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Free solution capillary electrophoresis of calcitonins and calcitonin tryptic digests

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Abstract

The aim of the present work was the development of a simple capillary electrophoretic strategy, complementary to high-performance liquid chromatography, for the separation of different calcitonins (CTs) and calcitonin tryptic digests. Capillary electrophoresis was carried out with a manual capillary electropherograph with "on column" UV absorbance detection at 200 nm. The separation was accomplished in a 70 cm × 50 μm I.D. bare silica capillary. About 6 nl was loaded into the capillary by means of a split-flow system. Except in particular cases, electric fields of 300 V/cm were used at constant voltage. Separations were carried out in 0.05 *M* citrate buffer pH 2.5 or, alternatively, in 0.05 *M* borate buffer pH 9.5. A complete resolution of salmon, ASU1,7-eel, and human calcitonins was obtained in citrate and borate buffers. Other CT analogues could be separated only in one of the two buffers. Capillary electrophoresis in citrate buffer was also successful in the separation of the four final trypsin cleavage fragments of salmon calcitonin and, at least tentatively, of the nine intermediate cleavage products.

1. Introduction

Calcitonin (CT) is a peptide hormone discovered in 1961 by Copp and Davidson [1]. Its main physiological role in man is the protection of the skeleton against calcium loss, especially during growth and pregnancy. Besides, CT has been hypothesized to be involved in the neuromodulation of pain and to have some functions in behavioral actions, and is thus being considered a neuropeptide.

CT (molecular mass 3500 Da) is composed of 32 amino acids, with a disulphide bridge between the cysteine residues 1 and 7, forming a 7-amino acid ring at the N-terminal end; at the C-terminal end a prolinamide is located. These characteristics are constant in the CTs of different species. The CT sequence has been relatively conserved in phylogenesis, *e.g.* the human and rat analogues have only two modifications; also, salmon and eel CTs have a high degree of homogeneity with amino acid changes only in three positions. On the other hand, human and salmon CTs differ by as many as 17 amino acid residues (ref. 2; see also the Sigma Catalog).

Synthetic CTs resembling the structure of the

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human, salmon and eel analogues are currently used for the treatment of osteoporosis, Paget's disease and hypercalcemia. Changes in the natural sequence of CTs have also been introduced; *e.g.* eel CT has been modified by the replacement of the disulphide bridge with an ethylene bridge, giving the ASU1,7-eel CT [2].

Separation of the different analogues and fragments of CTs is currently accomplished by high-performance liquid chromatography (HPLC) [3]. Quite recently, capillary electrophoresis (CE) has become a new "additional" approach to peptide and protein separation, which, being complementary to HPLC, is suitable for incorporation in the classical strategies used for peptide analysis.

In this respect, the family of CTs and related peptides offers a challenging model for evaluating the potential of CE; in fact, some authors have already reported applications in this field [4,5]. To the best of our knowledge, however, no specific investigation was carried out until now to separate the different commercial analogues of CT. The aim of the present work was the development and validation of a simple CE strategy for the separation of CTs from different species and of salmon CT (sCT) tryptic digests.

2. Experimental

2.1. Calcitonins

Salmon (sCT), rat (rCT) and human (hCT) CTs were obtained from Bachem (Bubendorf, Switzerland) and Sigma (St. Louis, MO, USA); chicken CT (cCT) from Peninsula Labs. (Belmont, CA, USA). ASU1,7-eel CT (elcCT) was a generous gift from SmithKline Beecham (Milan, Italy). Stock solutions of 1 mg/ml of each analyte in water–methanol (50:50, v/v) were stored at −20°C and diluted daily in the working buffers.

2.2. Electrophoretic methods and instrumentation

CE was carried out using a manual capillary electropherograph (Model 3850, Isco, Lincoln, NE, USA) fitted with an "on column" UV absorbance detector set at 200 nm. Electrophoresis was carried out in 65 cm long (45 cm to detector) bare silica capillaries (50 μm I.D.) obtained from Isco. For the study of CT tryptic hydrolysis the length of the capillary was halved, in order to reduce analysis time meeting the velocity of the reaction kinetics. Manual injections were accomplished by a split-flow system, with a split ratio of 1:830. About 6 nl was loaded into the capillary, by "injecting" with a syringe, via the splitter, 5 μl of sample. CE buffers were used after filtration through a 0.45-μm nylon 66 membrane (Alltech, Milan, Italy) and degassing under reduced pressure (water pump) with sonication. Electrophoretic conditions, except in particular cases, were as follows: electric field = 300 V/cm, at constant voltage; buffer: 0.05 *M* citrate pH 2.5 or, alternatively, 0.05 *M* borate pH 9.5, as detailed in the text. The resulting currents were less than 60 μA in both cases. After every run, the capillary was washed, at pH 9.5, with 0.1 *M* NaOH (3 drops) and conditioned with 0.05 *M* borate (5 drops); at pH 2.5, capillary washing was carried out simply with 5 drops of 0.05 *M* citrate buffer. The electroosmotic flow was calculated by injecting water and measuring the migration time of the "negative peak" of water detected at 190 nm.

2.3. Tryptic digestion of sCT

A tryptic digest of sCT was obtained using trypsin immobilized onto cross-linked agarose (TPCK-Trypsin, Pierce, Rockford, IL, USA), with a specific activity ⩾45 TAME units/ml of gel. In brief, sCT was dissolved in a buffer at pH 8.3 containing: 0.1 *M* sodium acetate, 0.01 *M* Tris and 0.1 m*M* calcium chloride. Five microliters of enzyme suspension (corresponding to 0.22 units) were added to 500 μl of a 500 μg/ml sCT solution, at room temperature under continuous mixing. At regular time intervals in the range from 0 to 60 min the suspension was centrifuged at 750 *g* for 3 min to separate the enzyme and a few microliters were collected and injected.

2.4. Degradation of sCT

An old standard of sCT, which had been stored for 3 years in water–methanol (50:50, v/v) at −20°C, was analyzed as sample likely to contain partially degraded sCT.

2.5. HPLC analysis of sCT and CT tryptic fragments

Reversed-phase chromatographic separations of fresh/degraded sCT and CT tryptic fragments were carried out on a C_4 wide-pore column (250 × 4.6 mm I.D.) (Bio-Rad, Richmond, CA, USA) and on a μBondapak C_{18} column (300 × 3.9 mm I.D.) (Waters-Millipore, Bedford, MA, USA), respectively. Gradient elution with mixtures of water–acetonitrile (A: 90:10; B: 20:80) containing a constant percentage (0.01%) of TFA (Carlo Erba, Milan, Italy) was used. Two gradient profiles were used in alternation; the first from 15% to 45% of B in 30 min, the second from 0% B to 30% B in 30 min. Detection was by UV absorption at 214 nm.

2.6. Calculation of charge on CTs

The charge of the different CTs, due to substitutions in the respective amino acid composition, in the working buffers was calculated according to the following equations:

$$C_{pos} = 1/1 + \alpha\ , \quad C_{neg} = \alpha/1 + \alpha \quad \alpha = 10^{(pH - pK)}$$

The pK_a values of amino acid residues (from ref. 6) were used without correction for in-peptide inclusion.

3. Results and discussion

3.1. CE separation of CTs

A complete resolution of salmon (sCT), ASU1,7-eel (elcCT) and human (hCT) CTs was obtained by CE in acidic and basic buffers, *i.e.* 0.05 *M* citrate pH 2.5 and 0.05 *M* borate pH 9.5. The migration pattern of the three CTs at pH 2.5 (Fig. 1) was consistent with the calculated

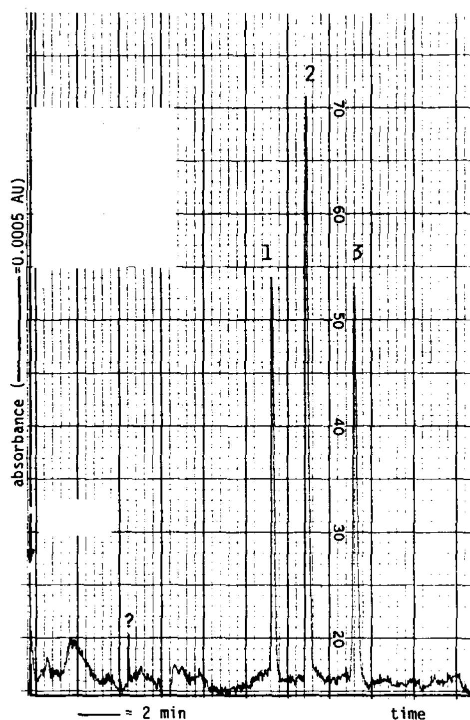

Fig. 1. CE migration pattern of sCT (1), elcCT (2) and hCT (3) (concentration *ca.* 30 μg/ml of each peptide) in 0.05 *M* citrate buffer pH 2.5. Electric field: 300 V/cm. UV detection at 200 nm, range: 0.005 AUFS. Chart speed: 0.5 cm/min. The arrow indicates injection, "?" indicates an unknown peak present in sCT. Other conditions are detailed in the text.

charges, as shown in Table 1. In fact, the fastest peptide was sCT, having the highest positive charge (+4.98), followed by elcCT and hCT with progressively lower calculated charges (*i.e.* +3.94 and +2.96, respectively). Accordingly, cCT with a charge (+4.94) very close to sCT

Table 1
Calculated charge on CTs at pH 2.5 and 9.5

CT	pH 2.5	pH 9.5
sCT	+4.98	+2.82
cCT	+4.94	+1.82
elcCT	+3.94	+0.86
hCT	+2.96	+0.86
pCT	+3.97	+1.90
rCT	+2.96	+0.86

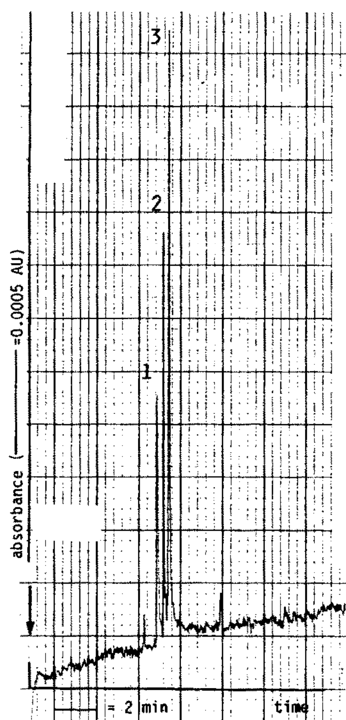

Fig. 2. CE migration pattern of sCT (1), elcCT (2) and hCT (3) (concentration *ca.* 30 μg/ml of each peptide) in 0.05 *M* borate buffer pH 9.5. Electric field: 300 V/cm. UV detection at 200 nm, range: 0.005 AUFS. Chart speed: 0.5 cm/min. The arrow indicates injection. Other conditions are detailed in the text.

overlapped the sCT peak, rCT (+2.96) co-migrated with hCT (+2.96) and pCT (+3.97) with elcCT (+3.94).

At a first glance, the same separation mechanism seemed to occur also in borate buffer, considering the higher electroosmotic flow (at pH 9.5), contributing to the crowding of peaks (Fig. 2). sCT migrated faster than elcCT and hCT, according to the highest calculated charge (+2.82 *vs.* +0.86) (Fig. 2). However, quite surprisingly, hCT (and rCT), still positively charged (+0.86), migrated more slowly than the electroosmotic flow (Table 2), and complete separation was observed between elcCT and hCT (Fig. 2), having the same calculated slightly positive charge of +0.86. In contrast, cCT (+1.82) and elcCT (+0.86) were completely superimposed (Fig. 3, left), but separation, although incomplete ($r = 0.5$), was achieved by halving the electric field (150 V/cm) (Fig. 3, right). These apparent inconsistencies may be explained in different ways, which, however, is well beyond the scope of the present study. Conceivably, subtle charge/shape changes induced by the pH in the capillary and/or by formation of cyclic diols by interaction of borate with threonine and serine OH residues could be suggested. Also, voltage-dependent Joule heating of the capillary, affecting viscosity and pH of the buffer [7], is probably an important factor in the observed phenomena.

The aims of the present work were mainly qualitative. However, a basic quantitative parameter in method validation, precision, was nevertheless studied. The reproducibility of the migration times and peak heights of sCT and hCT (relative to elcCT) is shown in Table 2: the

Table 2
Inter-day precision of migration times and peak heights of sCT and hCT ($n = 6$)

CT (30 μg/ml)	E.O. flow (mean ± C.V.) (min)	Migration time (mean ± C.V.) (min)	Peak height (mean ± C.V.) (ratio to elcCT)
pH 2.5			
sCT	35.0 ± 1.68%	11.6 ± 2.00%	0.68 ± 1.75%
hCT	35.0 ± 1.68%	15.6 ± 1.78%	0.69 ± 1.15%
pH 9.5			
sCT	6.25 ± 1.00%	6.05 ± 1.81%	0.70 ± 1.92%
hCT	6.25 ± 1.00%	6.65 ± 1.00%	0.47 ± 2.11%

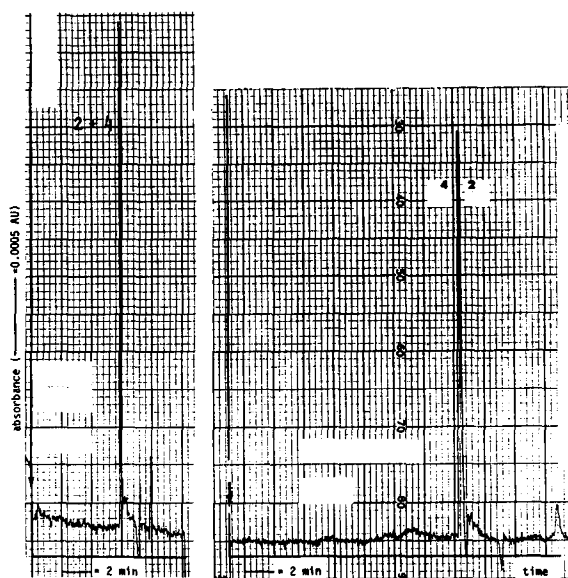

Fig. 3. Comigration of elcCT (2) and cCT (4) at an electric field of 300 V/cm (left) and partial separation of the two peptides at 150 V/cm (right). The arrows indicate injections. Other conditions are as in Fig. 2.

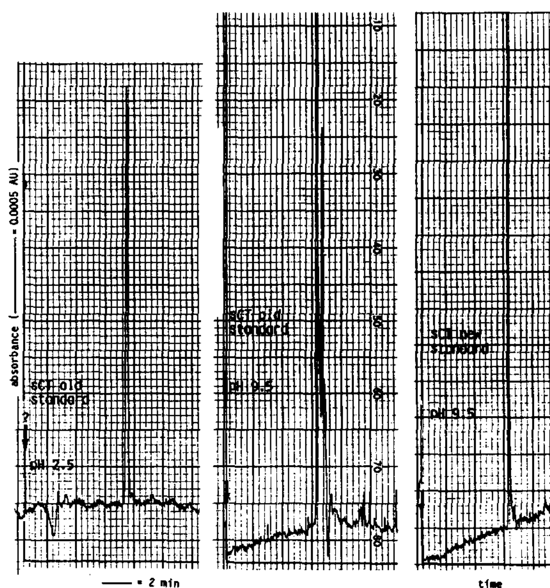

Fig. 4. Mildly degraded sCT assayed by CE either at pH 2.5 (left) or 9.5 (middle). Fresh sCT assayed at pH 9.5 (right). Conditions are as in Fig. 1 for pH 2.5 and as in Fig. 2 for pH 9.5. [Important notice: the electropherogram at pH 2.5 (left), for technical reasons, lacks the first part, with the injection point; thus, the arrow with "?" does not indicate injection, but the unknown peak constantly present in sCT, also appearing in Fig. 1 and there indicated with "?".]

results are comparable to those of other CE methods.

Pure sCT, stored in a freezer for 3 years in water–methanol (50:50, v/v), was assayed by CE under the above described basic and acidic conditions (Fig. 4). At pH 9.5, it was demonstrated that the peptide had degraded and several peaks appeared. On the other hand, at pH 2.5, only a single peak was observed. Of course, a fresh sCT standard gave a sharp and single peak at pH 9.5. The degradation of the 3-year-old sample was confirmed also by HPLC (data not shown), but the products were not further characterized.

3.2. CE of the peptide fragments from the tryptic digest of sCT

Trypsin cleaves sCT at 3 points, giving rise to as many as 9 intermediate and 4 final cleavage products (*i.e.* fragments 1–11, 12–18, 19–24 and 25–32). The tryptic pattern after 5 and 30 min of incubation with the solid-phase enzyme is shown in Fig. 5.

No particular attempts were made to identify the peaks. However, the electropherogram obtained after 30 min incubation, with 4 peaks nicely separated, meets the theoretical number of end-products of the reaction, and is substantially superimposable on that reported by Camilleri *et al.* [4]. The main difference is the presence, in Camilleri's electropherograms, of several small peaks, which could be ascribed to interfering compounds present in the enzyme solution. In our experiments, the solid-phase enzyme, conceivably, did not contaminate the reaction mixture, hence giving much cleaner electropherograms.

Because of the rapidity of separation, it was possible to accomplish repeated CE assays before completion of the cleavage. After 5 min of incubation, 10 almost completely resolved peaks could be distinguished; *i.e.* a number corresponding to the theoretical expectations and to a

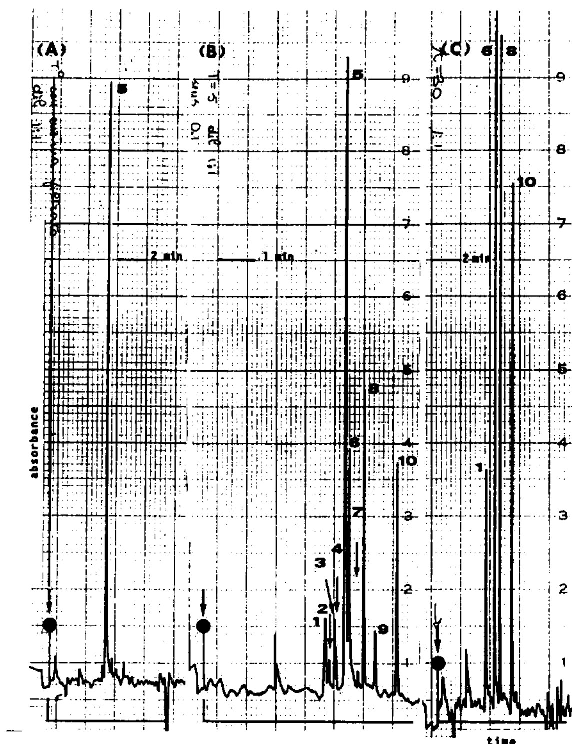

Fig. 5. Kinetic study of sCT tryptic degradation: (A) $t=0$; (B) $t=5$ min; (C) $t=30$ min. Conditions: (A) and (C) as in Fig. 1, except capillary length, halved; (B) as for (A) and (C), except detector range: 0.01 AUFS and chart speed: 1.25 cm/min.

previous HPLC study (Fig. 6). It must be pointed out that the full electropherogram was produced in less than 6 min, whereas the liquid chromatographic procedure required 35 min. No attempt was made to compare and interpret the HPLC and CE retention/migration patterns of the individual fragments. However, because of the substantial difference in the separation mechanisms, there is no reason to doubt that HPLC and CE give complementary results.

4. Conclusion

The above described results, once again demonstrate the usefulness of free solution CE in the study of peptide drugs and specifically of CTs. In particular, CTs provide challenging separation problems: in fact the different analogues show differences in the amino acid sequence, but have the same molecular mass and, in aqueous solution, a low degree of secondary structure [8].

CE carried out at pH 2.5 was able to separate the CT analogues differing in charge, such as sCT, elCT and hCT. However, cCT and sCT, the charge difference of which is minimal, comigrated. On the other hand, at pH 9.5 it was possible to separate elCT and hCT, despite their high similarity in charge, and some degradation products of sCT, which at pH 2.5 co-migrated. Although CE seems to be superior to HPLC, at least terms of rapidity, in the separation of sCT tryptic fragments, the combined use of both techniques seems the main innovation provided by CE in the study of bioactive peptides.

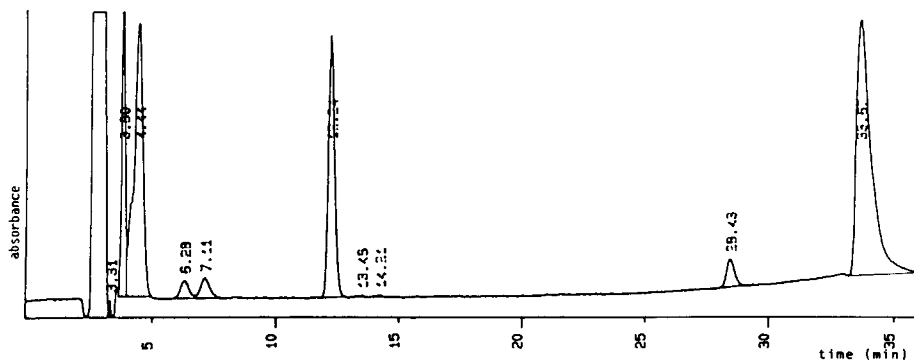

Fig. 6. HPLC chromatogram of sCT tryptic digest after 5 min of incubation (uncleaved sCT is the last peak), to be compared with Fig. 5(B). For analytical conditions, see text.

5. Acknowledgement

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