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Iontophoretic delivery of nafarelin across the skin

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Abstract

The iontophoretic delivery of the luteinizing hormone releasing hormone (LHRH) analogue, nafarelin ([D-Nal (2)^6]LHRH) across hairless mouse skin in vitro has been investigated. The initial range-finding studies showed that a pharmacologically significant amount of the decapeptide can be transported across the skin in a relevant period of time. However, metabolism of nafarelin was observed to take place during its transdermal delivery. It was further found that a reservoir of nafarelin could be established in the skin; for example, following current passage for a period of 12 h, the amount transported across the tissue was comparable to that which subsequently desorbed passively from the skin over the next 12 h. Different current profiles were tested in an attempt to improve drug delivery and minimize the apparent reservoir effect. In addition, the electro-osmotic transport (from both anode and cathode chambers of the in vitro diffusion cell) of an uncharged compound (namely, mannitol) with and without the anodal delivery of nafarelin was determined. It was found that iontophoresis of the peptide into the skin caused electro-osmotic flow to reverse in direction (from anode-to-cathode to cathode-to-anode), relative to the control (no peptide) situation. The anodal delivery of the cationic peptide into the current-conducting pathways of the skin is believed to result in an association of the drug with the fixed negative charges on the membrane; this neutralization and further concentration of the lipophilic peptide reverses the permselectivity of the skin and hence electro-osmotic flow is also reversed. Overall, therefore, optimization of nafarelin delivery by iontophoresis is a complex challenge which warrants considerable further study.

Keywords: Iontophoresis; Convective flow; LHRH analog; Nafarelin; Peptide; Transdermal delivery

1. Introduction

The long-term objective of this research is to identify a successful solution to the systemic delivery of peptide and protein drugs. An analogue of luteinizing hormone releasing hormone (LHRH), namely nafarelin, has been chosen for investigation. In addition to its inherent interest, nafarelin serves as a model for other polar and/or (singly or multiply) charged drugs. Systemic availability of nafarelin poses a real challenge to the pharmaceutical scientist. Chronic intravenous or intramuscular injection is clearly undesirable. Oral dosing presents stability and absorption problems. Other potential routes (e.g., rectal, nasal, sustained-released injectable) suffer from different, but equally significant, problems.

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We report here 'range-finding' studies to assess the feasibility of iontophoretically delivering nafarelin via the skin, an approach which has been considered for LHRH itself and for other of its analogues (Meyer et al., 1988, 1990; Miller et al., 1990; Heit et al., 1993; Lu et al., 1993). An important advantage of iontophoresis is that offers the potential to provide flexible control of drug input rate through modulation of the current profile employed (constant current versus pulsed, for example). Such an ability would be particularly useful for LHRH and its analogs which elicit distinctive pharmacological effects when delivered in a pulsatile as opposed to a continuous fashion (Nestor et al., 1984).

Nafarelin ([D-Nal (2)^6]LHRH) is a potent superagonist of LHRH (Ho et al., 1984; Nestor et al., 1984). The increased efficacy of this hydrophobic analogue is believed to be due to a combination of its high binding affinity to LHRH receptor and its relatively long biological half-life (Ho et al., 1984; Nestor et al., 1984). Currently, an intranasal formulation is approved for use in the treatment of endometriosis. The recommended dose is 200 μg twice a day, with a typical bioavailability of 3.6% (Chan et al., 1988). Thus, this route of delivery provides 10–20 μg/day; it follows that a transdermal delivery formulation which could deliver ≈ 1 μg/cm^2 per h would only need to be 10–20 cm^2 in size to be 'bioequivalent' in terms of dose. The reasonableness of this target is a strong motivation for examining the feasibility of nafarelin iontophoresis.

2. Materials and methods

2.1. Chemicals

Nafarelin acetate was a generous gift from Syntex Research (Palo Alto, CA). [^{14}C]Mannitol (specific activity = 55 mCi/mmol; purity 98.2%) was obtained from NEN Research Products (Wilmington, DE). All other chemicals used were analytical grade (Aldrich Chemical Co., Milwaukee, WI; Sigma Chemical Co., St. Louis, MO) and were used as received. Solutions were prepared with deionized water (resistivity ≤ 18 MΩ cm).

2.2. Nafarelin analysis

Nafarelin, which had been delivered iontophoretically across the skin into the receptor phase of the diffusion cell, was analyzed by high-performance liquid chromatography (HPLC) (HP 1090 Liquid Chromatograph, Hewlett Packard Inc., Palo Alto, CA) (Miller et al., 1990). The mobile phase, which consisted of 71% water, 29% acetonitrile, and 0.1% trifluoroacetic acid, was passed through a Vydac C18 peptide-protein column (4.6 mm i.d., 25 cm long, 5.6 μm particle size) (Rainin Instrument Co., Woburn, MA). The peptide was detected by UV absorbance at 223 nm. Receptor phase samples (0.5 ml) were passed through a Millex-HV$_4$ filter (Millipore Corp., Bedford, MA) prior to injection into the HPLC.

2.3. Iontophoresis procedure

Iontophoretic delivery of nafarelin across hairless mouse skin (HMS) was assessed in vitro. Skin, freshly excised from 8–12 week old females (HRS/hr hr, Simonsen Laboratories, Gilroy, CA) immediately after killing, was used. The full-thickness tissue was clamped between the two halves of standard side-by-side diffusion cells (Laboratory Glass Apparatus, Inc., Berkeley, CA) (Fig. 1). Donor and receptor phase volumes were 3.25 ml; the area of skin available for transport was 0.64 cm^2. The solution in the receptor chamber was magnetically stirred; however, to prevent nafarelin aggregation (Powell et al., 1991), the donor solution was not agitated. The electrodes used in the iontophoresis experiments were

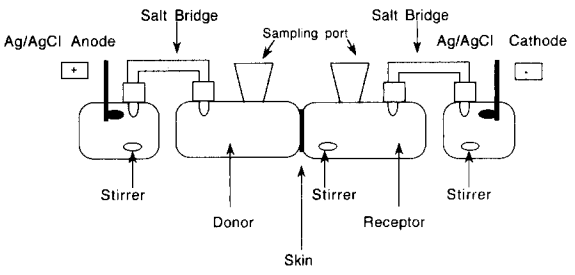

Fig. 1. Schematic diagram of the iontophoretic diffusion cell set-up used in this study.

Ag/AgCl, prepared as previously described (Green et al., 1991). The electrodes were isolated from the donor and receptor solutions via salt bridges (1 M NaCl in 3% agarose) to ensure that neither electrochemical degradation nor adsorption of the peptide on the electrode surface was possible. The salt bridges connected the donor and receptor compartments to identical solutions (volume = 9 ml) in which the electrodes were immersed. Constant current was provided to the electrodes by a custom-built power supply (Professional Design and Development Services, Berkeley, CA) interfaced to a Macintosh IIfx computer (Apple Computers, Inc., Cupertino, CA) running Labview software (National Instruments Inc., Austin, TX). In most experiments the anode was located in the donor compartment, to which the epidermal surface of the skin was exposed. Other configurations (see below), however, were also studied. Typically (though, again, not always), current was applied for 24 h and then terminated. Samples (0.5 ml) were withdrawn from the receptor solution at 6, 8, 10, 12, 14, 24, 26 and 28 h and were analyzed for permeated nafarelin by HPLC (see above). Volume withdrawn from the receptor was immediately replaced with an identical amount of fresh solution. All experiments were performed in at least triplicate and, in all cases, the corresponding passive (i.e., no current) controls were conducted.

2.4. Experiments

A number of studies were performed to examine the influence of key variables on the delivery of nafarelin. Except where specifically stated, the background electrolyte in the donor and receptor compartments was phosphate-buffered saline (50 mM phosphate in the donor, 100 mM in the receptor; 77 mM NaCl in both) at pH 5 (Anik and Hwang, 1983). The current density applied in all experiments was 0.63 mA/cm^2; the initial nafarelin concentration in the donor chamber was 1 mg/ml.

2.5. Ionic strength

Nafarelin delivery from a donor solution of 'low' ionic strength (14 mM NaCl) was compared with that from the standard electrolyte. Current was passed for 24 h and transport was monitored for a further 4 h post-iontophoresis.

2.6. Receptor phase composition

Transport into receptor phase at pH values 5, 6 and 7 was compared. The donor solution was low ionic strength (14 mM). Subsequently, at pH 7 the separate presence of three aminopeptidase inhibitors (0.3 mM bestatin, 0.3 mM amastatin and 1 mM puromycin) was examined. Current was passed for 24 h and peptide flux was monitored for a further 2 h after iontophoresis.

2.7. Nafarelin accumulation in the skin

The uptake of nafarelin into the skin during iontophoresis was assessed. Current was passed for 12 h and transport monitored. Donor and receptor solutions were then removed and replaced with fresh electrolyte. 12 h later the amount of peptide released by passive desorption into the receptor phase was determined. To evaluate the importance of the dermal side of the skin to the accumulation of peptide, delivery of nafarelin through 'inverted' skin (i.e., dermis side facing the donor, anodal chamber) was measured.

2.8. Effect of current

First, the effect of current pretreatment on the skin's passive and iontophoretic permeability to nafarelin was evaluated. In the absence of peptide, current was passed for 12 h. At this point, nafarelin was introduced into the donor compartment and its flux over the next 14 h (either 12 h current + 2 h passive, or simply 14 h passive) was assessed. The impact of different current profiles was also evaluated. Constant anodal current (24 h) was compared with an 'alternating' profile (6 h anodal, 6 h cathodal, 6 h anodal, 6 h cathodal) and a 'pulsed' profile (6 h anodal, 6 h 'off', 6 h anodal, 6 h off).

2.9. Electro-osmosis

Because of the perceived importance of electro-osmosis on the enhanced transport (with ion-

Table 1
Effect of donor solution ionic strength (I) and receptor solution pH on the iontophoretic transport of nafarelin

Donor/receptor solutions	Accumulated nafarelin [a] (nmol/cm^2)			
	6 h [b]	12 h [b]	24 h [b]	28 h [b]
'High' I-pH 5 [c]	1.9 ± 1.3	5.7 ± 3.3	19.1 ± 8.7	28.2 ± 11.6
'Low' I-pH 5 [d]	1.3 ± 1.2	5.1 ± 3.4	17.2 ± 8.1	22.4 ± 10.3
'Low' I-pH 6 [e]	0.0 ± 0.0	0.5 ± 0.9	1.6 ± 1.4	1.8 ± 1.7
'Low' I-pH 7 [f]	0.4 ± 0.5	1.2 ± 0.9	4.3 ± 2.3	5.2 ± 3.0

[a] Mean ± standard deviation.
[b] Constant current was applied for 24 h followed by 4 h of passive transport.
[c] $n = 11$.
[d] $n = 9$.
[e] $n = 4$.
[f] $n = 7$.

tophoresis) of certain peptides (Pikal, 1992; Lu et al., 1993; Delgado-Charro and Guy, 1994) an evaluation of electro-osmotic flow was made. In the presence and absence of nafarelin (1 mg/ml) in the donor (anodal) chamber, the electro-osmotic transport of mannitol (^{14}C-labeled; concentration 1 mM) in both the anode-to-cathode and cathode-to-anode directions was determined. Samples of the receiver solution were collected and were analyzed for ^{14}C radiolabel: the 0.5 ml samples were mixed with 5 ml of scintillation fluid (Ready Gel, Beckman Instruments, Irvine, CA) and radioactivity was then determined in a liquid scintillation counter. Six replicates of these experiments were performed.

2.10. Statistics

The significance of difference between experimental results was assessed by ANOVA and Student's t-test as appropriate.

3. Results

First, it should be noted that, at the level of sensitivity of the HPLC assay, no measurable passive permeation of nafarelin could be detected. Likewise, iontophoresis from the cathode was completely unsuccessful. With constant-current anodal iontophoresis, however, significantly

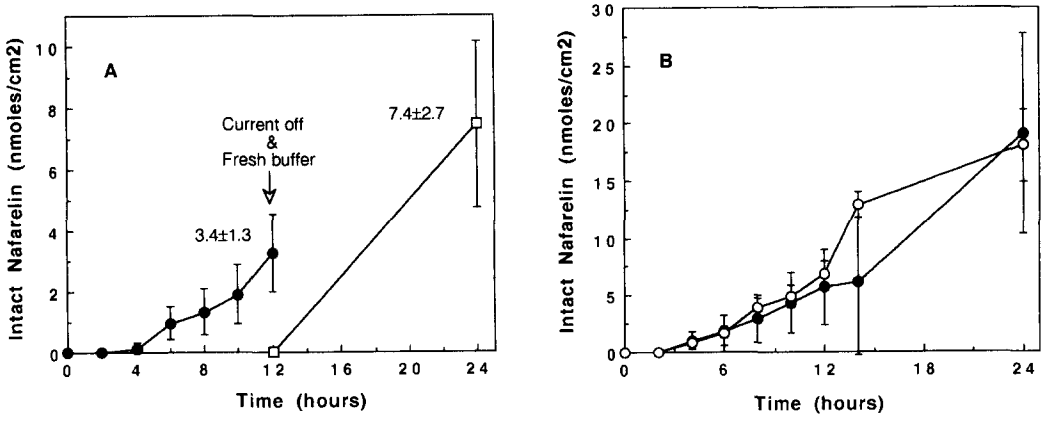

Fig. 2. (a) Cumulative nafarelin delivery across the skin during 12 h of constant current iontophoresis, followed by 12 h of passive 'desorption' into fresh receptor solution. (b) Cumulative nafarelin delivery across the skin during 24 h of constant current iontophoresis with either the epidermis (●) or the dermis (○) facing the anodal, 'donor' peptide compartment of the diffusion cell. The results shown are the means (± standard deviation) of at least four separate measurements.

Table 2
Effect of current profile on the iontophoretic transport of nafarelin

Current profile	Accumulated nafarelin [a] (nmol/cm^2)				
	6 h [b]	12 h [b]	18 h [b]	24 h [b]	28 h [b]
Anodal iontophoresis [c]	1.9 ± 1.3	5.7 ± 3.3	ND	19.1 ± 8.7	28.2 ± 11.6
'Alternating' profile [d]	0.0 ± 0.0	1.2 ± 0.2	7.3 ± 1.3	10.8 ± 4.8	12.0 ± 4.1
'Pulsed' profile [d]	0.5 ± 0.9	1.5 ± 0.4	3.2 ± 1.7	8.7 ± 1.3	9.8 ± 1.3
Cathodal iontophoresis [d]	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

[a] Mean ± standard deviation.
[b] The current profile (see text for details) was applied for 24 h followed by 4 h of passive transport.
[c] $n = 11$.
[d] $n = 3$.

measurable transport of the peptide was observed (Table 1). With pH 5 buffer in the receptor solution, nafarelin delivery was on the order of 20 nmol/cm^2 per 24 h, independent of the ionic strength of the donor electrode chamber. Increasing the pH of the receptor phase to 6 or 7 caused a significant reduction (relative to pH 5) in the appearance of nafarelin (Table 1) and a concomitant increase in the amount and number of peptide metabolites observed in the HPLC chromatograms (data not shown). Attempts to inhibit metabolism with any of the aminopeptidases inhibitors used were totally ineffective.

Fig. 2a shows that, while a 12 h period of iontophoresis results in delivery of nafarelin to the receptor phase, there is a significant amount of peptide which has also became associated with the skin during this time and which can subsequently desorb passively over the next 12 h. The uptake of the peptide was deduced to be primarily by the epidermis from the data in Fig. 2b, which shows that nafarelin is delivered equally efficiently through the skin mounted either with the epidermal surface facing the donor anodal chamber or with the tissue inverted (i.e., dermis facing the donor compartment).

Exposure of the skin to iontophoresis for 12 h prior to nafarelin delivery resulted in no statistically significant effect on either the passive or the iontophoretic permeability of the peptide. No passive transport in the 16 h post-iontophoresis was detected. Furthermore, the iontophoretic de-

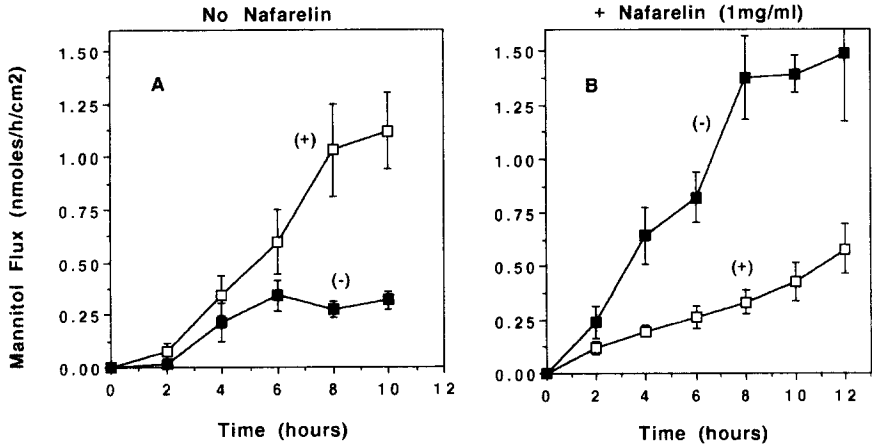

Fig. 3. Electro-osmotic transport of ^{14}C-labeled mannitol, under conditions of constant current iontophoresis, from either the anodal (□) or cathodal (■) chamber of the diffusion cell in (a) the absence, and (b) the presence of nafarelin (at an initial concentration of 1 mg/ml) in the donor, anodal chamber. The results shown are the means (± standard deviation) of at least four separate measurements.

livery in the 12 h period following pretreatment (3.1 ± 2.8 nmol/cm^2) was not statistically different from the control (5.7 ± 3.3 nmol/cm^2) (i.e., iontophoretic delivery without pretreatment).

The effects of different current profiles on the delivery of nafarelin are summarized in Table 2. The cumulative amount of peptide recovered in the receptor phase after 12 h of the alternating profile (6 h anodal + 6 h cathodal iontophoresis) was not significantly different from either that after 12 h of the pulsed protocol (6 h anodal + 6 h passive) or that after 6 h of anodal direct current. Similarly, nafarelin delivery after 24 h of either the alternating or the pulsed profiles did not differ significantly from that after 12 h of anodal direct current.

The impact of nafarelin delivery on electro-osmotic flow across the skin during iontophoresis is shown in Fig. 3. Using ^{14}C-labeled mannitol, it was first confirmed (in the absence of peptide) that electro-osmotic flow occurs preferentially in the anode-to-cathode direction ($p < 0.01$) reflecting the cation permselectivity of skin due to the presence of a net negative charge of the membrane at physiological pH. However, if nafarelin is present in the anodal chamber, the electro-osmotic transport of mannitol, from either the anode or cathode, is dramatically affected. Both anodal and cathodal mannitol fluxes differ significantly from the control values ($p < 0.01$) and there is a reversal in the direction of preferred electro-osmotic flow to the cathode-to-anode direction.

4. Discussion

As anticipated, anodal iontophoresis of the cationic peptide resulted in successful delivery of a quantity (approx. 20 nmol/cm^2 per 24 h) which would be pharmacologically significant (Table 1). However, the susceptibility of nafarelin to metabolic breakdown increased with increasing pH in the receptor phase. Addition of aminopeptidase inhibitors failed to prevent the degradation of the peptide. While the relatively poor overall transport at pH 7 is disappointing, it should be noted that, in human epidermis, the level of metabolism is significantly less, whereas delivery is comparable (Rodríguez-Bayón and Guy, manuscript in preparation).

No effect of altered donor solution ionic strength on the iontophoretic delivery of nafarelin was observed. Although this was at first somewhat surprising, measurements of the osmolality of the donor solution at the end of a iontophoretic run yielded values that were independent of initial ionic strength. The reason was almost certainly leakage of high concentration NaCl from the salt bridges employed. Given the initial concentration gradient across the salt bridge-donor solution interface, 'contamination' of the donor solution ionic composition would have been rapid, therefore, serving to eliminate any possible effects of different ionic strengths on nafarelin delivery.

In the 24 h iontophoresis experiments, peptide transport into the receptor phase continued at an appreciable level during the 4 h following current termination, suggesting that a reservoir of nafarelin had been established in the skin. To investigate this phenomenon further, at times when the in vitro skin barrier could be expected to be fully viable, the experiment, whose results are displayed in Fig. 2a, was performed. After peptide delivery by iontophoresis for 12 h, the current was stopped, the donor and receiver chambers emptied and then refilled with blank electrolyte, and the subsequent passive 'desorption' of nafarelin into the receptor phase was measured. Clearly, a large amount of the drug was associated with the skin, indicating an interaction between the positively charge peptide and the membrane. The kinetics of iontophoretic nafarelin delivery were identical independent of the orientation of the skin in the diffusion cell (Fig. 2b), suggesting the location of the peptide-membrane interaction was most likely in the transport-rate limiting part of the barrier (i.e., the epidermis) as has been revealed by skin-sectioning experiments (Rodríguez-Bayón and Guy, manuscript in preparation). If the dermis had been primarily responsible for the association, a low initial rate of drug delivery would have been anticipated when the transport through inverted skin took place. Furthermore, the large association phenomenon

could not be attributed to an exceptional effect of 12 h current passage on the skin. Pre-iontophoresis of the membrane, followed by either passive or iontophoretic transport, revealed no difference from the control measurements. We note, however, that for smaller peptides, pre-iontophoresis may result in subsequently enhanced delivery (Green et al., 1992).

Because of the importance of the 'reservoir' effect observed, the effects of different current profiles on the efficiency of nafarelin delivery were examined. The results (Table 2) revealed that peptide transport following 12 h of constant current anodal iontophoresis was not significantly different from either (a) that following a pulsed current profile, or (b) that following an alternating current sequence. The latter finding, coupled with the knowledge that 12 h of iontophoresis concentrates an appreciable amount of nafarelin in the skin, led to an investigation of the effects of the peptide on the membrane properties of the skin and the role of electro-osmosis in the mechanism of enhanced drug delivery by iontophoresis (Delgado Charro and Guy, 1994). Under the conditions of the present study, the impact of nafarelin iontophoresis from the anodal, donor compartment on the electro-osmotic transport of radiolabelled mannitol was evaluated (Fig. 3). The control experiments (no nafarelin present) showed the expected outcome (Fig. 3a): electro-osmotic delivery in the anode-to-cathode direction is favored, consistent with the net negative charge on the skin and its cation permselectivity, therefore (Burnette and Ongpipattanakul, 1987; Pikal and Shah, 1990). Introduction of nafarelin into the anode chamber completely reverses this behavior, however (Fig. 3b). From a systematic assessment of this phenomenon (Delgado-Charro and Guy, 1994), we have deduced that anodal delivery of the positively charged peptide into the current-conducting pathways of the skin results in a strong association of the lipophilic cation with the fixed negative charges on the membrane. This neutralization and further concentration of the peptide causes the permselectivity of the skin to be reversed such that electro-osmosis flows preferentially in the opposite (i.e., cathode-to-anode) direction. A similar effect can be achieved by decreasing the pH of donor and receptor solutions below 4 (Kim et al., 1993).

It follows that optimization of nafarelin transport by iontophoresis is a complex challenge because delivery of the peptide into the skin alters the barrier's permeability properties such that a major mechanism of the drug's enhanced permeation (namely, electro-osmosis) is impeded. Thus, increasing nafarelin concentration in the donor phase may lead to more peptide being iontophoresed into the skin but may reduce the amount which actually transports through the membrane. Considerable additional study of this fascinating problem is therefore warranted and forms the basis of further communications (Delgado Charro et al., manuscript in preparation; Rodríguez-Bayón and Guy, manuscript in preparation).

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