to a longer incubation time (>240 min), this reduction in activity was significant. When the urea was removed by dialysis and its concentration gradually decreased in the system, the quantity of active hGH improved and it looks like to native state (**Fig. 3A- B**).

When this reaction is performed by adding HP-β-CD, it seems that protein structure becomes more resistant to change and the denaturating effect of urea was reduced. This effect remained until 240 minutes and then a significant decrease in activity was observed **(Fig. 3D**)

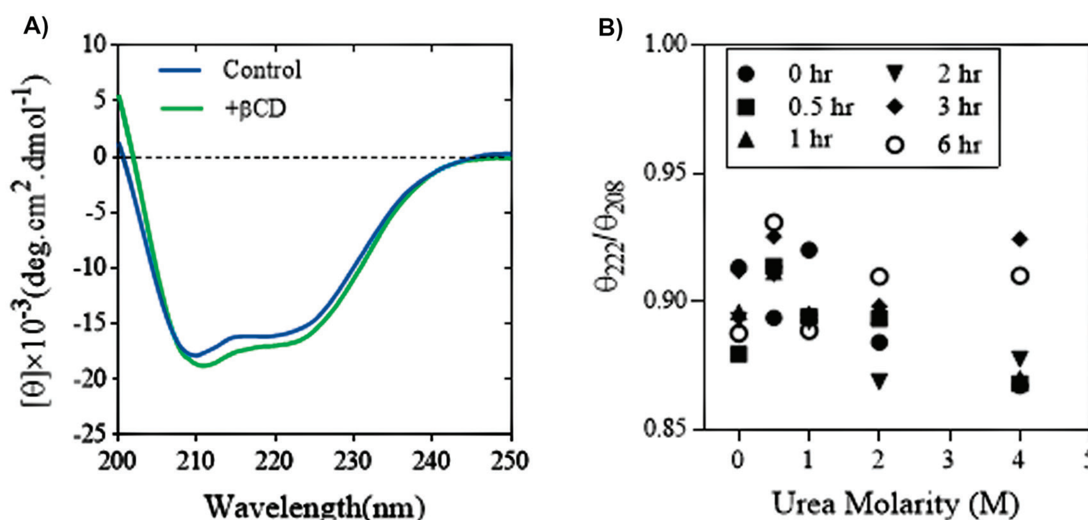

Figure 2. CD spectra of hGH **A)** in the presence and absence of HP-β-CD **B)** θ 218 /θ 222 diagram for CD spectra of hGH in the presence and absence of urea.

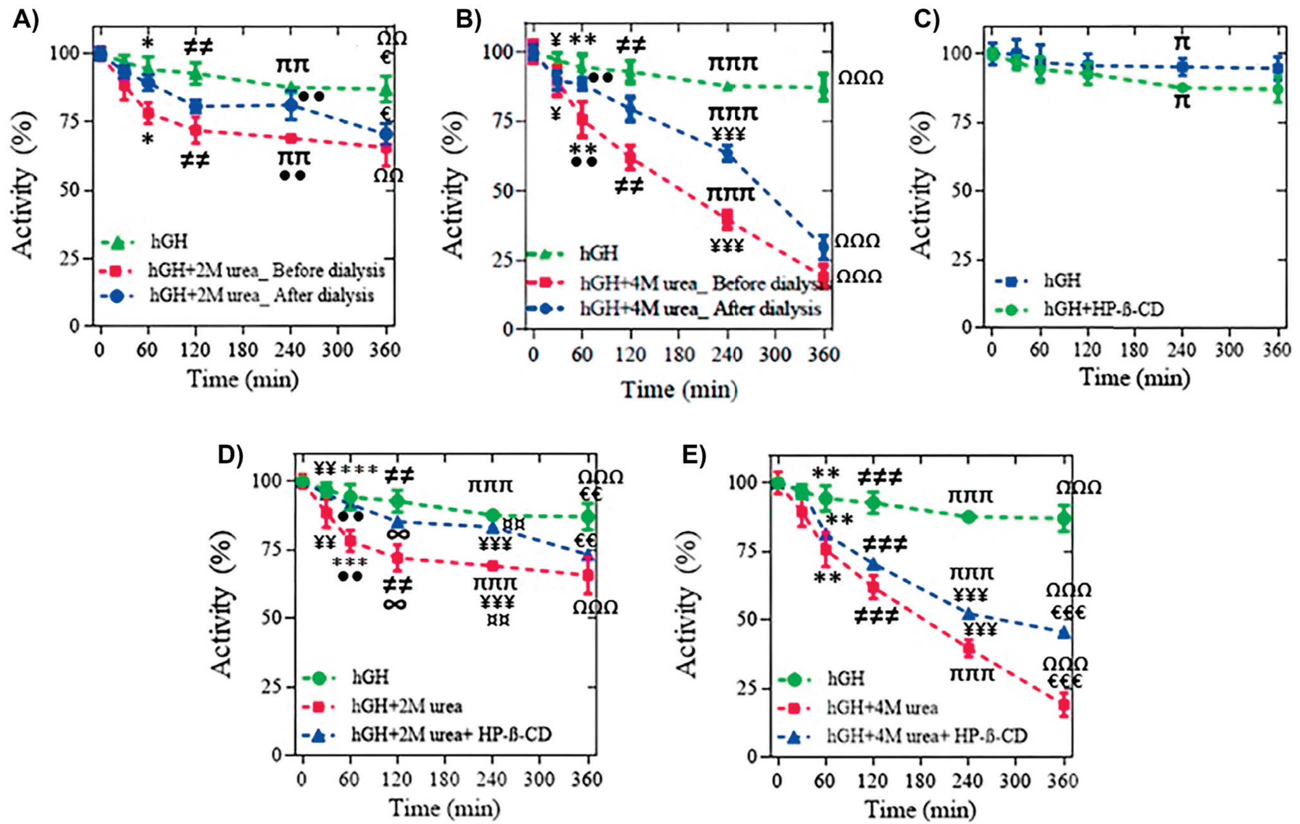

Figure 3. Biological activity of hGH in the presence of **A)** 2 M urea, **B)** 4 M urea **C)** HP-β-CD, **D)** 2 M urea and HP-β-CD **E)** 4 M urea and HP-β-CD (Mean±SD, N=3, *,€,¥,π,∞P<0.05, ≠≠,ππ,••,ΩΩ,**,¥¥,¤¤,€€P<0.01, πππ, ¥¥¥, ΩΩΩ,***,≠≠≠,¥¥¥,€€€ P<0.001)

Figure 3E shows that testing at 4M urea will significantly reduce hGH activity at all times with or without the presence of HP-β-CD.

In order to confirm that reduction in the observed activity is due to conformational change and not degradation or aggregation of the protein, gel electrophoresis was performed on hGH samples incubated (6h) with different concentrations of urea (0-4M). The results are presented in **Figure 4**. As shown in **Figure 4**, there is no visible degradation or aggregation of hGH on the SDS-PAGE, in test conditions. According to the mentioned results, in the presence of urea and HP-β-CD, the structural status and protein conformation of hGH have been determined, thus its transdermal delivery can be carried out.

4.3. Transdermal Delivery Study

In each formulation, the amount of drug passing through the surface area of the skin (Q) was measured using an ELISA kit based on the activity of the hGH. The result for different formulations is summarized in **Table 1.** The amount of growth hormone intake per unit area of skin was zero. As long as the measured penetrated hGH is based on its activity, the zero detected hGH in the presence of urea can be due to the adverse effect of urea on the activity of penetrated hGH. Penetration of hGH for formulation A (hGH control sample) is only slightly higher than formulations containing urea (E and F), while maximum penetration for hGH is observed at120 min equal to 23 $ng.mL^{-1}.cm^2$. After 120 min, the amount of detectable hGH decreases which can be caused by the loss in the activity of the hGH over time. This low amount of penetrated hGH is due to the size of the protein molecule. The presence of HP-ß-CD in formulation D leads to a dramatic increase in the amount of hGH penetrated through the skin up to 1331 $ng.mL^{-1}.cm^2$ after 240 min and remains almost constant until 360 min which is about 58 times more than the hGH control. This increase in the amount of detectable hGH is due to its enhancing effect of penetration (40).

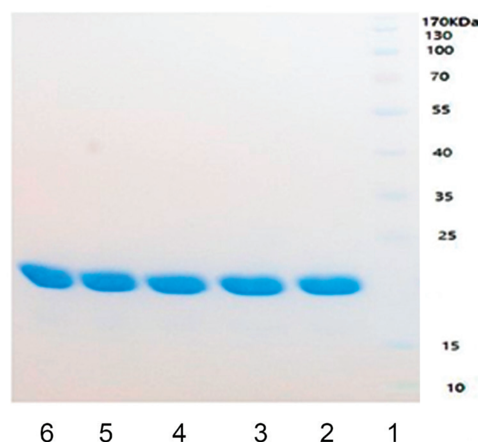

Figure 4. SDS_PAGE of urea treated hGH 1-MW standard 2-6:samples incubated with 0,0.5,1,2,4 M urea respectinely

In formulation B containing hGH + urea (2M) + HP-ß-CD the highest penetration rate equal to 1426 ng.mL^{-1}. cm² is achieved after 120 min. This penetration level being the highest in all formulations shows more than a 63-fold increase compared to hGH control.

5. Discussion

The skin is an appealing route for the dermal and transdermal delivery of drugs. It has been reported that growth hormone uptake is increased using cyclodextrins including α-CD, β-CD and HP-βCD (30). It has been proved that HP-β-CD has the highest enhancing effect on hGH skin permeation and the growth hormone has the highest activity with HP-β-CD which increases the bioavailability of the hormone at the biological membrane surface (30). However, due to the removal of biomembrane molecules by native β-CD that result in the formation of highly insoluble cholesterol-containing complexes, and the toxicity associated with the extended exposure of such complexes, the use of β-CDs is not recommended (41). Nevertheless, such toxicity can be minimized by changing the affinity of β-CDs for biomembrane components through the synthesis of β-CD derivatives. Currently drug combinations that contain HP-β-CD have been approved for different drug delivery systems (42).

Analysis of the native hGH's structure has shown that most of its 21 amino acid residues are completely or partially hidden, and only six of its fully-exposed residues (phe 25, Tyr 42, phe 44, phe 92, Tyr 103, and phe 139) may be involved in interactions with the native form of the HP-β-CD. When denatured by acid treatment, a greater number of residues can become accessible to solvents. Considering steric interactions, only a small proportion of the sites may become occupied at a particular point in time (36).

Due to the denaturing properties of urea, it could probably have similar effects (43). The current results suggested that hydrophilic HP-β-CD can effectively inhibit the unfolding of hGH. Thus urea and HP-β-CD may be potentially useful excipients for penetration of hGH.Urea molecules directly interact with hydrophobic and polar groups of proteins. The intake of water and urea into protein 3-D structure causes instability and disturbance of the regular conformation of the protein. At high concentrations (6-8 M), urea changes the chemical and physical interactions, and thus affects the folding of the protein structure. These kinds of changes cause the exposure of hydrophobic moieties on the surface, which are generally located within the internal part of the 3-D structure (38).

Considering the denaturing effect of urea (34, 35), it is very important to optimize its concentration to minimize this effect while taking advantage of its Co-delivery with hGH. By diluting proteins to low concentrations of denaturants, they fold to the native state and compete with the aggregated forms. In fact, native protein yield has been observed to enhance when CDs are added to the dilution buffer (36). Apart from causing the folding process to occur, the CD and aromatic amino acid interactions may provide the right conditions to inhibit the nucleation process and other inter molecular relationships. The reason for this is that although being weak, such interactions are sufficiently vigorous to let

proteins adopt various intramolecular conformations whilst preventing the establishment of interfaces with other molecules. At a higher concentration of urea (4M), hGH delivery is significantly decreased, which can be a result of either no transportation or biological activity decreation and difficulty in hGH detection. Since the quantification of hGH delivery is based on the biological activity of hGH with normal conformation, any change in 3-dimensional structure, reduce the expected response. The result of this study for a formulation containing hGH, HP-β-CD, and urea (2 M) shows that the maximum amount of hGH is passed through the skin after 120 min equal to 1426.35 ng.mL $^{-1}$.cm². Several studies support the capacity of urea to enhance penetrations of drugs (33) in to the skin. A study by Grice *et al* (44). Kinetics of permeation into the horny layer of the skin is changed in the presence of urea due to alteration of the binding capacity that increases retention time while decreasing penetration (45). It has also been reported that electroporation and urea used together leads to synergistic enhanced transdermal delivery (46). The natural moisturizing factor presumed to be present in corneocytes, is comprised of glycerol and urea. The latter stimulates transdermal permeation by enabling the hydration of the stratum corneum and forming hydrophilic diffusion channels in the skin (47). Urea also use in skin products to soften dry, rough and scaly skin. It shows that the cream containing 3% to 10% of urea has a better effect than the placebo. No water loss was observed during treatment with a cream containing 3% urea. Also, water loss was decreased using creams containing 10% of urea (44). In this study, the optimum concentration of urea for penetration of hGH was 2 M, which is equivalent to 12.1%. At this optimal concentration, the activity of hGH is preserved, and the penetration level is elevated remarkably.

6. Conclusion

In this study, Co-delivery of urea and hGH using HP-ß-CD as enhancer was studied. Results indicate that controlling the urea content in the formulation will prevent denaturation of hGH while it does not affect the enhancing activity of HP-ß-CD. Best results were obtained in case of the formulation containing 2 M urea and HP-ß-CD, which shows the rising trend up to 120 min after sampling. The maximum permeability was observed at 120 min after sampling (1424.35 ng.mL^{-1}.cm^{-2}), which is much higher than the control sample. Using a higher concentration of urea in the formulation will significantly decrease the level of hGH delivery. For the formulation using 4 M urea and HP-ß-CD, only a negligible amount of delivery was observed at 120 min (9.86 ng.mL^{-1}.cm $^{-2}$). According to results, this strategy can be considered as a successful method for enhanced Co-delivery of hGH and urea.

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