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Transdermal iontophoresis of insulin. Part 1: A study on the issues associated with the use of platinum electrodes on rat skin

Omathanu Pillai, Naresh Kumar, Chinmoy Sankar Dey, Suresh Borkute, Sivaprasad Nagalingam and Ramesh Panchagnula

Abstract

We have studied the issues associated with the use of platinum electrodes for transdermal iontophoretic delivery of peptides, using insulin as a model peptide. Insulin permeation was studied using full-thickness rat skin by varying the donor solution pH as a function of electrode polarity. The stability of insulin under the iontophoretic conditions was studied using TLC, SDS-polyacrylamide gel electrophoresis and HPLC. Large pH shifts were observed during anodal iontophoresis (AI), when the donor solution pH was above the isoelectric point of insulin and in cathodal iontophoresis (CI), when the donor solution pH was below the isoelectric point of insulin. The direction and magnitude of electroosmotic flow was influenced by pH of the donor solution and the electrode polarity. On the other hand, the buffer used to maintain the pH governed the contribution of electrorepulsion to the overall transport of insulin. Electrochemical degradation of insulin was significant during AI at pH 7.4. Among the pH investigated, AI of insulin at pH 3.6 and CI at pH 8.35 were better, as the pH shift was relatively less and electrochemically more stable during iontophoresis as compared with other pH. In summary, the pH shift caused by platinum electrodes had a significant influence on the permeation and stability of insulin.

Department of Pharmaceutics, National Institute of Pharmaceutical Education and Research (NIPER), S.A.S. Nagar – 160062, Punjab, India

Omathanu Pillai, Ramesh Panchagnula

Department of Biotechnology, National Institute of Pharmaceutical Education and Research (NIPER), S.A.S. Nagar – 160062, Punjab, India

Naresh Kumar, Chinmoy Sankar Dey

Radiopharmaceuticals Program, Board of Radiation and Isotope Technology, BARC Vashi Complex, Navi Mumbai-400075, Maharashtra, India

Suresh Borkute, Sivaprasad Nagalingam

Correspondence:
R. Panchagnula, Department of Pharmaceutics, National Institute of Pharmaceutical, Education and Research (NIPER), Sector-67, SAS Nagar – 160062 (Punjab), India.
E-mail: panchagnula@yahoo.com

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Introduction

The 'biotech era', which started with the introduction of recombinant human insulin in the 1980s, has since then resulted in more than 50 approved biotech drugs. However, the non-invasive delivery of peptide drugs remains the main bottleneck in realizing the therapeutic benefits of these new generation medicines (Pillai et al 2001). Among the various routes under investigation, transdermal is one of the attractive options in terms of patient compliance and lack of (or presence of low) proteolytic activity (Pillai et al 1999). Passive transdermal delivery is limited to small (<500 Da), lipophilic molecules and hence, the delivery of peptide drugs without a physical enhancement strategy is difficult (Naik et al 2000).

Iontophoresis, which uses a small electric current, is one of the physical enhancement strategies, which underwent a renaissance with the introduction of peptide drugs. The iontophoretic delivery of peptide drugs is governed by a number of physicochemical and electronic parameters, which have been reviewed elsewhere (Pillai et al 1999). Recently, Panchagnula et al (2000) highlighted the unresolved issues related to the development of iontophoretic systems. In this context, a series of systematic and comprehensive studies were undertaken using insulin as a representative peptide for the molecular weight range of 3–7 kDa. Since pH is one of the foremost physicochemical parameters which influences the peptide charge, skin charge and the polarity of the delivery electrode, the first part of our study focused on the issues associated with the use of platinum electrodes for the iontophoretic delivery of insulin.

In general, two types of electrode systems are used for iontophoresis; namely reversible and irreversible electrodes (Scott et al 2000). Silver–silver chloride is a widely used reversible electrode system, but the drug should be in chloride salt form to

provide the necessary 'fuel' for the electrode reaction. The use of this electrode is limited by the fact that the silver ions are known to interact with certain peptides (Langkjaer et al 1998). Metallic electrodes (platinum, stainless steel etc), on the other hand, although considered to be inert (with respect to taking part in electrochemical reactions), cause shift in pH due to the electrolysis of water (Kari 1986; Meyer et al 1989). A literature review showed that both types of electrodes had been used for iontophoresis (Pillai et al 2001). However, the platinum electrodes were simpler than Ag/AgCl and hence it was hypothesized that, by suitable choice of pH and electrode polarity, it might be possible to control the pH shift.

Insulin was chosen as the model peptide because of its therapeutic importance and due to the fact that the isoelectric point (pI) of insulin (5.3) was close to the pI of skin (4–5). Therefore, insulin provided an opportunity to understand the mechanism of iontophoretic transport at pH above and below the pI of skin as well as the peptide simultaneously. There are a few reports on the influence of pH on insulin iontophoresis (Siddiqui et al 1987; Mao et al 1995); however, there has been no comprehensive study on the combined influence of electrode polarity and pH on insulin iontophoresis. This study was carried out with various pH at two different electrodes.

Materials and Methods

Materials

Bovine insulin was a gratis sample from Knoll Pharmaceuticals (Mumbai, India). [^{125}I]Insulin (80–100 μCi g^{-1}) and [^{14}C]glucose (250 μCi g^{-1}) was provided by the Board of Radiation and Isotope Technology (Mumbai, India). Streptozotocin was procured from Calbiochem (USA). All other chemicals and reagents used were of analytical grade. Ultrapure water with a resistivity of 18 MΩ was used in all experiments.

Skin preparation

All animal experiments were conducted according to the protocol approved by the Institutional Animal Ethics Committee of NIPER. Male Sprague-Dawley rats (200–250 g) obtained from the central animal facility at NIPER were euthanized by excessive ether anaesthesia. The hair was removed from the dorsal portion of the rat using an animal hair clipper (Aesuclap, Germany) and, subsequently, the full-thickness skin was harvested. Fat adhering to the dermis side was removed by using a blunt scalpel and cleaning with cotton soaked in isopropyl alcohol. The skin was then rinsed in tap water and checked for any gross physical damage. Skin was stored at −20 °C and used within a week.

Donor and receptor solution composition

The donor solution was prepared by dissolving bovine insulin (2 mg mL^{-1}) in different buffer solutions and [^{125}I]insulin (80–100 μCi g^{-1}) was added. Buffer composition at different pH is given in Table 1. Receptor solution consisted of phosphate-buffered saline (0.1 M; pH 7.4). Urea (2 mg mL^{-1}) was added to both the donor and receptor solution as deaggregating agent and to prevent the adsorption of insulin to glass surfaces (Sato et al 1983). Sodium azide (0.0025%, w/v) was added to prevent microbial growth.

Ex-vivo permeation studies

Phosphate-buffered saline was sonicated for 30 min to remove any dissolved gases and was placed in the receptor compartment of unjacketed Franz diffusion cells (diffusion area 0.79 cm^2). The solution was equilibrated for 1 h at 37 ± 0.5 °C and a stirring speed of 900 rev min^{-1} in a heating-stirring module (Pierce, USA). Thawed skin pieces (37 °C) were mounted with the stratum corneum side facing the donor compartment. Insulin solution (500 μL) was applied to the donor compartment and samples (200 μL) were periodically withdrawn from the receptor compartment up to 48 h. Subsequently, the samples were analysed in an automatic gamma scintillation counter (1470, Wallac, Finland). At end of the study, pH of donor and receptor solutions was measured using a glass microelectrode (Eutech Instruments, USA). Skin area which was exposed to the drug solution was washed with water, dried, cut and weighed. The skin piece was then digested using tissue solubilizer (NCS-II, Amersham, UK) in a shaking water

Table 1 Composition and conductivity of buffer solutions at various pH used in skin permeation studies.

pH[a]	Buffer	Conductivity[b] (ms)
2.8	Glycine–HCl (0.20 M)	3.83
3.6	Citric acid–phosphate (0.24 M)	6.74
5.3	Sodium acetate–glAcOH (0.16 M)	6.34
7.4	Disodium hydrogen phosphate–potassium dihydrogen phosphate (0.10 M)	2.53
8.3	HEPES (0.025 M)	1.69

[a]pH indicates the initial donor solution pH used in skin permeation studies. [b]Conductivity measurements were made at room temperature (25 °C). The ionic strength of the buffers is mentioned in parentheses. HEPES is (n-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]).

bath (Julabo, Germany) at 37 °C and 100 rev min^{-1} overnight. From the skin homogenate, 200 μL was withdrawn for measuring the radioactive counts. Based on the ratio of radiolabelled to non-labelled insulin, the amount of insulin in the samples was calculated. Glucose was used as a marker for studying the electroosmotic flow at various pH during iontophoresis. For these experiments, the skin pieces were soaked overnight in glucose solution (2.5%, w/v) to saturate the glucose metabolizing enzymes (Rao et al 1993). Donor solution was composed of 2 mg mL^{-1} insulin, glucose (50 mg mL^{-1}) and [^{14}C]glucose (250 μCi g^{-1}). The samples were withdrawn from the donor and receptor compartments at the end of iontophoresis and were analysed in a liquid scintillation counter (1409, Wallac, Finland) after addition of 3 mL scintillation fluid (BCS 104, Amersham, UK).

Constant current iontophoresis

A custom made six-channel constant power supply unit (Ultrapure Scientifics, Mumbai, India) was used to apply a current of 0.5 mA cm^{-2} through platinum electrodes (2 cm × 0.5 mm diameter) for 6 h. During anodal iontophoresis (AI), the anode was placed in the donor compartment and the cathode in the receptor compartment. For cathodal iontophoresis (CI), the polarities were reversed. Passive permeation studies were carried out without the application of current and served as a control for comparison.

Analytical methods

Conductivity measurements

The conductivity of buffer solutions was measured using a conductivity meter (Eutech Instruments, USA) after calibration with standard electrolyte solutions at room temperature using one as the cell constant.

HPLC analysis

For assessing the stability of insulin during iontophoresis at different pH and electrode polarity, insulin solution (1 mg mL^{-1}) was applied in the donor compartment of unjacketed Franz diffusion cell and was separated from the receptor solution by Parafilm (American National Can Co., USA). Parafilm was chosen as the membrane, as it allowed the passage of electric current, but did not allow any insulin to pass through. The pH of both the donor and receptor solutions were measured after iontophoresis and the sample from the donor compartment was analysed by TLC, SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and HPLC in addition to testing for hypoglycaemic activity.

A modified HPLC method (Khaksa et al 1998) was followed for analysis of insulin. The mobile phase was composed of acetonitrile and sodium sulfate (0.2 M) in the ratio of 25:75. The HPLC system consisted of a dual solvent delivery pump (Waters 500, USA) connected to C-18 RP column (Deltapak, Waters, USA; 300Å, 100 μm; 150 × 2 mm) through a precolumn (Deltapak, Waters, USA). Samples were injected manually using a Rheodyne injector connected to a 50-μL injection loop and the detection was done at 214 nm using a Photo Diode Array detector. The mean retention time for insulin was 9 min and the flow rate was 0.7 mL min^{-1}. Calibration curves were prepared in different buffer solutions in the concentration range of 1–100 μg mL^{-1} and the peak area was integrated by using Millennium^{32} chromatography Manager software (version 3.0, Waters, USA). Correlation coefficient for insulin in the concentration range of 1–100 μg mL^{-1} was more than 0.99 (statistically significant at $P < 0.05$ when tested by *t*-test) at all pH used in the study. Inter-day and intra-day variation was less than 2% and the limit of detection was 0.2 μg mL^{-1}, while the limit of quantification was 1 μg mL^{-1}.

SDS-PAGE

Samples were prepared in Laemmli sample buffer (Laemmli 1970) and were separated on 18% acrylamide/bis acrylamide gel by SDS-PAGE. A sample volume equivalent to 5 μg insulin was applied to each lane and the gel was run at 20 mA constant current in Mini-PROTEAN II Cell (Bio-Rad, USA). The gel was subjected to silver staining using a procedure reported by Merril et al (1981), and the densitometric analysis of the stained gels was carried out using a GS-670 Imaging Densitometer (Bio-Rad, USA) and Molecular Analyst software (version 1.3). The relative intensity of insulin sample bands was determined by giving a value of 100 to the standard insulin (of same concentration) reference band.

Thin layer chromatography

The stability of insulin during skin permeation studies was qualitatively assessed using a TLC method developed in our laboratory. A pre-coated silica plate (Merck, Germany) was used as the stationary phase and the mobile phase consisted of n-butanol:glacial acetic acid:pyridine:water in the ratio of 37.5:25:7:30. The developed chromatogram was visualized by spraying with ninhydrin reagent (0.2% w/v in ethanol). To test the radiochemical purity of [^{125}I]insulin, at the end of the experiment, a sample obtained from the receptor compartment was eluted using the above-mentioned mobile phase. The TLC plate was then cut into 1-cm pieces which were separately counted on a gamma scintillation counter (1470, Wallac, Finland). Percent counts corresponding to the R_f of insulin was calculated from the total count of all the pieces.

Hypoglycaemic activity

Hypoglycaemic activity of insulin solution subjected to iontophoresis was tested in diabetic rats. Diabetes was induced in female Sprague-Dawley rats by intraperitoneal injection of streptozotocin (65 mg kg^{-1}). Insulin samples (obtained after passage of current) were injected subcutaneously (1 IU kg^{-1}) and the plasma glucose levels were measured using a commercial glucose estimation kit. The plasma glucose levels were then compared with the plasma glucose levels obtained from a freshly prepared insulin solution. Hypoglycaemic activity was tested only at those pH values where significant degradation was observed with HPLC.

Data treatment

Permeation profiles of insulin through full-thickness rat skin was obtained by plotting the cumulative amount

permeated vs time. Fluxes were calculated from the regression slopes of the line fitted to the linear portion of each of the permeation profiles (Ritschel et al 1989). The intercept of the straight line on the x-axis was taken as the lag time. For calculating the skin affinity values, the method reported by Panchagnula & Patel (1997) was followed. Drug concentration in the skin ($\mu g\,mg^{-1}$) was divided by the drug in the receptor compartment ($\mu g\,mg^{-1}$ – density of receptor solution was considered as $1\,g\,mL^{-1}$) to get the skin affinity values. In glucose permeation experiments, the electroosmotic ratio was calculated by dividing the amount of glucose in the receptor compartment by the amount of glucose in the donor compartment at the end of iontophoresis. The percent decrement ($D_{(0-1\,hr)}$) of plasma glucose levels was calculated by a method described by Tozaki et al (1997).

All the experiments were performed in triplicate unless specified and the values were expressed as mean ± s.d. The influence of electrode polarity and pH on the skin permeation parameters of insulin, pH shift and electroosmotic ratio were statistically analysed (Sigmastat Jandel Scientifics, USA) using Kruskal–Wallis test. In all cases the post-hoc analysis was performed using Nemenyi's test at a significance level of $P < 0.05$.

Results

Insulin permeation

Figure 1 shows the permeation profiles of insulin at different pH during AI and CI. The passive permeation of insulin was low compared with iontophoretic permeation at all pH. There was no significant difference ($P > 0.05$) between AI and CI at any of the tested pH except at pH 5.3 (Figure 1). This was also reflected in the skin permeation parameters namely flux, cumulative amount permeated, lag time, skin affinity and enhancement ratio (Table 2 and Figure 2). On the other hand, there was a significant difference ($P < 0.05$) in the skin permeation parameters at pH above and below the isoelectric point of insulin. However, there was no significant difference ($P > 0.05$) between pH 2.83 and 3.6 below the isoelectric point and between pH 7.4 and 8.35 above the isoelectric point of insulin (Table 2 and Figure 2).

During AI, flux at pH 7.4 was significantly high ($P < 0.05$) compared with lower pH values (Table 2). At pH 8.35, the flux during CI was significantly high ($P < 0.05$) compared with lower pH values but was not significantly different ($P > 0.05$) from pH 7.4. The cumulative amount permeated in 48 h was not significantly different ($P > 0.05$) at any of the pH tested except at pH 8.3 and 5.3 during AI (Table 2). Lag time was consistently low ($P < 0.05$) during CI at all the pH tested, and the amounts of insulin in the skin as observed from the skin affinity values were significantly different ($P < 0.05$) only during AI between pH 5.3 compared with pH 7.4 and 8.35 (Figure 2A, B).

At the isoelectric point of insulin (pH 5.3), CI showed a significantly ($P < 0.05$) higher permeation compared with AI (Figure 1C) and similarly iontophoretic flux enhancement was significantly high ($P < 0.05$) during CI at pH 5.3 compared with other pH values (Figure 2C). The high enhancement observed at this pH seems to be 'fictitious' as there was a large shift in pH ($P < 0.05$), which changed the pH from 5.3 to 7.4 (Figure 3). Similarly significant ($P < 0.05$) pH shift was observed at pH 2.83 during CI and at pH 7.4 during AI. Overall the pH shift during AI was large ($P < 0.05$) above the isoelectric point of insulin, while the pH shift during CI was large ($P < 0.05$) below the isoelectric point of insulin (Figure 3). This shift in pH observed during iontophoresis resulted in the high variability in the permeation of insulin through the skin.

Electroosmosis and electrorepulsion

Among the various buffers used, citrate (pH 3.6) and acetate (pH 5.3) buffers showed high conductivity, while HEPES (pH 8.3) showed the least conductivity (Table 1). Conductivity measurements give an indirect measure of the contribution of electrorepulsion to the overall iontophoretic flux of insulin. On the other hand, the electroosmotic contribution to the flux was measured by using glucose as a marker, as it is expected to be transported mainly by electroosmosis.

The glucose permeation from the donor compartment to the receptor compartment was found to be less ($P < 0.05$) for AI at pH < pI of insulin (Figure 4), as the electroosmotic flow was expected to be in the counter direction from cathode to anode below the pI of skin (pH < 4.5–5.0). In contrast, the direction of electroosmosis was found to be in the direction of insulin permeation during CI at pH < pI of insulin, as evidenced by the relatively higher electroosmotic ratio compared with AI (Figure 4). At pH 5.3 (which is close to the pI of skin), the electroosmotic flow was expected to be minimal (as was the case with AI), but the pH shift during CI resulted in an electroosmotic flow comparable ($P > 0.05$) with that observed during CI at pH 7.4 (Figure 4). When pH > pI of insulin (pH 7.4), it was observed that there was no significant difference ($P > 0.05$) in the electroosmotic flow between AI and CI, due to the large variability caused by the pH shift (Figures 3 and 4).

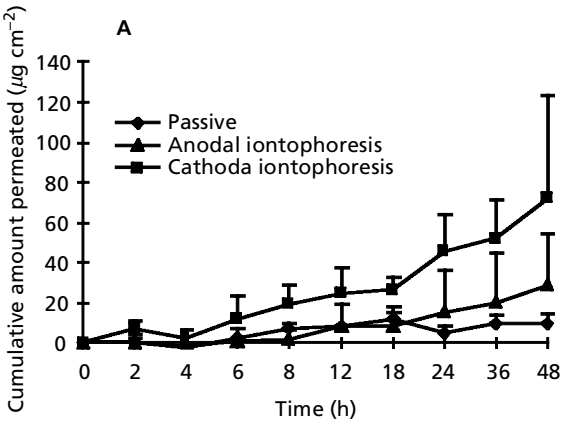
Stability of insulin

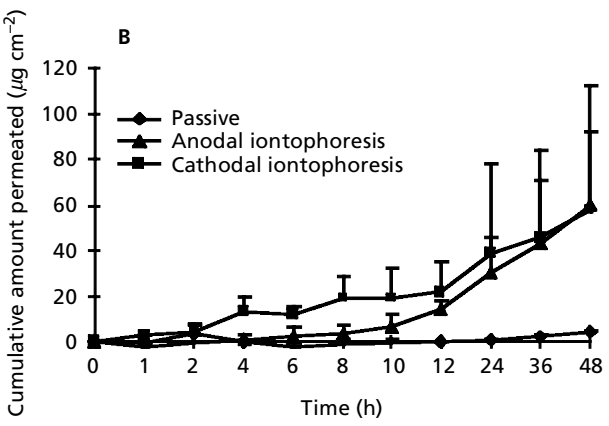
The HPLC analysis showed that there was significant degradation during AI at pH 7.4 and 8.3 (Figure 5A). At the same time, when the samples were analysed by SDS-PAGE, the intensity of insulin bands matched with the results of HPLC only at pH 3.6 (Figure 5A and B). Such differences due to the analytical method have been reported for other methods (Siddiqui et al 1987). Nevertheless, the large pH shift observed during AI at pH 7.4 and 8.3 was detrimental to the stability of insulin. In this regard, it is important to mention that the greater amount of insulin detected during permeation studies with AI at pH 7.4 and 8.3 may include the degraded fragments that may be more permeable than intact insulin, as the radiotracer method was not a stability indicating method to detect such changes. This was evidenced by the fact that the radioactivity recovery was 70% from the receptor compartment at the end of permeation studies, when analysed by TLC.

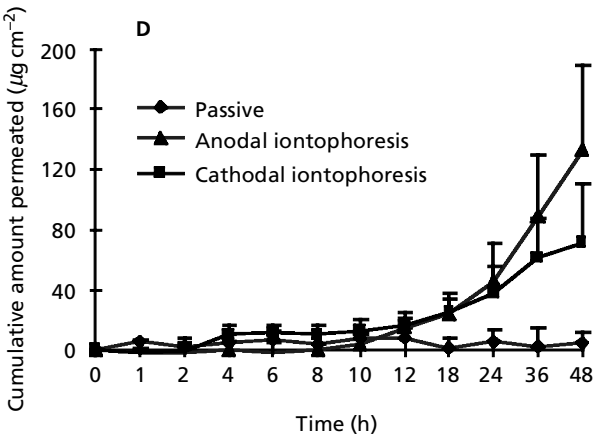
To test the hypothesis whether the insulin degradation (as detected by HPLC and SDS-PAGE) had any effect on the biological activity of insulin, the samples subjected to iontophoresis were tested for hypoglycaemic activity in diabetic rats. It was found that the samples from pH 7.4 AI showed comparable hypoglycaemic activity to freshly prepared insulin solution. For pH 5.3, the biological activity was less, probably as a result of change in conformation of insulin and/or different degradation pattern. Due to the small number of animals used (n = 2) in this exploratory study, further studies would be required to confirm the significance of this finding.

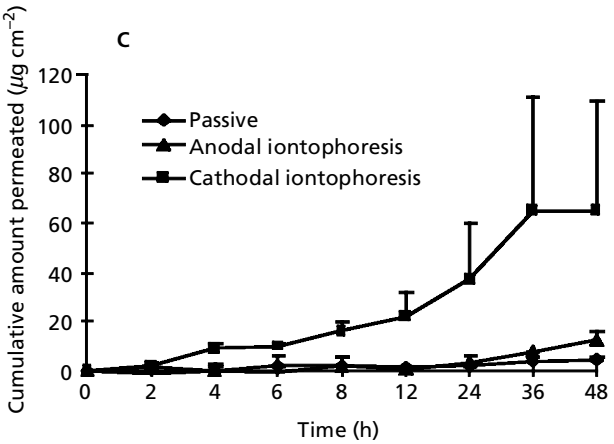
Discussion

For iontophoretic delivery, it is desirable that the peptide to be delivered remains charged over the entire pH range of the skin from 4 to 7 (Pikal 1995). Insulin is a typical peptide with an isoelectric point (pI 5.3) close to the isoelectric point of skin (pI 3–5) and, hence, the charge of insulin may change during transport across the skin. As a









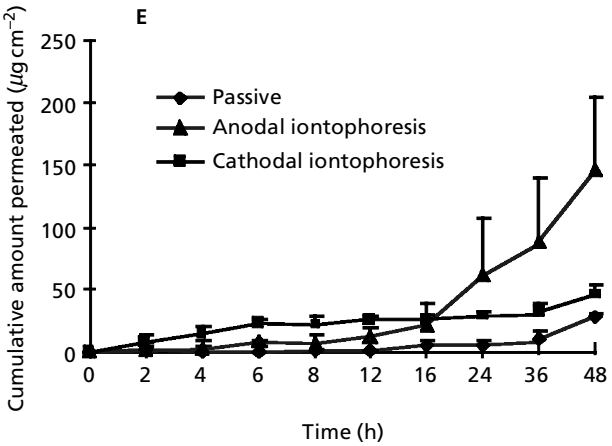


Figure 1 Permeation profiles of insulin at different pH as a function of electrode polarity. A, pH 2.83; B, pH 3.6; C, pH 5.3; D, pH 7.4; E, pH 8.3. Each data point is a mean of three measurements and the error bars represent the standard deviation. Current was applied for six hours (0.5 mA cm^{-2}).

Table 2 Skin permeation parameters for insulin as a function of pH and electrode polarity.

pH[a]	Electrode polarity	Flux ($\mu g\,cm^{-2}h^{-1}$)	Cumulative amount permeated[b] (μg)
2.8	Passive	0.19 (0.04)	7.8 (2.66)
	AI	1.16 (1.18)	22.56 (19.95)
	CI	1.34 (0.60)	56.78 (39.87)
3.6	Passive	0.13 (0.09)	3.27 (0.31)
	AI	1.39 (0.80)	46.98 (25.01)
	CI	0.93 (0.84)	44.97 (43.02)
5.3	Passive	0.14 (0.03)	3.77 (0.38)
	AI	0.36 (0.12)	9.67 (2.75)
	CI	1.63 (0.13)	51.17 (34.67)
7.4	Passive	0.86 (0.52)	7.54 (7.56)
	AI	3.73 (1.56)	104.00 (45.17)
	CI	1.84 (0.64)	56.30 (36.04)
8.3	Passive	0.55 (0.35)	20.11 (2.18)
	AI	2.78 (1.32)	112.41 (46.61)
	CI	3.66 (0.48)	34.44 (5.82)

[a]pH values are the initial donor solution pH. [b]Cumulative amount permeated after 48 h. Values are given as mean (n = 3) with s.d. in parentheses. AI, anodal iontophoresis. CI, cathodal iontophoresis.

result, at some depth inside the skin, iontophoretic forces may disappear and insulin will be transported due to diffusional forces (Sage et al 1995). The high lag time and skin affinity values of insulin amply testify to this fact (Figure 2A and B). Moreover, the high skin affinity values (Figure 2B) support the fact that insulin forms a depot in skin and is slowly released after stopping iontophoretic current as reported by several workers (Kari 1986; Siddiqui et al 1987; Banga & Chien 1993). Langkjaer et al (1998) demonstrated, with respect to the pI of insulin, that monomeric insulin analogues with pI < 4 or > 7.4 were better permeable than native insulin.

Overall the flux during iontophoresis is explained in the most simplified form by the equation:

$$J_{ion} = J_p + J_{er} + J_{eo}$$

where J_{ion} is the total iontophoretic flux, J_p is the flux due to passive permeation, J_{er} is due to electrorepulsion and J_{eo} is due to electroosmosis. The relative contribution of these three components to the overall iontophoretic flux depends on the molecular size, peptide charge, local pH and skin charge (Pillai et al 2001). Flux due to electroosmosis originates from the charged (negative) nature of skin, which is permselective to cations at physiological pH, and hence the electroosmotic flow is predominantly in the direction of anode to cathode (Burnette & Ongipipattanakul 1987). For small ions, electroosmotic flow may be important, but not dominant, while for large permeates (>1 kDa), the relative contribution of electroosmotic flow to flux enhancement increases (Pikal 1995). In other words, the flow of a solute with a solvent stream is independent of solute size, whereas the flow of a solute relative to solvent stream is inversely proportional to size through Stokes–Einstein relationship (Pikal & Shah 1990). However, the contribution of delineation of electrorepulsion and electroosmosis to the overall iontophoretic transport is experimentally difficult (Guy et al 2001).

Apparently, the volume flow may be influenced by the ionic concentration, which develops in the unstirred layer adjacent to the skin epithelium during the passage of a current (Gangarosa et al 1980). Since the ionic composition of buffers in this study was different, the contribution of ion volume flow to overall electroosmotic flow cannot be ruled out. However, the influence of buffer ions may be approximated by conductivity measurements to understand the relative role of electrorepulsion and electroosmosis to the overall flux of insulin.

When pH < pI, the predominance of electroosmotic flow was in the direction of cathode to anode, which partly explained the relatively high lag time (though not significant at $P > 0.05$) observed with AI compared with CI at pH 3.6 (Figures 2A and 4). At the same time, the contribution of electrorepulsion seemed to be minimal at pH 3.6, as the citrate and phosphate buffer ions were more mobile than insulin (Table 1). In contrast, the greater enhancement achieved with HEPES buffer (pH 8.3) during CI was attributed to the predominance of electrorepulsion, as HEPES ion was relatively less competitive to insulin species compared with other buffers (Table 1 and Figure 2C). However, it is difficult to comment about the relative contribution of electrorepulsion and electroosmosis at other pH, as the results were confounded by the generation of highly mobile hydrogen and hydroxyl ions from the electrode surface due to the large pH shift.

Kari (1986) and Meyer et al (1989) reported the shift in pH with platinum electrodes. Earlier on, other authors had studied the permeation of insulin using single polarity at different pH and found that the pH shift did not cause any

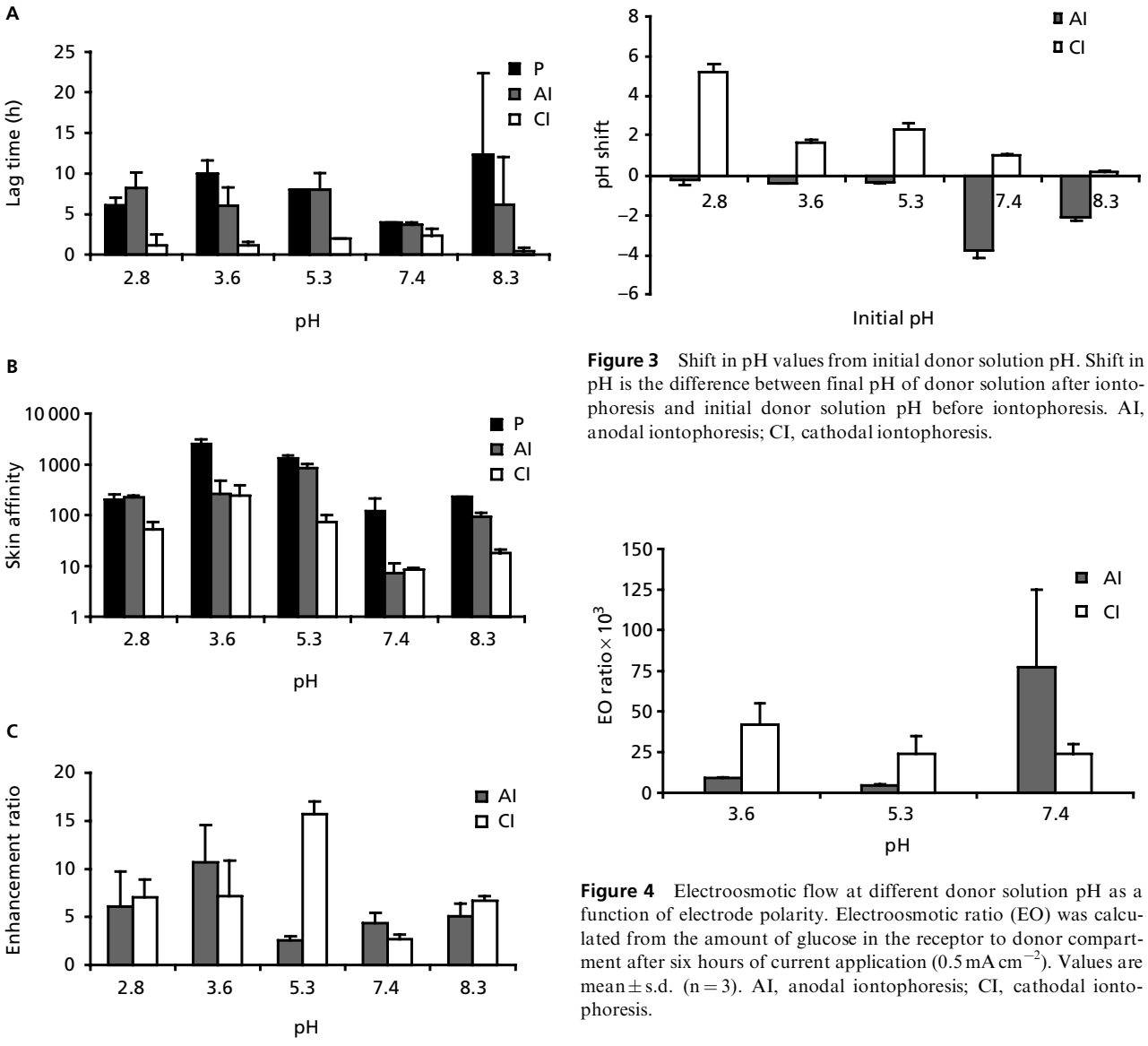


Figure 2 Influence of donor solution pH and electrode polarity on (A) lag time and (B) skin affinity. The y-axis is represented in logarithmic scale as the skin affinity values differed by more than one order of magnitude. C. Flux enhancement of insulin. Values are mean ± s.d. (n = 3). P, passive; AI, anodal iontophoresis; CI, cathodal iontophoresis. Current was applied for six hours (0.5 mA cm^{-2}).

Figure 3 Shift in pH values from initial donor solution pH. Shift in pH is the difference between final pH of donor solution after iontophoresis and initial donor solution pH before iontophoresis. AI, anodal iontophoresis; CI, cathodal iontophoresis.

Figure 4 Electroosmotic flow at different donor solution pH as a function of electrode polarity. Electroosmotic ratio (EO) was calculated from the amount of glucose in the receptor to donor compartment after six hours of current application (0.5 mA cm^{-2}). Values are mean ± s.d. (n = 3). AI, anodal iontophoresis; CI, cathodal iontophoresis.

significant degradation of insulin. In our study, the pH shift was studied at both electrode polarities and was higher during AI than during CI at pH 7.4 (Figure 3). There was a significant degradation of insulin during AI at pH 7.4 (Figure 5A) and the extent of degradation was comparable with that reported by Huang & Wu (1996). The earlier authors attributed the degradation to temperature (37 °C) and proteolytic degradation (using rabbit skin) in addition to the electrochemical degradation. Though proteolytic stability of insulin was not determined in our study, it has been reported to be 22% degradation at pH 3.6 with rat skin homogenates (Banga & Chien 1993).

On the other hand, Srinivasan et al (1989) did not find any proteolytic degradation in human skin at pH 7.4 using gel electrophoresis. Therefore, it is difficult to compare the results of those studies with our study as the experimental conditions were different, but it seems that the degradation of insulin during iontophoresis was dependent on the pH and the shift in pH during iontophoresis, type of skin used and the susceptibility of insulin to proteolytic enzymes (intact skin vs skin homogenate).

In general, the electrochemical degradation was a result of oxidation/reduction of insulin at the platinum electrode surface, which was exacerbated by the shift in pH (Langkjaer et al 1998). This degradation resulted in generation of smaller fragments, which were expected to be more permeable than intact insulin and probably accounted for the high amount detected by the non-specific radiotracer

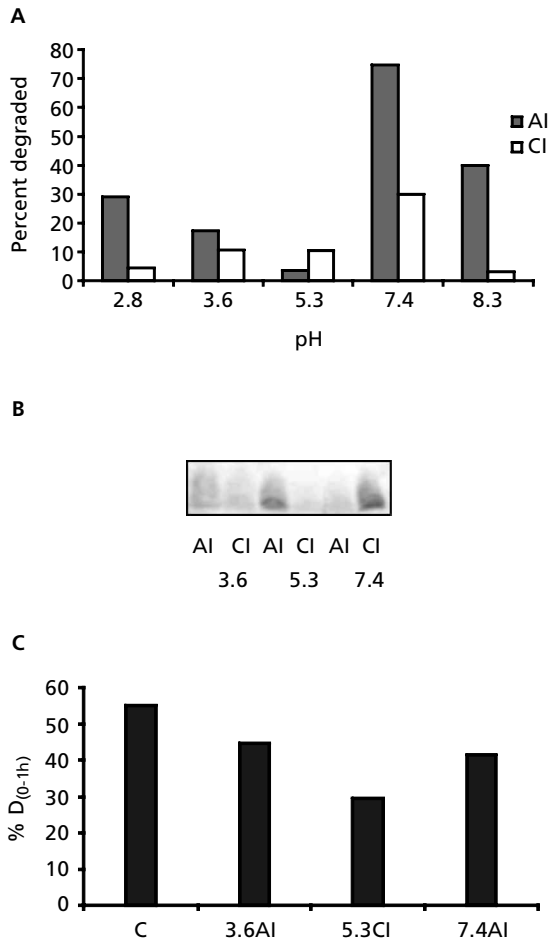


Figure 5 A. Percentage of insulin degraded at different donor solution pH as a function of electrode polarity after passage of electric current by HPLC. AI, anodal iontophoresis; CI, cathodal iontophoresis. B. The intensity of insulin bands in SDS-PAGE at three pH values as a function of electrode polarity. C. Percent decrement in plasma glucose levels up to 1 h at different donor solution pH as a function of electrode polarity after passage of electric current. Values are mean of two measurements. C. Freshly prepared insulin solution in saline.

method. Several authors have reported the difference in insulin permeation as detected by radiotracer and radioimmuno assay (Siddiqui et al 1987; Banga & Chien 1993; Huang & Wu 1996; Langkjaer et al 1998). A similar observation has been found in our laboratory, where there was a 200–3000-times difference in the amount of insulin measured by RIA and radiotracer method during skin permeation studies (unpublished data) and was in close agreement with the findings of Langkjaer et al (1998). Therefore, the skin permeation parameters detected using the radiotracer method may be apparent rather than real values.

In spite of the significant degradation, we found to our surprise that hypoglycaemic activity was not significantly compromised compared with fresh insulin solution. In this regard, previously, it has been reported in the literature that the products formed by hydrolysis of insulin possessed full or nearly full biological potency independent of the species of insulin and the site of deamidation (Carpenter 1966; Brange et al 1987). Another possible explanation would be that the insulin levels required to induce a hypoglycaemic response may be much less for rats. This implies that even a small amount of intact insulin in the samples subjected to iontophoresis may be sufficient to lower the blood glucose levels. Kari (1986) reported that there was only a single peak in HPLC and there was no change in hypoglycaemic activity after CI at 0.4 mA cm^{-2} for 3 h. Similarly, a single peak was observed with HPLC in our study, but the peak area was reduced significantly after AI at pH 7.4 and there were no additional peaks attributable to any degradation products, unlike the findings of Huang & Wu (1996). These variations are attributed to the difference in analytical methods and experimental conditions. To clarify the issue of proteolytic stability, hypoglycaemic activity and the importance of analytical method in determining the stability of insulin, further studies are necessary.

However, our results indicated that AI at pH 3.6 was preferable as the pH shift and electrochemical degradation was less, while the enhancement was high below the pI of insulin. This pH has been shown to result in greater hypoglycaemic activity than CI at pH 7.4 (Siddiqui et al 1987). At this pH (pH < 4) insulin tends to remain in the less aggregated state (Sato et al 1983). The pH shift observed at this pH (0.6 U) with platinum electrodes was comparable with that reported earlier with the Ag/AgCl (0.3 U) electrodes (Langkjaer et al 1998). Similarly, CI at pH 8.35 seemed to be better for the same reason as mentioned above. The low pH shift observed at pH 3.6 and 8.35 was due to the high buffering capacity of the buffer salts used to maintain the pH, indicating that it was possible to control the pH shift observed with platinum electrodes. At the same time, it may not be overlooked that the buffer type and concentration needs to be optimized, as they may compete with insulin for carrying the charge and influence the transport efficiency of insulin. We reported on these issues associated with the optimization of buffer type and concentration in a previous publication (Pillai et al 2003).

Conclusions

The findings from this study have highlighted the issues associated with the use of platinum electrodes for iontophoresis of large peptides like insulin. The pH shift was influenced by the electrode polarity, drug solution pH and the buffer used. The pH shift had an influence on the direction and magnitude of electroosmotic flow and electrorepulsion to the overall flux. In addition, the latter was influenced by the type and size of buffer ion used to control the pH. The degradation of insulin was high at pH 7.4 during AI and there was a difference in the amount of degradation detected by different analytical methods. In this regard, the radiochemical method overestimated the amount of insulin permeated, as it detected both intact and degraded insulin. Below the pI of insulin, AI at pH 3.6

was better due to the high enhancement ratio and the minimal shift in pH shift, in addition to the relatively greater stability of insulin. Above the pI of insulin, CI at pH 8.35 was better, for the same reasons. However, the skin irritation at high alkaline pH might be an issue limiting the use of this pH. As the pH shift was mainly dependent on the electrode polarity and buffering capacity of the buffer salts used, ways to reduce the pH shift by increasing the buffering capacity (type and amount of buffer salts) and alternating the electrode polarity need to be investigated further.

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