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CHARACTERIZATION OF IN VITRO TRANSDERMAL IONTOPHORETIC DELIVERY OF INSULIN

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ABSTRACT

In-vitro studies were conducted to characterize the transdermal iontophoretic delivery of insulin, thus avoiding potential complications from various biological variations, which may be encountered during in-vivo studies. The proteolytic degradation of insulin in skin homogenates and degradation under the experimental conditions used was investigated. Appropriate adjuvants were incorporated to minimize the potential degradation problems of insulin. ^{125}I-Insulin was observed to penetrate into and accumulate in the skin during the iontophoresis period. It was then released gradually from this depot, as a mixture of intact and ^{125}-I labelled fragments, into the receptor medium. Drug desorption

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studies supported the theory of skin depot or reservoir formation. It was found that an electric field could be used to facilitate the desorption of drug from the depot. The post-application flux of insulin (or its fragments) from the skin depot formed during iontophoresis was monitored to study the factors affecting the iontophoretic delivery of insulin. Stripping and delipidization of the skin were noted to increase the skin permeation rate of insulin. The cumulative radioactivity permeated and accumulated in the skin was higher at pH 3.6 than at pH 7.4. The iontophoresis-facilitated transdermal delivery was observed to increase with increasing duration of current application and increasing donor concentration of insulin. Modulation of drug delivery by multiple applications was also found to be feasible.

INTRODUCTION

A non-invasive route for the systemic delivery of insulin has been actively investigated by biomedical researchers. Transdermal delivery could be a possibility, especially since the skin has a very low level of proteolytic activity, unlike most other non-parenteral routes. Although transdermal delivery has gained increasing recognition in recent years, this route has received very little attention for the delivery of insulin and other peptide/protein drugs (1). The reason could be that on an intuitive reasoning and thinking, macromolecules have not been expected to permeate the skin due to their large molecular size and hydrophilic nature.

However, the recent renewed interest in the technique of iontophoresis has led several investigators to explore the possibility of transdermal iontophoretic delivery of peptide/protein drugs (2). The feasibility of delivering insulin iontophoretically has also been investigated (3-8). Srinivasan et al. performed some limited in-vitro experiments and found that insulin could be delivered through intact, but alcohol-treated, human skin and polyacrylamide gel electrophoresis of the receptor fluid seems to suggest that insulin is not metabolized while passing through the skin (7). Most of the other investigations

were in-vivo studies on laboratory animals though the results have been equally encouraging. Reduction of blood glucose levels was observed after iontophoretic delivery of insulin. Kari (3) felt that the stratum corneum has to be stripped for achieving insulin delivery while others could deliver insulin through the intact skin (4,5). In a recent investigation by Meyer et al (6), transdermal absorption of physiological doses of human insulin across the intact skin of albino rabbits was demonstrated, using low levels of electrical current.

While these in-vivo studies have demonstrated that insulin can be delivered iontophoretically through the skin, it is difficult to fully characterize the permeation kinetic profiles of insulin delivery based on these studies. This is because blood glucose levels as well as plasma insulin levels can fluctuate due to a wide variety of causes including, but not limited to, circadian rhythms. In-vitro studies, on the other hand, can permit a direct measurement of the permeation kinetics with better control of the experimental variables and also minimize the interference of most biological variables. This work was carried out to conduct in-vitro investigations on the iontophoretic delivery of ^{125}I- Insulin and to characterize the permeation profiles and make general observations on the iontophoretic delivery of insulin in an in-vitro set-up.

MATERIALS AND METHODS

Materials

Porcine insulin, Gentamicin sulfate and bacitracin were procured from Sigma Chemical Co. (St. Louis, MO). For radiotracer work, ^{125}I-Tyr^{A14}-Insulin (porcine) was obtained from NEN, Dupont (Wilmington, DE). Radioimmunoassay was performed using Coat-A-Count^{R} Insulin kit obtained from Diagnostic Products Corporation (Los Angeles, CA). Tritiated water (25.0 mCi/g) was procured from NEN (Boston, MA). Platinum wire was procured from Aesar for making the platinum electrodes. Urea was obtained from Fisher Scientific Co. All other chemicals used for making buffers were of reagent grade quality. All solutions were made with deionized water purified through a Nanopure water purification system (Sybron/Barnstead, Boston, Ma.). The water

had a resistivity of 16 megohm or greater. Hairless rats (HRS Strain) were obtained from Armed Forces Institute of Pathology, Walter Reed Army Medical Center, Washington, D.C.

Analytical Techniques

For radiotracer work, the cold (unlabelled) insulin in the donor solution was spiked with an adequate dose of the radiolabelled insulin and the samples taken from the receptor compartment were counted using a Gamma Counter (TM Analytic, IL). Since insulin was found to permeate through the skin only to a very small extent, the counting in insulin permeation studies was conducted for 3-9 minutes to accumulate counts and the counts were then converted to CPM in the calculation. Radioimmunoassay was performed using Coat-A-Count^R insulin kits. Since the kit was used to assay insulin in the buffers (receptor solution), instead of serum, the standards were prepared in the receptor solution being used in the experiment, rather than using the calibrators supplied with the kits. It was observed that the use of calibrators supplied with the kit would lead to erroneous results. Tritiated water was counted on a liquid scintillation counter (LSC). HPLC assay was performed using a Waters (Milford, MA) solvent delivery module (Model 590) and WISP (Model 712), attached to a integrator (Spectra-Physics, SP 4270, San Jose, CA) and a variable wavelength UV-detector (Spectraflow 773) using a method modified from Szepesi and Gazdag (9). Separations were performed on a μBondapak C_{18} column at a flow rate of 2.0 ml/minute and the wavelength of detection was set at 215nm. The mobile phase consisted of a combination of Buffer:Methanol: Acetonitrile (40:50:10). The buffer was prepared from $NaH_2PO_4.H_2O$, 0.05M (adjusted to pH3.2 with phosphoric acid) with sodium sulfate, 0.05M. The high concentration of salt was added to control the degree of ionization on the insulin molecule.

Transmembrane Potential

Reference electrodes (Microelectrodes, Inc., Londonderry, N.H.) were used to measure the transmembrane potential across the skin. The electrodes had

a Ag/AgCl wire enclosed in a flexible tube containing a saturated KCl solution and a porous frit at the measuring tip. The electrodes were placed on either side of the skin and the potential difference across the skin was measured by a Voltmeter (Elenco, M-7000).

Permeation Apparatus

The Valia-Chien skin permeation system was modified to accommodate a pair of platinum electrodes connected to a constant current power source. Unless otherwise indicated, the experiments were conducted with pulse current at a frequency of 2KHz and a on:off ratio of 1:1. A current intensity of 0.4 mA, equivalent to a current density of 0.62 mA/cm^2, was used in all cases.

Permeation Medium

The least solubility of insulin is at its isoelectric point, which is at pH 5.3. Thus the working pH can be at either end of this pH. However, the charge density is higher at acidic pH. Also, the aggregation is lower and stability is better at acidic pH. The pH was thus selected to be on the acidic side for this study. A pH of 3.6 was used for most studies as this has been reported to be the pH of optimal delivery in previous in-vivo work on insulin delivery (4).

The donor solution thus consisted of a citrate buffer at pH 3.6 with an ionic strength of 0.1M. Insulin, in a concentration of 10-30 IU/ml was dissolved in the donor solution. The receptor fluid was a phosphate buffer, pH 7.4 (Ionic strength 0.15M), to simulate the physiological pH. The osmotic pressure of the receptor solution was measured by a μOsmette (Precision Systems, Inc., MA) and was found to be 295 mOsm/Kg. The osmotic pressure of donor was slightly lower (241 mOsm/Kg) and thus 1% mannitol was added to the donor solution to make it iso-osmotic with the receptor solution.

Insulin is known to undergo adsorption to glass and this can be prevented or minimized by the use of additives such as albumin or urea at low concentrations. Urea was chosen as the additive to prevent adsorption because it has been reported to have the additional benefit of preventing or minimizing the self-aggregation of insulin molecules (10). Thus, urea at the concentration of

2mg/ml was added to both donor and receptor solutions. Insulin, being a protein molecule, is also susceptible to microbial degradation and thus both the donor and receptor solutions were preserved by addition of gentamicin sulfate and bacitracin (50μg/ml each). The combination gives a broad spectrum protection.

Skin Permeation Studies

Full-thickness skin specimen was freshly excised from the abdominal region of hairless rat and mounted between the donor and receptor half-cells of the Valia-Chien skin permeation system. The area of the skin available for permeation was 0.64 cm^2. All experiments were performed in triplicate. The donor and receptor solutions, previously equilibrated to 37°C, were filled into the donor and receptor compartments, respectively, and the temperature was maintained at 37°C by an external circulating water bath. A pair of platinum electrodes connected to the power source were immersed in the donor and receptor solutions and samples were taken at predetermined intervals over a period of 24-60 hours and analyzed.

Delipidization and Stripping of Skin

For delipidization of the skin, the skin was mounted on V-C cells and the donor compartment was filled with a combination of chloroform:methanol (2:1) and the stratum corneum surface was exposed to the delipidizing medium for 2 hours. The solvent was then drained and replaced with the donor solution after air drying the skin. Stripping of the skin was accomplished by the use of adhesive tape (Scotch brand Magic Tape, 3M Co., St. Paul, MN). The adhesive tape was placed firmly and evenly on the stratum corneum surface and then peeled away. The process was repeated 30 times to totally remove the stratum corneum layer after layer.

Preparation of Skin Homogenates

Freshly-excised full-thickness abdominal skin from hairless rats was taken and homogenized over a ice bath in citrate buffer (pH 3.6) to get a 10% skin

homogenate. The homogenate was allowed to stand for 2 hours (at 5°C) and then centrifuged at 2500 rpm for 30 minutes (at 5°C). The opalescent liquid was separated and filtered through a PTFE filter 0.2 μm to get a clear skin extract.

RESULTS AND DISCUSSION

Degradation of Insulin in the Experimental Setup

One of the most formidable problems while working with peptide/protein drugs is the extensive loss that could occur by several possible mechanisms. In the present setup, the loss could be attributed to: *1*. Adsorption to the large exposed glass surface of the V-C cells; *2*. Aggregation under the stirring; *3*. Microbial degradation of insulin, especially when experiment was run over prolonged periods of time; *4*. Possible electrochemical interactions at electrode surfaces, and *5*. Degradation due to proteolytic enzymes present in the skin.

Care was exercised to control or minimize the loss. As outlined earlier in the experimental section, low concentrations of urea were added to reduce adsorption and self-aggregation. Gentamicin and bacitracin were incorporated to prevent microbial degradation. Platinum electrode, which are known to be the most inert, were used. Ag/AgCl electrodes could not be used as these were observed to cause precipitation of insulin under the experimental conditions used.

The transdermal route has lower proteolytic enzyme activities than other absorptive mucosae such as the nasal mucosa. However, proteolytic enzymes are known to be present in the skin as well. Investigations were thus carried out to determine the degradation of insulin in skin homogenates and protease inhibitors were evaluated for their potential use to prevent or minimize proteolytic degradation of insulin during iontophoretic transport. The skin extract was prepared as described earlier and 2.5% v/v of this extract was added to an insulin (10 IU/ml) solution and incubated at 37° C for 3 hours. The amount of insulin remained was determined by the HPLC assay. No degradation was observed in the Control, to which no skin homogenate was added. In contrast, the solution containing the skin homogenate lost 22.0% of the insulin dose. The possibility

of using protease inhibitors to possibly prevent this degradation was then evaluated. Two agents, i.e., phenylmethylsulfonyl fluoride (PMSF) and aprotinin were investigated as potential protease inhibitors. PMSF, at 0.5 and 2.0 mM, failed to protect insulin from proteolytic degradation. One possible explanation is that PMSF could be unstable in buffer solutions. In contrast, aprotinin was found to give some protection against proteolytic degradation. It was observed that the concentration of aprotinin required for the protective effect has to be carefully worked out for any given set of conditions and the amount of protection obtained is not a linear function of the aprotinin concentration. A concentration of 0.25 mM of Aprotinin was observed to reduce the degradation from 22% to 5%. No benefit was observed by increasing this concentration further.

The protection afforded by aprotinin during permeation experiments was evident when radioimmunoassay (RIA) was used for insulin assay. Many of the degradation products of [^{125}I]-Insulin still carry the I-125 label (11,12) and thus the radiotracer assay is not capable of differentiating the degradation products from the initial insulin molecule. In the present study, the amounts of insulin permeating were too low to be within the sensitivity of the HPLC assay used but RIA was able to detect insulin in the receptor solutions. In the presence of aprotinin, insulin could be detected in the receptor compartment by RIA in the first few hours, but not at later time points, suggesting the occurrence of further degradation. This aspect is discussed in the following sections.

Insulin Permeation and Desorption Profiles

Figure 1 shows the passive and iontophoretic permeation profiles of ^{125}I-Insulin across excised hairless rat skin. Insulin, being a macromolecule, is not expected to permeate through an intact skin. Since no permeation could be detected by radioimmunoassay under passive conditions, it is likely that the radioactivity picked up in the receptor solution under passive conditions could be the degradation products of insulin. Application of iontophoresis was found to enhance the permeation and the cumulative radioactivity permeated increased as the duration of application of current is increased. It is interesting to note that

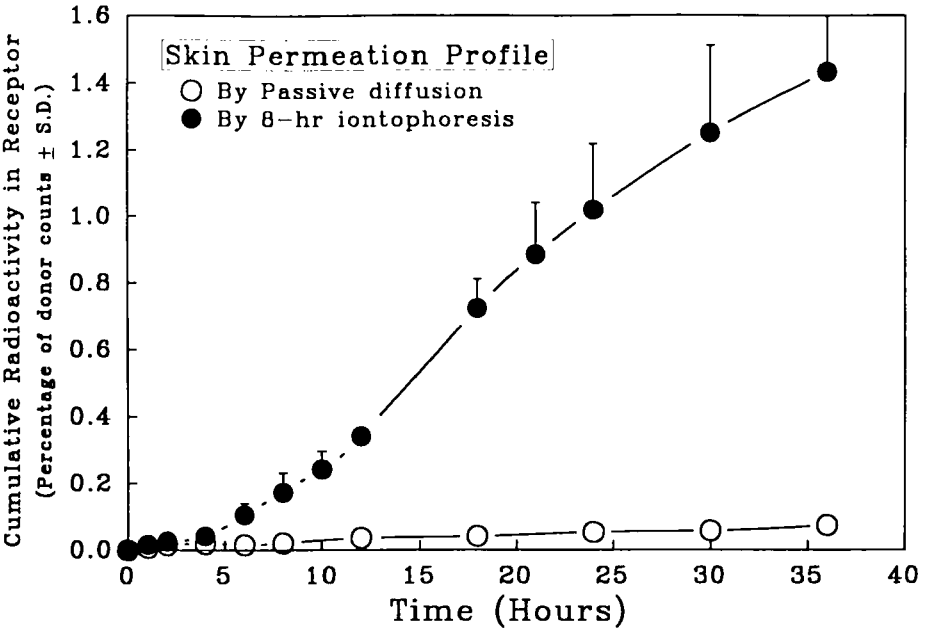

FIGURE 1

Typical skin permeation profiles of ^{125}I-Insulin under passive and iontophoretic conditions.

the cumulative radioactivity permeated continues to increase even after the termination of iontophoresis treatment. This is probably because ^{125}I-Insulin is accumulating in the skin. This observation seems to be in agreement with earlier in-vivo work by Liu et al. in which blood glucose levels were found to remain decreased for several hours following a relatively short iontophoresis treatment (13). Kari (3), using alloxan-induced diabetic rabbits, had also observed that blood glucose levels continued to decrease and serum insulin concentrations continued to increase after stopping iontophoretic application. He suggested that insulin is accumulating in the skin and subcutaneous tissues. Other drugs such as verapamil hydrochloride have also been shown to undergo strong binding in the skin and Wearley et al (14,15) investigated this phenomenon and related it to the reversibility of skin permeability. Factors that may be contributing to this depot or reservoir formation could be physical entrapment of the insulin

macromolecule as the monomer or self-associated polymeric forms, charge interactions with the skin and/or adsorption on tissue surfaces.

An interesting observation in the present study is that although the cumulative amounts of insulin permeating through the skin continued to increase as determined by the radiotracer measurements, no such increase was seen by the radioimmunoassay. This difference could be attributed to the degradation of the insulin reservoir in the skin and the gradual desorption of the products of enzymatic degradation from the skin with the passage of time. These degradation products of the insulin molecule still carry the radiolabel (^{125}I) but these would not be detected by the highly specific radioimmunoassay. Since iontophoresis was discontinued after 2-4 hours, the cumulative amounts permeating beyond the period of iontophoresis application are indicative of the amount of insulin deposited in the skin during the iontophoresis application. Some of these degradation products may still show hypoglycemic activity (16), and this may explain why several researchers observed a continued drop in blood glucose levels (as discussed earlier) long after the iontophoresis treatment was discontinued. Also, biological variations might have compromised some of the reported in-vivo results. Another explanation could be that there was more degradation in our in-vitro experimental set-up as compared to the in-vivo set-up of other workers.

During an investigation of the factors influencing insulin iontophoresis, no influence of any factors could be discerned in the first 2-4 hours during the iontophoresis period. It was only after the insulin and/or its fragments were allowed to desorb for over 24-36 hours, that one could discern the trends of the initial deposition. Radiotracer assay technique was thus used to generate the data reported in Figs 1-7. For the most part, radioimmunoassay could detect insulin in the samples from the receptor solution only in the first 1-4 hours. Possible reasons for this phenomenon have been discussed earlier. In several instances, a burst of radioactivity was also observed after the iontophoresis was stopped. This most likely resulted from the permeation of some fragments carrying a negative charge, which may have been repulsed by the cathode in the receptor solution until the termination of current application.

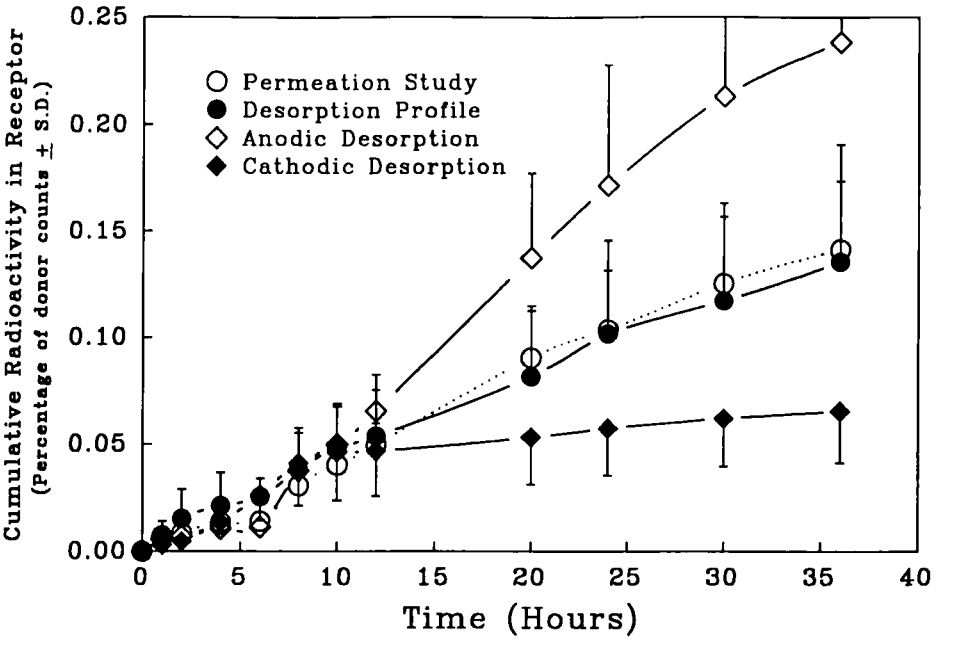

FIGURE 2

Desorption profiles of ^{125}I-Insulin released from the depot formed in the skin tissues during iontophoretic transdermal delivery of insulin.

It could be argued that the continued permeation is resulting from the damage to the integrity of the skin caused by application of current. In order to rule out this possibility, desorption studies were carried out. For desorption studies, the insulin-containing donor solution was totally withdrawn after iontophoresis treatment and the stratum corneum surface was then rinsed with distilled water to remove any residual insulin on the surface. A piece of aluminum foil was then used to cover the stratum corneum and the covered skin was again mounted in the Valia-Chien skin permeation system, with the donor half-cell remaining empty. Figure 2 compares the desorption profile after 1-hr current application with iontophoresis-facilitated transdermal permeation study, i.e., one in which donor solution is not removed following the 1-hr current application. The desorption profile is practically superimposed with the permeation profile, suggesting that the continued appearance of insulin after

termination of the current application has its origin in a skin depot and not in the donor solution. This observation lends support to the hypothesis of a reservoir formation in the skin and rules out the possibility of skin damage as the cause for the continued permeation observed.

In Figure 2, we can also see the results of active desorption facilitated by iontophoresis under anodic and cathodic currents. For active desorption studies, the donor solution was removed after 1-hr current application and replaced with the same donor solution with no insulin in it. The current was then again applied for 6 hours, with the anode in the donor phase for anodic desorption study and the cathode for cathodic desorption. The results indicate that anodic desorption increases the amounts of insulin desorbed into the receptor solution. This could be because the anode pushed the insulin reservoir (with positive charge) and/or positively charged fragments in the skin to the receptor phase, while cathodic desorption reduced the desorption.

Factors Influencing Insulin Delivery

As has been discussed above, insulin was observed to form a depot in the skin during iontophoretic application, from which it was released gradually over a period of time. It was found that the post-application release flux of insulin (or its fragments) from the skin depot could be used to study the relevant factors involved, since the depot is formed during and in proportion to the iontophoresis application parameters. Effect of several factors which were observed to affect transdermal iontophoretic delivery of insulin will be discussed in the following sections:

Effect of pH and Skin Reservoir Formation

As discussed earlier, the working pH for the donor solution can be at either side of the isoelectric point. An acidic pH was used for most experiments as it provided an opportunity for higher density of positive charge on the insulin molecule, reduced aggregation, better stability and greater delivery. Figure 3 compares the Skin depot formation and cumulative permeation of ^{125}I-Insulin at

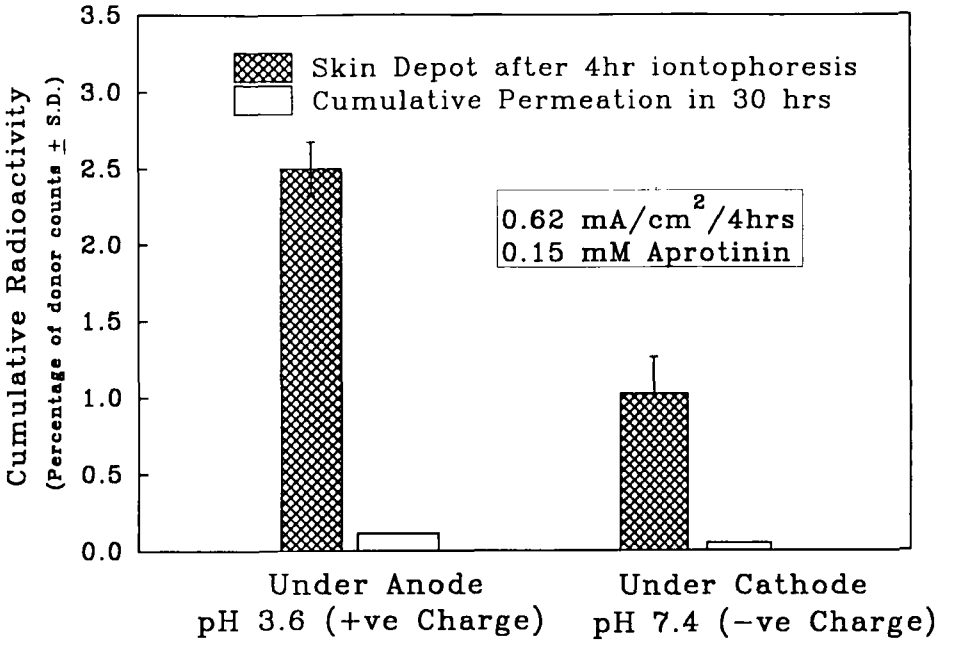

FIGURE 3

Effect of pH on the skin depot of ^{125}I-Insulin formed after 4 hours of iontophoresis application and the cumulative amounts permeated over 30 hours on a per unit volume basis.

two pH values: pH 3.6, at which the insulin molecule is positively charged and was delivered by anode, and pH 7.4, at which insulin is negatively charged and was delivered by cathode. It is evident that the amount of transdermal permeation is greater at lower pH than at higher pH. It was further observed that the amount of ^{125}I-Insulin retained in the skin depot, on a per unit volume basis, is much higher than the amount permeating over 30 hours, at both pH 3.6 and 7.4. In terms of the actual weight of the skin used in the studies and the total cumulative amount permeating into receptor solution, it was observed that a significant amount (50% or more) of the skin depot gets desorbed over the period of study (data not shown).

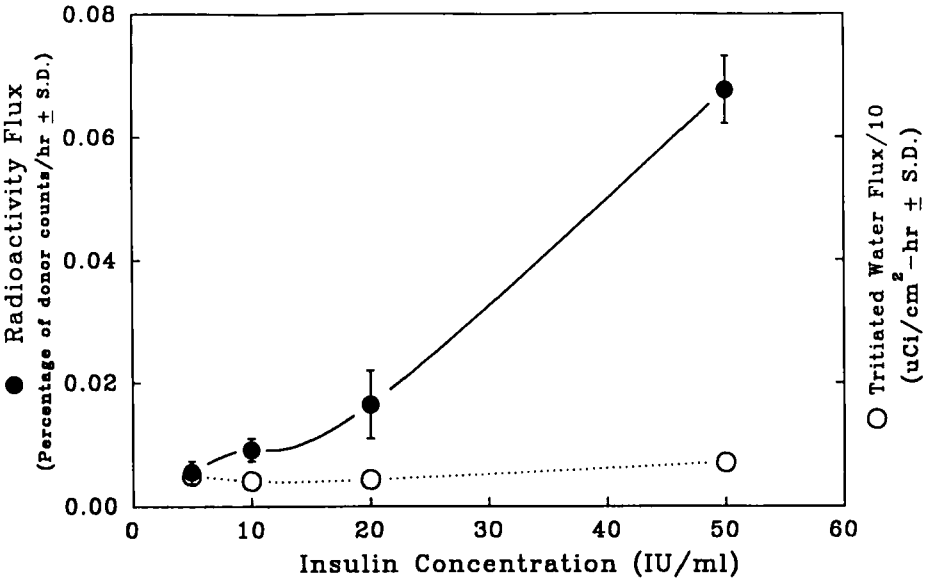

FIGURE 4

Effect of increasing concentration of ^{125}I-Insulin in donor solution on the iontophoresis-facilitated skin permeation flux of insulin and on the corresponding skin permeation flux of tritiated water.

Effect of Donor Concentration

The iontophoresis facilitated skin permeation flux of insulin was observed to increase linearly with the increase in the donor concentration of insulin in the concentration range investigated, i.e., 5 to 50 IU/ml (Figure 4). The corresponding flux of tritiated water at these concentrations was also measured and found not to be affected by increasing insulin concentration, which suggests that water flow in the system was not affected. This observation could be attributed to the fact that only a very small fraction of current is actually carried by insulin, its transport number being in the order of 10^{-6}, in the experimental set-up used (The transference number of an ionic species is the fraction of the total current carried by the ionic species).

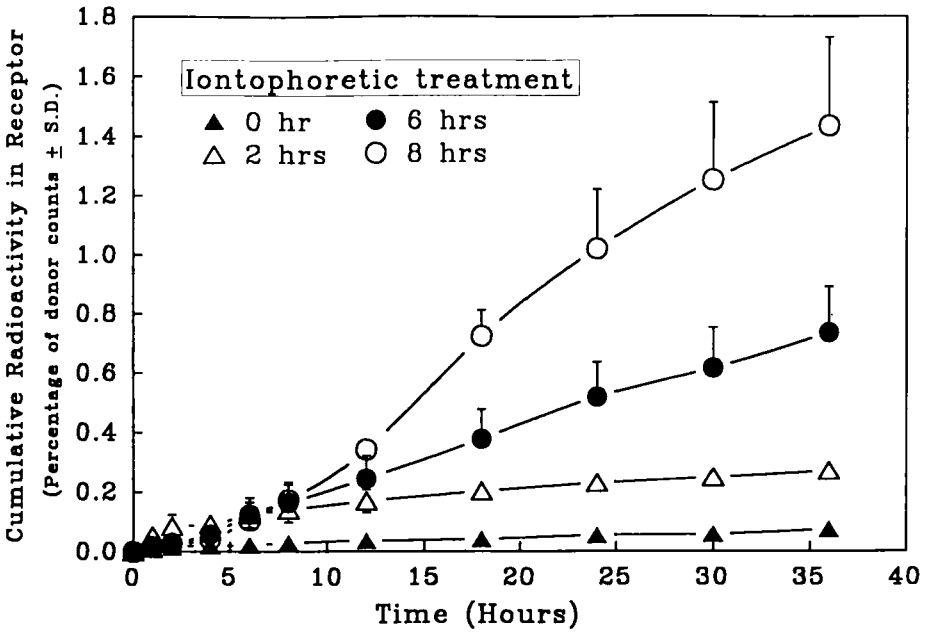

FIGURE 5

Effect of the duration of iontophoretic application on the cumulative amounts of ^{125}I-Insulin permeated or desorbed over a 36-hour period.

Modulation of Drug Delivery

In theory, iontophoretic delivery provides a means for an accurate regulation of the amount of medication delivered by controlling the supply as well as the quantity of electric current applied and also by turning the iontophoretic device on and off. This can be utilized to deliver the drug at programmable rates, so that the dosage regimen can be tailored to the needs of individual patients. This can also provide for an application in the biofeedback or circadian-modulated controlled drug delivery. In practice, however, one of the factors which is critical for this type of therapeutic application is the reversibility of skin permeability. Although insulin molecules form a skin depot during application, some modulation of drug delivery was still found feasible for insulin. Figure 5 shows that the cumulative amounts of ^{125}I-Insulin permeated through the skin over

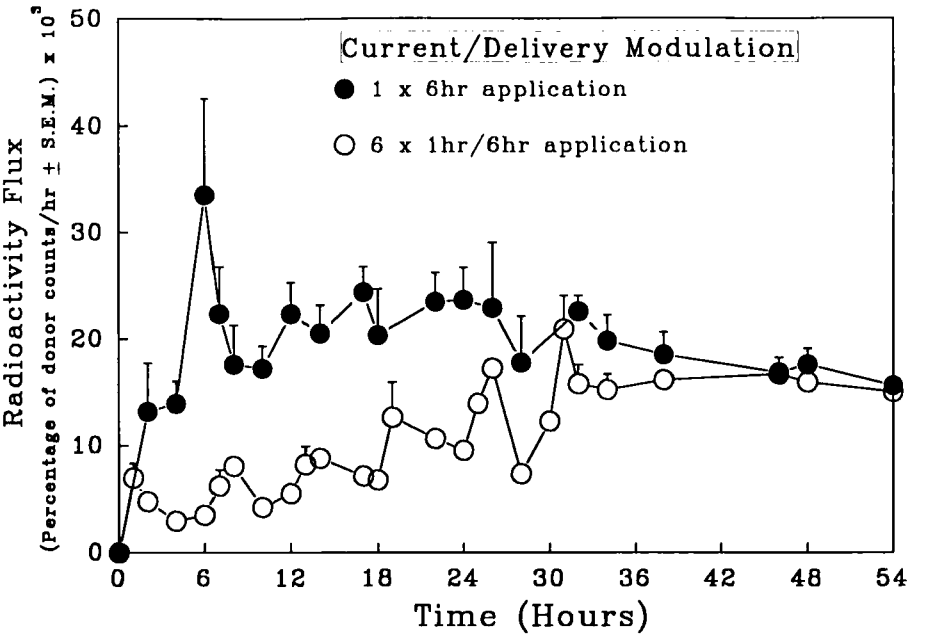

FIGURE 6

Comparison of the skin permeation rate profile of ^{125}I- Insulin between single and multiple applications of current (with the same total duration of current application).

36 hours increases as the duration of iontophoretic application is increased to 8 hours. Figure 6 compares the instantaneous permeation flux between a single 6 hour application and six applications of 1 hour each, applied every 6 hours. The results suggest that the intermittent applications gradually build up the skin depot. Therefore, the flux after the final application is the same as that achieved with a single continuous application of equivalent duration.

Stripping and Delipidization of Skin

Figure 7 compares the passive, iontophoretic and desorption profiles of insulin across the intact, stripped and delipidized skin. Both stripping and delipidization increase the skin permeation of insulin by passive diffusion. The observation of higher iontophoretic transport in Figure 7, as reflected by the

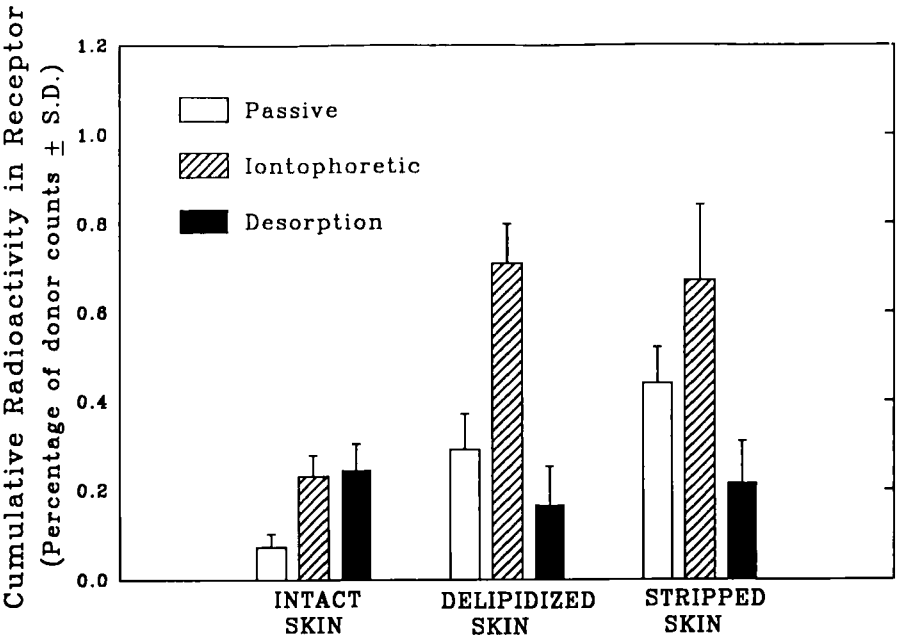

FIGURE 7

Comparison of permeation and desorption profiles of ^{125}I-Insulin by passive diffusion and iontophoretic transport across the intact, delipidized and stripped skin. Data indicates cumulative amounts of insulin permeated over a 36-hour study period. For iontophoretic transport, the pulse current was applied for 2 hours.

cumulative amounts permeated over the study period, could be attributed to greater contribution from the passive component as the donor solution was not removed after stopping current application. However, no increase in the iontophoretic transport of insulin was observed over the 2-hr current application period (data not shown). This could be attributed to the fact that removal of the stratum corneum also reduces the potential gradient drop across the skin, which acts as a driving force in accordance with the Nernst-Planck equation. The desorption profile of insulin from the stripped and delipidized skin is somewhat lower than that from the intact skin. However, the presence of a significant desorption from the stripped and delipidized skin suggests that the reservoir (or

depot) is not localized to the lipid domains in the stratum corneum, but is also distributed in other skin components.

CONCLUSIONS

^{125}I-Insulin was driven into the skin during the iontophoresis period of 1-4 hrs to form a depot or reservoir, from which ^{125}I-labelled fragments of the molecule were desorbed slowly throughout the sampling period of 36-60 hrs. Although no in-vivo studies were performed in these experiments, it seems that substantial degradation took place during iontophoretic studies and the amount of insulin delivered is likely to be inadequate to control diabetes. An improved study design could be evaluated in the future to determine the viability of this technique for control of diabetes. There is a need to use modified insulin with a high charge density. Also, the formulation should be designed such that there are no buffer ions which might be delivered preferentially, thus effectively increasing the delivery rate of insulin. The use of a radiochemical detector linked to an HPLC system would be a better way to analyze the permeation of ^{125}I-Insulin, rather than the use of gamma or liquid scintillation counter, and will be used in future studies.

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