Drug Reservoir Composition and Transport of Salmon Calcitonin in Transdermal Iontophoresis

Patrizia Santi,[1,3] Paolo Colombo, Ruggero Bettini, Pier Luigi Catellani, Antonia Minutello, and Nadia Maria Volpato[2]

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Purpose. The aim of the work was to study iontophoretic transdermal administration of salmon calcitonin (sCt) in rabbits, with particular attention to drug reservoir composition. A dry sCt disc, to be dissolved on the application site, was used for preparing the reservoir for transdermal iontophoresis. As a reference drug reservoir, a pad wetted with drug solution was used.
Methods. Experiments were done in rabbits depositing 100 IU of salmon calcitonin on skin and applying anodal iontophoresis. Serum calcium concentration was measured during iontophoresis, passive diffusion and after i.v. administration. Parameters such as pH value and reservoir type were examined.
Results. Transdermal iontophoresis of sCt elicited a decrease in the serum calcium level, whereas, in the absence of electric current, no significant fall was measured. Using the reservoir prepared from drug solution, anodal iontophoresis at pH 4.2 was more effective than at pH 7.4, probably due to higher sCt net positive charge. Using the reservoir prepared from dry disc, similar kinetics and extent of drug effect were observed at both pH values. The reservoir prepared from solid drug deposit concentrated sCt next to the skin.
Conclusions. Anodal iontophoresis for transdermal calcitonin administration shows therapeutical applicability. The type of reservoir is an important parameter affecting sCt transdermal iontophoresis.

KEY WORDS: iontophoresis; transdermal delivery; calcitonin; reservoir; peptide.

INTRODUCTION

Calcitonin is a peptide hormone that lowers calcium plasma concentration in mammals by diminishing the rate of bone reabsorption. It is used for the treatment of Paget's disease and established post-menopausal osteoporosis (1,2). Salmon calcitonin (sCt), 42 times more potent than human calcitonin, is usually administered by the parenteral route. However, nasal spray dosage forms are available in various countries (3,4) and alternative routes for administration are currently under investigation; for example a synthetic analogue of eel calcitonin has been transdermally absorbed using enhancers (5,6).

Iontophoresis is a way to enhance the transdermal transport of drugs, including peptides. Both human (7) and salmon calcitonins (8,9) have been administered in rats by transdermal iontophoresis, providing evidence of peptide absorption. So, iontophoresis could be used for administering calcitonin through skin, particularly because significant hypocalcemic responses are measurable at very low plasma levels (10).

The transdermal iontophoretic technique requires a drug deposit in the form of an aqueous conductive gel or a pad wetted with drug solution in contact with skin. The drug deposit constitutes a part of the reservoir of the Iontophoretic Drug Delivery System (IDDS). In the case of sCt the obvious concerns as to its stability in solution have brought about the development of a drug deposit capable of maintaining drug integrity during storage.

The aim of this work was to study the anodal iontophoretic administration of salmon calcitonin in rabbits, focusing on drug reservoir composition. A solid sCt formulation, to be dissolved on the application site, was designed for preparing the reservoir of the Iontophoretic Drug Delivery System. A thin dry disc was manufactured by the gentle compression of a freeze-dried mixture of sCt and gelatin. The sCt iontophoretic delivery system was assembled by placing the dry disc directly onto moistened skin and covering it with a wetted cellulose pad fixed to the skin by means of an adhesive patch containing the electrode. The wetted pad allowed the immediate dissolution of the disc on the skin. A pad wetted with the drug solution was used as reference drug reservoir. The performance of both types of reservoir was studied in iontophoretic in vivo experiments in rabbits.

MATERIALS AND METHODS

Materials

Synthetic salmon calcitonin BP 1993 (minimum calcitonin potency: 4000 IU/mg); hydrolyzed gelatin (Sigma Chemical Co., St. Louis, MO, USA); trifluoro acetic acid (Romil ltd, Cambridge, UK); acetonitrile (Carlo Erba, Milan I); sodium chloride, sodium acetate, acetic acid, monopotassium phosphate, disodium phosphate were of analytical grade.

For iontophoretic reservoir preparation, pH 4.2 buffer solution (85 mM sodium chloride, 24 mM sodium acetate, 34 mM acetic acid) and pH 7.4 buffer solution (13 mM monopotassium phosphate, 54 mM disodium phosphate) were used.

sCt powder was prepared by freeze-drying a solution containing sCt (100 IU/ml) and hydrolyzed gelatin (20 mg/ml); the dried powder was gently compressed to form a thin disc, with a diameter 13 mm and thickness 0.2 mm.

Methods

Iontophoretic Administration

Animal experimentation adhered to the "Principles of Laboratory Animal Care". New Zealand male rabbits, weighing about 4 kg (Charles River Italia, Calco, I) were made to fast for 24 hours prior to using them in order to eliminate any interference from dietary calcium (11). Fur was carefully removed from the back of the rabbit with an electric clipper 18 hours before calcitonin application.

The IDDS was assembled as follows: the dry disc deposit, containing 100 IU of salmon calcitonin, was placed on the moist, shaved rabbit skin and covered with a cellulose pad

[1] Pharmaceutical Department, University of Parma, Via delle Scienze, 43100 Parma, Italy.
[2] Permanent address: Departamento de Tecnologia Farmacêutica, Faculdade de Farmácia, UFRJ, Rio de Janeiro, Brazil.
[3] To whom correspondence should be addressed (email: santipat@ipruniv.cce.unipr.it).

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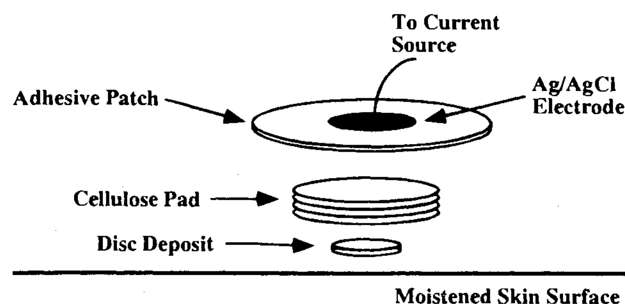

Fig. 1. Sketch of the iontophoretic delivery system containing the disc reservoir.

wetted with 0.3 ml of buffer solution, forming the drug reservoir (12) (Figure 1): the cellulose pad consisted of four discs of filter paper (Whatman 40, Maidstone, Kent, UK; area 2.5 cm^2, thickness 0.2 mm). An adhesive patch containing the electrode (Disposable Ag/AgCl electrodes for electrocardiography, Blue Sensor Q-00-S, Medicotest, Olstykke, DK) was placed over the pad and fixed to the skin; the electrode was connected to the positive pole of the current generator (Neuromed IBR2, IREM, Parma, I). The negative electrode, assembled without calcitonin deposit, was applied within 3–4 cm of the positive one. When sCt solution was used as drug deposit, a cellulose pad, wetted with salmon calcitonin (100 IU) dissolved in 0.3 ml of buffer, was applied to skin. The electrodes were as above.

The experiments were performed at the reservoir pH values of 7.4 and 4.2. Pulsatile direct current, trapezoidal-shaped, frequency 100 Hz and duty cycle 60%, was applied for 30 minutes, corresponding to 20 minutes of effective current treatment.

The maximum intensity of the current was limited to 2 mA, corresponding to a current density of 0.8 mA/cm^2. During all the experiments, the treated skin, visually inspected, never exhibited irritation and/or burns after the iontophoretic treatment.

The experiments in calcitonin administration were repeated on at least three animals. For control purposes, one experiment was carried out using current without calcitonin in order to check the effect of electric current on serum calcium levels. Another experiment on calcitonin passive absorption (without current application) was conducted. The i.v. administration of 25 IU/Kg of sCt was done according to Provasi *et al.* (13).

Prolonged exposure of rabbits to salmon calcitonin reduces the hypocalcemic response (4). In this paper the length of wash-out period after sCt administration by iontophoresis was previously checked. A wash-out period of two weeks was considered enough to recover normal sensitivity to the drug.

Assay Methods

The hypocalcemic response following sCt administration was measured during iontophoretic experiments. Venous blood samples (0.5 ml) were taken from the marginal ear vein by a puncture wound. Collection was carried out by inducing spontaneous bleeding in order to minimize hemolysis. Bleeding was facilitated by applying xylene to the tip of the ear in order to dilate the blood vessels. A blood sample was taken before the beginning of each experiment to determine the basal serum calcium concentration. After spontaneous clotting at room temperature, and within 3 hours of collection, the samples were centrifuged (3500 r.p.m., 5 minutes). The resulting sera were kept frozen until assay. Ionized calcium was measured using a colorimetric method based on the complexation with o-cresolphtalein complexon, in alkaline medium, and spectrophotometric assay at 575 nm (Kit 586-C, Sigma Diagnostics, St. Louis, MO, USA).

The amount of calcitonin in the freeze-dried powder and in solution was determined by HPLC (14) using a reversed phase column (Waters, NovaPak C18 3.9 × 150 mm, Millipore Corporation, Milford, MA, USA) and a Perkin Elmer HPLC System (Isocratic LC pump 250, Perkin Elmer, Norwalk, CT, USA). The mobile phase was acetonitrile: water 30:70 (v/v), both containing 0.1% of trifluoro acetic acid, pumped at a flow rate of 1.5 ml/min. sCt peak was detected at 214 nm (UV/VIS Spectrophotometric detector LC290, Perkin Elmer) with a retention time of about 12 minutes. Standard sCt solutions (15 μg/ml) were prepared in pH 4.2 buffer solution just before use to avoid any degradation of the peptide in aqueous solution.

The stability of the salmon calcitonin solution was checked in the presence of electric current, at both pH values. The salmon calcitonin solution (50 IU/ml) was submitted to 30 minutes of anodal pulsatile current, set at 2 mA. The Ag/AgCl electrode (cathode) was connected to the calcitonin solution by a salt bridge (1 M NaCl gelled with agar 3% w/v).

At the end of the experiment the distribution of calcitonin remaining in the reservoir of the iontophoretic system was checked: sCt remaining on the skin was recovered by wiping the surface with filter paper. Both this residue and each disc of the cellulose pad were extracted with 200 μl of a mixture of water and acetonitrile (1:1) and analyzed for individual sCt content.

RESULTS AND DISCUSSION

In this work the iontophoretic sCt delivery system was assembled on the skin placing a dry disc containing the drug, a wetted cellulose pad and the electrode on top of each other (Figure 1). Three potential advantages were expected: i) stability of the drug stored in solid form and dissolved just before current application, ii) avoidance of direct contact of peptide with Ag electrode, iii) a more concentrated drug solution in direct contact with the skin than with electrode.

Salmon calcitonin (pI 10.4 (15)) is protonated at pH 4.2 and can be electrotransported by anodal iontophoresis (a net positive charge of +3.8 was calculated from the pKa values of each ionizable amino acid residue). At pH 4.2, however, the negative skin charge is partially neutralized (16), and consequently a reduced electroosmotic component of iontophoretic drug transport is expected. The hypocalcemic responses of iontophoretic administration of 100 IU of sCt (pulsatile current, 30 min, 0.8 mA/cm^2), deposited on skin both as disc and solution reservoirs at pH 4.2, were measured.[4] Passive transport of sCt, electric current without sCt, and intravenous administration (25 IU/Kg) were carried out as control experiments. The results,

[4] sCt in solution showed maximum stability at acidic pH values (17). In this paper, after current application (30 min, pulsatile, 0.8 mA/cm^2) to a solution at pH 4.2, the degradation of sCt was measured as about 30% of initial amount.

Table I. Experimental Conditions for sCt Iontophoretic and i.v. Administration and Hypocalcemic Effects (mean value ± sem)

Type of administration	Reservoir Type and pH	Maximum Response (%)	AAIC[a] (% min)
Iontophoresis	disc, pH 4.2	20.8 ± 1.8	2894 ± 212
Iontophoresis	solution, pH 4.2	19.3 ± 4.2	2599 ± 962
Iontophoresis	disc, pH 7.4	17.8 ± 1.6	2405 ± 212
Iontophoresis	solution, pH 7.4	9.9 ± 2.2	1223 ± 856
intravenous (25 IU/Kg)	—	31.0 ± 3.5	9147 ± 628

[a] Area Above Intensity Curve measured with trapezoidal method (10).

as serum calcium variation versus time, are displayed in Figure 2 and summarized in Table I. The absence of hypocalcemic response of passive transdermal transport indicated that sCt was not able to cross the skin, presumably due to the high molecular weight and/or hydrophilicity of the drug. Electric current alone did not modify the serum calcium profile. On the contrary, the application of electric current to pH 4.2 reservoirs containing sCt placed on skin gave clear hypocalcemic responses. A steep decrease in serum calcium level was observed already at current switch off (30 min). The hypocalcemic responses were not significantly different between the two reservoirs used. The maximum lowering of serum calcium, obtained between 60 and 90 minutes, was 20.8 ± 1.8% and 19.3 ± 4.2%, for disc and solution reservoirs respectively (Table I). The calcium level returned to basal value approximately four hours after the beginning of the experiment. Thysman et al. (7) obtained similar results (i.e. calcemy decreasing even after the end of current application) using human calcitonin in rats. They hypothesized that the peptide accumulated in the skin and was slowly released thereafter. Figure 2 also shows the serum calcium profile following intravenous administration of 25 IU/Kg of sCt. Intravenous administration gave deeper and more prolonged hypocalcemic response as compared to iontophoresis. After injection the time required to achieve the maximum lowering of serum calcium of 31.0 ± 3.5% was 4 hours. Therefore, the serum calcium behavior measured after i.v. injection indicated that the continuous calcium level decrease after iontophoresis was typical of calcitonin pharmacodynamics.

Anodal iontophoresis was also performed in experiments at pH 7.4, where sCt still has a positive charge of +2.0 and skin a net negative charge. During transdermal iontophoresis at this pH, electroosmotic volume flow from anode to cathode could assume more relevance for the overall transport of peptide than at pH 4.2. Iontophoretic experiments were performed using the two different reservoir types, disc and solution, in the same conditions of current type, density and application time, as in the previous pH 4.2 experiments. The results obtained are reported in Figure 3 and summarized in Table I. At pH 7.4, the hypocalcemic response obtained with solution reservoir resulted less than with disc reservoir. In fact, the maximum hypocalcemic responses were 17.8 ± 1.6% and 9.9 ± 2.2%, and Area Above Intensity Curve (AAIC) were 2280 ± 373% min and 1223 ± 1156% min, for disc and solution reservoirs, respectively.

Comparing the results obtained at the pH values of 4.2 and 7.4, the two reservoirs, disc and solution, showed different performances depending on the pH. At pH 4.2, the hypocalcemic response obtained with the solution reservoir was significantly higher than that obtained at pH 7.4, both as maximum response and AAIC. On the contrary, with the disc the responses measured at both pH did not differ significantly. These differing responses of disc and solution at both pH values could not be explained in terms of sCt stability. Indeed stability studies showed that 30% of sCt degraded in solution at pH 7.4 under the effect of electric current (30 min, pulsatile, 0.8 mA/cm^2), almost the same result as that obtained at pH 4.2. The lower response at pH 7.4 using solution reservoir could be attributed to the lower net positive charge of peptide at this pH. Nevertheless, using the disc reservoir, the lower positive charge did not affect the hypocalcemic response measured at pH 4.2.

With iontophoretic experimental conditions the same for both disc and solution reservoirs and sCt stability equivalent, the different behavior of the two reservoirs could be due to the

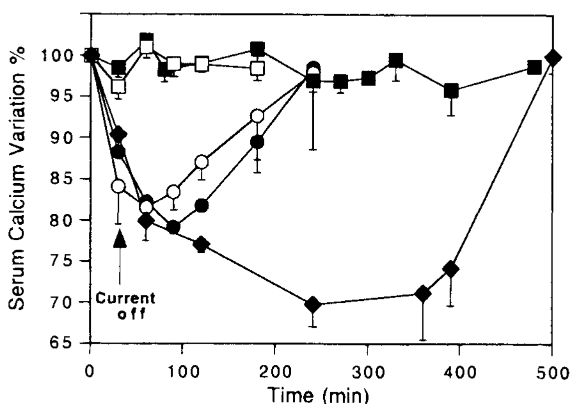

Fig. 2. Serum calcium variation after iontophoretic application of 100 IU of sCt at pH 4.2 (30 min, pulsatile current, 0.8 mA/cm^2: disc reservoir (—●—); solution reservoir (—○—); passive (—□—); control (—■—) and intravenous injection of 25 IU/Kg of sCt (—◆—). Each point represents the mean value ± s.e.

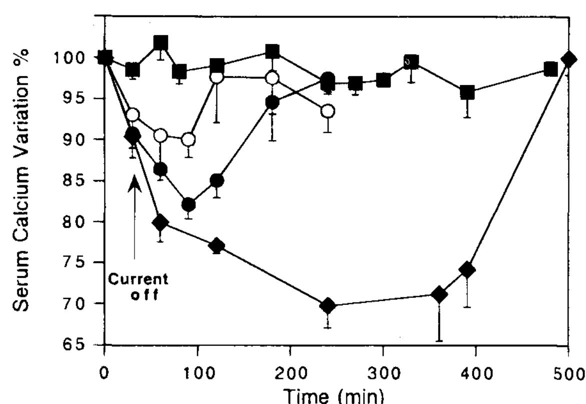

Fig. 3. Serum calcium variation after iontophoretic application of 100 IU of sCt at pH 7.4 (30 min, pulsatile current, 0.8 mA/cm^2: disc reservoir (—●—); solution reservoir (—○—); control (—■—) and intravenous injection of 25 IU/Kg of sCt (—◆—). Each point represents the mean value ± s.e.

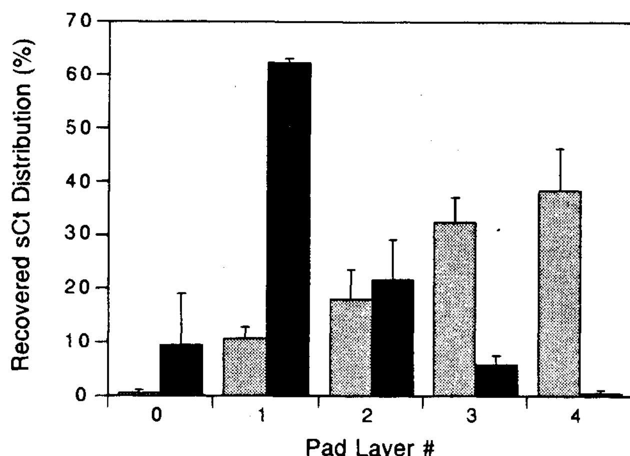

Fig. 4. Recovered sCt distribution in the four layers of cellulose pad and on the skin surface after an iontophoretic experiment (100 IU of sCt, pH 4.2, 30 min, pulsatile current, 0.8 mA/cm^2). # 0 was the skin surface, # 1 was the disc in contact with skin and # 4 was the disc in contact with electrode. Black bars represent the disc reservoir, grey bars represent the solution reservoir (mean value ± s.e).

particular assemblage of the disc reservoir. A more concentrated solution was expected in contact with the skin in case of disc reservoir as compared to solution reservoir. So, distribution of sCt in the reservoir was checked at the end of anodal iontophoresis experiments at pH 4.2. The measurement of the amount of sCt present in the four pad layers of the disc reservoir revealed that more than 70% of the recovered calcitonin remained close to the skin surface after current application (Figure 4). On the contrary using the solution deposit (pad uniformly wetted with drug solution), sCt distribution after current application, was substantially different, showing 10% of drug recovered in contact with skin and about 40% in contact with the electrode.

A more concentrated solution of sCt in contact with the skin, determined by the reservoir prepared from dry disc, improves both electric and electroosmotic transport and limits the effect of a lower ionization of sCt at pH 7.4.

CONCLUSIONS

The results obtained showed that the application of electric current promoted the permeation of salmon calcitonin through rabbit skin, whereas, in the absence of current, a significant decrease in calcium plasma levels was not observed.

The sCt deposit can affect the hypocalcemic response. Using the disc reservoir, a more intense hypocalcemic response was observed at pH 7.4, as compared to the solution. At pH 4.2 both reservoirs behaved in the same way.

Using the reservoir prepared from solution, anodal iontophoresis at pH 4.2 was more effective than at pH 7.4, probably due to the higher sCt net positive charge. On the contrary, using the reservoir prepared from the dry disc, similar kinetics and extent of drug response were observed at both pH values.

The type of reservoir resulted an important parameter affecting sCt transdermal iontophoresis, because solid deposits determine a higher concentration of sCt next to the skin as compared to the solution.

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REFERENCES

1. *SCRIP*, **1565**, 28 (1990).
2. R. Lauro and G. Palmieri, *Acta Toxicol. Ther.* **14**(2):73–83 (1993).
3. M. Hanson, G. Gazdick, J. Cahill, and M. Augustine, in Davis S. S., Illum L., Tomlinson E. (Eds.) *Delivery systems for peptide drugs*, Plenum Press, New York, NY, pp 233–242 (1986).
4. N. G. M. Schipper, S. G. Romeijn, J. Verhoef, and F. W. H. M. Merkus, *Calcif. Tissue Int.* **54**:50–55 (1994).
5. T. Ogiso, M. Iwaki, I. Yoneda, M. Horinouchi, and K. Yamashita, *Chem. Pharm. Bull.* **39(2)**:449–453 (1991).
6. Transdermal route for elcatonin?, *SCRIP*, **1766**:24 (1992).
7. S. Thysman, C. Hanchard, and V. Preat, *J. Pharm. Pharmacol.* **46**:725–730 (1994).
8. K. Morimoto, Y. Iwakura, E. Nakatani, M. Miyzaki, and H. Tojima, *J. Pharm. Pharmacol.* **44**:216–218 (1992).
9. A. K. Banga and Y. W. Chien, *Pharm. Res.* **10**(5):697–702 (1993).
10. P. J. Sinko, C. L. Smith, L. T. McWhocter, W. Stern, E. Wagner, and J. P. Gilligan, *J. Pharm. Sci.* **84**(11):1374–1378 (1995).
11. D. L. Goad, M. E. Pecquet Goad, and H. B. Warren, *JAVMA*, **194**(11):1520–1521 (1989).
12. P. Colombo, P. Santi, Italian Patent Application, MI94A002047 filed 7/10/1994.
13. D. Provasi, A. Minutello, P. L. Catellani, P. Santi, G. Massimo, and P. Colombo, *Proceed. Intern. Symp. Control. Rel. Bioact. Mater.* **19**:421–422 (1992).
14. I. H. Lee, S. Pollack, S. H. Hsu, and J. R. Miksic, *J. Chrom. Sci.* **29**:136–140 (1991).
15. M. L. Heinitz, E. Flangan, C. Orlowski, and F. E. Regnier, *J. Chromatogr.* **443**:229–245 (1988).
16. A. Kim, P. Green, G. Rao, and R. H. Guy, *Pharm. Res.* **10**(9):1315–1320 (1993).
17. K. C. Lee, Y. J. Lee, H. M. Song, C. J. Chun, and P. P. De Luca, *Pharm. Res.* **9**:1521–1523 (1992).