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Cation-exchange high-performance liquid chromatography of synthetic salmon calcitonin

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ABSTRACT

Several species of calcitonin, namely, porcine, human, and salmon, are of clinical importance. Previous studies have shown that reversed-phase high-performance liquid chromatography (HPLC) can be used for the analysis of synthetic calcitonin. To confirm the homogeneity of synthetic HPLC purified salmon calcitonin, an analytical method, based on cation-exchange HPLC with a linear sodium chloride gradient in phosphate buffer was developed. The method is capable of selectively analyzing salmon calcitonin in complex synthetic matrices, separating it from synthetic by-products and degradation products, and provides a rapid and precise chemical assay for determining calcitonin content and quality.

INTRODUCTION

Salmon calcitonin (SCT) is a single-chain polypeptide, consisting of 32 amino acid residues. It contains a disulfide bridge at the amino terminus, resulting in a seven-residue cyclic structure. The peptide has a carboxyl-terminal proline. Calcitonin is produced by parafollicular cells, wich are located in the thyroid in mammals but are associated with the ultimobranchial body in lower animals, such as the salmon. The major action of calcitonin is to inhibit osteoclast-mediated bone resorption. The hormone is used in the treatment of Paget's disease of the bone, post-menopausal osteoporosis, and in the treatment of hypercalcemia.

Synthetic SCT is prepared by the Merrifield solid-phase peptide synthesis technology [1] with modifications, and purified by ion-exchange, reversed-phase and gel chromatography.

Peptides manufactured by chemial synthesis require exacting definition of their identity. It is necessary to show that the end product of the synthesis has the intended structure and that undesirable synthetic by-products, if detected, are removed from the target peptide, or at least, that quantities of by-products are limited. Finally, it is necessary to show that the characteristics of successive production lots are consistent.

In recent years, reversed-phase high-performance liquid chromatography (RP-HPLC) has become the method of choice for the separation and analysis of a wide variety of peptides and proteins [2–8]. The ability of RP-HPLC to separate deletion analogues and derivatives of SCT has been described [3]. Porcine (PCT), human (HCT), and SCT are resolved easily. The ability to resolve calcitonins from various

species has been noted by Corran and Zanelli [9] and justifies the use of RP-HPLC, not only for identification, but also for determining the level of impurities.

The impurities generated in the synthetic salmon calcitonin production process may differ from the parent molecule in either size, charge, or hydrophobicity.

RP-HPLC is used to demonstrate the homogeneity of SCT preparations. This technique separates SCT from peptide impurities based on relative hydrophobicity. Since RP-HPLC purification is employed as part of the manufacturing process for SCT, another method employing a different separation principle than RP-HPLC is needed. Experiments that have led to the development of cation-exchange chromatography are described.

EXPERIMENTAL

Materials

PCT and SCT were obtained from the Manufacturing Laboratories of Armour Pharmaceutical Company. HCT was obtained from Sigma (St. Louis, MO, U.S.A.). UV-grade acetonitrile (Burdick & Jackson, Muskegon, MI, U.S.A.), analytical-grade reagents, and distilled, deionized Mill-Q water (Millipore, Bedford, MA, U.S.A.) were used.

Method

Cation-exchange HPLC. Cation-exchange HPLC of SCT was performed on a Bio-Gel TSK, SP-5PW (75 × 7.5 mm I.D.) column (Bio-Rad, Richmond, CA, U.S.A.). The flow-rate was 1.0 ml/min. The mobile phases consisted of 20 mM potassium phosphate buffer (pH 6.8), containing 5% (v/v) acetonitrile and 20 mM potassium phosphate buffer, 500 mM NaCl (pH 6.8), containing 5% (v/v) acetonitrile. A linear gradient from 0–100% B in 20 min was employed. The injection volume was 200 μl and the peptides were detected at 220 nm and 0.1 a.u.f.s.

Instrumentation. A Beckman Model 322 liquid chromatograph, equipped with 112M pumps, and a Hewlett-Packard Model 1000F series computer, equipped with a Beckman computer automated laboratory system was used. The pumps were connected to a SP8500 dynamic mixer prior to a WISP (710B) autosampler. A Kratos Spectroflow 773 provided UV detection at 220 nm.

RESULTS

Comparison between RP-HPLC and cation-exchange HPLC

A comparison of the cation-exchange chromatograms (Fig. 1) and the reversed-phase chromatogram (Fig. 2) for lot D520-148A, shows that the cation-exchange method is capable of separating SCT from peptide impurities with a resolution very similar to RP-HPLC.

The resolving power of cation-exchange HPLC is further emphasized by Fig. 3, which shows the separation of three deletion peptides from SCT. The delection analogues, des(Asp-3)SCT, des(Leu-16)SCT, and (22–32)SCT fragment are easily separated from each other and from SCT.
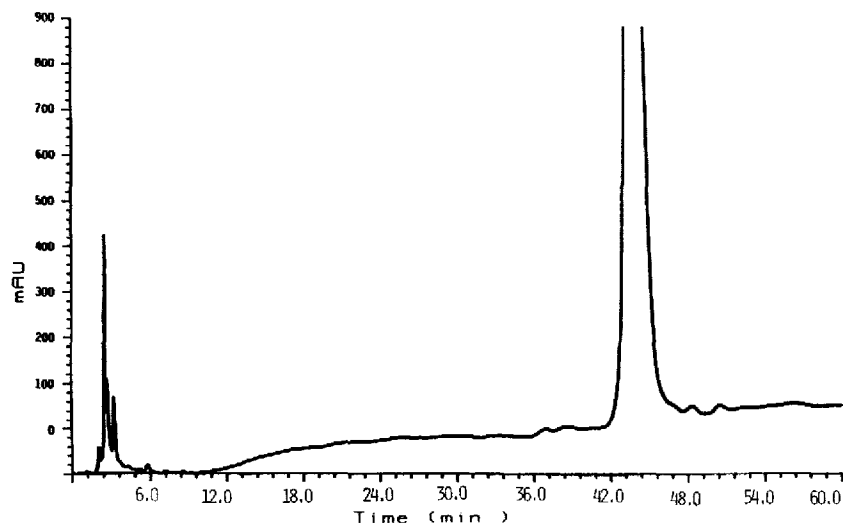
Fig. 1. RP-HPLC of SCT. Column, B&J OD5 C_{18} (250 × 4.6 mm I.D.). Linear AB gradient (1% B/min), where solvent A is 0.054 M KH_2PO_4 in water–acetonitrile–methanol (75:20–5, v/v/v) (pH 2.86) and solvent B is acetonitrile–methanol (80:20, v/v), flow-rate, 1 ml/min; injection volume 200 μl, concentration 0.2 mg/ml. Detection at 220 nm.
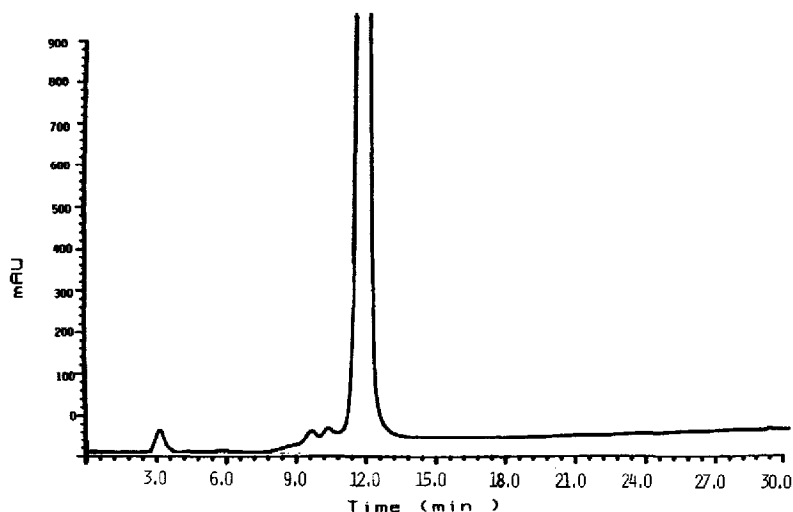

Fig. 2. Cation-exchange HPLC of SCT. Column Bio-Rad Bio-Gel TSK, SP-5PW (75 × 7.5 mm I.D.). Linear AB gradient (5% B/min), where solvent A consisted of 20 mM potassium phosphate buffer (pH 6.8), containing 5% (v/v) acetonitrile and solvent B is 20 mM potassium phosphate buffer, 500 mM sodium chloride (pH 6.8), containing 5% (v/v) acetonitrile. Injection volume 200 μl, concentration 0.2 mg/ml. Detection at 220 nm.
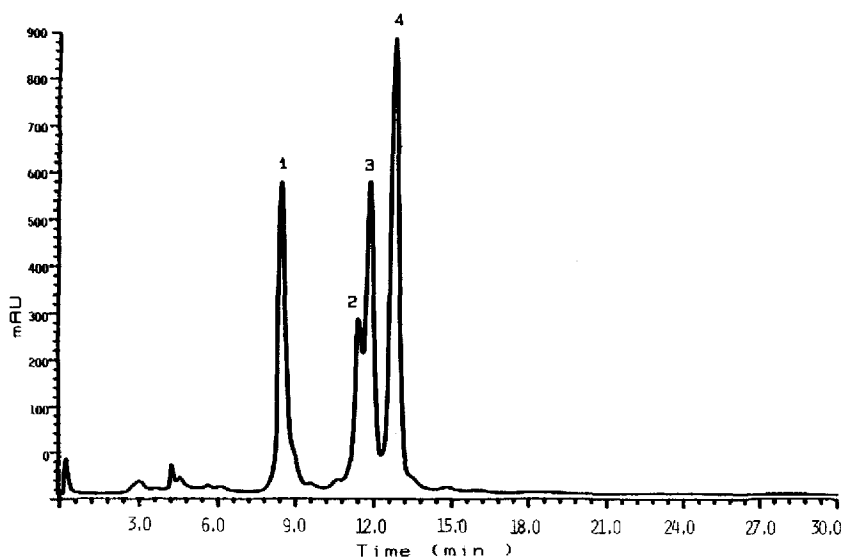
Fig. 3. Cation-exchange HPLC of a mixture of SCT analogues. Peaks: 1 = des(Asp³)SCT; 2 = des-(Leu¹⁶)SCT; 3 = SCT; 4 = fragment (32–22)SCT. Conditions as in Fig. 2.
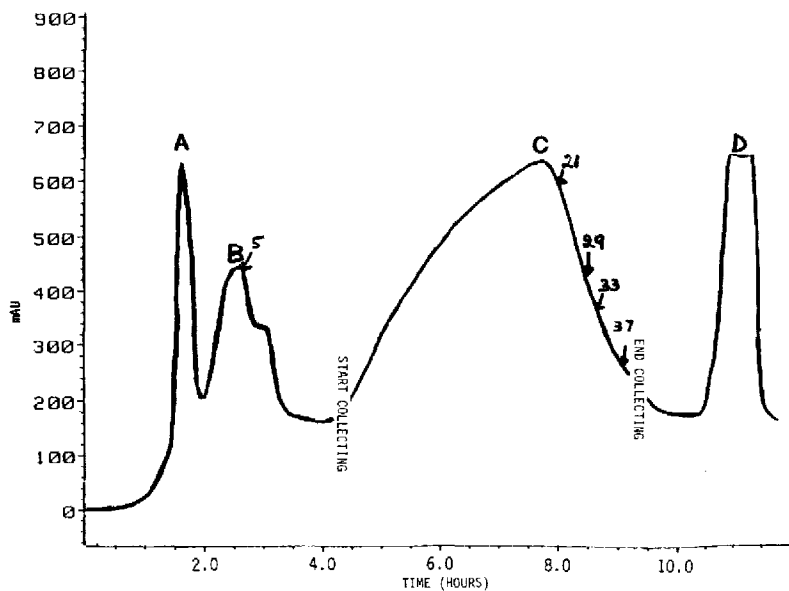

Fig. 4. Production ion exchange (column 3) chromatography of crude calcitonin solution. Fractions between 4.5 and 9.0 h are normally saved for further purification by RP-HPLC. Detection at 220 nm.
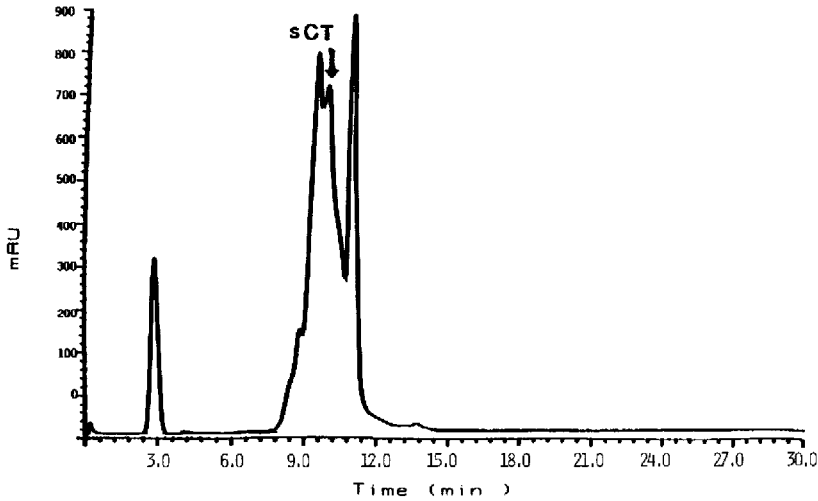
Fig. 5. Cation-exchange HPLC of a mixture of peptides from position B-5 of process ion-exchange column 3. Fraction retention time = 2.7 h. Column, gradient and detection as in Fig. 2. Injection volume 20.0 μl. Concentration (1–1000) of process ion-exchange column 3 eluent.

Resolution and selectivity

The ability of cation-exhange HPLC to separate SCT from by-products of the manufacturing process was investigated by chromatographic analysis of in-process samples obtained during the purification steps (*e.g.*, the eluent from a production in-exchange column). The elution profile of this column monitored at 220 nm shows four peaks, A, B, C, and D as identified in Fig. 4. Figs. 5–7 show the cation-exchange
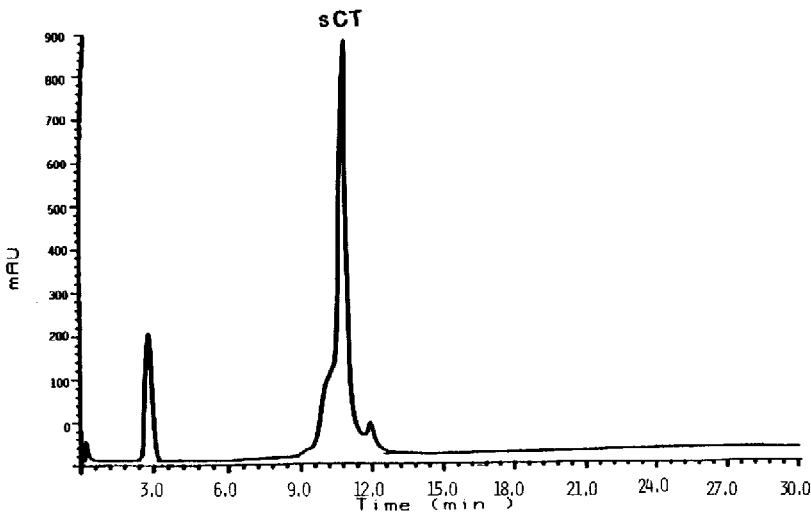

Fig. 6. Cation-exchange HPLC of peptides from position C-21 of process ion-exchange column 3. Fraction retention time = 8.2 h. Column, gradient and detection as in Fig. 2. Injection volume 20.0 μl.
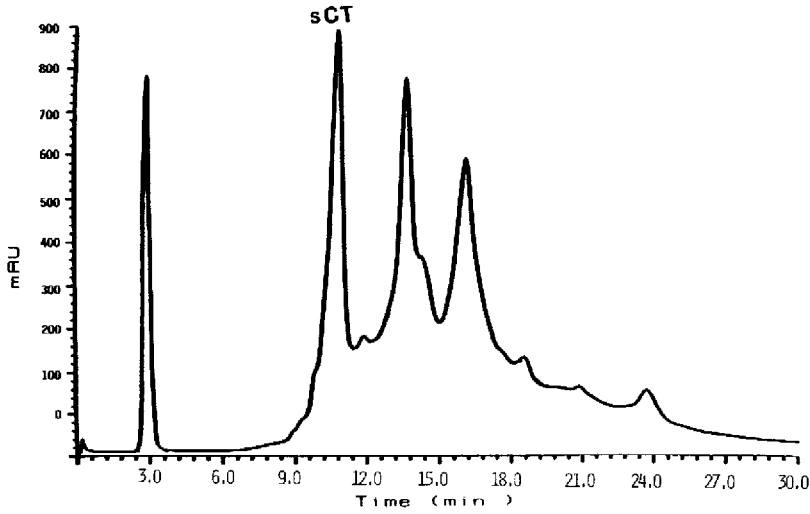
Fig. 7. Cation-exchange HPLC of peptides, polymers, dimers, and trimers from peak D of process ion-exchange column 3. Fraction retention time = 11.0 h. Conditions as in Fig. 2. Concentration (1–1000) of process ion-exchange column 3 eluent.
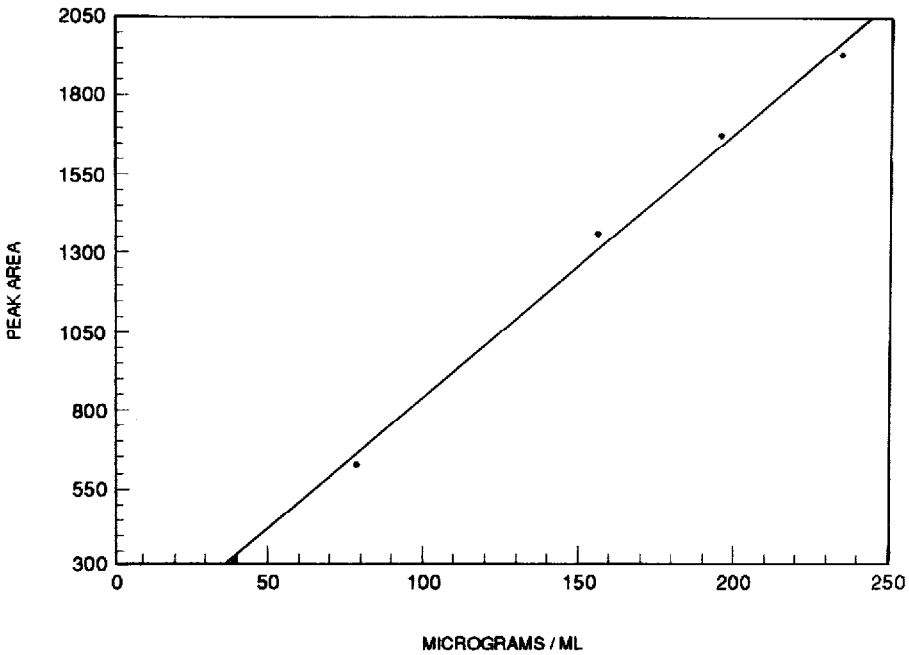

Fig. 8. Calibration plot for SCT. Peak area percent is used to correct for actual concentration of SCT peptide in the samples. Correlation coefficient = 0.999. x-intercept = 0.85 mg/ml, y-intercept = −0.156 area counts.

chromatograms for the analysis of several fractions taken from peaks B, C, and D of the eluent from the production column of the purification process. These chromatograms show that cation-exchange HPLC has the sufficient resolving power to separate SCT from a complex synthetic matrix, which contains fragments, deletion, and chemically modified sequences of SCT.

Calibration plot

The linearity of response has been investigated from 50–150% of the operating range of the assay. The pertinent statistical parameters as calculated for the peak response and the calibration plot are shown in Fig. 8. In general, the data exhibit excellent linearity over the specified range regardless of whether quantification is performed by peak area (correlation coefficient greater than 0.998) or peak height (data not shown).

DISCUSSION

Our studies show that cation-exchange HPLC is capable of separating and selectively analyzing HPLC-purified salmon calcitonin from its process by-products, impurities, and related peptides with resolution similar to that obtained by RP-HPLC [3,10].

These studies with cation-exchange HPLC further confirm the homogeneity of HPLC-purified salmon calcitonin. As shown, cation-exchange HPLC easily identifies SCT analogues which are similar in molecular weight, but have different molecular charges. Variation of the acetonitrile content and buffer salt concentration suggests that the cation-exchange HPLC system separates the peptides mainly by the net charge of the peptides.

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