In Vitro Transdermal Iontophoretic Delivery of Leuprolide—Mechanisms Under Constant Voltage Application

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ABSTRACT: The transdermal iontophoretic delivery of Leuprolide, a nonapeptide LHRH agonist was studied with the aim of understanding the mechanisms of iontophoresis. Permeation studies were carried out at pH 4.5 and 7.2, at which the average ionic valence of the drug molecule was roughly 2 and 1, respectively. Heat-separated human epidermal membrane was subjected to constant voltage within the range of 250 to 1000 mV during the iontophoretic phase. Iontophoretic enhancement at pH 7.2 was greated than at 4.5. A model for iontophoretic enhancement was developed that takes into consideration the membrane alterations caused by iontophoresis depicted as increased porosity and the permeation through lipid pathways of the stratum corneum. Model-based evaluation yielded that first, the porosity increased with the applied voltage to as much as three times the original at 1000 mV. Second, the lipid pathways contributed approx. 20% to the total permeation during the passive phase. Third, the electro-osmotic flow contributed significantly to the enhancement and its direction was from anode to cathode at pH 7.2 and the opposite at pH 4.5. The magnitude of the electro-osmotic flow was at pH 4.5 somewhat lower than at pH 7.2. Addition of a negatively charged water soluble peptide, Acetyl leucine leucinolyl phosphate as an adjuvant led to twofold increase in the enhancement factor at pH 4.5 and a decrease in the magnitude of the electro-osmotic flow from cathode to anode. Repeated iontophoretic applications of 250 mV on the same skin specimen resulted in same enhancement every time and did not cause any barrier alterations when applied for 1 h every 24 h, which was not the case if the duration between the two iontophoretic applications was only 3 h.
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INTRODUCTION

Iontophoresis involves the transport of molecules through skin membrane under the influence of an electric field and has been investigated rather extensively as a potential technology for the transdermal delivery of drugs[1–3] including peptides and proteins.[4] The transport process was modeled early on, based on the Nernst-Planck equation using the constant field approximation or the electroneutrality approximation for solving the equation and obtaining an analytical expression for the concentration profile in the membrane and for the flux.[5,6] It was also recognized that human skin carries a net negative charge at physiologic pH, which gives rise to permselectivity favorable to positive ions[7] and convective solvent flow due to electro-osmosis upon application of the electric field. Therefore, the Nernst-Planck equation was modified to incorporate the effect of solvent flow

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on drug flux.[8,9] The magnitude of the electro-osmotic flow depends on skin pore surface charge density and pore radius. Attempts to estimate this quantity were based on the concepts of irreversible thermodynamics involving measurements of streaming potential or electro-osmotic volume flow in combination with transference number data in the skin.[9–11] Furthermore, hindrance of diffusional and convective flow-affected transport of drug in skin pores, the latter also expressed in terms of the reflection coefficient, was taken into account using respective empirical restriction functions.[12,13]

Potential drug candidates for iontophoretic delivery including peptides are commonly weak electrolytes, sometimes with amphoteric properties whose ionization depends on pH. These compounds may permeate skin through the aqueous and/or lipoidal pathways,[14] the significance for each pathway depending on the ionic state of the compounds. Thus, the effectiveness of iontophoresis for promoting the transdermal permeation of these drugs will depend on their ionization and consequently on pH for at least two reasons: first, because the effect of the electric field varies with the net charge of the compound, and second, because drug permeating by the lipoidal route will not be affected by iontophoresis.

In this report, a model for iontophoretic enhancement under constant voltage application is presented as an extension of the model developed earlier in our laboratory, which started from the modified Nernst-Planck equation and was expanded to encompass the relative permeation through the lipid and aqueous domains of skin and the pH difference between bulk and aqueous skin domain.[15] One drawback of the Nernst-Planck equation is that it disregards any changes in the membrane at the applied voltage, whereas it is widely accepted that at higher applied voltages ($\geq$500 mV), alterations in the membrane do take place.[16,17] Therefore, the earlier model was applied to the analysis of data obtained at 250 mV only. To overcome this drawback, the model presented here takes into consideration the alterations in the membrane depicted as change in the porosity, this being dependent on the voltage. This model is then used to analyse the results of iontophoresis of Leuprolide, a bioactive nonapeptide employed here, that is a potential candidate for transdermal iontophoretic delivery. The mechanistic insights provided by this analysis are used as starting point for developing and specifically synthesizing a rather lipophilic, water-soluble and negatively charged dipeptide to be used as an adjuvant. The effect of this adjuvant on iontophoretic enhancement upon addition to the drug vehicle is studied. The gained understanding of the process is further applied in the evaluation of the feasibility of delivery of the drug with constant current iontophoresis, which is the subject of a separate manuscript. Finally, transdermal permeation and enhancement under repeated iontophoretic application to the same skin sample is investigated.

THEORETICAL MODEL FOR THE ENHANCEMENT FACTOR

For the development of a model it is assumed that, in mechanistic terms, drug can permeate the epidermis through aqueous and lipid domains of the tissue denoted by the subscripts "ad" and "ld", respectively. The aqueous domain is responsible for the conductance of electric current, while the lipid domain is practically nonconducting and their respective effective volume (or surface area) fraction is ε and $1-\varepsilon$, respectively. Further, it is considered that the permeant is present in two ionic forms "1" and "2," which holds for compounds with one ionizable group or for those with two ionizable groups when the respective pK_a values differ by more than five units. The diffusion coefficient of both ionic forms in each domain is D_{ad} and D_{ld}, respectively, and the pH dependent apparent partition coefficient of the drug between the aqueous domain and the bulk solution is $K_{ad/b}$ and between the lipid domain and the bulk solution is $K_{ld/b}$.

$K_{ld/ad}$ is the apparent drug partition coefficient between the lipid and the aqueous domain and is given by:

$$K_{ld/ad} = \frac{K_{ld/b}}{K_{ad/b}} \tag{1}$$

Assuming the new porosity arising due to voltage dependent alteration in the membrane to be ε', the fraction of the aqueous and lipid domains is ε' and $1-\varepsilon'$, respectively. It is reasonably assumed that the lipid domain conducts no significant electric current and does not contribute to solvent flow, which is aqueous. The total iontophoretic flux (J) under these conditions can be expressed as:

$$J_{total}^{\Delta\Psi} = \varepsilon' J_{ad,1} + \varepsilon' J_{ad,2} + (1-\varepsilon') J_{ld,1} + (1-\varepsilon') J_{ld,2} \tag{2}$$

Aqueous domain fluxes are expressed using the modified Nernst-Planck equation while lipid domain fluxes are solely due to passive diffusion. Substituting the individual fluxes integrating and rearranging[15] gives the following iontophoretic flux at steady state:

$$J_{\text{total}}^{\Delta\Psi} = \frac{K_{\text{ad/b}}\left[-\frac{D_{\text{ad}}\bar{z}_{\text{ad}}F}{RT}\frac{\Delta\Psi}{h}\varepsilon' + \varepsilon'\nu\right]}{1-\exp\left[\frac{-\frac{D_{\text{ad}}\bar{z}_{\text{ad}}F}{RT}\frac{\Delta\Psi}{h}\varepsilon' + \varepsilon'\nu}{\varepsilon' D_{\text{ad}} + K_{\text{ld/ad}}(1-\varepsilon')D_{\text{ld}}}h\right]} C_D \quad (3)$$

where

$$\bar{z}_{\text{ad}} = \alpha_{\text{ad},1}z_1 + \alpha_{\text{ad},2}z_2 \quad (4)$$

i.e., the weighted average net ionic valence of the permeant in the aqueous domain, $\alpha_{\text{ad},1}$ and $\alpha_{\text{ad},2}$ are molar concentration fractions of the species "1" and "2," respectively, in the aqueous domain ($\alpha_{\text{ad},1} + \alpha_{\text{ad},2} = 1$), z_1 and z_2 are the corresponding net ionic valences, $\Delta\Psi/h$ is the electrical potential gradient applied across the membrane, h is the thickness of the membrane, ν is the convective flow velocity, F is the Faraday constant, R is the gas constant, T is the absolute temperature and C_D is the concentration in the donor.

Under passive conditions, the steady state flux is given by:

$$J_{\text{total}}^{\text{passive}} = \left[\frac{K_{\text{ad/b}}\varepsilon D_{\text{ad}}}{h} + \frac{K_{\text{ld/b}}(1-\varepsilon)D_{\text{ld}}}{h}\right]C_D \quad (5)$$

where the first and second terms in the bracket correspond to permeation by the aqueous and the lipid pathway, respectively.

The enhancement factor (E) attained by iontophoresis is given for identical donor concentrations by the ratio of fluxes, otherwise by the ratio of permeability coefficients under iontophoretic and passive conditions:

$$E = \frac{J_{\text{total}}^{\Delta\Psi}}{J_{\text{total}}^{\text{passive}}} \quad \text{or} \quad E = \frac{P_{\text{total}}^{\Delta\Psi}}{P_{\text{total}}^{\text{passive}}} \quad (6)$$

Combining eq. 3, eq. 5, and eq. 6 yields:

$$E = \frac{\frac{K_{\text{ad/b}}\left[-\frac{D_{\text{ad}}\bar{z}_{\text{ad}}F}{RT}\frac{\Delta\Psi}{h}\varepsilon' + \varepsilon'\nu\right]}{\frac{K_{\text{ad/b}}\varepsilon D_{\text{ad}} + K_{\text{ld/b}}(1-\varepsilon)D_{\text{ld}}}{h}}}{1-\exp\left[\frac{-\frac{D_{\text{ad}}\bar{z}_{\text{ad}}F}{RT}\frac{\Delta\Psi}{h}\varepsilon' + \varepsilon'\nu}{\varepsilon' D_{\text{ad}} + K_{\text{ld/ad}}(1-\varepsilon')D_{\text{ld}}}h\right]} \quad (7)$$

Rearranging, the following equation is obtained:

$$E = \frac{\frac{-\bar{z}_{\text{ad}}B\frac{\varepsilon'}{\varepsilon} + \frac{\varepsilon'}{\varepsilon}\text{Pe}}{1+\frac{K_{\text{ld/b}}(1-\varepsilon)D_{\text{ld}}}{K_{\text{ad/b}}\varepsilon D_{\text{ad}}}}}{1-\exp\left[-\frac{-\bar{z}_{\text{ad}}B + \text{Pe}}{1+\frac{K_{\text{ld/b}}(1-\varepsilon')D_{\text{ld}}}{K_{\text{ad/b}}\varepsilon' D_{\text{ad}}}}\right]} \quad (8)$$

where

$$B = \frac{F\Delta\Psi}{RT} \quad (9)$$

and

$$\text{Pe} = \frac{\nu h}{D_{\text{ad}}} \quad (10)$$

At the voltages used, B is $\ll -1$; furthermore, the ratio of passive permeabilities of lipid to aqueous skin domain for rather hydrophilic molecules is ≤ 1 and for all practically resulting Pe values the argument of the exponential term is $\ll -1$, and therefore, the exponential term in the denominator can be neglected. The final equation used for further calculations is:

$$E = \frac{\frac{\varepsilon'}{\varepsilon}[-\bar{z}_{\text{ad}}B + \text{Pe}]}{1+(P_{\text{ld}}/P_{\text{ad}})^{\text{passive}}} \quad (11)$$

where $(P_{\text{ld}}/P_{\text{ad}})^{\text{passive}}$ is the ratio of permeability coefficients for the lipid and the aqueous domains under passive conditions.

The apparent partition coefficient of drug between aqueous domains and bulk, $K_{\text{ad/b}}$ is given by:[15]

$$K_{\text{ad/b}} = \frac{10^{(\text{pK}_a - \text{pH}_{\text{ad}})} + 1}{10^{(\text{pK}_a - \text{pH}_b)} + 1}\left(10^{(\text{pH}_b - \text{pH}_{\text{ad}})z_2}\right) \quad (12)$$

where pK_a refers to the ionization constant of the drug, pH_b is the pH of the bulk solution, pH_{ad} is the calculated pH in the aqueous membrane domain and z_2 is the valence of the drug ionic species "2," for which it holds $z_2 = z_1 - 1$.

MATERIALS AND METHODS

Materials

Active Substances

Leuprolide diacetate (84.9% base) was donated by Bachem AG (Bubendorf, Switzerland). Its amino

acid sequence is Pyr-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-NHEt and has a molecular weight of 1209.4 (base). It contains three ionisable amino acid residues—Histidine (pK_a 6.2), Tyrosine (pK_a approx. 10), and Arginine (pK_a approx. 12.5), and thus, depending upon the chosen pH of solution, can exist as singly and doubly ionized species. The pK_a of the Histidine residue was measured by potentiometric titration to be 6.2. Leuprolide concentration used in the donor was 5 mg/mL. To determine the effect of different charges on the transport of Leuprolide, pH 7.2 and 4.5 were chosen as working pHs at which the drug had a weighted average net ionic valence of 1.09 and 1.98, respectively.

Acetyl leucine leucinolyl phosphate (LLP), a derivatized dipeptide used as adjuvant, was synthesized and donated by Bachem AG. It has molecular weight of 352.4, and is negatively charged (ionic valence -1) at the employed pH value due to the phosphate group.

Buffer

Universal buffer was used at both pH 4.5 and 7.2. It composed of Citric and Phosphoric acid (6.67 mM each), and Boric acid (11.5 mM), all of which were dissolved in double distilled water and the mixture then titrated to the desired pH using Sodium hydroxide. Sodium chloride (0.125 mM and 0.140 mM) was added to the donor and acceptor solutions, respectively, to render them isotonic. All the chemicals used were of analytical reagent grade or better.

Skin

Heat-separated human epidermal membrane was used for permeation experiments. Full thickness human skin, excised from the abdominal region of female donors postmortemed within 24 h of demise, was provided by the Department of Pathology, University Hospital, Basel. These specimens were stored at -70°C until further use. A part of such segment was thawed, freed of fat, heated in water bath at 60°C for 1 min, and epidermis was peeled off it. This was then mounted vertically in the glass diffusion cell (described later) with Stratum corneum facing the donor, sandwiched between the two compartments and sealed with the help of a Teflon band. Area of the skin available for diffusion was approx. 2 cm^2. Every cell was then checked for leakage by filling about 5 mL of acceptor solution in the donor compartment and keeping it in upright position for 15 min. If the cell were not leak proof, the buffer would flow down into the acceptor compartment due to the gravitational pressure. Thus checked cells were then used for carrying out permeation experiments. Barrier properties of the membrane were checked electrically in due course of the experiment.

Iontophoretic Equipment

Custom-made glass diffusion cells with four-electrode system were used in which skin is sandwiched perpendicularly between the donor and acceptor compartments. These cells are symmetrical about the skin and have a volume of 7.5 mL each. Thin Luggin capillaries are built in the cell so that they reach very close to the skin but do not touch it. The other end of the capillary is broadened to hold the reference Ag/AgCl electrodes (Metrohm AG, Herisau) immersed in 3 M KCl solution. Two working electrodes were made from a sheet of silver (Aldrich Chemie, Buchs, Switzerland). The one in cathode was coated with silver chloride to prevent electrolysis of water. The anode was placed in the donor and the cathode in the acceptor compartment of the cells. The complete cell was immersed in a water bath maintained at 37°C, which could hold five such cells. The cells were stirred at 400 rpm with Teflon paddles, which were interconnected with a bead string and were driven by an electric motor. The current source (built in Department of Physics, University of Basel) supplied direct current only and could maintain at one time either current or voltage constant. In the experiments reported here, voltage was kept constant and the current was measured and recorded on a disk with Digital Chart Recorder (DCR 520, W + W Instruments AG, Basel, Switzerland).

Methods

Assay

Leuprolide was assayed by an HPLC instrument (Hewlett Packard 1100 system). A reversed-phase column (12×0.25 cm) Hypersil ODS 18, packed with 5 μm spherical particles (Macherey Nagel, Oensingen) was used with mobile phase 55/45 Methanol/0.25 M aq. Ammonium acetate using UV detection at 220 nm. The limit of detection with this system was 1 ng. The lowest quantified concentration was 25 ng/mL with an injection volume of 100 μL and a relative standard deviation of 4%.

Determination of Partition Coefficients

Partition coefficients were determined in n-Octanol/Buffer at both pH 4.5 and 7.2 at 37°C using shaking flask method.[18] Drug concentration in both the aqueous and the organic phases, both before and after partitioning, was determined against a set of standards. These coefficients were calculated as the ratio of the equilibrium drug concentration in Octanol phase to that in the buffer.

Typical Protocol of Permeation Experiments

A permeation experiment lasted typically for 72 to 75 h. Skin integrity during this time was monitored using the electrical resistance of the skin. The experiments consisted of three consecutive phases: Passive-1, Iontophoretic and Passive-2. All experiments started on day 0, when the cells were set up completely. Because Leuprolide is a large molecule and low transport rate was expected, regular samples were drawn only after 15–18 h. On day 1, during the passive-1 phase, baseline passive fluxes were measured. These fluxes served first for the calculation of Enhancement factor and second to assess the reversibility of the drug fluxes after iontophoresis. At the end of the passive-1 phase, potential difference of 250 mV was applied for 5 min to record the initial electrical resistance and also to check the integrity of the barrier. During the iontophoretic phase, on day 2, predetermined constant potential drop was applied for specified duration (250 mV for 3 h, 500 and 750 mV for 1 h, and 1000 mV for half an hour). The current was measured throughout this phase and recorded every minute. During the passive-2 phase on day 3, postiontophoretic passive fluxes were measured and compared with those of passive-1 phase; 250 mV were applied again for 5 min to check the electrical resistance of the skin.

During all these phases, typically 0.2-mL samples were drawn from the acceptor at regular predetermined intervals and the volume was replenished with their respective acceptor solutions. The donor samples (0.1 mL) were drawn only at the beginning and end of each of the phases, and were not replenished. All these samples were then analyzed in duplicate against a set of standard solutions. Sink conditions were maintained throughout and permeability coefficient (P) was calculated using the equation:

$$P = \frac{dQ}{dt}\frac{1}{AC_D} \tag{13}$$

where, dQ/dt is the slope of the cumulative permeating amount versus time curve, A is the area of diffusion, and C_D is the average donor concentration during the phase. Enhancement factors were then calculated using eq. 6.

The transference number (T_n), defined as the ratio of the current carried by the drug ions to the total current, was calculated for all the experiments from the slope of the iontophoretic phase using the equation:[27]

$$T_n = \frac{\overline{z}_{\mathrm{ad}} F\, dQ/dt}{M_r I} \tag{14}$$

where, dQ/dt is the slope of the cumulative permeating amount versus time curve during iontophoresis, M_r is the molecular weight, and I is the total current passed through the membrane and the other parameters were defined above.

Permeation Experiments with the Additive

Acetyl-leucine leucinolyl-phosphate (LLP) was found to be compatible with Leuprolide and did not affect the drug's solubility at the concentration used in these experiments. Because LLP has a negative charge owing to the phosphate, it would be transported from cathode to anode (acceptor to donor) during the iontophoretic phase. To maintain its presence in the membrane during all phases, it was added to both the donor and acceptor compartment. The concentration used was 2.4 mg/mL and three experiments were carried out using typical permeation protocol at pH 4.5 and applying 250 mV for 3 h during the iontophoretic phase.

Experiments with Longer Hydration Period

The skin was set up in the permeation cell as described above and buffer (acceptor solution pH 4.5) was filled in both the donor and acceptor compartments. After 14 h both these compartments were emptied, filled with their respective solutions, and permeation experiments were carried out applying 1000 mV for half an hour during the iontophoretic phase.

Repeated Voltage Applications

Studies with repeated iontophoretic application were carried out using donor concentration of

5 mg/mL. During the iontophoretic phase, 250 mV was applied as it had been shown that single application of this voltage does not cause any irreversible damage to the membrane. In these experiments, the same piece of skin was exposed repeatedly to 250 mV as opposed to typical experiments where a single piece of skin had had a single exposure to voltage. Enhancement factor for each iontophoretic application was calculated based on the immediately preceding passive phase. Two different protocols were used.

Protocol 1

To measure the baseline passive permeability and the passive peremability after the skin had been exposed to various iontophoretic phases and include as many iontophoretic phases in 1 day as possible, the following protocol was used: day 0—setting up the cell; day 1—passive-1 for approx. 9 h; day 2—iontophoretic-1, 1 h; passive-2, 3 h; iontophoretic-2, 1 h; passive-3, 3 h; iontophoretic-3, 1 h; day 3—passive-4, 8 h.

Protocol 2

With the aim of giving the skin enough time (approx. 24 h) within two iontophoretic phases and measuring both baseline and postiontophoretic passive permeability, the following protocol was used: day 0—setting up the cell; day 1—passive-1 for approx. 9 h, iontophoretic-1, 1 h; day 2—passive-2, 8 h; iontophoretic-2, 1 h; day 3—passive-3, 8 h.

RESULTS AND DISCUSSION

Single Voltage Application

Passive Permeability Through the Epidermal Membrane

Figure 1 shows the typical permeation profile of the experiments. Generally, the amount increased linearly with time and gave measurable fluxes during all phases. Preceding hydration of the skin for 14 h did not produce any significant differences in permeation (not shown). From slopes of these permeation profiles, permeability coefficients, P, were calculated using eq. 13. These results are shown in Table 1. The absolute passive P values are low but could be accounted for taking into consideration the large size and charges of Leuprolide. These values of permeability showed a wide range of both interindividual (within the skin segments obtained from different donors) and intraindividual (associated with different segments obtained from the same skin donor) variability. It has already been documented in literature that ionized compounds of fairly large size, having low permeability coefficients exhibit large scatter in their *in vitro* skin permeation.[19] An additional reason of the variability may be the skin uptake, which is quite high compared to the drug transported in the acceptor (up to 10 times). This is also consistent with previous reports where it was considered to be a major source of variation.[20]

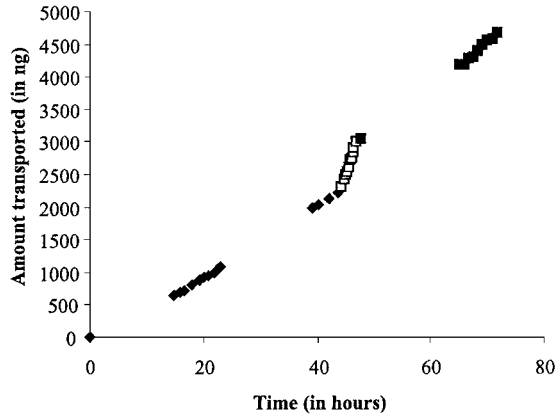

Figure 1. Cumulative amount permeating through skin over the duration of an experiment with Leuprolide concentration of 5 mg/mL at pH 4.5 and 250 mV. Filled symbols show the amount permeating during the passive phases both before and after iontophoresis, whereas empty symbols show transport during iontophoresis.

The measured passive permeability for the individual specimens at both the pHs was correlated to the electrical resistance of the epidermal membrane. The plot is shown in Figure 2. This log-log plot had a slope of −1.03 with a standard error of 0.1, which attests an inversely proportional relationship between passive permeability and resistance. This correlation shows that the transport during the passive phases takes place mainly through aqueous domains. This correlation also provides a reason for the observed variability of the passive permeability implying that at same pH, the samples with higher resistance would have lower permeabilities and vice versa. pH does not seem to affect the passive permeability. The permeability at pH 7.2 appeared to be somewhat higher than that at pH 4.5 but when all

Table 1. Average Permeability Coefficient, P, of Leuprolide with Standard Error of Mean, SEM, at both pH 4.5 and 7.2

		P and SEM $\times 10^9$ (in cm/s)							
		Passive 1		Iontophoretic		Passive 2		Passive 1[a]	
pH	Voltage (in mV)	P	SEM	P	SEM	P	SEM	P	T_n^b (in %)
4.5	250	0.89	0.42	3.79	1.91	1.05	0.52	1.64	0.09
4.5	1000	1.47	0.81	137.34	100.11	6.08	5.36	0.77	0.07
7.2	250	5.29	2.95	85.70	36.23	4.64	3.54	1.47	0.13
7.2	750	0.78	0.28	58.07	27.36	1.48	0.44	0.66	0.08
7.2	1000	9.33	5.63	3508.4	1920.1	30.13	25.8	1.20	0.35

[a]Permeability coefficients of Passive 1 phase interpolated to a membrane resistance of 60 kΩ*cm^2.
[b]Transference number T_n during the iontophoretic phase (average of experiments) calculated as the ratio of the current carried by the drug ions to the total current.

permeability values were numerically interpolated to the same resistance (60 kΩ*cm^2), the difference in the permeabilities vanished. Table 1 shows these adjusted permeability coefficient values at 60 kΩ*cm^2, which for all groups concentrated around 1×10^{-9} cm/s.

It is worth mentioning that this phenomenon of observing inverse proportionality between these two parameters is not universal. Reports have shown that such a correlation exists for polar molecules like Urea,[19] whereas for molecules like Corticosterone,[21] nonexistence of such a correlation has been reported. This can be explained on the basis of lipophilicity of the molecule. Because hydrophilic molecules diffuse through the aqueous domains of the membrane, their permeability electrically speaking would depend directly upon the conductance or inversely upon the resistance of the aqueous domains of the membrane, whereas the lipophilic molecules would pass through the lipid domains, their behavior being independent of the conductance of the aqueous channels.

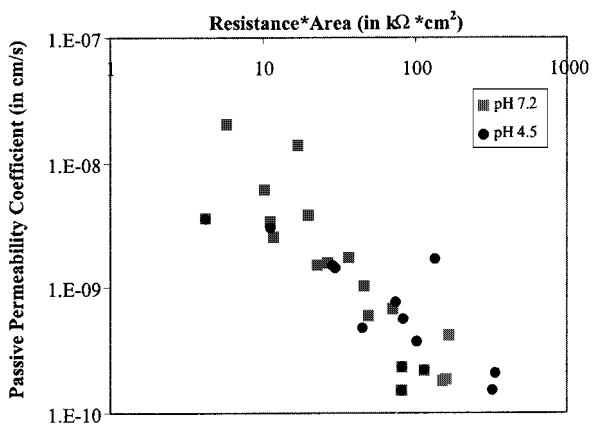

Figure 2. Correlation between Leuprolide permeability and electrical resistance. Each point represents the individual data collected from a single human epidermal membrane specimen. Regression through the points gave a slope of -1.03 and $r = 0.84$.

Iontophoretic Permeability Through Epidermal Membrane

The iontophoretic permeability profiles were also generally linear. At 1000 mV, a few skin samples showed tendency towards a nonlinear profile (slope increasing with time), but the duration of iontophoretic phase at this voltage was short enough (0.5 h) not to let this phenomenon manifest itself strongly. Permeation curves with standard error of their slope within 10% of its value were considered linear. The rate of permeation in the iontophoretic phase should depend upon the current passing through the specimen, which in turn, depends upon the applied voltage and the initial conductance of the skin. Hence, the measured iontophoretic permeability coefficients at both the pHs, under all voltage conditions were plotted against the measured average current during that phase. Figure 3 shows this log-log plot, which had a positive slope of 1.2 with standard error of 0.1 and correlation coefficient $r = 0.9$. This demonstrates a positive correlation between iontophoretic permeation rate and the current. This correlation implies that under same voltage conditions, permeability coefficients would vary due to the different currents that flow through different membranes depending upon their initial conductance. From this, the possibility to control Leuprolide permeation based on the applied current is inferred. The current measured, however,

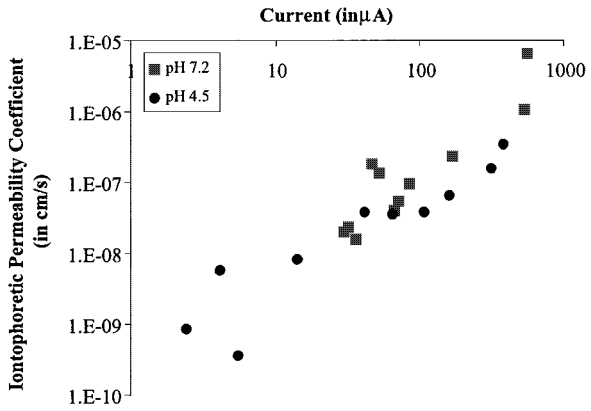

Figure 3. Correlation between iontophoretic Leuprolide permeability and the average current passed. Each point represents the average current passed through a single human epidermal membrane specimen during the individual iontophoretic phase. All the voltages applied and both the pHs are included.

is far from being all due to the transport of drug ions. The transference numbers, calculated from eq. 14, at both the pHs during the iontophoretic phase were in the range of 0.07 to 0.35% (Table 1) showing that a very small fraction of the current is carried by the drug. More than 99% is carried by the buffer ions owing to their smaller size and higher mobility. The deviation of the slope of the correlation line in Figure 3 from +1.0 suggests that the transference number may vary with the current or the initial membrane resistance.

Enhancement Factors and Membrane Alteration

Enhancement factors calculated using eq. 6 are shown in Figure 4. Expectedly, enhancement increased with increasing voltage. At high voltages,

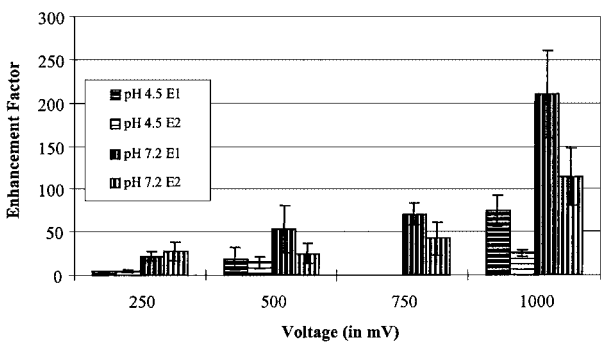

Figure 4. . Enhancement factors at both pH 7.2 and 4.5. E1 is calculated as the ratio of iontophoretic flux to baseline passive flux (passive-1), whereas E2 is calculated as the ratio of iontophoretic flux to the postiontophoretic passive flux (passive-2).

there is a nonconformity of the enhancement factors calculated on the basis of the baseline and the postiontophoretic passive fluxes. The differences between the two enhancement factors arise from a difference in the properties of the skin specimens before and after voltage was applied. At both the pHs at 250 mV and at pH 4.5 at 500 mV there is practically no difference between the two enhancement factors, showing that skin integrity was maintained. At pH 7.2 and 500 mV, however, the enhancement factor based on postiontophoretic passive flux dropped to about half of the baseline-based value. At and above 750 mV, this drop was much higher (>60%) at both the pHs. This drop is probably due to the fact that the applied voltage caused perturbations increasing the porosity of the membrane due to which the passive permeation rate did not completely return to its baseline value in the postiontophoretic passive phase. As enhancement factor is defined as the ratio between iontophoretic and passive flux, this ratio is lower when based upon the passive-2 phase. This shows that the threshold of the irreversible membrane alterations might be $\geq$500 mV (where the data showed recovery at pH 4.5 but not at 7.2). There have been other reports as well that show that the applied potential of 500 mV may be high enough to cause alterations in the human epidermis.[19,21]

Model-Based Evaluation

The model-derived eq. 11 presented in the theoretical section was used to evaluate the enhancement factor data determined based on the baseline passive permeability coefficients. The following conditions were considered to apply:

1. No alterations in the membrane properties take place at 250 mV, as demonstrated in the previous section and the porosity during iontophoresis remains the same as that during the passive phase. Hence, at 250 mV,

$$\frac{\varepsilon'}{\varepsilon} = 1 \qquad (15)$$

2. Peclet number (*Pe*) depends linearly on the voltage at both the pHs.[16,22,23]
3. The $(P_{ld}/P_{ad})^{passive}$ value is the same at both pHs based on the following reasoning. First, owing to the very small difference in the Octanol/buffer partition coefficients (0.5 at pH 7.2 and 0.4 at pH 4.5), the lipid domain permeability at both the pHs would almost be the same.

Second, the calculated aqueous domain/bulk partition coefficient of the drug, $K_{ad/b}$ (eq. 12), taking into account a pH difference between the bulk and the aqueous domains of 0.05 units.[15] resulting from a skin charge density of 0.0035 C/m^2, deviates slightly from unity (1.13 and 0.8 at pH 7.2 and 4.5 for net negative and positive skin charge, respectively; see below). Thus, although aqueous domain permeability at pH 7.2 would be expected to be somewhat higher than at pH 4.5, this difference is rather small and lies within the limits of the experimental variability of the passive permeabilities (Table 1). Therefore, the $(P_{ld}/P_{ad})^{passive}$ value is assumed to be practically the same at both pHs.

4. It was reasonably presumed that the porosity is an inherent property of the membrane and is independent of pH.

Values of the parameters of the theoretical expression for iontophoretic enhancement (eq. 11) were determined that yielded enhancement factors that approximated as closely as possible the experimentally determined values. Besides respecting the conditions established above, Pe was assumed to be different at the two pH values and ε'/ε to vary with the voltage. The parameter values were deduced based on a least squares fitting procedure with which a minimum of the sum of squared deviations between experimental and calculated enhancement factors is found simultaneously for all enhancement factors (software: MINSQ, MicroMath, Salt Lake City, UT). Grouped t-test performed on the enhancement factors calculated with the best fit parameters (given in Table 2) and the observed set of data showed that there were minimal differences between the two sets ($p = 0.999$). Thus, the deduced parameters of the model are shown to describe accurately the experimental results.

The obtained parameters are presented in Table 2 and are used below as a means to elucidate the mechanisms of iontophoresis. The peclet number at pH 7.2 is positive, indicating an electro-osmotic flow from donor to acceptor and is negative at pH 4.5, thereby assisting drug flux at pH 7.2 and opposing it at pH 4.5. This can be interpreted in terms of the isoelectric point of skin and the interaction of Leuprolide with skin. The isoelectric point of skin is between pH 3–4.5, being in the range of that of its constituents like keratin and fatty acids. At pHs higher than the isoelectric point like the physiologic pH, the epidermis carries fixed negative charges, and the positive counterionic atmosphere in the aqueous domains is responsible for electro-osmotic flow from anode to cathode. This situation is opposite at pHs lower than the isoelectric point where epidermis would be positively charged. It has been shown that this reversal of the electro-osmotic flow, which arises from the reversal of the sign of the fixed charges, takes place between pH 3 and 4.5.[15] Leuprolide and other positively charged peptides have been reported to adsorb strongly to the skin.[20,24–26] In this study, 15 to 20 μg of Leuprolide (relative standard deviation $\approx 10\%$, $n = 6$) were recovered from a 2-cm^2 piece of epidermis at pH 4.5 at the end of the permeation experiment. Adsorption of doubly positively charged Leuprolide on the epidermal membrane seems to be converting the bound skin charge from negative to neutral or positive and thus bringing about the reversal of the polarity of electrical double layer leading to reversed electro-osmotic flow at pHs higher than its isoelectric point. This results in lower iontophoretic fluxes at pH 4.5 than at pH 7.2 despite the fact that at pH 4.5 the ionic valence of the drug is almost double that at pH 7.2. The magnitute of the electro-osmotic flow is somewhat smaller at pH 4.5 than at pH 7.2, yet the difference is quite small.

Table 2. Parameters Calculated Using the New Model

pH	Voltage (in mV)	$(P_{ld}/P_{ad})^{passive}$	Pe	ε'/ε	E Calculated	E Observed
4.5	250	0.24	−10.4	1.00	6.52	3.83
4.5	500	0.24	−20.8	1.44	18.89	18.90
4.5	1000	0.24	−41.6	2.86	74.49	74.97
7.2	250	0.24	12.7	1.00	18.41	21.64
7.2	500	0.24	25.4	1.44	53.34	53.00
7.2	750	0.24	38.1	1.28	70.79	70.72
7.2	1000	0.24	50.8	2.86	210.34	210.14

P_{ld}/P_{ad} value for passive transport is 0.24, showing limited yet noticeable permeation through the lipid domains. Taking into account the lipophilic nature of several amino acids of Leuprolide namely Tryptophan, Tyrosine, D-Leucine, and Leucine and its octanol/water partition coefficient, this value seems to be reasonable. Thus, permeation through lipid membrane domains hampers to a small extent iontophoretic enhancement.

ε'/ε depicts the degree of increase in the porosity of the membrane during iontophoresis relative to that of the baseline passive phase at the specified voltage. The results at 500, 750, and 1000 mV show an increase (1.4, 1.3, and 2.9 times, respectively) at all these voltages. No clear difference is seen between 500 and 750 mV probably due to the scatter of the data. At 1000 mV, there is an approx. threefold increase in the porosity, which is clearly the greatest of all voltages and explains the enormous increase in the enhancement factors at this voltage at both pHs. Also, these results are quantitatively consistent with the differences between the two enhancement factors based on baseline and postiontophoretic passive permeability at high voltages discussed in Figure 4.

Effect of the Additive

The direction of the electro-osmotic flow from the cathode to the anode evidenced by the negative Pe number at pH 4.5 was attributed to the positive charges arising from the binding of Leuprolide to the skin. To test the validity of this mechanism, the effect of Acetyl leucine leucinolyl phosphate (LLP) on Leuprolide iontophoretic permeation was determined. It was postulated that due to its small size and relative lipophilicity, LLP would readily permeate and bind to the skin thereby adding negative charges to it due to its phosphate group, shifting the net charge of the skin and influencing electro-osmotic flow. The sole purpose of synthesizing LLP was to test its effect on electro-osmotic flow. The compound has no known biological activity.

Table 3 shows that the enhancement factor of Leuprolide at pH 4.5 increased by a factor of two in the presence of LLP. The difference in the baseline permeabilities between the experiments with and without LLP are due to the inherent properties of the skin specimens used. Indeed, when these values are adjusted to a skin electrical resistance of 60 $k\Omega^*cm^2$, this difference diminishes (Table 3). Because enhancement factors are determined on the same piece of skin, they constitute relative measurements, being considerably less sensitive to skin-to-skin variability than absolute permeability values.

The twofold increase of the enhancement factor by LLP corresponds to a decrease of the magnitude of electro-osmotic flow (Table 3). The value of Pe decreased in absolute terms in the presence of LLP, yet its sign remained negative, indicating that the direction of the electro-osmotic flow was from cathode to anode. Thus, one may hypothesize that the net surface charge density of the skin decreased (became less positive) with the addition of LLP yet the charge remained positive because of the binding of Leuprolide. This effect of LLP is relatively small but might be increased at higher concentrations. Because LLP was added to both compartments to maximize its effect, its own transport across skin could not be measured.

These results demonstrate a concept based on which iontophoretic enhancement may be augmented with the help of adjuvants and supports the postulated mechanism about the generation and the influence of electro-osmotic flow in iontophoretic skin permeation.

Repeated Applications

Because *in vivo* iontophoretic delivery is likely to involve multiple voltage exposures of the same

Table 3. Effect of the Additive LLP upon the Permeability Coefficient, P ($\times 10^9$ cm/s) of Leuprolide at 250 mV, Enhancement Factor, E, and Peclet Number, Pe; SEM: Standard Error of Mean.

	P (SEM)					P
	Passive 1	Iontophoretic	Passive 2	E (SEM)	Pe (SEM)	Passive-1[a]
Without Additive	0.89	3.79	1.05	3.83	−13.81	1.64
	(0.42)	(1.91)	(0.52)	(0.67)	(0.83)	
With Additive	0.48	4.03	0.98	8.13	−8.46	1.31
	(0.05)	(1.49)	(0.18)	(2.27)	(2.82)	

[a]Adjusted to 60 $k\Omega^* cm^2$.

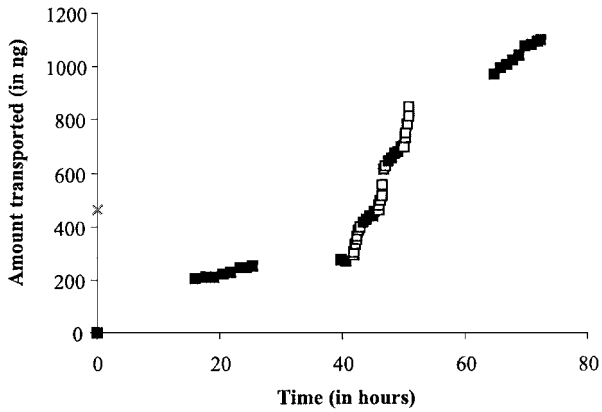

Figure 5. Cumulative amount permeating through the skin during repeated iontophoretic applications of 250 mV for 1 h, according to Protocol 1. The empty symbols show consecutive iontophoretic phases, whereas filled symbols show the consecutive passive phases.

skin site, studies with repeated iontophoretic application were carried out. A voltage of 250 mV was used, as it has been shown that this voltage in single application does not cause any irreversible alterations to the membrane. Figure 5 shows the typical permeation profile of repeated iontophoretic experiment. Table 4 shows the results of the experiments performed with protocol 1 at pH 7.2 according to which three 1-h iontophoretic phases were separated by two 3-h passive phases, besides the baseline (passive-1) and the final postiontophoretic (passive-4) passive phases. The first enhancement factors are generally in the area of that for single application. Consecutive enhancement factors, however, declined markedly. Analysis of the permeability coefficients reveals that this decrease in the enhancement factors is due to the increase in the successive passive permeability coefficients rather than decrease in the iontophoretic ones. The increase of the permeability of successive passive phases may, in theory, be either because of skin alteration caused by the multiple voltage applications (constrary to the single application that had no such effect) or because the duration of the passive phases, i.e., 3 h, was just not long enough to allow steady state passive permeation to be established. The latter hypothesis is supported by the fact that permeability coefficients of the final postiontophoretic passive phase (passive-4) were lower than those of the preceding passive phases and approached the baseline value.

This hypothesis was further tested by carrying out experiments with Protocol 2 according to which the duration between two iontophoretic phases was approx. 24 h. Table 5 and Table 6 show the results of these experiments at pH 4.5 and 7.2, respectively. The two enhancement factors of each experiment were generally comparable with each other, and were in the bulk part of the values obtained with the single voltage application. The only exception is experiment 4 at pH 4.5, which shows erratic results. Also, the permeability of the passive phases is quite constant.

These data demonstrate that repeated constant voltage iontophoresis at 250 mV with the same skin specimen gives consistent and reproducible results with respect to flux and iontophoretic enhancement. The skin is able to withstand repeated voltage exposure with no alterations under the conditions applied here, and the effect of iontophoresis does not seem to depend on the history of application. The schedule of application, however, appears to affect the passive permeation rate between iontophoretic phases. This can be

Table 4. Individual Permeability Coefficients ($\times 10^9$ cm/s) and the Subsequent Enhancement Factors of Leuprolide at pH 7.2 Measured with Repeated Applications Using Protocol 1

	Experiment 1	Experiment 2	Experiment 3	Experiment 4
Passive 1	0.15	0.22	0.23	3.61
Passive 2	0.61	0.78	0.39	4.15
Passive 3	0.79	1.30	0.98	5.28
Passive 4	0.53	0.58	0.33	3.58
Iontophoretic 1	3.20	5.13	2.71	31.10
Iontophoretic 2	5.32	4.11	2.64	22.50
Iontophoretic 3	4.83	3.91	2.57	27.60
E1	21.04	23.18	11.68	8.61
E2	8.69	5.25	6.83	5.42
E3	6.12	3.00	2.61	5.23

Table 5. Individual Permeability Coefficients ($\times 10^9$ cm/s) and the Subsequent Enhancement Factors of Leuprolide at pH 4.5 Measured with Repeated Applications Using Protocol 2

	Experiment 1	Experiment 2	Experiment 3	Experiment 4
Passive 1	0.61	0.96	2.02	0.38
Passive 2	1.61	1.30	2.75	1.08
Passive 3	0.07	1.37	1.07	0.89
Iontophoretic 1	6.54	5.72	19.90	28.40
Iontophoretic 2	11.70	6.63	29.60	21.10
E1	10.73	5.96	9.82	73.84
E2	7.23	5.10	10.73	19.59

explained in terms of the concentration gradient of the drug in the skin. During iontophoresis, the concentration gradient in the membrane follows a convex shaped curve, whereas it is linear in the passive phases, this being related to the different fluxes resulting under the two circumstances.[5,15] The lag time required for the transition from the iontophoretic steady state flux to the passive steady state flux appears in the present work to be more than three and less than 23 h. During this transition, passive fluxes differ from their baseline values.

CONCLUSIONS

The model presented here makes it possible from evaluating Leuprolide iontophoretic transport data to quantify (1) the change in permeability properties in terms of change of porosity of skin inflicted upon it by the electric field, (2) the contribution to passive permeation of the lipid skin domains that hampers iontophoresis, and (3) the magnitude of the electro-osmotic flow and determine the direction and thus the positive or negative role of the latter for enhancement. This model described well the experimental results. Skin charge, which controls the direction and magnitude of the electro-osmotic flow, is affected by the presence of chemicals, and can be manipulated with the addition of adjuvants for improving iontophoretic enhancement. Repeated iontophoretic application at the same skin site using 250 mV for 1 h is possible because it does not affect the barrier properties of the skin and provides reproducibly recurring flux data.

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Table 6. Individual Permeability Coefficients ($\times 10^9$ cm/s) and the Subsequent Enhancement Factors of Leuprolide at pH 7.2 Measured with Repeated Applications Using Protocol 2

	Experiment 1	Experiment 2	Experiment 3	Experiment 4
Passive 1	1.78	0.19	3.82	0.42
Passive 2	2.71	0.32	4.16	0.65
Passive 3	2.65	0.39	3.34	1.08
Iontophoretic 1	27.10	1.32	80.10	5.36
Ionotophoretic 2	54.60	1.88	76.40	10.10
E1	15.22	7.11	20.95	12.85
E2	20.14	5.85	18.34	15.50

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