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Stability of insulin under iontophoretic conditions

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The present study focuses on the physical and chemical stability of insulin under iontophoretic conditions using HPLC, SDS-PAGE, RIA and biological assay. Influence of pH, concentration of insulin, current strength and duration of current application on the stability of insulin was studied. Anodal iontophoresis at pH 7.4 caused more than 80% degradation of insulin, while the degradation was minimal at pH 3.6. The degradation was not influenced by insulin concentration, but increase in current strength above 0.75 mA/cm^2 or application of current for 12 h (at 0.5 mA/cm^2) led to 80 and 20% degradation respectively. All the samples showed biological activity comparable to intact insulin.

1. Introduction

Transdermal iontophoresis is a physical enhancement strategy, which delivers ions and charged molecules across the skin, into systemic circulation, at an enhanced rate in a controlled manner by use of a small electric current (Pillai et al. 1999, 2001). Its ability to deliver charged and hydrophilic drugs makes it a suitable approach for protein and peptide delivery. Transdermal iontophoresis is a complex process influenced by several parameters that include physicochemical characteristics of drug, parameters of the iontophoretic system and physiological parameters of the skin. For a given drug, concentration and charge of drug, strength and duration of current application can be altered to achieve a desired flux. For delivery of proteins and peptides by transdermal iontophoresis, platinum electrodes are generally preferable because silver-silver chloride electrodes cause precipitation. But, platinum electrodes cause a shift in pH, which may lead to instability of drugs, and in particular this may be detrimental for proteins and peptides. Many authors have studied transdermal iontophoretic delivery of drugs but stability studies are rare. To investigate this insulin was chosen as a model peptide. Insulin undergoes degradation mainly through deamidation at Asn^{A21} and Asn^{B3} depending on the pH of solution. It is more stable towards deamidation at neutral pH as compared to acidic or alkaline pH. Along with pH, stability of insulin is influenced by ionic strength and heat. Further, all these conditions prevail during transdermal iontophoresis i.e. pH to maintain PP in ionized form, buffers contributing to ionic strength and 37 °C at which transdermal iontophoresis is carried out. The electrochemical reaction occurring on the electrode may cause a shift in pH. Secondary structure of proteins and peptides is important for their biological activity. However, to the author's knowledge there is not a single published report regarding on the effect of transdermal iontophoresis on the conformation of proteins. According to the International Conference on Harmonization guideline Q5c on proteins and peptides stability, it is necessary to study their stability by more than one analytical technique and results should be correlated with its biological activity. In addition, due to multiple functional groups and multiple inactivation pathways no single technique can be uniformly recommended as a stability-indicating assay (Banga and Reddy 1994).

Physicochemical parameters such as pH and concentration of the permeant can be varied to optimize the iontophoretic flux. On the other hand, current strength and duration can be manipulated to control the drug input kinetics. During optimisation of these parameters, it is essential to ensure that the peptide/protein under investigation is stable. Though a number of studies have been reported on the influence of various parameters on iontophoretic delivery of peptides/proteins, not much attention has been focused on the stability of these drug molecules under iontophoretic conditions. Earlier, we optimized the conditions for transdermal iontophoretic delivery of insulin (Pillai et al. 2003a, b). In continuation, the present study focuses primarily on the physical and chemical stability of insulin under different iontophoretic conditions.

2. Investigations, results and discussion

During anodal electrophoresis (AI), there was a significant pH shift, when the donor solution pH was 7.4 (pH shifted to 2.0), while there was negligible pH shift (0.6 units), when the donor solution pH was 3.6. The pH shift varied from 1–2 units during cathodal electrophoresis (CI) depending on the donor solution pH. The shift in pH can be explained by the electrochemical reactions taking place due to the electrolysis of water at the platinum electrode surface. As oxidation occurs at the anode and reduction at the anode the following reaction takes place;

$$H_2O \rightarrow 2\,H^+ + {}^1/_2\,O_2 + 2e^- \text{ (at anode)}$$

$$2\,H_2O + 2e^- \rightarrow H_2 + 2\,OH^- \text{ (at cathode)}$$

The generation of hydronium ions at the anode causes a decrease in pH, while the hydroxyl ions generated at the cathode surface shift the pH to the alkaline side. The extent of pH shift is dependent on the buffering capacity of the buffer used in the donor and receptor solutions. In this regard, we have found that the pH shift can be reduced to a minimum by optimizing the buffer type and concentration (Pillai et al. 2003b). At pH 3.6, there was no significant difference in degradation ($p > 0.05$) during passive and AI conditions, when analyzed by RIA, SDS PAGE and HPLC (Fig. 1). In addition, shift in pH was also observed to be minimal at this pH as discussed above. On the other hand, during CI there was 25–30% degradation during 6 h of iontophoresis. Our findings are consistent with those of other investigators, where AI at pH 3.6 has been reported to be better in terms of stability and permeability (Kari 1986; Banga and Chien 1993).

From the analysis of insulin samples after iontophoresis at pH 7.4 (AI and CI), it was observed that there was significant degradation (80%) in 6 h (Fig. 2). With CI, the degradation was 30% in 6 h. These findings are consistent with findings of Huang and Wu (1996), who found 80% of insulin degraded in 6 h; however they had used skin as

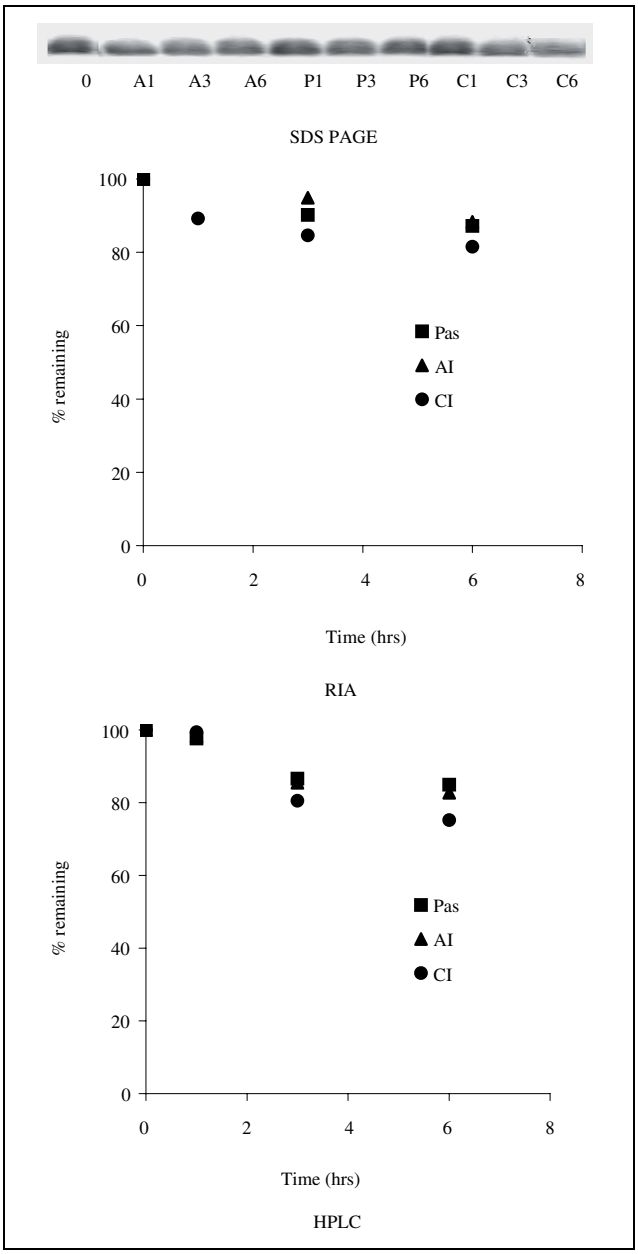

Fig. 1: Electrochemical stability of insulin during different iontophoretic protocol at pH 3.6 at 0.5 mA/cm^2 for a duration of 6 hrs when analyzed by SDS PAGE, RIA and HPLC. Pas represents the degradation profile during passive treatment, AI represents anodal iontophoresis and CI represents cathodal iontophoresis.
0 is 0 h sample for AI, CI and passive cells. A1, A3 and A6 represent sample withdrawn from AI cells after 1, 3 and 6 h. P1, P3 and P6 represent sample withdrawn from passive cells after 1, 3 and 6 h. C1, C3 and C6 represent sample withdrawn from CI cells after 1, 3 and 6 h. In case of RIA and HPLC, each value represents the mean (n = 3) and mean (n = 3), respectively

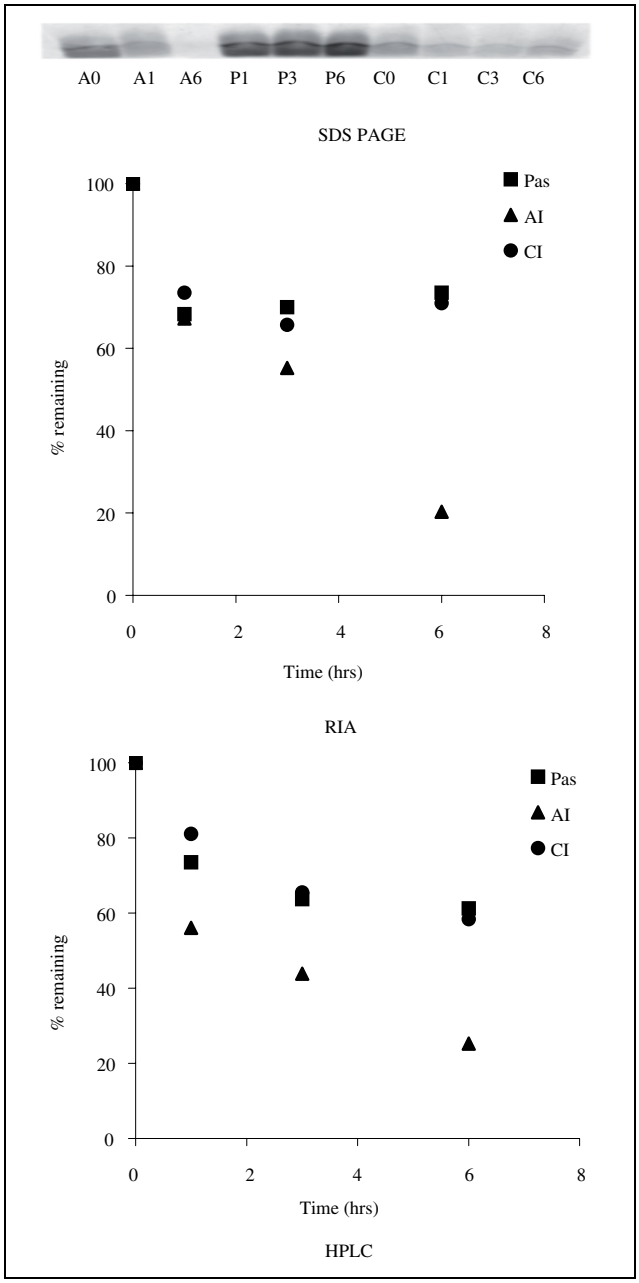

Fig. 2: Electrochemical stability of insulin during different iontophoretic protocol at pH 7.4 at 0.5 mA/cm^2 for duration of 6 h when analyzed by SDS PAGE, RIA and HPLC. Pas represents the degradation profile during passive treatment. CI represents cathodal Iontophoresis and AI represents anodal iontophoresis.
A0 and C0 are 0 h samples for AI and CI cells respectively. A1 and A6 represent sample withdrawn from AI cells after 1 and 6 h. C1, C3 and C6 represent sample withdrawn from CI cells after 1, 3 and 6 h. P1, P3 and P6 represent sample withdrawn from passive cells after 1, 3 and 6 h. In case of RIA and HPLC, each value represents the mean (n = 3) and mean (n = 3), respectively

rate limiting membrane and attributed degradation due to electrochemical reactions, temperature and proteolytic activity of enzymes present in the skin. In our case a synthetic membrane was used hence excluding the possibility of proteolytic degradation. This indicates that the majority of degradation taking place during tansdermal iontophoresis of insulin with platinum electrodes is due to electrochemical reactions at the electrode surface. As is evident from Figs. 1 and 2, all the three methods showed comparable results with respect to degradation of insulin. In order to observe conformational alterations of insulin at the pH where it showed maximum degradation (AI, pH 7.4), a FTIR spectrum was recorded. As shown in Fig. 3, there was a shift of both the peaks corresponding to β sheet at 1637 and 1689 cm^{-1} in amide I region. There was no shift in peaks corresponding to random coil, α helix, and β turn at 1649, 1659 and 1678 cm^{-1} respectively. However, further studies are required to investigate whether the physical instability leads to chemical instability or *vice versa* under iontophoretic conditions.

There was no significant difference in degradation as a function of insulin concentration ($p > 0.05$), when analyzed by HPLC and SDS PAGE (Fig. 4). On the other hand, RIA showed significant differences in the amount of insulin degraded ($p < 0.05$) as a function of insulin concentration. From the literature, it is known that the aggregation of insulin is concentration dependent, in addition to pH and ions (Brange and Langkjaer 1993; Pillai et al. 2003b). Hence, it is possible that the aggregation state of insulin has an effect on the analysis of insulin by RIA, while in SDS-PAGE and HPLC, insulin is mostly in the linear form due to the surfactant and the high concentration of ions respectively under the analytical conditions. Nevertheless, the findings show that the degradation is concentration independent during AI at pH 3.6. This is in agreement with Brange et al. (1992), where they suggested that degradation takes place by deamidation under acidic conditions and the reaction was concentration independent.

After application of current (0.5 mA/cm^2) for 12 h, there was 25–30% degradation, while increase in current density to 1 mA/cm^2 led to 80% of degradation in 6 h. Increase in current duration and strength leads to higher transdermal flux, but this may also lead to a higher rate of degradation as was observed in our case. All the three methods showed comparable results. Our earlier *in vitro* skin permeation studies showed a significantly high amount of insulin in the receiver medium at high current strengths, but as observed through this study there was a corresponding increase in insulin degradation. Hence, the increased transdermal permeation of insulin in our earlier study with high current strength and density using radiochemical method is mostly attributed to the radioactive counts from the degraded insulin fragments. In a study

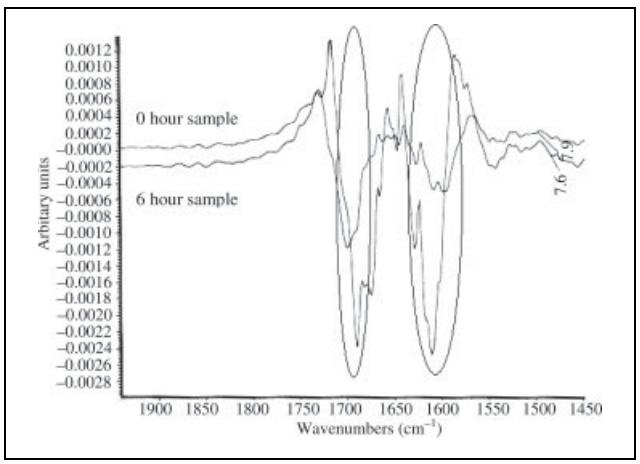

Fig. 3: Change in FT-IR spectra of insulin under the influence of TI conditions at pH 7.4 AI, with shift in peaks due to β sheet at 1637 and 1689 cm^{-1} (regions marked).
6 h sample obtained after TI was applied for 6 h at 0.5 mA/cm^2

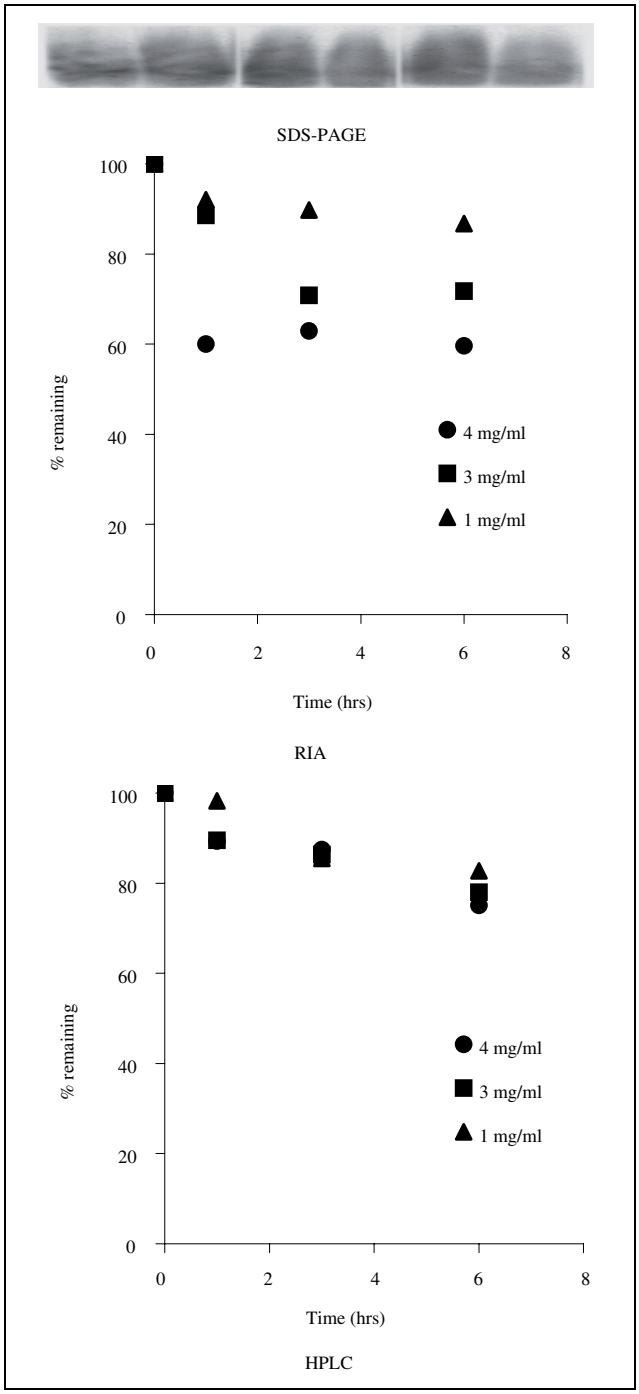

Fig. 4: Effect of concentration on degradation profile of insulin at pH 3.6 when analyzed by SDS PAGE, RIA and HPLC.
1–0 and 1–6 represents bands obtained from TI sample after 0 h and 6 h respectively with concentration of 1 mg/ml, at 0.5 mA/cm^2.
3–0 and 3–6 represents bands obtained from TI sample after 0 hr and 6 h respectively with concentration of 3 mg/ml, at 0.5 mA/cm^2.
4–0 and 4–6 represents bands obtained from TI sample after 0 h and 6 h respectively with concentration of 4 mg/ml, at 0.5 mA/cm^2.
1,3 and 4 mg/ml are TI sample after 6 h with concentration of 4, 3 and 1 mg/ml respectively at current strength of 0.5 mA/cm^2

with TRH (Huang and Wu 1996), it was observed that an increase in current strength beyond 0.32 mA/cm^2 resulted in significant degradation and they also reported that application of current increases the degradation rate. Similarly, Chiang et al. (1998) reported an increase in degradation of delta sleep inducing peptide with increase in current strength. In our case, an increase in current strength above 0.75 mA/cm^2 showed a significant increase in degradation.

In order to determine if the degraded compound showed any biological activity, blood glucose levels (BGL) were measured in diabetic rats after injecting the stability samples subcutaneously. The samples from the stability studies used for the study included pH 3.6 (0.5 and 1.0 mA/cm^2 and 12 h) and pH 7.4. Surprisingly all these samples caused a significant reduction of BGL (Fig. 5) and were comparable to passive samples. According to Brange and Langkjaer (1993), deamidation degradation products of insulin show biological activity to similar intact insulin, while high molecular weight transformation products have negligible biological activity. Hence, it is quite possible that the degradation products formed under the iontophoretic conditions might be deamidation products. Though there may not be a difference in the biological activity, the degradation products might show other adverse effects, which remains to be investigated.

In conclusions findings from the study demonstrate the importance of assessing the stability of peptide/protein during optimization of the iontophoretic conditions. The choice of the electrode can have a significant impact on the pH shift and the resulting effect of pH shift on physical and chemical stability of the peptide. Use of radiochemical method during skin permeation studies can be misleading and therefore the stability needs to be ensured by different analytical methods during the optimsation of the iontophoretic parameters for peptides/proteins.

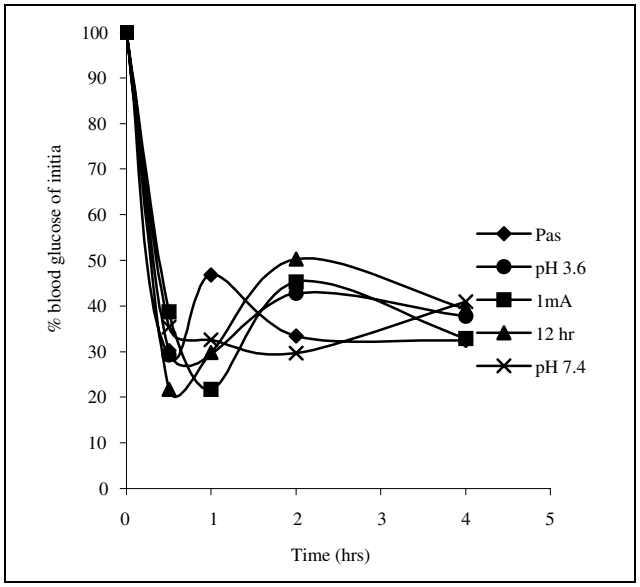

Fig. 5: *In vivo* blood glucose levels determined after the subcutaneous administration of insulin to diabetic rats (n = 6) samples subjected to different iontophoretic protocols. Pas depicts the profile of insulin samples, which were not subjected to any iontophoresis.
3.6, AI, 6 hr depicts the profile of insulin samples at pH 3.6 AI which were subjected to iontophoretic current of 0.5 mA/cm^2 for 6 h. 1.0 mA depicts the profile of insulin samples at pH 3.6 AI that were subjected to iontophoretic current of 1.0 mA/cm^2 for 6 h. 12 h depicts the profile of insulin samples at pH 3.6 AI which were subjected to iontophoretic current of 0.5 mA/cm^2 for 12 h. pH 7.4 depicts the profile of insulin samples at pH 7.4 AI which were subjected to iontophoretic current of 0.5 mA/cm^2 for 6 h

3. Experimental

3.1. Materials

Biosynthetic human insulin was a gratis sample obtained from Eli Lilly & Company (Indianapolis, USA). Human insulin specific RIA kit was procured from Linco Research Inc. (St. Charles, MO, USA). Glucose estimation kit was purchased from Accurex Biomedical Pvt. Ltd. (Mahim, Mumbai, India). To induce diabetes streptozotocin was obtained from Calbiochem (La Jolla, CA). Acrylamide, ammonium persulphate, bis acrylamide, silver nitrate, sodium dodecyl sulphate and TRIS were procured Amresco (Ohio, USA). *N*,*N*′-Tetramethyl ethylenediamine was obtained from Biorad Lab (Hercules, CA). All the other chemicals were of analytical grade and solvents used were of HPLC grade.

3.2. Radioimmunoassay

Insulin samples were analyzed using Human insulin specific RIA kit (Linco Research Inc., USA). It is based on the principle of competition between labeled and unlabeled antigen for limited and constant number of binding sites. The radioactivity counts were obtained by means of a gamma counter. The insulin concentration of the sample was calculated from a standard curve.

3.3. High performance liquid chromatography

Insulin was analyzed using a HPLC system (Waters^{TM} 510, Miliford, MA, USA) consisting of a PDA detector (996), a pump (600 controller) and a manual rheodyne injector (50 µl) using the method described by Reveir and McClintock (1983). The wavelength of detector was set at 214 nm and a Vydac protein C_4 column (4.6 × 250 mm, 5 µm, 300°A, USA) connected through a precolumn (300°A, Deltapak, Waters^{TM}, USA) was used. Mobile phase composed of eluant A (water) and eluant B (acetonitrile with 0.1% trifluroacetic acid), with a gradient of eluant B from 27 to 30% in 25 min at a flow rate of 1.5 ml/min (at ambient temperature) was used. It was always filtered and sonicated before use and samples were diluted to 0.25 mg/ml with 0.01N HCl just proir to analysis. The mean RT of insulin was found to be 17 min.

3.4. Sodium dodecyl polyacrylamide gel electrophoresis

Polyacrylamide gel was run on a Biorad (Protean III, Hercules, CA). Resolving gel (18%, 5 ml) was prepared from acrlyamide stock solution (30%), sodium dodecyl sulphate (10%, 50 µl), and *N*,*N*′-tetramethyl ethylenediamine (50 µl) in Tris buffer (1.5 M, pH 8.8), finally polymerization was initiated by addition of ammonium persulphate (10%, 50 µl). This mixture (3.2 ml) was added to assembled plates and allowed to polymerize. Stacking gel (3%, 2.5 ml) was prepared from acrylamide stock solution (30%), sodium dodecyl sulphate (10%, 25 µl), *N*,*N*′-tetramethyl ethylenediamine (2.5 µl) in Tris buffer (0.5 M, pH 6.8), polymerization was initiated by addition of ammonium persulphate (10%, 17.5 µl) and added on to resolving gel, further comb was inserted, and finally gel was allowed to polymerize. Samples were prepared in sample buffer (5×) using glycerol (50% v/v), SDS (10% w/v) and β-mercaptoethanol (5% v/v) with a final concentration of 6 µg equivalent of insulin solution. Bromophenol blue was also added to track the movement of gel. Wells were loaded with 20 µl of samples prepared and run with a running buffer which was prepared from Tris (3 g/l), SDS (1 g/l) and glycine (14.1 g/l). A current of 20 mA and 180 V was applied until the dye front travelled to the bottom of the gel. Then gels were silver stained using standard protocol as described in Meril et al. (1981).

In all the methods the 0 h sample was taken 100% and samples were compared to it. In case of SDS PAGE intensity of band due to 0 h was considered to be as 100% and background as intensity 0%, rest all the bands were compared densitometrically. During HPLC a decrease in area was seen with no separate peaks.

3.5. FT-IR

FT-IR spectra of insulin samples (20 mg/ml) in the respective buffer were collected on a Nicolet Impact 410 model spectrophotometer and loaded onto calcium fluoride windows with a 5 µm spacer. After correcting for blank (respective buffer), smoothed second derivative was determined in amide I region (1700–1590 cm^{-1}).

3.6. In vitro studies

Unjacketed Franz diffusion cells with platinum electrodes and six channel power source (Ultrapure Scientifics, Bombay, India) were used in the *in vitro* studies. Excised skin or synthetic membrane was sandwiched between the donor compartment. *In vitro* studies involving synthetic membrane were carried out at four pH values (2.8, 3.6, 5.3 and 7.4), three current strengths (0.5, 0.75, and 1.0 mA/cm^2), duration (1, 3, 6, 8, and 12 h) and concentrations (1, 3 and 4 mg/ml) (Pillai et al. 2003a). These conditions were chosen based on earlier experiments to study the effect of various parameters on permeability of insulin under transdermal ionto-

phoretic conditions. The donor compartment was filled with insulin solution (1 mg/ml) and the receptor compartment (5.3 ml) with phosphate buffer saline (pH 7.4) with 0.2% sodium azide and urea (2 mg/ml) to prevent microbial growth and adsorption of insulin to glass surfaces. Cells were maintained at $37 \pm 0.5\,^{\circ}C$ by heating the stirring module and stirred at 900 rpm using small magnetic beads. Samples were withdrawn at specified intervals of 0, 1, 3 and 6 h from the donor compartment and analyzed by RIA, SDS PAGE and HPLC.

Permeability studies were carried out using excised full thickness Sprague Dawley dorsal skin and *Stratum corneum* was placed facing the donor compartment. In case of *in vitro* studies involving membranes, an insulin concentration of 3 mg/ml and the membrane was allowed to equilibrate for a period of 12 h with receptor medium and samples were withdrawn from the receptor compartment for 48 h and analyzed by RIA.

3.7. Preparation of skin

Dorsal skin was excised from Sprague Dawley rats procured from the central animal facility, NIPER under the protocol approved by the institutional animal ethical committee and euthanised with excessive ether. Any adhering fat and subcutaneous tissues were carefully removed from excised skin using a scalpel. Skin was rinsed in normal saline and stored at $-20\,^{\circ}C$ until further use (within one week).

3.8. In vivo study

The biological activity of insulin was tested by measuring blood glucose reduction in Sprague Dawley rats (200 ± 50 g) in which diabetes was induced using streptozotocin. Animals with blood glucose level of more than 300 mg/dl were chosen for further study. The animals were fasted overnight prior to and during the experiment. On the day of the experiment, each animal received insulin injection (1 IU/ml/kg) subcutaneously and blood samples were withdrawn from the retroorbital plexus under light ether anaesthesia 5 min before injection and 30, 60, 120, 240 min after injection. Glucose levels were measured with Accurex (Autozyme, India) (Pillai and Panchagnula 2003c).

3.9. Data analysis

Data was subjected to statistical analysis by one way analysis of variance (Tukey test) at significance level of $p < 0.05$ using sigmastat® (Jandel Scientific, USA).

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