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Short communication

Reversed-phase high-performance liquid chromatography of salmon calcitonin and its degradation products in biological samples using column switching and flow-through radioisotope detection

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Abstract

For the determination of salmon calcitonin and its degradation products in biological samples, a reversed-phase HPLC method with column switching and flow-through radioisotope detection has been developed using high specific activity [^{125}I]salmon calcitonin. Effects of the precolumn packing material and washing solvent were examined in terms of [^{125}I]salmon calcitonin recovery. Spiked samples of [^{125}I]salmon calcitonin in plasma and kidney homogenate were injected onto a LiChroprep RP-8 precolumn after dilution with 0.1% trifluoroacetic acid. After washing the polar interfering compounds with 0.1% trifluoroacetic acid, the concentrated [^{125}I]salmon calcitonin and its degradation products were eluted and separated on a W-Porex C_{18} column with a gradient of 0.1% trifluoroacetic acid in acetonitrile–water. Detection and calibration of [^{125}I]salmon calcitonin were possible down to picogram levels. Reproducible kinetic data for the degradation of intact [^{125}I]salmon calcitonin by rat kidney homogenate could be traced.

1. Introduction

Salmon calcitonin (sCT) is a polypeptide comprised of 32 amino acid residues which is one of the most potent forms among the calcitonins (CT) available for clinical use. sCT is used for the treatment of postmenopausal osteoporosis and Paget's disease and in the management of hypercalcemia [1]. It has a short biological half-life of about 14 min [2].

There are several methods for the determination of sCT and [^{125}I]-sCT (sCT*) in biological samples: radioimmunoassay (RIA) [3,4], precipitation with trichloroacetic acid (TCA), gel permeation chromatography (GPC) and chromatoelectrophoresis [5–13]. Because of its sensitivity, RIA is primarily used for the determination of sCT in plasma; however, the

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accuracy depends on the specificity of the antiserum, analytical artefacts and interferences. RIA also does not distinguish between bioactive and non-bioactive forms of sCT. Other methods are insufficiently sensitive, time-consuming and unable to separate intact sCT* from some of its degradation products.

Although the usefulness of reversed-phase HPLC has been reported for the study of the stability, potency and purity of various CT drugs [14–18], there is little information on HPLC separation of sCT* and its degradation products in biological samples. This study describes a reversed-phase HPLC method with column-switching technique [19–24] and flow-through radioisotope detection [23] for the simultaneous determination of sCT* and its degradation products in rat plasma and tissue homogenates and characterizes the degradation of sCT* by rat kidney homogenate.

2. Experimental

2.1. Materials and reagents

Synthetic sCT was obtained from Bachem (Torrence, CA, USA). sCT* was obtained from Peninsula Laboratories (Belmont, CA, USA) at a specific activity of 1176 Ci/mmol and radioiodinated at tyrosine residue with the Chloramine T method. HPLC-grade trifluoroacetic acid (TFA) and acetonitrile were obtained from Sigma (St. Louis, MO, USA) and Baxter (Muskegon, MI, USA), respectively. All solutions and buffers were prepared with distilled deionized water from a Milli Q water purification system (Millipore-Waters, Milford, MA, USA) prior to use. All other materials were of reagent grade.

2.2. Chromatographic system

The HPLC system consisted of a Beckman 126 pump (Fullerton, CA, USA), a Waters 510 pump (Milford, MA, USA), an Altex 210 valve (San Ramon, CA, USA), a Rheodyne 7000 switching valve (Cotati, CA, USA), and a Beckman 171 radioisotope detector equipped with a 125-μl solid cell. Data handling was performed by System Gold using the Beckman 406 analog interface module. The instrumental arrangement for the six-port column-switching system was exactly as described in our previous papers [23,24].

The precolumn (20×3.9 mm I.D.) was tap-filled with LiChroprep RP-8 (25–40 μm, Merck, Darmstadt, Germany) and was changed after injection of 40 samples. The guard column was Nova-Pak C_8 guard insert (4.0×10 mm I.D., Waters, Milford, MA, USA) and the analytical column was a W-Porex 5 C_{18} packed column (250×4.6 mm I.D., 300 Å, 5 μm, Phenomenex, Torrance, CA, USA).

The washing solvent was 0.1% TFA in water and the flow-rate was 0.5 ml/min. The mobile phase consisted of (A) 0.1% TFA in water and (B) 0.1% TFA in acetonitrile. A linear gradient was used as the mobile phase: from 25% B in A to 55% B in A (0–20 min), 55% B to 100% B (20–25 min). The flow-rate was 1.2 ml/min. The radioactivity of the effluent was monitored using the Beckman 171 radioisotope detector. The column temperature was ambient.

2.3. Analytical procedure

sCT*-spiked samples in rat plasma and tissue homogenates were diluted ten-fold with 0.1% TFA in water, and the aliquot of the mixture was injected. The prepared samples were kept on ice before injection. The sample analysis included the following steps and required about 35 min.

Step I (0–5 min). The diluted biological sample was injected onto the precolumn. Polar interfering plasma components were washed out to waste with washing solvent using pump 1. The guard column and the analytical column were equilibrated with the mobile phase of starting eluent of the gradient elution using pump 2.

Step II (5–30 min). The washing solvent passed directly to waste. The retained components were eluted in the back-flush mode from the precolumn to guard column/analytical column by gradient elution after switching the valve.

Step III (30–35 min). The precolumn and

analytical column were re-equilibrated with the washing solvent and the initial mobile phase composition, respectively.

2.4. In vitro degradation study

sCT* (140 pg) was incubated in 1 ml of 0.1 *M* Tris·HCl (pH 7.4) containing rat kidney homogenate at 37°C in a shaking waterbath. Samples (200 μl) were removed at timed intervals. Aliquots (100 μl) were diluted ten-fold with 0.1% TFA in water and analyzed by reversed-phase HPLC with column switching. To other aliquots (100 μl) 1 ml of 10% TCA was added, and the radioactivity of supernatant and precipitate was measured using a γ-counter. The TCA-soluble fraction was analyzed by reversed-phase HPLC.

Kidney homogenate was prepared in 0.1 *M* Tris·HCl (pH 7.4) using a glass-Teflon homogenizer and used immediately.

3. Results and discussion

Reversed-phase HPLC was used for the analysis of sCT* and its degradation products in plasma and tissue homogenate samples. sCT* and its degradation products were well separated using a gradient of 0.1% TFA in acetonitrile–water on a W-Porex 5 C_{18} column (Fig. 1).

In the column-switching technique [19–24], the choices of precolumn packing material, washing solvent, washing time and flow-rate are important to achieve the quantitative recovery of sCT* and its degradation products in biological samples and to remove the interference components from the precolumn.

Table 1
Effect of the precolumn packing materials on the recovery of [^{125}I]salmon calcitonin (50 pg/ml) from plasma ($n = 3$)

Packing material	Recovery (%)
LiChroprep RP-8 (25–40 μm)	92.9 ± 4.8
μBondapak C_{18}/Corasil (37–50 μm)	82.1 ± 6.4
μBondapak Phenyl/Corasil (37–50 μm)	34.6 ± 6.9

Table 1 shows the effect of the precolumn packing materials on the recovery of sCT* from plasma. Optimal recovery was obtained with LiChroprep RP-8 (25–40 μm), a non-polar octylsilane bonded phase sorbent.

A mixture of citrate–phosphate buffer (pH 3.3), in which sCT was most stable [18], and 0.1% TFA was examined as the washing solvent and sample dilution buffer. As shown in Table 2, the recovery of sCT* from plasma exhibited a high dependence on the washing solvent and sample dilution buffer. It is shown that 0.1% TFA in water is adequate as the washing solvent, and sCT* will be quantitatively adsorbed on LiChroprep RP-8. The dilution of plasma samples with 0.1% TFA in water increased the

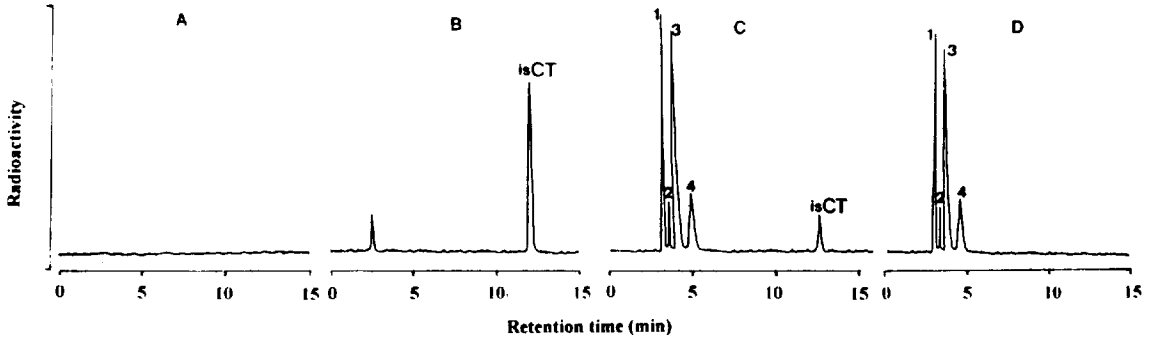

Fig. 1. Chromatograms of (A) a blank plasma, (B) a plasma spiked with [^{125}I]salmon calcitonin (50 pg/ml), (C) a degradation pattern of [^{125}I]salmon calcitonin incubated with rat kidney homogenate for 10 min, and (D) TCA-soluble fraction of [^{125}I]salmon calcitonin incubated with rat kidney homogenate for 5 min. Peaks 1, 2, 3, and 4 are degradation products of [^{125}I]salmon calcitonin.

Table 2
Effects of dilution buffer and washing solvent on the recovery of [^{125}I]salmon calcitonin (50 pg/ml) in rat plasma using column-switching technique ($n = 3$)

Washing solvent	Dilution buffer (dilution ratio 1:9)	Recovery (%)
Direct injection		100
Citrate–phosphate buffer (pH 3.3)	Citrate–phosphate buffer (pH 3.3)	69.1 ± 5.5
	0.1% TFA	66.2 ± 4.9
0.1% TFA	Citrate–phosphate buffer (pH 3.3)	71.0 ± 4.4
	0.1% TFA	92.9 ± 4.8

recovery of sCT* from 61.7% (no dilution) to 92.9% (10-fold dilution).

The effect of washing solvent volume on the recovery of sCT* is shown in Table 3. A 2.5-ml volume of washing solvent was adequate to obtain a good recovery of sCT* and a clean chromatogram from a plasma sample. The flow-rate of washing solvent was 0.5 ml/min because the higher the flow-rate, the less the recovery of sCT* (data not shown).

The correlation of peak area with the concentrations of sCT* in plasma was linear in the range of 5–200 pg/ml. The correlation coefficients were better than 0.99. The detection limit was determined as the concentration of compound giving a signal-to-noise ratio greater than 4:1. The limit of detection of sCT* was 2.5 pg/ml (equivalent to 500 cpm) for an injection of 100 μl of plasma.

The mean recovery of sCT* in plasma was 92.3 ± 5.1%. The precision (defined as the coefficient of variation of replicate analysis) and the accuracy (defined as the deviation between added and found concentration) of the assay for sCT* were evaluated over the plasma concentration range of 5–200 pg/ml. The results are shown in Table 4. The coefficient of variation ranged from 4.6% to 7.6% of the added amount in the spiked plasma samples.

Fig. 1 illustrates the chromatograms of sCT* incubated with rat kidney homogenate at 37°C for a specific time. Incubation resulted in rapid and substantial degradation to several metabolites (Figs. 1, 2). Most studies of sCT degradation performed using GFC, RIA, and precipitation with TCA have shown that the degradation of sCT* was tissue-specific, and isCT was severely degraded to TCA-soluble fragments by rat kidney in vivo and in vitro [5–11]. Using these methods, sCT* degradation products were indistinguishable from the intact sCT. However, the present method allows a faster and more efficient determination of sCT* and its degra-

Table 3
Effect of washing solvent volume on the recovery of [^{125}I]salmon calcitonin (50 pg/ml) from plasma ($n = 3$)

Washing solvent volume (ml)	Recovery (%)
2.5	92.9 ± 4.8
3.5	72.6 ± 5.9
5.0	67.2 ± 6.2

Flow-rate of washing solvent: 0.5 ml/min.

Table 4
Reproducibility of [^{125}I]salmon calcitonin in plasma samples ($n = 4$)

Concentration added (pg/ml)	Concentration found (pg/ml)	C.V. (%)
Within-day		
5	5.2	6.8
50	49.0	4.8
100	95.2	4.6
200	199.6	5.2
Day-to-day		
5	5.3	7.6
50	46.2	6.1
100	96.0	4.9
200	202.5	5.8

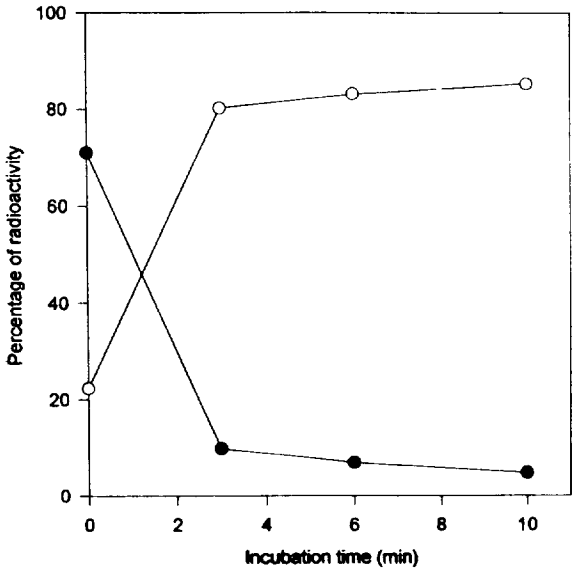

Fig. 2. Percentages of total radioactivity attributable to [^{125}I]salmon calcitonin (●) and its unknown degradation products (○) when [^{125}I]salmon calcitonin was incubated with rat kidney homogenate.

dation products produced in the degradation process.

Since sCT* was radioiodinated at the tyrosine residue, sCT* degradation products may contain the tyrosine residue. To characterize further sCT* degradation products separated by reversed-phase HPLC with column switching, TCA precipitability of each fraction was evaluated. All sCT* degradation products in Fig. 1C were TCA-precipitable and identical to HPLC peaks of the TCA-soluble fraction (Fig. 1D). The structures of sCT* degradation products were not identified in this study.

4. Conclusions

Reversed-phase HPLC with column switching and flow-through radioisotope detection was developed for the determination of sCT* and its degradation products in biological samples. This method allows the accurate evaluation of pharmacokinetics of sCT* and the rapid characterization of the fate of sCT* and its degradation products in vitro and in vivo.

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