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Human Calcitonin Delivery in Rats by Iontophoresis

SOPHIE THYSMAN, CECILE HANCHARD AND VERONIQUE PRÉAT

Université Catholique de Louvain, Ecole de Pharmacie, Unité de Pharmacie Galénique, Av. E. Mounier 73/20, 1200 Brussels, Belgium

Abstract—In-vitro iontophoresis (0·33 mA cm^{-2}) of calcitonin (50 $\mu g\,mL^{-1}$, pH 4) was performed with the hairless rat skin model. Direct current was as potent as pulse current (2·5 kHz on/off 1/1) iontophoresis in promoting transdermal permeation of calcitonin. Increase in duration of current application from 20 min to 1 h did not increase calcitonin flux. Results suggest that calcitonin can be blocked in the skin pores through which it travels or can accumulate in the skin and be progressively released from the depot. In-vivo experiments showed that transdermal iontophoretic administration of calcitonin induced a hypocalcaemic effect in rats.

Peptides with potent pharmaceutical activity are likely to be significant drugs of the next century. The advent of genetic engineering has resulted in a proliferation of new biopharmaceuticals that are orally inactive and need to be administered by subcutaneous injection or intravenous infusion. Indeed, rapid degradation or metabolism in the gastrointestinal tract and low permeability through biological membranes often present a significant barrier to oral delivery. Peptides have an extremely short biological half-life when administered parenterally and repeated injections are often needed (Banga & Chien 1988).

The transdermal route has been thought to have little proteolytic enzyme activities compared with other non-parenteral routes and thus may avoid delivery problems with bioactive peptides. However, because of the low permeability of the skin and the large molecular size of peptides and their ionic character, a means to increase skin permeation must be found. Iontophoresis and permeation enhancers have the potential to overcome the barriers presented by natural biological membranes and avoid the trauma of subcutaneous or intravenous injection (Pitt 1990). Iontophoresis is a technique which allows the delivery of ions or charged molecules into or through the skin using an electrical current. Since peptides and protein molecules can be charged by controlling solution pH below or above the isoelectric point, they may thus penetrate through the skin with the assistance of iontophoresis (Chien et al 1990). Some studies have been performed on the iontophoretic delivery of insulin (6000 Da) (Chien et al 1987; Siddiqui et al 1987; Liu et al 1988; Thysman & Préat 1991), vasopressin (1084 Da) (Lelawongs et al 1989, 1990; Iwakura & Morimoto 1991; Morimoto et al 1992a), TRH (362 Da) (Burnette & Marrero 1986), LHRH (Miller et al 1990), growth hormone releasing factor 1–29 derivative (3929 Da) (Kumar et al 1992a) and 1–44 derivative (5039 Da) (Kumar et al 1992b), salmon calcitonin (3500 Da) (Morimoto et al 1992b), leuprolide (1209 Da) and cholecystokinin-8 (1150 Da) (Srinivasan et al 1990).

Human calcitonin, a polypeptide of 32 amino acids (3527 Da), is one of the calcitropic hormones along with parathyroid hormone and 1,25-dihydroxyvitamin D_3. The major biological effects of calcitonin are the inhibition of bone resorption, increase in the urinary excretion of calcium and inhibition of the absorption of calcium in the gastrointestinal tract.

Calcitonin is currently formulated as a sterile solution for intramuscular or subcutaneous injection in the management of several bone-related diseases requiring long-term therapy. Those include Paget's disease, postmenopausal osteoporosis and malignant hypercalcaemia. Calcitonin is subjected to digestive degradation and has a short half-life in the body of 14 min (Lee et al 1991). Its isoelectrical pH is about 9.

The aim of the present study was to investigate parameters affecting in-vitro iontophoresis of calcitonin through hairless rat skin and to analyse the pharmacodynamic activity of human calcitonin delivered to rats by iontophoresis. Some studies on mechanisms of iontophoretic transport were also performed.

Materials and Methods

Chemicals and animals

Human calcitonin (Cibacalcin) was supplied by Ciba (Basel, Switzerland). Tritiated water and ^{125}I-human calcitonin (3(^{125}I)iodotyrosyl ^{12}calcitonin) were purchased from Amersham International plc (Aylesbury, UK). Aprotinin (6×10^6 int. units mg^{-1}) was provided by ICN Biomedicals (Costa Mesa, CA, USA). The salts used to prepare the buffer (analysis grade) were obtained from UCB (RPL, Leuven, Belgium). All solutions were prepared in ultrapure water (Sation 9000).

The experiments were carried out with 2–3-month-old hairless rats (mutant rat Iops hairless from Iffa Credo, Saint Germain les Abresles, France).

Apparatus and procedures

A two-chamber polycarbonate (Makrolon, Obra, Liège, Belgium) horizontal cell with stirring in the receptor compartment was used for in-vitro studies. The surface area of membrane was 3 cm^2. The freshly excised, full thickness, abdominal skin of hairless rats was removed and mounted between the two compartments with the stratum corneum facing the donor phase. Platinum electrodes (1 × 1 cm)

Correspondence: S. Thysman, Université Catholique de Louvain, Ecole de Pharmacie, Unité de Pharmacie Galénique, Av. E. Mounier 73/20, 1200 Brussels, Belgium.

(platinum pure, SA Johnson Matthey, Brussels, Belgium) were immersed in the solutions (the anode in the donor solution and the cathode in the receptor solution). The electrodes were connected to a constant or a pulse positive square-wave power source.

For all studies, the receptor compartment (7·5 mL) was filled with phosphate buffer (0·024 M) at pH 7·4 made isotonic with glucose. The upper reservoir was filled with 1·5 mL donor solution. Human calcitonin (50 $\mu g\,mL^{-1}$) and [^{125}I]human calcitonin (0·2 $\mu Ci\,mL^{-1}$) were introduced in an acetate buffer (0·01 M) at pH 4. The mean density current (direct and pulsed 2·5 kHz on/off 1/1) applied was 0·33 $mA\,cm^{-2}$. Current was applied for one hour or for 5, 20, 30 or each hour. Samples were taken from the receiver compartment at regular intervals for 7 h and replaced with an equal volume of drug-free buffer.

The radioactivity in the samples was measured by gamma counting (Autogamma Scintillation Spectrometer, Packard). The cumulated quantities were plotted against the time and the fluxes were calculated from the linear portion of the curve. Significant differences were assessed by analysis of variance.

Iontophoresis was performed for 1 h (direct current 0·33 $mA\,cm^{-2}$) with and without human calcitonin (50 $\mu g\,mL^{-1}$) in the donor solution (acetate buffer (0·01 M) pH 4). After stopping the current, donor solution was removed and replaced with a solution of metoprolol as the tartrate salt (7·8 $mg\,mL^{-1}$) in a citrate buffer (0·01 M) at pH 3·5. Direct current was applied (0·17 $mA\,cm^{-2}$) for 6 h. Samples were withdrawn from the lower reservoir and were analysed for metoprolol using HPLC (Thysman et al 1992). Cumulated metoprolol in the receptor compartment after iontophoresis with and without calcitonin were statistically compared using a two-way analysis of variance.

To study the modification of skin permeability to water after iontophoresis, the current was applied (0·33 $mA\,cm^{-2}$) for 30 min or 1 h with a calcitonin solution (50 $\mu g\,mL^{-1}$ in acetate buffer (0·01 M) at pH 4) in the donor compartment. After stopping the current, the upper solution was removed and replaced by tritiated water (1 $\mu Ci\,mL^{-1}$). Water diffusion was measured for 6 h and compared with water flux obtained after calcitonin diffusion performed for the same duration. Liquid scintillation fluid (Ready Safe, Beckman, Belgium) was added to samples and counting was performed in a β-counter (Wallac 1410, LKB, Pharmacia). Diffusion fluxes of water were calculated from the linear portion of the plot of cumulated quantities against time. Statistical comparisons were performed using a variance analysis.

For in-vivo studies, platinum electrodes were clamped in a polyurethane hydrophilic foam of 6 cm^2 (Allevyn, Smith and Nephew, Hull, UK) (Thysman & Préat 1993). The cathode and the anode poles were respectively soaked with 0·9% NaCl and with an acidic solution of human calcitonin (150 $\mu g\,mL^{-1}$) in an acetate buffer (0·01 M) at pH 4. The device was applied on the abdominal skin of the rat anaesthetized by intraperitoneal injection of pentobaritone solution (60 $mg\,kg^{-1}$). Direct or pulsed current (2·5 kHz on/off 1/1) was applied for 30 min or for 3 h at a mean current density of 0·17 $mA\,cm^{-2}$ (1 mA) or 0·50 $mA\,cm^{-2}$ (3 mA). Blood samples were taken by tail incision and sera were frozen until analysed for calcium. Calcium serum concentrations were determined either by atomic absorption (Perkin Elmer 375) or by colorimetric analysis after complexation of calcium with *o*-cresolphthalein (Ca^{2+} test colorimetric, Boerhinger Mannheim, Germany). Changes in serum calcium levels were expressed as percentage of variation ± s.e.m. relative to the value before iontophoresis. The area above the hypocalcaemic effect curve (AAC) was calculated using the trapezoidal rule. Statistical significance of data was assessed using one-way analysis of variance.

Stability studies of calcitonin were conducted with the peptide solution (500 μg/10 mL in acetate buffer (0·01 M) at pH 4) exposed to a direct current (7 mA). An HPLC procedure was used to analyse the samples. The HPLC system consisted of two solvent-delivery systems (model 6000A Waters), a gradient controller (Waters) and a variable wavelength UV detector (model 491, Waters). Human calcitonin and its degradation products were separated on a reversed phase column (μBondapak C18, 30 cm × 3·9 mm, Millipore, Waters) and detected at 230 nm. A linear gradient was effected in 25 min from 25 to 50% of solvent B. Solvent A was an aqueous solution of trifluoroacetic acid (0·1%) (99% Merck, Belgolabo, Overijse, Belgium). Solvent B was trifluoroacetic acid solution (0·1%) in acetonitrile (HPLC grade, for UV analysis, Labscan Ltd, Dublin, Ireland) (Lee et al 1991, 1992).

Results

In-vitro iontophoresis of calcitonin

In-vitro assays were performed to investigate the influence of current profile and duration of current application. Human calcitonin (50 $\mu g\,mL^{-1}$) was introduced in an acetate buffer (0·01 M) at pH 4 because of its higher stability in aqueous solution at this pH (Lee et al 1992) and because Morimoto et al (1992b) have reported the efficiency of in-vivo iontophoretic administration in these conditions.

The sum of ^{125}I recovered in the skin, in the donor and in the receptor compartment was 96% of the total radioactiv-

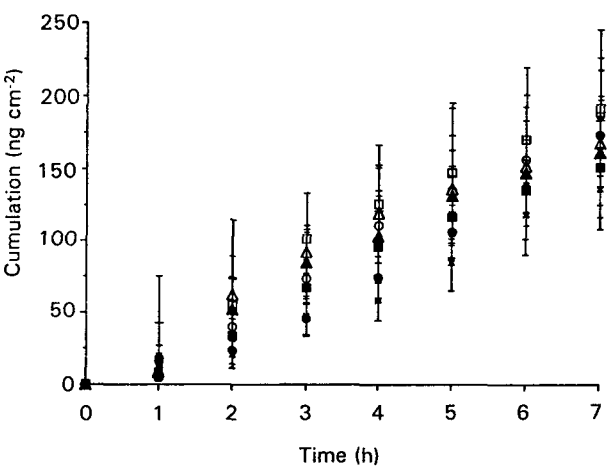

FIG. 1. In-vitro cumulated quantity of calcitonin delivered across hairless rat skin in the receptor compartment vs time. Iontophoresis (0·33 $mA\,cm^{-2}$) was performed with direct current for 5 min each hour (○), for 20 min (□), for 30 min (△) and for 60 min (+), and with pulsed current (2·5 kHz, on/off 1/1) for 5 min each hour (●), for 20 min (■), for 30 min (▲) and for 60 min (×) (mean ± s.e.m, n = 7 to 10).

Table 1. In-vitro fluxes of calcitonin after iontophoresis (0·33 mA cm^{-2}) (n = 7 to 10).

	Flux of calcitonin (ng cm^{-2} h^{-1})	
Duration of current application	Direct current	Pulsed current (2·5 kHz on/off 1/1)
5 min hourly	27·62 ± 3·48	31·60 ± 4·59
20 min hourly	23·26 ± 5·61	23·04 ± 4·64
30 min hourly	17·24 ± 0·90	20·93 ± 3·94
60 min	25·94 ± 5·50	21·35 ± 5·23

ity of the donor solution before iontophoresis (30 min direct current 0·33 mA cm^{-2}), followed by 6 h 30 min of diffusion.

No degradation was detected during the first 2 h of the stability study. Nevertheless, after 3 and 7 h of electrical field exposure (1 mA/1·5 mL or 1 mA/75 μg), the percentages of degradation were 10 and 75%, respectively, and degradation products appeared.

Direct and pulsed current iontophoresis was performed at a mean current density of 0·33 mA cm^{-2} for 5 min at the beginning of each hour, for 20 min or 30 min or for 1 h. The accumulation of the peptide in the receptor compartment was recorded for 7 h. The diffusion of the ionic peptide through the skin was 0·7 ± 0·4 ng cm^{-2} h^{-1}. During iontophoresis the cumulated quantity in the receptor compartment increased linearly with time (Fig. 1). No difference was observed between fluxes obtained with direct or pulse current iontophoresis ($P < 0·05$) (Table 1). The flux was not influenced by the duration of iontophoresis as 20 min, 30 min, 60 min or 5 min hourly current applications induced the same flux and the same cumulated quantities in the receiver (Table 1). The flux of calcitonin was constant over a period of 7 h, although current application lasted for a maximum of 1 h, suggesting that the peptide could be accumulated in the skin reservoir and progressively leak out. The cumulated quantity of peptide in the receptor compartment was significantly slowed (< 5 ng cm^{-2} h^{-1}) when the current was applied for 7 h without interruption. However, calcitonin is strongly degraded by prolonged iontophoresis.

Calcitonin could be degraded by proteolytic enzymes. To check whether enzymatic degradation could affect the iontophoretic kinetics, aprotinin was added to the peptidic solution (1·2 × 10^6 and 3·6 × 10^6 int. units mL^{-1}). This enzymatic inhibitor was reported to protect calcitonin against proteolytic degradation (Morimoto et al 1992b). However, aprotinin did not modify the kinetics of transdermal iontophoresis of radioactive calcitonin performed for 5 min each hour (data not shown).

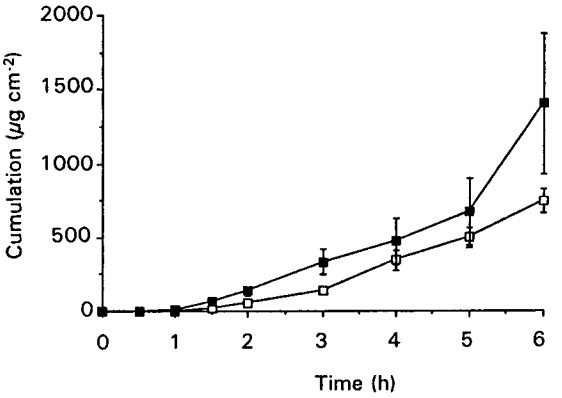

FIG. 2. Effect of calcitonin iontophoresis on the iontophoresis of metoprolol through the same skin sample. Iontophoresis (direct current 0·33 mA cm^{-2}, 1 h) was performed with (□) and without (■) calcitonin. Direct current iontophoresis (direct current 0·17 mA cm^{-2}, 6 h) was then processed with metoprolol (n = 4).

The enhancement of current application duration does not induce an increase in the skin permeation of the peptide. To check whether human calcitonin was able to block the route of passage, iontophoresis was performed in-vitro and skin permeability was then analysed.

Metoprolol iontophoresis was performed after iontophoresis made with and without calcitonin in the donor compartment. Metoprolol was chosen as the second drug because its iontophoretic transport has been previously optimized (Thysman et al 1992). The iontophoretic permeation of metoprolol through the skin after calcitonin iontophoresis was slow compared with the metoprolol permeation observed after acetate buffer (0·01 M, pH 4) iontophoresis. Cumulated quantities of metoprolol detected in the receptor compartment were significantly different at 1 and 3 h ($P < 0·05$). This suggests that calcitonin could be stopped in the skin and could decrease the iontophoretic transport of another drug or calcitonin itself (Fig. 2).

To confirm the modification of skin permeability after calcitonin iontophoresis, other experiments were performed. The measurement of water flux through the skin is a simple method and a good model to study skin permeability as this solvent and electrolytes could take the same route for transdermal migration (Siddiqui et al 1985; Clemessy et al 1991). The diffusion fluxes of water through hairless rat skin were measured after iontophoresis. The results were compared with intrinsic permeability. Direct current was applied for 30 min or 1 h at a mean density current of 0·33 mA cm^{-2}. Compared with intrinsic skin permeability, water flux through the membrane was enhanced after 30 min ionto-

Table 2. Effect of iontophoresis duration on skin permeability for water.

	Water flux (μL cm^{-2} h)		
Duration of iontophoresis	Direct current 0·33 mA cm^{-2}	Pulse current 2·5 kHz on/off 1/1	Calcitonin diffusion
30 min	10·5 ± 7·0*	8·3 ± 1·1*	1·5 ± 0·7
60 min	12·5 ± 0·8*	10·9 ± 1·0*	1·7 ± 0·2

Water fluxes were measured for 6 h after calcitonin iontophoresis performed with direct current, with pulse current or after calcitonin diffusion (mean ± s.e.m, n = 5, *$P < 0·05$ vs diffusion).

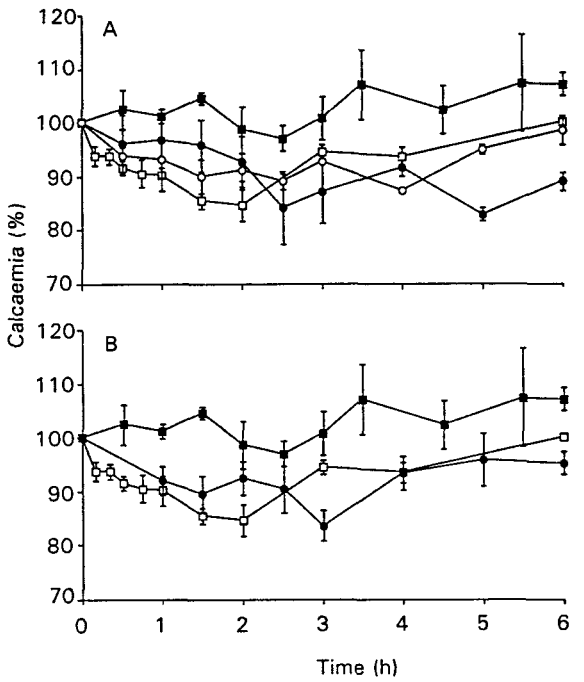

FIG. 3. Effect of calcitonin iontophoresis on the change in serum calcium levels in rats. A. Pulsed current (2·5 kHz on/off 1/1) (●) or direct current (○) was applied for 30 min at a mean density of 0·50 mA cm^{-2}. B. Pulsed current (2·5 kHz on/off 1/1) was applied for 3 h at a mean density of 0·17 mA cm^{-2} (●). Results were compared to control (■) and intravenous injection (7 μg kg^{-1}) (□) (mean ± s.e.m., n = 3).

phoresis (0·33 mA cm^{-2}). No difference between enhancement of skin permeability after iontophoresis with direct or pulsed current was observed. The prolongation of iontophoresis to 1 h did not modify the permeability for water compared with 30-min iontophoresis (Table 2).

In-vivo administration of calcitonin in rats

In-vivo iontophoretic administration of calcitonin was performed to study the influence of current density and duration of current application and to verify the efficiency of this process in inducing hypocalcaemic effects in rats.

Hypocalcaemic effects induced by iontophoresis were compared with those obtained after anaesthesia (control) or intravenous injection of human calcitonin (7 μg kg^{-1}). Without current application, no hypocalcaemic effect was observed (control) (Fig. 3).

Intravenous injection induced a 15% decrease in the calcium serum concentration after 2 h. Afterwards, calcaemia increased to control values, 6 h after injection.

Short iontophoresis (30 min) with low current density (0·17 mA cm^{-2}) did not produce any calcaemia variation compared with control values (not shown). Nevertheless, an increase in duration or in current density induced an important biological effect. The area above the curve obtained after various iontophoresis experiments is shown in Table 3.

A short period of iontophoresis (30 min) induced a significant decrease in calcaemia only if the current density was enhanced to 0·5 mA cm^{-2}. Both current profiles were used. The areas above the curve after direct or pulsed current iontophoresis were not significantly different but pulsed current induced more prolonged effects since calcaemia observed 5 and 6 h after the beginning of the pulsed current iontophoresis was significantly lower ($P < 0·05$) than those measured at the same time after direct current application (Fig. 3).

After 3-h pulsed current iontophoresis (0·17 mA cm^{-2}), the maximum decrease in calcaemia appeared at the end of current application. The calcaemia diminution was the same as after intravenous injection but it was prolonged. Direct current iontophoresis could not be performed for 3 h because of the enhancement of skin resistivity.

Iontophoresis for 30 min with a pulsed current density of 0·5 mA cm^{-2} was as efficient as iontophoresis for 3 h with a pulsed current density of 0·17 mA cm^{-2} in inducing hypocalcaemia in rats. Nevertheless, the total quantity of electrical charge travelling through the skin was reduced with short iontophoresis. Indeed, the product of current density and iontophoresis duration is one-half that after the shorter treatment.

Discussion

The aim of this study was to investigate in-vitro transport of calcitonin and the influence of parameters on transdermal kinetics and mechanisms of transport. Iontophoretic administration of calcitonin in rats was performed to assess the potentiality of this process for the transdermal delivery of peptides.

The first parameter analysed was current profile. Direct and pulsed current were equally potent in promoting in-vitro iontophoretic transport of calcitonin.

After in-vivo iontophoresis with direct or pulsed current, the areas above the curve of percentage of calcium level against time were not significantly different. However, the pharmacological effects seem to be more prolonged after pulsed current iontophoresis. This highest efficiency of pulsed current was also reported in-vivo for insulin by Chien et al (1987), Liu et al (1988) and Thysman & Préat

Table 3. Area above curve (% calcaemia h) of calcaemia variation vs time (from 0 to 6 h).

Control			−9·28 ± 13·9 (n = 4)
Intravenous (7 μg kg^{-1})			48·38 ± 6·15 (n = 4)*
Iontophoresis		Direct current	Pulsed current
0·17 mA cm^{-2}	3 h		48·96 ± 15·23 (n = 3)*
0·17 mA cm^{-2}	30 min	−0·42 ± 21·43 (n = 4)	−3·57 ± 12·96 (n = 4)
0·50 mA cm^{-2}	30 min	45·75 ± 3·40 (n = 3)*	62·20 ± 21·83 (n = 3)*

*$P < 0·05$ vs control, t values were determined by analysis of variance followed by Scheffé's test.

(1991), and for vasopressin by Chien et al (1990). However, pulsed current was as potent as direct current for the in-vitro iontophoresis of arginine-vasopressin (Lelawongs et al 1990) and somaturelin (Kumar et al 1992a). The difference between the two behaviours could be related to the size of the peptide. The modification of skin permeability after iontophoresis can not be involved in the difference between the profiles since direct and pulsed current application induced the same enhancement of skin permeability for water.

The peptide flux was constant over a longer period (7 h) than current application (max. 1 h) suggesting that peptide could be retained in the skin and progressively released at a constant rate. The enhancement of the resistivity of the skin could be related to the accumulation of peptide in the pores of the tissue since it has been shown that current and electrolytes move through the same skin channels (Burnette & Ongpipattanakul 1988; Cullander 1992).

Surprisingly, the transdermal flux of calcitonin did not increase with the prolongation of iontophoresis, although iontophoretic flux of small mol. wt drugs usually increases with the prolongation of iontophoresis (Préat & Thysman 1993; Thysman et al 1994). This could be due to the fact that the peptide is driven into the skin channels and it could be blocked in the pores through which it travels or accumulated in the skin reservoir. When the current was switched off, the peptide slowly diffused out of the skin. The blockage of the skin channel and the loading of the skin depot could be induced by peptide accumulation following precipitation, tissue fixation or aggregation. The transdermal delivery of peptides could be reduced by their low mobility because of their size. This observation could be explained, in part, by the large size of the peptide or by its precipitation in the tissue because of the higher pH of the skin compared with that of the donor solution (Cullander 1992). Morimoto et al (1992b) have reported hypocalcaemic effects of salmon calcitonin iontophoresis performed in rats for 2 h. However, the calcaemia was measured only for 2·5 h.

The slowing of iontophoretic movement of metoprolol through the skin exposed to previous calcitonin iontophoresis also suggests that calcitonin can accumulate in the skin and affect the iontophoretic transport of metoprolol which utilizes the same route across the skin.

The prolongation of current application from 30 min to 1 h did not modify the water skin permeability. However, the prolongation of current application is usually linked with an enhancement of skin permeability (Préat & Thysman 1993; Thysman et al 1994). Calcitonin iontophoresis modified the skin permeability when current was applied for a short time. If the process was prolonged, the enhancement of skin permeability induced by electrical treatment could be balanced by the obstruction of skin channels following peptide accumulation in the skin.

The use of an enzymatic inhibitor did not enhance the in-vitro flux of calcitonin in contrast to the results obtained by Morimoto et al (1992b) during the in-vivo iontophoresis of salmon calcitonin in rats. The enzymatic barrier of peptidases could be less important in limiting the transdermal delivery of peptides during in-vitro permeations than in in-vivo studies.

The hypocalcaemic effects of calcitonin iontophoresis in rats confirm the potential of this process for transdermal peptide administration. The control of the current profile, the current density and duration of iontophoresis allows us to modify the rate of transdermal delivery and also to adjust kinetics of administration to the needs of the patient. In conclusion, our results demonstrate the potential of iontophoresis as a transdermal sustained-release system for calcitonin.

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