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Synergistic effect of anionic lipid enhancer and electroosmosis for transcutaneous delivery of insulin

S. Narasimha Murthy [1], Ya-Li Zhao, Sek-Wen Hui, Arindam Sen *

Department of Cancer Biology, Roswell Park Cancer Institute, Buffalo, NY 14263, United States

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Abstract

A lipid formulation consisting of 1,2-dimyristoyl-sn3-phosphatidylserine (DMPS) in a 0.2% sodium dodecylsulfate (SDS) solution was tested as an in vivo enhancer for the transcutaneous delivery of insulin. The formulation when applied to for 15 min was found to permeabilize porcine epidermis and prolong the permeable state as evidenced by electric resistance measurement. The formulation enhanced the transport of insulin through the epidermis by 40- to 100-fold, as compared to epidermis that was treated with SDS or DMPS alone. Application of electroosmosis across the formulation-treated epidermis enhanced the transport of insulin by an additional 10-fold. Pharmacokinetic studies were carried out in Sprague-Dawley rats. Transcutaneous delivery of insulin with formulation treatment and electroosmosis increased the plasma level of insulin by ~10-fold over delivery by formulation treatment alone. With the above protocol plasma insulin concentration remained relatively constant for up to 4 h. The synergistic application of anionic lipid formulation and electroosmosis offers a promising non-invasive technique to deliver insulin transcutaneously.

Keywords: Anionic lipid; Insulin; Porcine epidermis; Rat skin; Transcutaneous delivery; Electroosmosis; Pharmacokinetics

1. Introduction

Insulin is a 51 amino acid peptide generally administered parenterally for treating diabetes mellitus (Chien, 1996). Injections generally are not ideal methods for the administration of drugs, especially for chronic conditions. There have been many attempts to deliver insulin by non-invasive methods. These include oral, colonic, rectal, ocular, buccal, pulmonary, uterine and transdermal routes (Hoffman and Ziv, 1997). Oral delivery of insulin is affected due to degradation by the proteolytic enzymes in the G.I.T. (Aboubakar et al., 1999). Mucosal delivery is not effective because of its poor absorption by mucosae of nasal, rectal, pulmonary and ocular route (Damge et al., 1997). Transdermal delivery has many advantages over other delivery methods, the most important being its convenience. The skin also serves as a reservoir for controlled release. Transdermal delivery of insulin is limited by the low permeability of the stratum corneum (SC), which consists of flat, enucleated cells filled with keratin fiber surrounded by lipid bilayers. Several approaches such as iontophoresis and sonophoresis have been taken to bypass the skin barrier properties (Tomohira et al., 1997; Kankkannan et al., 1999; Langkjaer et al., 1998; Smith et al., 2003; Boucaud et al., 2002). However, the flux for even monomeric insulin resulting from iontophoresis was low and variable (Langkjaer et al., 1998), because iontophoresis works best only for small, charged molecules that pass through the appendage routes of the skin. The time required to achieve therapeutically relevant concentrations was relatively long. Pillai et al. (2004) applied iontophoresis onto chemically treated or permeabilized skin for improved transport of insulin. Unless significant passages for solutes are created and maintained in the SC to allow larger molecules to transit, transdermal delivery of insulin will remain a challenge.

We reported the phenomenon of prolonged permeabilization of mammalian epidermis on incorporation of an anionic lipid, dimyristoyl-phosphatidylserine (DMPS), in the SC lipid domain with the aid of electrical pulses (Sen et al., 2002a, 2002b). The perturbation of SC lipids by joule heating associated with applied electrical pulses was demonstrated recently Martin et al., 2002; Pliquett et al., 2005). It is likely that the exogenous lipids

* Corresponding author. Tel.: +1 716 845 8911; fax: +1 716 845 1349.
E-mail address: Arindam.sen@roswellpark.org (A. Sen).
[1] Current address: Department of Pharmaceutical and Biomedical Science, Raabe College of Pharmacy, Ohio Northern University, Ada, OH 45810, United States.

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like DMPS get incorporated in the SC lipid lamellae due to the phase transition of SC lipid brought about by the joule heating and the incorporated DMPS retard the reformation of the SC barrier. In this work, we tried to incorporate DMPS in the SC lipid lamellae without the application of electrical pulses but with the aid of external heating to 40 °C which corresponds to the first transition temperature of the SC lipids (Golden et al., 1987) and by using a small amount of SDS as a vehicle. We found that treating porcine epidermis with a lipid formulation consisting of DMPS (in 0.2% SDS) at 40 °C for 15 min renders the skin permeable for prolonged duration. The treatment is referred to as "formulation treated" in this paper. We further tested for synergy between formulation treatment and iontophoresis in the delivery of insulin across the skin. Our mechanistic studies have revealed that at physiological pH, insulin is delivered mainly by electroosmosis and only minimally by electromigration (Murthy et al., 2006). Therefore, we use the term "electroosmosis" in all the experiments involving application of dc (the anode was placed in the donor and cathode in the receiver compartment) in this study.

2. Materials and methods

2.1. Chemicals

Fluorescein isothiocyanate (FITC) labeled insulin and other chemicals used were from Sigma–Aldrich and Fisher Chemical Company (St. Louis, MO). FITC-insulin was dialyzed for 48 h using 3500 Da dialysis membranes (Spectrapor®) to remove any free dye. Centrifree filtered ^{125}I-labeled porcine insulin was purchased from Amersham Biosciences (Piscataway, NJ). Dulbecco's phosphate buffered saline (PBS pH 7.2, free of calcium) was purchased from Gibco (Grand Island, NY).

Dimyristoyl-phosphatidylserine (DMPS) was purchased from Avanti Polar Lipids (Birmingham, AL). Lipid dispersions were prepared by drying the lipids from chloroform solutions, and then vortexing in PBS buffer at a final lipid concentration of 2 mg/ml. Sodium dodecylsulfate (SDS) was added to the suspension at 0.2% (w/w). This is referred hereon as the "formulation".

2.2. Skin for in vitro studies and animals for in vivo studies

Porcine belly skin was excised from freshly euthanized experimental animals. Pieces of the skin wrapped in aluminum foil were heated to 60 °C for 2 min in a water bath and the epidermis was gently peeled off the skin. The fresh epidermis was placed on glass microscope slides and kept dry at 4 °C until used. Prior to use, the epidermis was hydrated with normal saline (0.9% (w/v) sodium chloride) for 1 h. The procedure follows that used by Chizmadzhev et al. (1998) for preserving human epidermis.

Sprague-Dawley rats were purchased from Jackson Laboratory (Bar Harbor, ME). Animals weighing about 250–300 g were used in the study. Animals were provided free access to food and water and were allowed at least 5 days to recover after transportation. All animals were kept in the Institute Animal Facility before and during experiment.

2.3. In vitro experimental setup

Franz type vertical diffusion apparatus (Crown Glass Company Inc., Somerville, NJ) was used for all resistance and transport measurements across porcine epidermis. The temperature of the chamber was regulated by water circulation to 40 °C. A piece of porcine epidermis was placed between two compartments of the diffusion apparatus, one serving as the donor and the other as the receiver compartment. The area of epidermis available for diffusion was 0.64 cm^2. The volume of donor and receiver compartment was 0.5 and 5 ml, respectively. Ag/AgCl electrodes of 5 mm diameter (InVivo Metric, Healdsburg, CA) were placed 2 mm away from the skin in both the donor and the receiver compartments.

2.4. Electrical measurements

The resistance of the epidermis was measured by placing a load resistor R_L (4.7 kΩ) in series with the epidermis. The voltage drops across the whole circuit (V_O) and across the epidermis (V_S) were measured using a recording digital oscilloscope (Fluke 99 Scopemeter series II, Eindhoven, The Netherlands). Epidermis resistance (in kΩ) was approximated from the formula:

$$R_S = \frac{V_S R_L}{V_O - V_S}$$

where R_S is the epidermis resistance and R_L is the load resistor in kΩ. A piece of porcine epidermis was used only if it had a resistance greater than 50 kΩ/cm^2.

During electroosmosis, the electrodes were connected to a regulated dc constant current supply with the anode in the donor compartment and the cathode in the receiver compartment.

Four sets of experiments were carried out involving the following treatment. Porcine epidermis was maintained at 40 °C with the buffer for 10–15 min until the resistance became constant ($R_S = R_O$). Then the epidermis was treated for 15 min with one of the following: (1) buffer (control/untreated), (2) SDS (0.2% (w/w)), (3) DMPS (2 mg/ml) alone and (4) the formulation. Time-dependent changes in the electrical resistance $R_S(t)$ of the epidermis with the different treatments were measured and used to calculate the relative resistance $R_S(t)/R_O$.

In skin recovery studies, the epidermis was transferred to a new diffusion cell maintained at 37 °C and the electrical resistance of the epidermis was measured over a time period. The relative resistance, the ratio of resistance at different times at 37 °C $R_S(t)$ to the initial resistance of the epidermis measured at 40 °C before any treatment (R_O) was calculated.

2.5. In vitro insulin transport

The receiver compartment of the diffusion chamber was filled with PBS and FITC-insulin solution (2 mg/ml solution in PBS) was added to the donor chamber. For electroosmosis, a constant dc (0.5 mA/cm^2) was applied across the epidermis. The amount of insulin transported across the epidermis was determined from the measured fluorescence intensity of the FITC-insulin in the receiver compartment. The insulin solutions present in both

compartments were tested by chromatography on Sephadex® G-25 column to confirm that no free FITC was liberated at the experimental conditions. FITC-insulin fluorescence was measured using a SLM 8000 spectrofluorimeter with excitation and emission set at 494 and 520 nm, respectively.

2.6. Pharmacokinetic studies

Rats used in the study were divided into three groups ($n = 3$). They were administered with ketamine (80 mg/kg) + xylazine (20 mg/kg) anesthetic 15 min prior to the start of the experiments. The animals were placed on heating pad to maintain the body temperature. Shaved back skin of the rats were folded and clamped on to a custom-made in vivo transdermal cell (Murthy et al., 2005). The cell contains a donor compartment with Ag/AgCl electrode. A stainless steel plate clamped across the skin fold serves as the counter electrode. The donor compartment contained 2 mg/ml of insulin solution (^{125}I-insulin mixed with cold insulin to give a concentration 2 mg/ml prepared in PBS).

The control group and the electroosmosis group received no prior treatment with the formulation. The third and fourth groups were treated with the formulation. A cotton pad soaked with the formulation was placed on the skin and was heated locally using a metal block heated by circulating water. Constant current source was connected to the electrodes to the rats in the fourth group immediately after placing the insulin solution in the donor chamber.

Blood samples (200 μl) were drawn from by retro-orbital bleeding before starting with any treatment for baseline reading and after 0.5, 1, 3, 4 and 5 h after starting the insulin delivery. The blood samples were centrifuged at 12,000 rpm for 15 min. Fifty microlitres of plasma was collected and measured for radioactivity. The rats were administered with 1 ml of PBS by peritoneal injection after each withdrawal of blood sample. The samples were measured using a Packard COBRA II gamma counter (Perkin-Elmer Life Science Div., Boston, MA).

A positive control group was administered 2 units/kg of insulin by tail vein injection. The blood samples were collected 15, 45, 60 and 120 min after injection.

3. Results and discussion

3.1. The effect of DMPS/SDS formulation on the electric resistance of porcine epidermis

The electric resistance is a good indicator of the permeability of the skin or the epidermis (Sen et al., 2002a; Chizmadzhev et al., 1998). We therefore first measured the effect of DMPS, SDS and the combination treatments on the epidermis to determine if there is any synergistic effect of these two chemical treatments on the electric resistance. DMPS alone in aqueous suspension is in vesicular form and is not expected to penetrate the skin or the epidermis. However, if introduced into the epidermis by electric pulses, DMPS prolongs the lifetime of electropores and enhances the electroporation-induced transport by orders of magnitude (Sen et al., 2002a). Being a surfactant

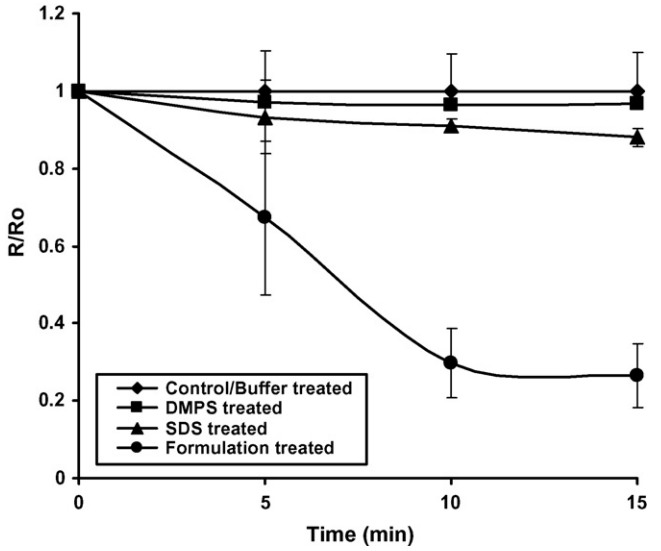

Fig. 1. Change in the electrical resistance of porcine epidermis on treatment with buffer, SDS, DMPS and the formulation. The relative resistance at 40 °C was measured during the treatment for 15 min. The relative resistance is the ratio of resistance $R_S(t)$ at time t to the resistance of the epidermis R_O before any treatment.

and transdermal transport enhancer, SDS will partition into the stratum corneum (Patil et al., 1995) and reduce the electric resistance following iontophoresis (Kalia and Guy, 1997). However, at the concentration used (0.2%), only a 10% reduction in resistance was found after 4 h of treatment (Murthy et al., 2004). Our basic hypothesis was that if SDS at this low concentration can assist the penetration of DMPS into the SC then one can expect a synergistic effect on the reduction of skin resistance, and also on transdermal transport.

As expected, there was no significant change in electrical resistance in porcine epidermis treated with buffer or DMPS alone (Fig. 1). The drop in resistance was about 10% with SDS treatment which is in agreement with previously published data (Murthy et al., 2004). Interestingly, the resistance dropped by 85% within 10 min when the epidermis was treated with the formulation. After that initial drop, there was no further decrease in resistance even after prolonged treatment with the formulation.

The recovery of porcine epidermis electrical resistance after treatment with buffer, SDS, DMPS and the formulation was also assessed by measuring the change in electrical resistance (Fig. 2). The SDS-treated epidermis recovered within 10 h. The recovery was significantly slower in case of the formulation-treated epidermis. Slow recovery started after 10 h and was still incomplete after 24 h.

The resistance measurements show that in the formulation-treated epidermis the reduction of the electrical resistance is much greater than the sum of SDS and DMPS alone, i.e. the effect is synergistic. The results thus support our hypothesis that DMPS, with SDS acting as a vehicle, can permeabilize and prolong the permeabilized state of the stratum corneum by partitioning and incorporating into the lipids of the stratum corneum.

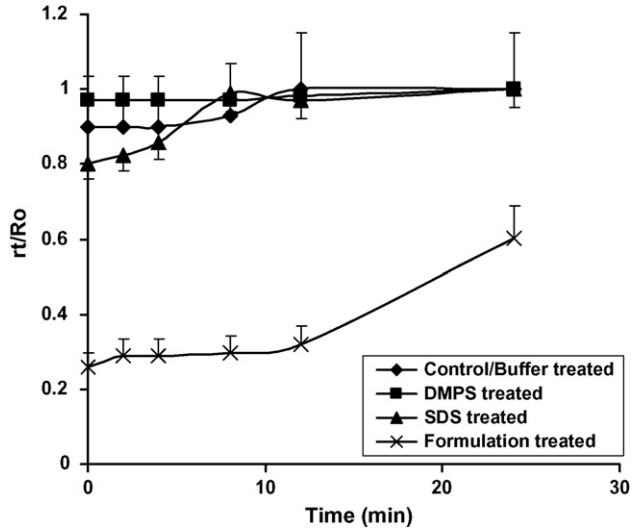

Fig. 2. Recovery of electrical resistance of porcine epidermis after treating with buffer, SDS, DMPS and formulation for 15 min. The relative resistance is the ratio of resistance $R_S(t)$ noted at different times t at 37 °C to the initial resistance of the epidermis at 40 °C before any treatment (R_O).

3.2. The effect of DMPS–SDS formulation on the transport of insulin through porcine epidermis with or without electroosmosis

We measured the transport of insulin following the treatment of DMPS or SDS alone, as well as the combination DMPS–SDS formulation. The transport studies revealed that the formulation treatment was more efficacious in terms of delivery of significant amounts of insulin across the porcine epidermis, as compared to that treated with DMPS or SDS alone (Fig. 3). The total transport of insulin across formulation-treated epidermis was ~100-fold and 40-fold, respectively, as compared to the transport across DMPS- or SDS-treated epidermis, respectively.

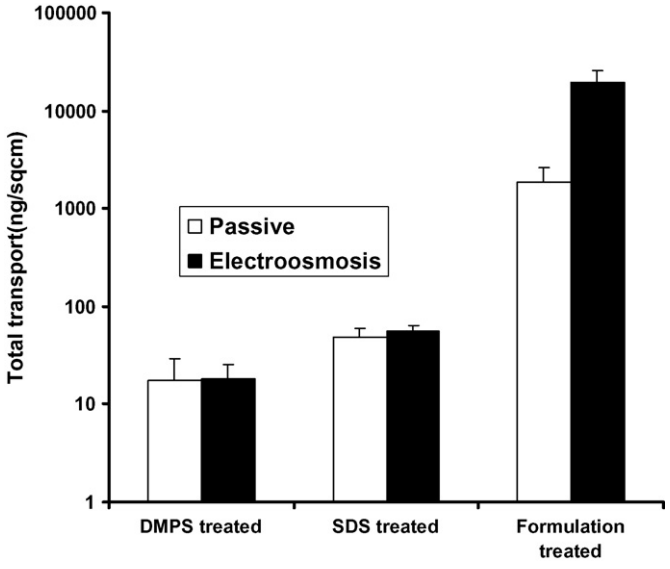

Fig. 3. Transport of insulin across porcine epidermis after treatment with SDS, DMPS and the formulation. The blank bar indicates the passive transport of insulin across the treated epidermis and the filled bar indicates the effect on coupling electroosmosis. The transport across the PBS-treated epidermis was negligible (not shown in the graph).

To further increase the insulin transport, by taking advantage of the prolonged permeabilized state of the epidermis following treatment with the formulation, we applied a dc current at the level as that for conventional iontophoresis. Under a dc potential, neutral molecules such as insulin are transported through the skin mainly by the mechanism of electroosmosis (Guy et al., 2000). Coupling electroosmosis during the post-treatment diffusion period (4 h) did not cause any significant increase in insulin transport in case of epidermis treated with buffer, DMPS and SDS. In contrast, the transport across the formulation-treated epidermis increased by about 10-fold with added electroosmosis (Fig. 3). These results show that electroosmosis alone is not effective in transporting insulin across control, untreated epidermis. However, when electroosmosis is applied to formulation-permeabilized epidermis, there is a very significant enhancement of insulin transport. This enhancement could be due to additional pathways created by formulation treatment similar to electropores (Fang et al., 2004). It is possible that an electrolysis product of SDS, dodecanol, contributes to the enhanced transport of insulin. We did not check the amount of dodecanol in the formula. Judging from the similarity of passive and electroosmosis-assisted transports of insulin through SDS-alone treated samples (Fig. 3), we believe that possible impact on insulin transport by SDS hydrolysis, caused by current and pH effects upon electroosmosis, is not significant.

3.3. Pharmacokinetic measurements of transdermal insulin delivery by combined formulation treatment and electroosmosis as compared to i.v. bolus injection in rat

The time profile for plasma concentration of insulin administered by i.v. bolus in rats, as indicated by ^{125}I radioactivity, was measured. The result fits well to a mono-exponential curve ($r^2 = 0.9955$) (Fig. 4). The elimination half-life of insulin was 17.46 ± 4.3 min. The clearance rate ($V_d K_{el}$) was found to be 1.08 ml/min.

For comparison, insulin was delivered by passive diffusion through untreated skin, formulation-treated skin, as well as by electroosmosis alone, or electroosmosis with formulation-treated skin of rats. Passive diffusion through untreated skin, as well as by electroosmosis alone resulted in negligible plasma concentrations. Passive diffusion after formulation treatment (no electroosmosis) alone was found to deliver relatively small amounts of insulin after 4 h (Fig. 5). In rats treated with the formulation followed by application of electroosmosis, the concentration remained relatively constant up to 4 h (until the electroosmosis was turned off). The concentration also remained constant during the next one hour after the cessation of electroosmosis (Fig. 5). This is most likely due to the formation of an insulin reservoir in the skin. The results echo that of in vitro measurement using porcine epidermis, that formulation treatment plus electroosmosis enhanced the delivery by an order of magnitude. Without the formulation treatment, electroosmosis has very little effect in enhancing the delivery of insulin.

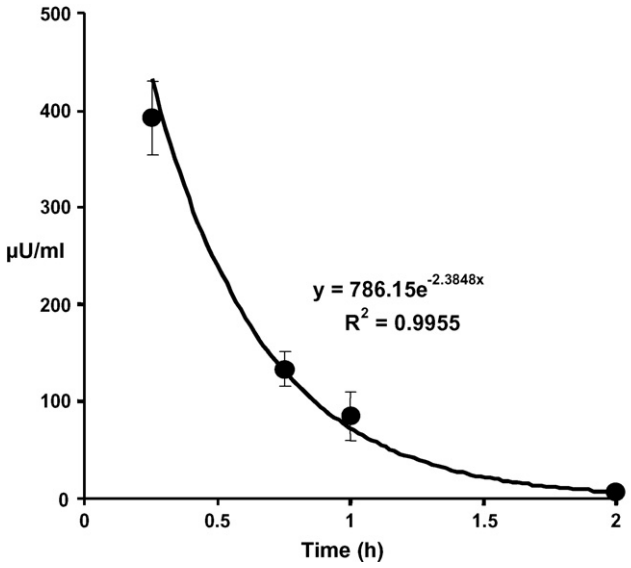

Fig. 4. Plasma concentration profile of ^{125}I-insulin as indicated by ^{125}I measurements after i.v. bolus administration in Sprague-Dawley rats.

Transdermal delivery of insulin has been a challenge because the molecular size of insulin (MW ∼ 6000) is not amendable to be delivered through appendegeal routes that are the paths for most iontophoresis applications. So far other enhancers do not afford a significant transport of insulin, possibly due to restricted transdermal paths they create. DMPS has been found to be able to sustain the opening of electropores (Sen et al., 2002a). The mechanism is believed to be that DMPS partitions into the SC lipids. The electric charge carried by DMPS creates and maintains an aqueous space in the SC lipid lamellae and thus facilitates the transport of solutes by diffusion or by electroosmosis. The pores or paths maintained by DMPS are sufficiently large for the passage of 10 kDa molecules (Murthy et al., 2004). As an amphiphile of very low critical micelle concentration, DMPS forms vesicles (liposomes) in aqueous dispersions, and will not readily penetrate the skin surface. In past applications, DMPS was driven into the epidermis using electroporation to elicit its delivery enhancement effect (Sen et al., 2002a, 2002b). In this work, SDS was used as a vehicle to transfer DMPS into the epidermis. This vehicle worked well in reducing the epidermal electric resistance and enhancing insulin transport in vitro. The transport enhancement effect was found both in porcine epidermis in vitro and in rat skin in vivo. It should be noted that in vivo data could be an over-estimation because the results were based on radioactivity measurements; the metabolic breakdown of insulin was not taken into account (Pillai and Panchagnula, 2003). In a separate study (Murthy et al., 2006), we have determined that the iontophoretic current alone did not cause insulin breakdown. For the purpose of this study, the synergistic effect in facilitating transcutaneous transport by the formulation and electroosmosis is demonstrated. Pharmacodynamic studies on transdermal delivery of insulin using the anion surfactant formulation described here are being planned.

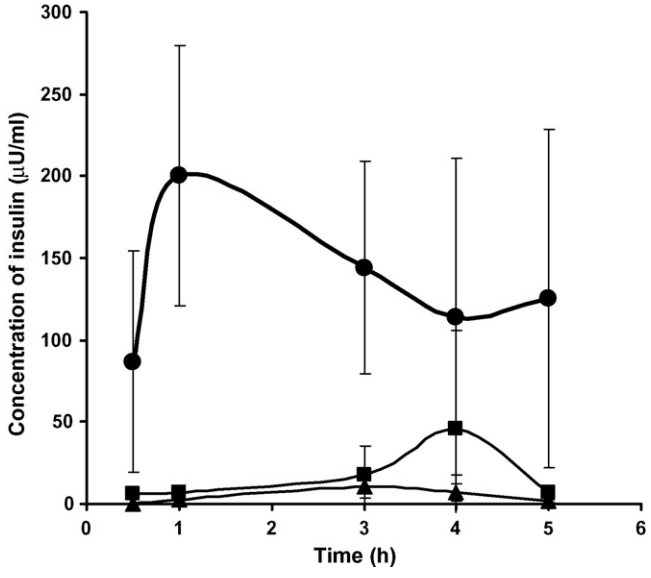

Fig. 5. Plasma concentration profile of ^{125}I-insulin as indicated by ^{125}I measurement after transdermal delivery. In the case of the control group (passive diffusion through untreated skin), no insulin could be detected in the plasma. The electroosmosis group (triangles), formulation-treated group (squares) and the group treated with the formulation followed by electroosmosis (circles) are shown in the figure.

4. Conclusions

The experimental results indicate a significant increase in the transdermal transport of insulin with the application of electroosmosis across formulation-treated rat skin. Incorporation of DMPS with the aid of mild heating and in the presence of surfactants prolongs the permeable state of skin. The delivery level achieved by coupling formulation treatment and electroosmosis could be improved to meet therapeutic needs.

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