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RESEARCH PAPER

Effects of pH, Electric Current, and Enzyme Inhibitors on Iontophoresis of Delta Sleep-Inducing Peptide

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ABSTRACT

Delta sleep-inducing peptide (DSIP), a peptide of nine amino acid residues, was used as a model drug to investigate the effects of pH, electric current, and enzyme inhibitors on the transdermal iontophoretic delivery of peptide drugs. DSIP was fairly stable in pH 4–9 buffer solutions but was cleaved by the skin enzymes during iontophoretic delivery. Enzyme inhibitors, such as o*-phenanthroline, ethylenediaminetetraacetic acid (EDTA), dilucine, and sodium deoxycholate, could inhibit the degradation of DSIP to a certain extent in the skin homogenate. Our results showed that metalloproteases were probably more important enzymes for DSIP hydrolysis. By using 0.2 mM* o*-phenanthroline in the iontophoretic delivery of DSIP at pH 4, we were able to significantly enhance the penetration of DSIP. The flux was about eight times as much as control (without* o*-phenanthroline) at pH 7.4.*

INTRODUCTION

In recent years, peptide and protein drugs have been grossly produced and used in the treatment of diseases (1–3). After oral administration, most peptides and proteins are readily metabolized in the intestinal tract and lose their activity (4,5). Peptides and proteins, with large molecular weights and hydrophilic properties, do not usually permeate easily through the biological membranes to produce therapeutic effects. Until now, most peptide drugs were used as parenteral preparations, the administration of which is invasive. Therefore, other routes for peptide delivery, such as ocular, nasal, and pulmonary, have been studied (6–8).

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Iontophoresis has been shown to be most promising in facilitating the percutaneous absorption of insulin and some other bioactive agents (9–11). Thus, an iontophoretic delivery system may offer an expedient alternative to deliver drugs into the target sites (12). However, in iontophoresis, because the electrical potential is applied on the skin and the drug reservoir, the drugs might be degraded. Currently density, pH, and additives of drug reservoir are known to have a profound effect on the stability and permeation of peptides. Therefore, understanding these influential factors might be beneficial for designing the iontophoretic transdermal delivery systems of peptides.

Delta sleep-inducing peptide (DSIP) is an oligopeptide of nine amino acid residues (Try-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu). It is described as a sleep-promoting substance to induce delta or slow-wave sleep (13). Some studies reported that DSIP might have potential in the treatment of emotional stress and chronic insomnia (14,15). DSIP has a molecular weight of 848.8 D and has a zwitterionic property, and like other peptides, its charge varies according to pH. Thus, DSIP was a good representative drug to study the iontophoretic delivery of peptides. In this paper we investigated the effect of pH, current, and enzyme inhibitors on the percutaneous absorption of DSIP, which might provide further information for the design of an iontophoretic delivery system for oligopeptide drugs.

MATERIALS AND METHODS

DSIP was ordered from Sigma (St. Louis, MO). Nadolol (E.R. Squibb & Sons, Princeton, NJ) and terbutaline (USP grade) were used as internal standards in HPLC analysis. Sodium deoxycholate, *o*-phenanthroline, diisopropyl fluorophosphate (DFP), iodoacetic acid, and dilucine were used as enzyme inhibitors and purchased from E. Merck (Darmstadt, Germany). All other chemicals used in this study were reagent grade.

Buffers

Aqueous solutions of 0.02 M Na_2HPO_4 and 0.02 M NaH_2PO_4 were used to prepare various pHs of phosphate buffers. Three different pHs of HEPES (2-hydroxyethyl-1-piperazinyl-ethanesulfonic acid) buffers were prepared to dissolve 1.489 g of HEPES in 200 ml of Milli-Q water, then the solutions were adjusted with 1 N NaOH or 1 N HCl to pH 3.0, 4.0, and 7.4, respectively. Finally, sodium chloride was added to adjust isotonicity and the solutions were made to 250 ml volume with Milli-Q water.

Electrode Preparation

Silver wire (0.5 mm i.d. × 12 cm, Aldrich, Milwaukee, WI), having been cleaned with 1 N HCl and coiled, was connected to a strip of copper wire. Then, two pieces of silver wire were placed in 0.5 M KCl solution and the other two ends were connected to either the cathode or the anode of a power supply. Silver/silver chloride electrodes were generated after applying 1 mA of current for 12 hr on this system.

Stability of DSIP for pH Effect

DSIP samples were prepared in the phosphate buffers with the concentration 0.6 μg/ml. Then, the drug solutions were sealed in 10-ml ampules and placed in a circulation water bath at 32°C and shaken at 80 rpm. At certain time intervals, samples were collected and 50 μl, 0.34 mg/ml of terbutaline was added as internal standard, then samples were analyzed by an HPLC method.

Stability of DSIP in the Presence of Electrodes with Varied Currents

DSIP was prepared at a concentration of 1.5 μg/ml in phosphate buffer (pH 7.4). Twenty-five milliliters of the drug solution was placed in a 50-ml beaker. After the silver/silver chloride electrodes were installed, the drug stability was studied at various current intensities (0.2, 0.6, 0.8, and 1.0 mA). Samples were withdrawn at 1, 2, 3, 5, 7, 9, and 11 hr and determined by an HPLC method to measure the effect of currents on the stability of DSIP.

Skin Preparation

White New Zealand rabbits, either sex, weighing 2.0–3.0 kg, were injected with an overdose of ketamine through the marginal ear vein. After euthanasia of the rabbit, the skin auricles was isolated, and washed with normal saline. Then, the auricles were packaged in parafilm, stored at 4°C, and used within 3 days.

Effects of Skin Enzyme on DSIP Degradation

Skin homogenate was prepared in pH 4.0 and 7.4 HEPES buffers. Auricle skin was dissected into 1 × 1 mm fragments using a pair of scissors; the fragments

were then placed in the HEPES buffer and disrupted by a homogenizer at a speed of 7000 rpm for 1 min, three times. During the homogenizing process, the sample was immersed in ice water. The dispersed mixture was centrifuged at 2000 $\times g$ for 20 min. The supernatant of skin homogenate was collected for further study.

One hundred microliters of 200 μg/ml of DSIP was added into 9.9 ml of pH 7.4 or 4.0 skin homogenates. After the mixtures were mixed, they were placed in a 32°C water bath at a shaking speed of 80 rpm. At certain time intervals, a 5-ml sample aliquot was withdrawn and 0.5 ml of 2% trifluoroacetic acid was added. After the sample was centrifuged at 2000 $\times g$ for 20 min, the supernatant of skin homogenate with DSIP was collected and analyzed by an HPLC method.

Enzyme inhibitors with different concentrations, including ethylenediaminetetraacetic acid (EDTA), sodium deoxycholate, dilucine, iodoacetic acid, DFP, and *o*-phenanthroline were prepared in pH 7.4 HEPES buffer to determine the effect of inhibitors on skin enzyme activities. DSIP solution (0.5 ml, 200 μg/mL) was added to 2 ml of 0.5% skin homogenate, then different concentrations of inhibitors were added, and finally the volume was adjusted to 10 ml by addition of pH 7.4 HEPES. The mixture was placed in a water bath with a shaking speed of 80 rpm at 32°C. At 0, 1, 2, 4 and 6 hr, an aliquot of 0.5 ml was withdrawn and 0.5 ml of 2% trifluoroacetic acid was added into the sample. After the sample was centrifuged at 2000 $\times$ g for 20 min, the supernatant was obtained and analyzed by an HPLC method.

Iontophoretic Studies

A horizontal diffusion cell was used in this study and maintained at 32°C with a circulation water bath. After the skin was mounted in the diffusion cell (active surface area 0.785 cm^2), 3.5 ml of 0.2 mg/ml DSIP in HEPES buffer was loaded in the donor compartment (stratum corneum side), and the same volume of buffer solution without drug was loaded in the receptor compartment. The stirring speed was controlled at 600 rpm. Then, a pair of silver/silver chloride electrodes was installed with one electrode in the donor compartment as cathode and the other electrode in the receptor compartment as anode. In the study, three different currents of 0.2, 0.4, or 0.6 mA were applied in each separate experiment. At 1, 2, 3, 4, 5, 6, 7, 8, and 9 hr, 0.15 ml of sample was collected from the donor compartment and compensated with an equal volume of HEPES buffer. Samples were determined by an HPLC method using nadolol as internal standard. To investigate the effect of the enzyme inhibitor on the iontophoretic delivery of DSIP, 0.2 mM *o*-phenanthroline was combined in the media of pH 4 and pH 7.4 HEPES buffers. Then, a similar procedure as stated above was followed; a constant current of 0.6 mA was applied in the iontophoretic studies.

HPLC Analysis

DSIP and tryptophan (a degradant of DSIP) were analyzed by an HPLC method. HPLC equipment including a reciprocating pump (Jasco 880-Pu, Japan), an autosampler (Jasco 851-AS) with a 50-μl injection loop, a UV detector (Jasco 875-UV), and an integrator (SIC Chromatocorder 12) were used. A reversed-phase C18 column (7 μm, 4.5 × 250 mm, Nucleocil) was used to analyze DSIP. A mobile phase was composed of acetonitrile and Milli-Q water (containing 0.1% of trifluoroacetic acid) with a ratio of 10:90 (v/v). The flow rate was 1.0 ml/min. The UV detector was set at 217 nm. Two internal standards were used: terbutaline for stability and nadolol for all the other studies. Standard curves were constructed and used to determine the DSIP concentrations for samples in stability, enzyme inhibition, and iontophoretic studies.

RESULTS AND DISCUSSION

In this study, DSIP had a retention time of 7.5 min in the HPLC system as shown in Fig. 1. Terbutaline, with a retention time of 4.5 min and nadolol, with a retention time of 14.5 min, were used as internal standards for the stability and iontophoretic transdermal studies, respectively. Because of the interfering skin components released in the percutaneous study, terbutaline was used as the internal standard only for stability study in HPLC analysis. The calibration curves were $y = 0.538x - 0.0028$ ($r^2 = 0.999$) and $y = 2.781x + 0.033$ ($r^2 = 0.9996$), for stability and iontophoretic transdermal studies, respectively. The results demonstrated that the calibration curves had good linearity between 0.05 and 2 μg/ml with a detection limit of 0.025 μg/ml.

pH and Current Effects on DSIP Stability

In the phosphate buffers with varied pHs of 4.4, 7.4, and 9.2, the percent remains of DSIP at 9 hr were still greater than 90% (Fig. 2). These results suggested that

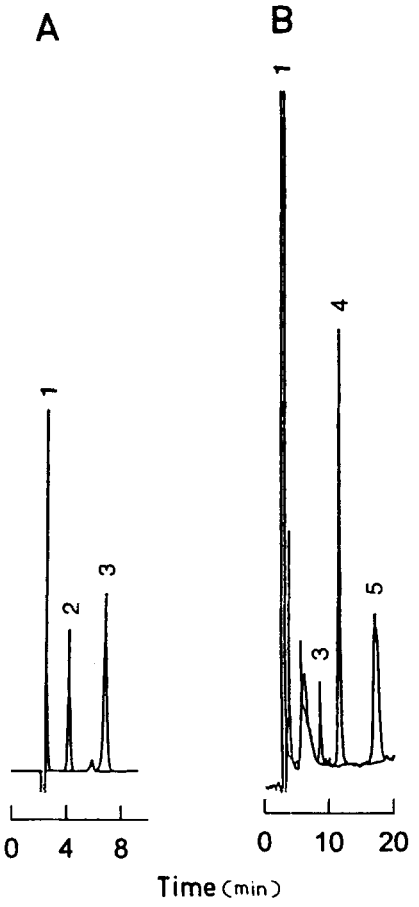

Figure 1. High-pressure liquid chromatograms of DSIP for stability study (panel a) and transdermal iontophoretic delivery study (panel b). Key: peak 1: solvent front; peak 2: terbutaline; peak 3: DSIP; peak 4: tryptophan; and peak 5: nadolol.

the stability of DSIP in the aqueous pH 4–9 solutions was not significantly affected. When current >0.2 mA was applied in pH 7.4 phosphate buffer, the DSIP solution tended to increase the degradation of DSIP with an increase in current density (Fig. 3). DSIP was fairly stable if the current applied was less than 1 mA. Under this condition, the remaining DSIP was more than 90% at 8 hr. The results demonstrated that iontophoresis with lower current in a short period of time would not significantly affect the stability of DSIP.

Enzyme Activities and pH

Enzyme activities of skin homogenate at pH 4 and 7.4 were different. At neutral pH, the enzyme activity for the degradation of DSIP was significantly higher

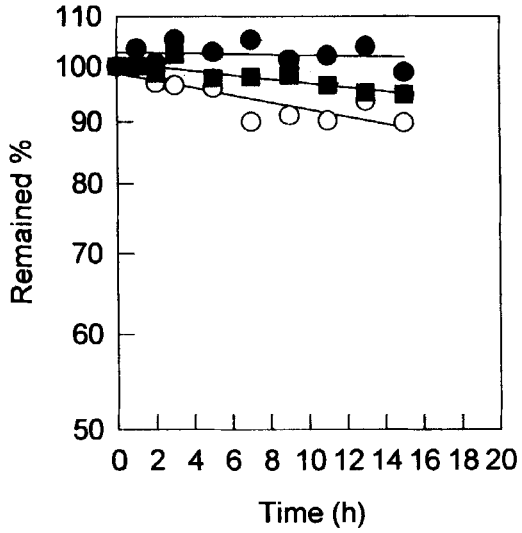

Figure 2. DSIP stability profiles in different pHs of phosphate buffers. Key: (●), pH 4.4; (○), pH 7.4; and (■), pH 9.2.

than that at lower pH (Fig. 4). The apparent first-order degradation rate constants at pH 4 and pH 7.4 were 0.176 and 0.241 hr^{-1}, respectively. In this study, the degradation of DSIP was significantly increased with increasing pH. Aspartic protease usually has higher activity in lower pH. Thus, it could not be a major enzyme in the degradation of DSIP.

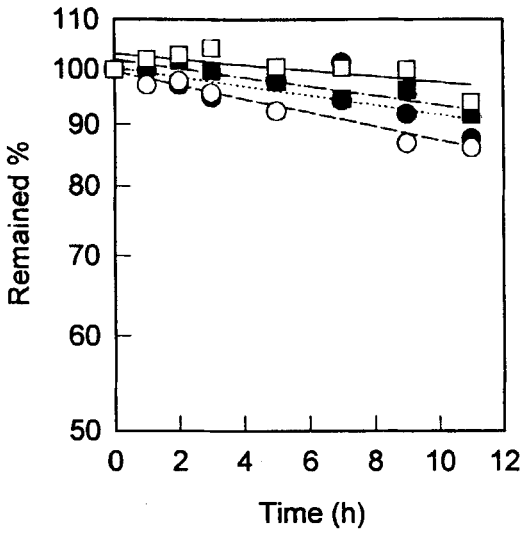

Figure 3. DSIP stability profiles for different electric currents in pH 7.4 of phosphate buffer. Key: (···●···), 1 mA; (– –○– –): 0.8mA; (– · ■· –), 0.6mA; and (—□—), 0.2 mA.

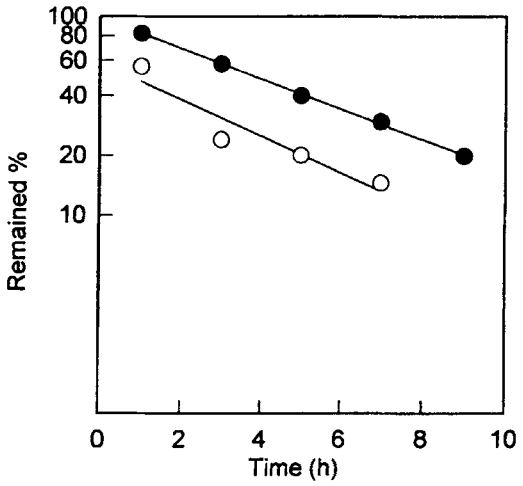

Figure 4. DSIP degradation profiles in pH 4.0 and pH 7.4 HEPES buffers containing 5% skin homogenate. Key: (●), pH 4.0 and (○), pH 7.4.

Effect of Enzyme Inhibitors

Four enzyme inhibitors with the concentration of 1 mM demonstrated some effects on the inhibition of degradation of DSIP in homogenate in pH 7.4 HEPES buffer (Fig. 5). At 1 mM, *o*-phenanthroline and EDTA were most effective among the six agents, followed by

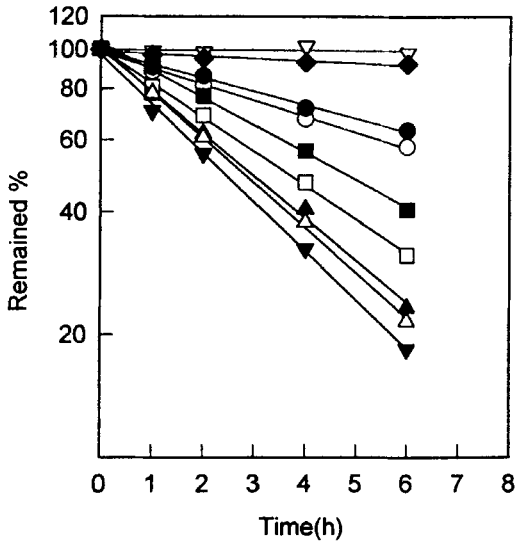

Figure 5. Effects of enzyme inhibitors (1 mM or 10 mM concentration) on the degradation of DSIP in 1% skin homogenate. Key: (●), *o*-phenanthroline, 1 mM; (○), EDTA, 1 mM; (■), dileucine, 1 mM; (□), sodium deoxycholate, 1 mM; (▲), iodoacetic acid, 1 mM; (△), DFP, 1 mM; (▼), control; (▽), DFP, 10mM; and (◆), iodoacetic acid, 10 mM.

dilucine and sodium deoxycholate. Iodoacetic acid and DFP were almost void of inhibition. Nevertheless, they were good inhibitors if the concentration of 10 mM was used.

Our study has shown that the enzymes of skin homogenate were not specific in catalyzing the degradation of DSIP. Skin enzymes could be inhibited by different types of inhibitors. In the study, tryptophan, the major degradation product, was found in the skin penetration medium. It was produced by cleavage of the first amino acid of DSIP from the N-terminal with amino protease. It has been reported that DSIP in spinal cord fluid was hydrolyzed by amino protease and the two major metabolites were tryptophan and glycine (16). We have studied four different peptidase inhibitors including those of metalloprotease, cysteine protease, aspartic protease, and serine protease. Metalloproteases are inhibited by chelating agents EDTA and *o*-phenanthroline; serine proteases are inhibited by DFP (17). We found that these enzymes might possibly be involved in the degradation of DSIP during skin penetration. Of them, metalloproteases were considered more important, and were significantly inhibited by EDTA and *o*-phenathroline. Deschaft-Lanckman et al. found that metallopeptidase was responsible for cleaving the N-terminal tryptophan of CCK-8, which was also inhibited by *o*-phenanthroline (18). The result is similar to that from our study.

Iontophoretic Study

The influences of pH and electric current on the percutaneous absorption of DSIP are shown in Table 1. The represented flux during the entire experimental course was obtained from the slope of the amount of cumulative drug versus time plot. In the same pH conditions, an increase in the electric current intensity also increased the penetration rate of DSIP. The results were consistent with the modified Nernst Planck equation (19). For iontophoresis, the flux was contributed from the passive diffusion component, both the electrical term and convective term. In this study, without applying current, the flux could not be determined because the skin penetration was below the detection limit. Thus, the skin penetration of DSIP was dominantly contributed by the electrical term and the convective term. Furthermore, the highest penetrating rate was obtained at pH 4 for iontophoresis of DSIP. One example of the DSIP absorption curves for application of 0.6 mA at three pHs (3, 4, and 7.4) is shown in Fig. 6. Fluxes could be determined from the slopes of the absorption curves

Table 1

Penetration Rates of DSIP for Iontophoretic Delivery Through Rabbit Auricle Skin with Different pHs and Electric Currents

Currents (mA)	Flux (μg/hr/cm²)		
	pH 3.0	pH 4.0	pH 7.4
0.2	0.177 ± 0.035[a]	0.100 ± 0.031	0.060 ± 0.036
0.4	0.200 ± 0.039	0.368 ± 0.075	0.087 ± 0.023
0.6	0.327 ± 0.052	0.506 ± 0.126	0.192 ± 0.038

[a]Standard deviation, $n = 3$.

which might not be constant during the entire experimental period, such as pH 3 decreasing with time, pH 4 increasing with time, and pH 7.4 maintaining constant. The flux patterns of these three pHs were possibly related to the charges of DSIP and skin. DSIP is a peptide composed of nine amino acids with four free functional groups in three residues: one amino group in tryptophan ($pK_a = 9.4$) at N-terminal, one carboxyl group in aspartic acid ($pK_a = 3.9$) at the fifth residue, and two carboxyl groups in glutamic acid ($pK_a = 2.3$ and 4.3) at C-terminal. The isoelectric point (pI) of peptide was usually estimated from the individual pK_a of free functional groups as follows (20).

$$pI = (pK_1 + pK_2 + \ldots pK_n)/n \quad (1)$$

According to Eq. (1), the calculated pI of DSIP is near 5.0. If so, DSIP should carry a positive charge at pH 4. However, the donor compartment was connected with an anode or an anodic delivery, and DSIP could not be detected in the receptor compartment even after 5 hr. In our study, the donor compartment was connected with a cathode or a cathodal delivery and DSIP was delivered through the skin. It is possible that the pI of DSIP might deviate from the calculated value (5.0). An alternative approach was employed to obtain the carried charge of DSIP by summation of individual functional group charges. Each functional group charge was estimated from the Henderson–Hasselbach equation using pH and pK_a of the four functional groups in DSIP. Figure 7 is the profile for plotting the DSIP charge versus pH. It suggests that the pI of DSIP is near 3. The reason for the higher pI value of DSIP is that the application of Eq. (1) was probably limited to proteins and peptides with about equal numbers of free acidic and basic functional

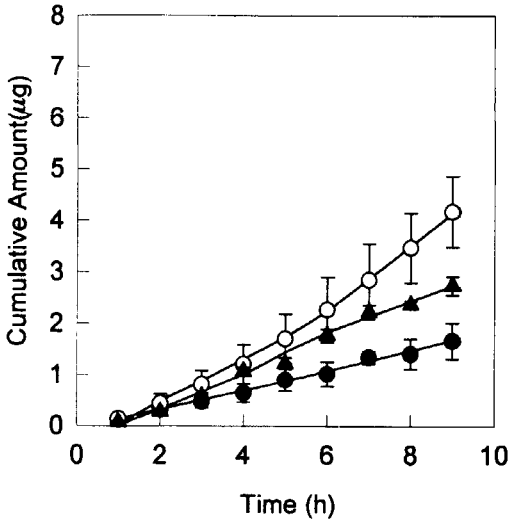

Figure 6. Iontophoretic delivery of DSIP penetrating through the rabbit auricle skin with application of 0.6 mA of electric current in pH 3.0, pH 4.0, and pH 7.4 HEPES buffers. Key: (○), pH 4.0; (▲), pH 3.0; and (●), pH 7.4.

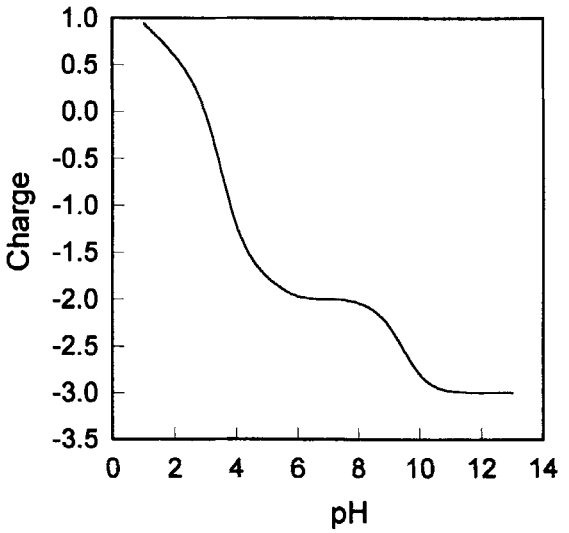

Figure 7. DSIP charge versus pH plot. Charges are calculated by Hasselbach equation using the pK_as of four free functional groups of DSIP and pHs.

groups. DSIP has three acidic and one basic group, which does not suit eq. (1). Lower pH could reduce the dissociation of carboxyl groups to balance the charge; therefore, a smaller value of pI was obtained. The result was consistent with our study in that DSIP in the donor compartment was delivered from the cathode, because DSIP had a negative charge at pH > 3. The flux of DSIP at pH 3.0 was still higher than that at pH 7.4. This might have arisen from electroosmosis. The bulk fluid flow by electroosmosis is considered to be an important factor to transport neutral species in iontophoresis (21). At pH 3, the skin carried a positive charge. By means of cathodal delivery, anions of the medium in the donor compartment penetrated through the skin, which also produced a fluid flow to bring neutral species such as DSIP permeating through the skin.

Effects of Inhibitors on Iontophoresis

In the iontophoretic study of DSIP at pH 7.4, we found that after 8 hr, the remaining drug in the donor compartment was less than 60%. Two factors, current intensity and enzymatic degradation, could be noticed. Without skin, the degradation of DSIP was less than 10%, as mentioned above in the stability study. The major degraded reaction might be attributed to the enzymatic cleavage. Therefore, the combination of enzyme inhibitors might possibly increase the penetration rate of DSIP (Table 2). Figure 8 shows the percutaneous profiles of DSIP at pH 4 and 7.4 containing 0.2 mM *o*-phenanthroline in the media of donor and receptor compartment and by applying 0.6 mA current in the iontophoretic study. The flux of inhibitor group is about three times as much as control (without inhibitor) at pH 4. However, the flux of inhibitor group at pH 7.4 was similar to that of control. An explanation is that the stratum corneum of skin is composed of keratin with pI near 3–4 (22). Thus, in pH 7.4, both skin and DSIP

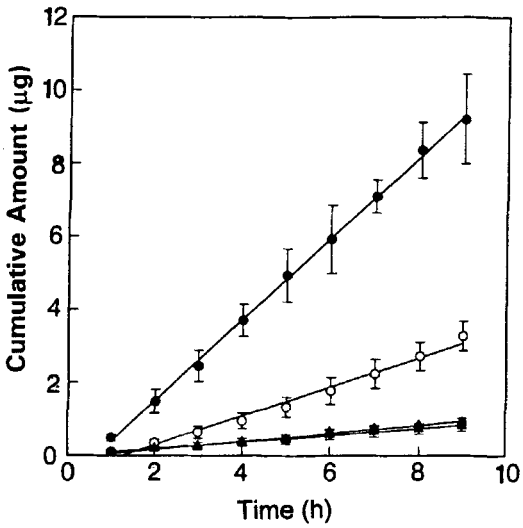

Figure 8. Iontophoretic delivery of DSIP penetrating through the rabbit auricle skin with application of 0.6 mA of electric current in pH 3.0, pH 4.0 and pH 7.4 HEPES buffers containing 0.2 mM *o*-phenanthroline as an enzyme inhibitor. Key: (●), pH 4.0 with inhibitor; (○), pH 4.0 without inhibitor; (▲), pH 7.4 with inhibitor; and (Δ), pH 7.4 without inhibitor.

carried negative charges and the repulsive effect of the same charges increased the resistance of DSIP in penetrating skin. With an inhibitor, the enzyme activity was decreased and the degradation of DSIP could be decreased. However, the inhibitor probably also acted as a competitor with DSIP in iontophoretic delivery and further decreased the skin penetration of DSIP. Thus, the overall effect caused no significant difference between control and inhibitor groups. At pH 4, skin carried the charge near zero and thus the repulsive effect of charges was minimized. This factor, along with the lower enzyme activity, might highly improve the penetration of DSIP at pH 4 with an enzymatic inhibitor. The flux is about eight times that of control of pH 7.4. In this study, we used the same pH in the donor and

Table 2

Pentration Rates of DSIP for Iontophoretic Delivery Through Rabbit Auricle Skin with 0.6 mA of Current for Combining 0.2 mM of o-*Phenanthroline in the Diffusion Media of Isotonic HEPES Buffers*

	Flux (μg/hr/cm²)	
Inhibitor	pH 4.0	pH 7.4
0.2 mM *o*-phenathroline	1.42 ± 0.017[a]	0.145 ± 0.008
Control	0.506 ± 0.126	0.192 ± 0.038

[a]Standard deviation, $n = 3$.

receptor compartments; in the real system, other pHs (except pH 7.4) might not be achieved in the receptor compartment. However, using the same pH could minimize the effect of pH difference between both compartments and more accurate results could be obtained. In the skin homogenate we disrupted the epidermis, which contained the most enzymes of the skin. The skin homogenate was somewhat different from the enzyme system of the penetration study, but nevertheless could reflect the enzyme activity in the iontophoretic study. The approach was successfully used to screen the most effective inhibitor.

In this study, we added an enzyme inhibitor in the receptor and donor compartments. Because skin enzymes are distributed not only in the epidermis but also in the dermis. Thus, the receptor compartment also contained skin enzymes in the iontophoretic study. It is reasonable to introduce the enzyme inhibitor in both compartments to measure their effect on penetration, although the approach might not be practical.

On the whole, DSIP is fairly stable in our study conditions. By using optimal pH and enzyme inhibitors and a current as low as 1 mA, we were able to deliver DSIP through the skin by iontophoresis. The optimal penetration rate of 1.4 $\mu g/hr/cm^2$ could be obtained by using 0.2 mg/ml DSIP concentration with 0.2 mM *o*-phenanthroline and 0.6 mA electric current at pH 4. The flux, however, can be further enhanced by increasing the concentration of DSIP (solubility of 5 mg/ml). We suggest that except currents, other factors including pH and enzyme inhibitor also played important roles in the iontophoretic delivery of peptides.

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