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Reversed-phase high-performance liquid chromatography of radioiodinated salmon calcitonins

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Abstract

Reversed-phase HPLC conditions for simultaneous separation of salmon calcitonin, mono- and di-radioiodinated salmon calcitonins and their tryptic digested fragments have been developed. Salmon calcitonin was radioiodinated with $Na^{125}I$ by the iodo-beads method. After solid-phase extraction from the reaction mixtures using C_{18} Bond Elut cartridges, mono- and di-radioiodinated salmon calcitonins were separated from each other, as well as from unlabeled salmon calcitonin, on a Bondclone 10 C_{18} column (300×7.8 mm I.D.) by isocratic elution with 0.1% trifluoroacetic acid in 34% aqueous acetonitrile. The characteristics of either iodinated peptides or unlabeled salmon calcitonin were evaluated on the basis of UV absorbance (215 and 280 nm), fluorescence (λ_{ex}=282 nm, λ_{em}=310 nm) and measurement of specific radioactivity by means of a flow-through radio-isotope detector. HPLC separation of a tryptic digest of iodinated salmon calcitonin fraction on a W-porex 5 C_{18} 300 Å column (250×4.6 mm I.D.) and subsequent amino acid analysis, led to the conclusion that radioiodination took place at the Tyr residue and not at the His moiety.

Keywords: Calcitonins

1. Introduction

Calcitonins (CT) are polypeptide hormones present in various species comprised of 32 amino acid residues having a molecular mass of about 3400 u depending on the species, which forms a disulfide bridge between Cys[1] and Cys[7] (see Fig. 1), and causes hypocalcemia inhibition of the release of calcium from bone and a stimulation of the urinary calcium excretion. Among the CTs available for clinical use (e.g., human, salmon, porcine, chicken and eel CT, etc.), salmon calcitonin (sCT) is one of

Cys-Ser-Asn-Leu-Ser[5]-Thr-Cys-Val-Leu-Gly[10]-Lys-Leu-Ser-Gln-Glu[15]-Leu-His-Lys-Leu-Gln[20]-Thr-Tyr-Pro-Arg-Thr[25]-Asn-Thr-Gly-Ser-Gly[30]-Thr-Pro-NH_2

Fig. 1. Primary structure of salmon calcitonin.

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the most potent forms and is used primarily for the treatment of postmenopausal osteoporosis and Paget's disease [1,2].

Radioiodinated CTs have generally been used in CT metabolism [3–5] and receptor binding studies [6] and in radioimmunoassay [7–9], under the assumption that the radioiodinated CTs (I-CTs) extensively exhibit the same biological properties as native sCT. However, the data available on the structural and biological characterization of the radioiodinated sCTs remain very limited. In many cases, radioiodinated peptides assumed to be indistinguishable from the native forms, actually showed divergences in their biological properties. This has recently been reported for insulin [10], vasopressin analogues [11] and epidermal growth factor [12].

In the present study, the iodo-beads method was employed for the radioiodination of sCT. The labeled products were separated from each other as well as from unlabeled sCT, by HPLC and further chemical characterization was performed on the basis of amino acid sequencing.

2. Experimental

2.1. Materials and reagents

Salmon calcitonin (synthetic, cyclic, sCT) was purchased from Bachem (Torrance, CA, USA) and $Na^{125}I$ from Dupont NEN (Boston, MA, USA). The following analytical grade reagents were purchased from Sigma (St. Louis, MO, USA): $Na^{127}I$, trypsin, trifluoroacetic acid (TFA), monoiodotyrosine, diiodotyrosine, phenylisothiocyanate (PITC), thioglycolic acid and glucose. HPLC grade acetonitrile, 2-propanol and cyclohexane were purchased from J.T. Baker (Phillipsburg, NJ, USA) and iodo-beads reagent from Pierce (Rockford, IL, USA). All other materials were of reagent grade.

2.2. Radioiodination of sCT

To a mixture of 0.1 mCi $Na^{125}I$ and 100 μl of 1 μmol ml^{-1} $Na^{127}I$ in 0.1 *M* phosphate buffer solution (pH 7.0, PBS), the following additions were made: 500 μl sCT in 0.05 *M* PBS (1.6 mg ml^{-1}) and two iodo-beads. After 10 min the reaction mixture was transferred to a C_{18} Bond Elut cartridge (Varian, Harbor City, CA, USA) pretreated with 20 ml of 2-propanol–water (90:10, v/v) followed by 20 ml of 0.1% TFA in water. The cartridge was washed with an additional 5 ml of 0.1% TFA in water to remove free iodine and sCT and I-sCTs were eluted with 2 ml of 90% acetonitrile. An aliquot of 500 μl of effluent was directly subjected to HPLC. The fractions of sCT and I-sCTs eluted from the column were mixed with 3 volumes of 0.1% TFA and transferred to a C_{18} Bond Elut cartridge pretreated with 20 ml of 2-propanol–water (90:10, v/v) followed by 10 ml of 0.1% TFA in water. The cartridge was washed with an additional 5 ml of 0.1% TFA and then sCT and I-sCTs were eluted with 2 ml of 2-propanol–Ringer's solution (90:10, v/v) containing 0.2% D-glucose. The effluent was then mixed with four volumes of cyclohexane and the aqueous-phase was isolated, flushed with nitrogen until no 2-propanol could be smelled, and then freeze dried.

2.3. Tryptic digestion of sCT and I-sCTs

The tryptic digestion of sCT and I-sCTs was performed by adding 20 μl of trypsin (5 mg ml^{-1} in water) to 100 μl of sCT (1 mg ml^{-1} in 0.1 *M* phosphate buffer, pH 7.0) and allowed to incubate at 39°C for 5 h. Digestion products were separated from trypsin by centrifugal ultra-filtration using a Centricon-10 concentrator (Amicon, Beverly, USA) at 3000 rpm for 30 min and the filtrates were stored below −20°C [13]. The fragments from tryptic digestion of sCT and I-sCTs were separated by reversed-phase HPLC as described below.

2.4. Amino acid analysis of tryptic digested fragments

Amino acid analyses were performed reversed-phase HPLC, using the PITC derivatizing method [14]. In brief, fractions of tryptic digested sCT and I-sCTs were hydrolyzed in constant boiling 6 *M* HCl in the presence of 4% thioglycolic acid at 110°C for 24 h in a pyrex tube (60×5 mm I.D.) and dried under vacuum. A 10-μl volume of redrying reagent (ethanol–water–triethylamine, 2:2:1, v/v/v) was added to

the dried residues, followed by nitrogen purging and freeze-drying. Then 20 μl PITC derivatizing reagent (ethanol–triethylamine–water–PITC, 7:1:1:1) was added to the re-dried residues and incubated for 20 min at room temperature. The unreacted derivatizing reagents were flushed off with nitrogen gas and freeze-dried. The derivatized hydrolysates were dissolved in 250 μl of 0.05 *M* sodium acetate solution (pH 7.2) and analyzed as described below.

2.5. HPLC system

The HPLC system consisted of two model 307 pumps, a model 234 autoinjector, a model 118 UV–visible detector and a model 712 system controller with a 506C interface module (Gilson, Villiers-le-Bel, France). A model F4010 fluorescence spectrophotometer (Hitachi, Tokyo, Japan) equipped with flow-cell for HPLC was connected next to the UV detector. Radioactivity of the column effluent was also measured by on-line detection using a Ramona 2000 flow-through radio-isotope detector (Raytest, Straubenhardt, Germany) equipped with a 100-μl gamma scintillator flow cell.

Separation of 500-μl aliquots of radiolabeled peptides from each other, as well as from unlabeled sCT, was done on a Bondclone 10 C_{18} (300×7.8 mm I.D., 10 μm-particles) column from Phenomenex (Torrance, CA, USA) at a flow-rate of 4 ml min^{-1} with 0.1% TFA in 34% aqueous acetonitrile as the eluent. The UV responses were measured at 215 nm, whereas fluorescence was monitored at λ_{ex}=282 nm and λ_{em}=310 nm.

For separation of fragments from tryptic digests of sCT and I-sCT (50 μl) a W-porex 5 C_{18} 300 Å column (250×4.6 mm I.D., 5 μm-particles) and a Bondclone 10 C_{18} guard column (30×3.9 mm I.D., 10 μm-particles) from Phenomenex were used and operated at 40°C. Gradient elution was carried out at a flow-rate of 1.2 ml min^{-1} with solvent A (0.1% TFA in water) and solvent B (0.1% TFA in acetonitrile). The following gradient profile was chosen: 0% B for 8 min, 0% B to 55% B in 16 min. After an additional elution for 20 min with 100% solvent A, the system was ready for the next injection. Signal responses from UV absorbance (215 and 280 nm), fluorescence (λ_{ex}=282 nm and λ_{em}=310 nm) and radioactivity were measured.

Separation of amino acids obtained after hydrolysis of tryptic digestion fragments (20 μl) was done on a Cosmosil 5 C_{18}-AR column (150×4.6 mm I.D., 5 μm-particles) from Nacalai (Kyoto, Japan) at a flow-rate of 1 ml min^{-1} by gradient elution HPLC using solvent A (50 m*M* sodium acetate, pH 7.2) and solvent B (100 m*M* sodium acetate solution (pH 7.2)–acetonitrile–methanol, 46:44:10, v/v/v). The following gradient profile was applied: 0% B to 100% B in 25 min, 100% B for 5 min. After an additional elution for 15 min with 100% solvent A, the system was ready for the next injection. Signal responses were measured at 254 nm.

3. Results and discussion

Depending on the labelling procedure, the products exhibited a more or less marked impact on their biological activities. As a consequence, studies of both receptor-binding and metabolism are only meaningful if the labeled peptides are biologically active. Therefore, it may be reasonable to generate biologically active species with defined positions of the radiolabel. In general, investigators rarely test whether the radioiodinated sCT preparations behave in the same way as the natural ligand in receptor binding or biological activities, although receptor affinity and capacity estimates can be seriously affected when there is no equivalence between the labeled and the native ligand.

sCT, in particular, contains two potential radioiodination sites, i.e., tyrosine at position 22 and histidine at position 17, but in general, it is strongly preferable to use a monoiodinated radiolabel to reduce potential steric hindrance of binding by bulky multiple iodinated residues.

Radioiodination of sCT has been carried out by oxidation of the radioiodide in the presence of sCT performed either chemically by Chloramine T [4] or electrolytically [3], with Chrolamine T being preferred. However, the Chloramine T procedure is known to be prone to damage the polypeptide to be iodinated because the oxidizing agent is in solution. Therefore, we applied the iodo-beads method for iodination of sCT, in which direct contact between the oxidizing agent and the peptide to be radiolabeled is minimized by using the Chloramine T

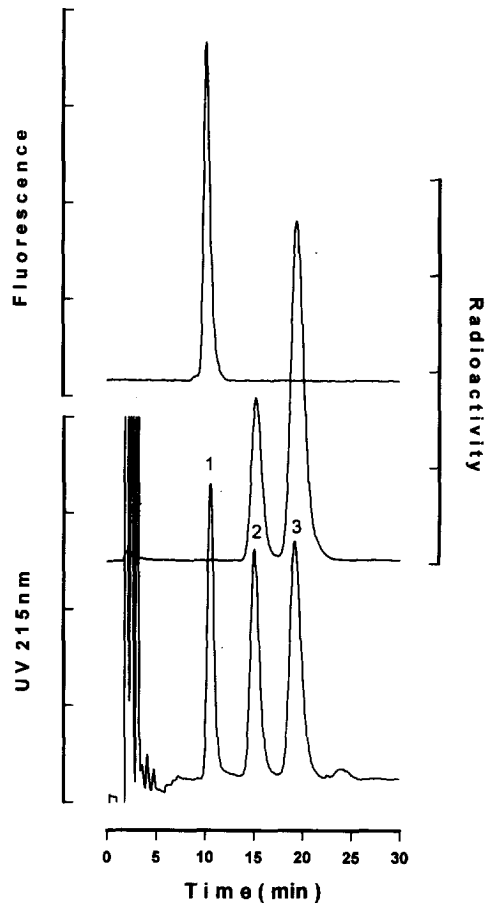

Fig. 2. Reversed-phase isocratic HPLC separation of salmon calcitonin and radioiodinated salmon calcitonins from the reaction mixture. (for conditions, see text) 1: salmon calcitonin, 2: mono-radioiodinated salmon calcitonin, 3: di-radioiodinated salmon calcitonin.

coupled to a solid support. After solid-phase extraction from the reaction mixtures using C_{18} cartridge, sCT and I-sCTs were subjected to HPLC separation.

As shown in Fig. 2, the radioiodination reaction mixture contained unlabeled sCT and mono- and di-radioiodinated sCT (mono-I-sCT, di-I-sCT) which were well-resolved under the described reversed-phase HPLC conditions. Retention times of the sCT invariably increased with the number of iodine moieties incorporated into Tyr residues of sCT (sCT: 10.7, mono-I-sCT: 15.2, di-I-sCT: 19.2), reflecting the increasing hydrophobicity of sCT due to the fact that iodine is markedly hydrophobic [15].

The comparison of specific radioactivities of peaks in Fig. 2 for identification of the substitution of iodine into sCT were sufficient to ascertain the presence of mono- and di-I-sCT as shown in Table 1.

There are two amino acid residues in sCT that can be iodinated, e.g., a tyrosyl and a histidyl residue in a molecule. Tryptic digestion of sCT and I-sCTs were performed to confirm the iodination site. sCT contains an argininyl and two lysyl residues so that a tryptic digest is expected to give four fragments of 1 (1–11), 2 (12–18), 3 (19–24) and 4 (25–32). As shown in Fig. 3, four fragments are clearly separated by using the described reversed-phase gradient HPLC method. Neither absorbance at 280 nm nor fluorescence detected peaks a, b and d whereas in contrast, peak c was detected. This means that this peptide fragment contains a Tyr residue because no other amino acid in sCT exhibited absorption at 280 nm or intrinsic fluorescence. Almost no fluorescence was observed on peak c2 and c3 from I-sCTs due to high quenching effect iodine exerts on the Tyr moiety. Although retention times of peaks a, b and d are identical, regardless of whether the peptide is labeled or not, those of peak c1, c2 and c3 from sCT, mono- and di-I-sCT, respectively, increase due to the increasing hydrophobicity by incorporation of one and two iodine atoms into the Tyr moiety of sCT as impressively shown in the chromatogram of I-sCT in Fig. 2. Furthermore, the amino acid composition of

Table 1
Characterization of radioiodinated salmon calcitonins from HPLC separations

Peak no.	Retention time (min)	Peak area (%)	sCT (μg)	Specific activity (cpm/μg)	Identity
1	10.7	28.6	6.72	–	sCT
2	15.2	30.8	7.24	$5.17 \cdot 10^5$	Mono-I-sCT
3	19.2	40.6	9.54	$1.14 \cdot 10^6$	Di-I-sCT

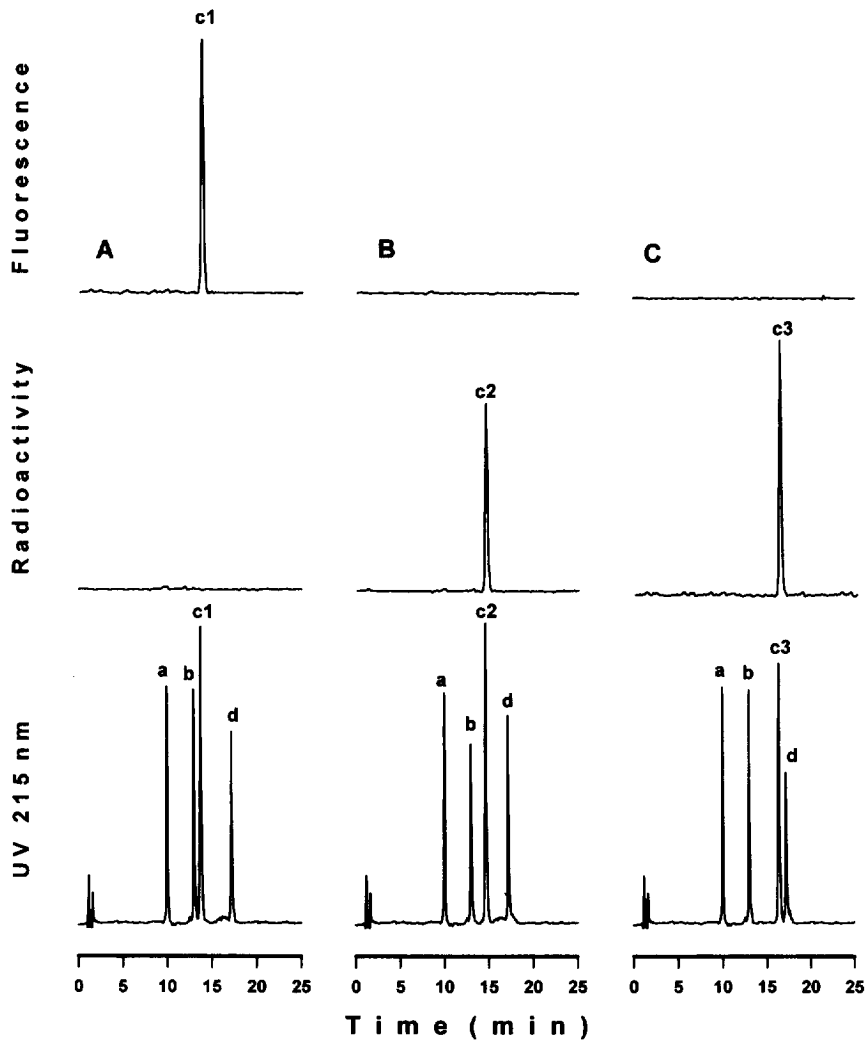

Fig. 3. Reversed-phase gradient HPLC of the tryptic digested fragments of salmon calcitonin and radioiodinated salmon calcitonins. (for conditions, see text) A: salmon calcitonin, B: mono-radioiodinated salmon calcitonin, C: di- radioiodinated salmon calcitonin. Peaks c1, c2 and c3 stand for Tyr-containing fragments of unlabeled sCT, mono- and di-iodinated sCT, respectively.

each tryptic digested fragment was assessed by amino acid analysis as shown in Table 2 and Fig. 4.

The His residue presents a further iodination site and up to two iodine atoms per molecule can be incorporated in addition to Tyr residue in sCT, particularly at higher pH values. However, only the Tyr-containing fragments, i.e., peak c2 and c3 from both mono- and di-I-sCT, respectively, showed radioactivity, whereas no radioactivity was observed on peak b containing the His moiety. These results provide evidence that radioiodination take place only at the Tyr residue and not on His moiety when the reaction is carried out at pH 7.0.

In conclusion, the present HPLC study has shown the separation of mono-I-sCT and di-I-sCT from intact sCT using isocratic reversed-phase HPLC. Their structural characteristics were deduced from the comparison of the chromatographic and spectrometric data of UV absorption, fluorescence and specific radioactivity, as well as from analysis of fragments obtained by tryptic digestion of I-sCT, which are all well-separated by reversed-phase gra-

Table 2
Characterization of tryptic digested fragments of sCT and I-sCTs from HPLC separations

Fragment	Peak[a]	Absorption, 280 nm	Fluorescence	Radioactivity	Amino acid analysis
1	d	No	No	No	2 Cys, 2 Ser, Asn, 2 Leu, Thr, Val, Lys, Gly
2	b	No	No	No	2 Leu, Ser, Gln, Glu, His, Lys
3					
sCT	c1	Yes	Yes	No	Leu, Gln, Thr, Tyr, Pro, Arg
Mono-I-sCT	c2	Yes	No	Yes	Leu, Gln, Thr, Tyr[b], Pro, Arg
Di-I-sCT	c3	Yes	No	Yes	Leu, Gln, Thr, Tyr[b], Pro, Arg
4	a	No	No	No	3 Thr, Asn, 2 Gly, Ser, Pro

[a] From Fig. 3.
[b] Tyr residue of mono- and di-I-sCT was confirmed using monoiodotyrosine and diiodotyrosine, respectively.

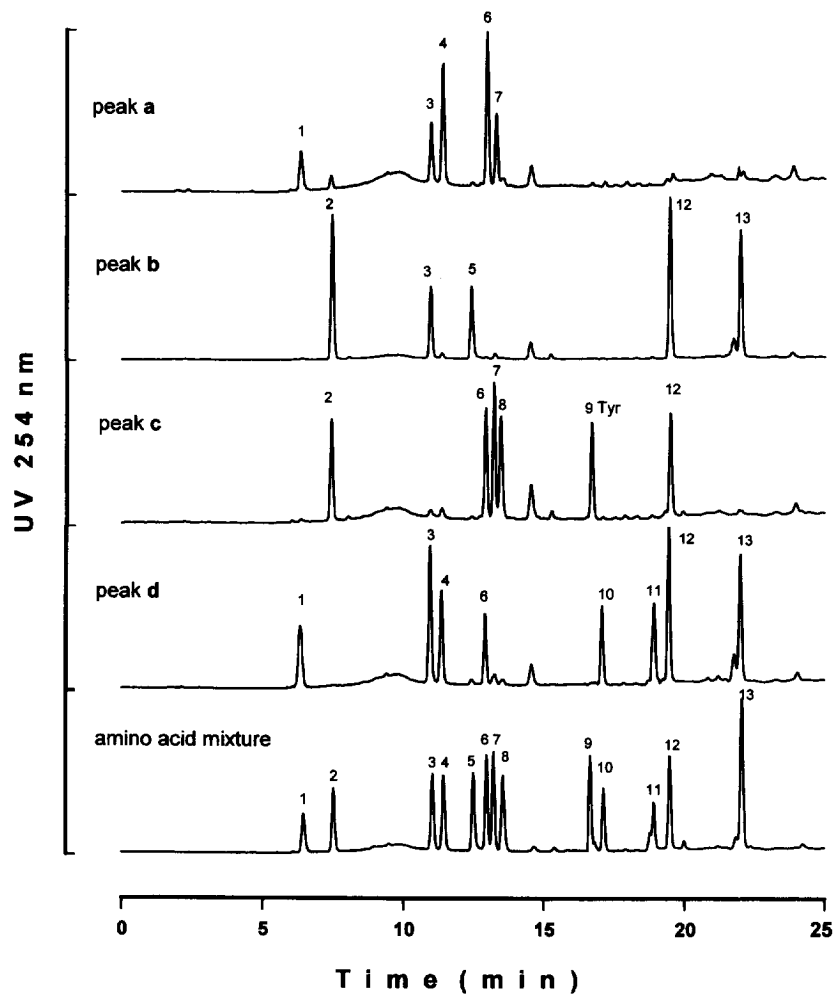

Fig. 4. Amino acid analysis of hydrolyzed tryptic digests after derivatization with PITC and separation by reversed-phase gradient HPLC from peaks a to d in Fig. 3A (for conditions, see text). 1: Asn, 2: Glu, 3: Ser, 4: Gly, 5: His, 6: Thr, 7: Pro, 8: Arg, 9: Tyr, 10: Val, 11: Cys, 12: Leu, 13: Lys.

dient HPLC. The latter investigations clearly revealed that radioiodination take place only at the Tyr residue and not at the His moiety, at least at pH 7.0. The comparison of sCT, mono- and di-I-sCT with respect to their stability, as well as the use in receptor-binding experiments and metabolic studies, are currently under investigation.

Acknowledgments

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