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Quantitative analysis of synthetic human calcitonin by liquid chromatography–mass spectrometry

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Abstract

The quantitative analysis of synthetic human calcitonin (hCT) by micro-liquid chromatography–electrospray ionization mass spectrometry (micro-LC–ESI-MS) is reported. hCT was extracted from plasma by an immobilized antibody column and separated by a micro-LC system. The molecular mass of hCT is 3417, and m/z 1140, corresponding to the $[M+3H]^{3+}$ ion, was observed by ESI-MS. This ion was monitored in the selected-ion monitoring mode; rat calcitonin, which is highly homologous to hCT, was used as an internal standard. The calibration curve for the quantification of hCT was linear in the range 10 ng/ml to 1 μg/ml of plasma.

1. Introduction

Calcitonins (CTs), single-chain polypeptide hormones, have been isolated from many different species, and synthetic salmon and eel CTs are used clinically for treating osteoporosis [1] and hypercalcemia [2]. CTs, especially human CT (hCT), in biological fluids are commonly determined by radioimmunoassay (RIA) with polyclonal antibodies [3,4]. Measurement of CTs by RIA has the merits of high sensitivity and simplicity, but it is accompanied by several problems. One of its disadvantages is low selectivity; because immunoreactive peptides derived from CT are detected variably in plasma [5], it is not possible to estimate the basal CT. A further shortcoming of RIA is its relatively low precision. Measurements of CTs by high-performance liquid chromatography with ultraviolet detection (HPLC–UV) and bioassay have also been reported [6–11]. These are conventional and popular methods, but the disadvantage of HPLC–UV analysis in this application is low sensitivity due to the weak UV absorption of CTs, while bioassay has similar disadvantages to RIA. We decided, therefore, to try measuring synthetic hCT added to plasma by liquid chromatography–mass spectrometry (LC–MS), another sensitive and selective analytical method.

In the case of quantitative analysis by MS, quadrupole spectrometry (QMS) is widely used for its convenience. Applications in which QMS coupled with electrospray ionization (ESI) measures high-molecular-mass compounds, such as polypeptides and proteins, are becoming possible [12–15] because ESI produces multiply charged ions; for example, hCT was observed mainly as a triply charged ion. Here, we demonstrate a new

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technique for the measurement of hCT by a selected-ion monitoring (SIM) method, through which we monitor the triply charged ion of synthetic hCT.

2. Experimental

2.1. Reagents

Synthetic hCT and rat calcitonin (rCT) were obtained from Peptide Institute (Osaka, Japan). The amino acid sequences of hCT and rCT are shown in Fig. 1. An Affigel protein A MAPS-II kit was obtained from Bio-Rad (Richmond, CA, USA), and Sepharose 4B was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Methanol and acetonitrile of HPLC grade (Wako Pure Chemical Industries, Tokyo, Japan) were used. Trifluoroacetic acid (TFA), human serum albumin (HSA) and other reagents were purchased from Sigma (St. Louis, MO, USA). These reagents were of analytical grade and were used without further purification.

2.2. Preparation of solutions of synthetic hCT and rCT

Synthetic hCT and rCT were dissolved in 0.1% TFA aqueous solution, 50 μg/ml each, immediately before use in order to minimize the adsorption on the container and self-adduct formation. These solutions were used for the measurement of ESI spectra of hCT and rCT.

Synthetic hCT and rCT were dissolved in TFA–water–methanol (0.1:50:50, v/v/v), 50 μg/ml each, and were stored at 4°C. These stock solutions were used for the hCT standard solutions for the preparation of a calibration curve.

2.3. Preparation of an immobilized antibody column

The anti-hCT antiserum was prepared in the Neuroscience Research Laboratories, Sankyo (Tokyo, Japan). It was raised directly against synthetic hCT in rabbits.

An immobilized affinity column for hCT was prepared according to the modified conventional method [16,17]. The antiserum was affinity-purified by a protein A-Sepharose column to obtain the immunoglobulin G (IgG) fraction, according to the Bio-Rad kit, and dialysed against 0.1 *M* carbonate buffer (pH 9.0). The anti-hCT IgG fraction was coupled to cyanogen bromide-activated Sepharose 4B through the N-terminus. Cyanogen bromide-activated Sepharose 4B was suspended in 0.1 *M* carbonate buffer (pH 9.0), and the anti-hCT IgG fraction was added to the suspension. The mixture was stirred gently using a rotary evaporator at 4°C for coupling. Any remaining chemically active sites on Sepharose 4B were blocked by reaction with free amino groups using Tris–HCl buffer (pH 8.0). The antibody-coupled Sepharose 4B gel thus obtained was stored in a phosphate-buffered saline solution (pH 7.4, PBS) at 4°C.

2.4. Extraction of synthetic hCT from rat plasma by the immobilized antibody column

The immobilized antibody gel (1 ml) was packed in a polypropylene column (Sepacol-mini-pp, Seikagaku-kogyo, Tokyo, Japan) and handled at room temperature during the clean-

Fig. 1. Amino acid sequences of human calcitonin (hCT) and rat calcitonin (rCT). The amino acid sequence of rCT differs only at two positions from that of hCT: phenylalanine-16 in the human versus leucine-16 in the rat, and alanine-26 in the human versus serine-26 in the rat.

up steps. The column was charged with 1 ml of rat plasma supplemented by the hCT solution and 0.1 μg of rCT solution as an internal standard (I.S.). After washing the column with water (3 × 5 ml), hCT and I.S. were eluted with 100% methanol (5 ml). The eluent was evaporated in vacuo, and the residue was redissolved in the LC mobile phase. The sample solution thus obtained was subjected to LC–MS analysis.

2.5. Micro-liquid chromatography

A schematic diagram of the apparatus is shown in Fig. 2. The HPLC gradient system consisted of two LC-9A pumps (Shimadzu, Kyoto, Japan), a splitter (Acurate, LC Packing, Zürich, Switzerland) and a Model 8125 injector (Rheodyne, Cotati, CA, USA). A micro-column (C_{18}, 150 × 0.32 mm I.D., LC Packing) was connected to the system with fused-silica capillary (I.D. 50 μm). The mobile phase was composed of a 0.1% TFA aqueous solution (solvent A) and a TFA–water–acetonitrile (0.1:40:60, v/v/v) solution (solvent B). Preconcentration and separation were accomplished by increasing solvent B from 0% to 100% linearly in 10 min. The total flow-rate of 0.45 ml/min was generated by the gradient pumps, and a constant flow at 3 μl/min was separated from the LC column by the splitter. Although the maximum sample injection volume with a conventional injector was 20 μl, for this work only a 5-μl aliquot was injected, using a 5-μl sample loop, to minimize the dead volume.

2.6. Mass spectrometry

A triple-stage quadrupole mass spectrometer (TSQ-700, Finnigan MAT, San Jose, CA, USA) equipped with an Analytica model ESI source (Finnigan MAT) was used for LC–MS. The manifold temperature was set at 70°C, the voltage was set at −3.5 kV, and the drying gas for ESI was nitrogen (ca. $6.8 \cdot 10^4$ Pa, 80°C). The gas sheath, nitrogen (ca. $10.2 \cdot 10^4$ Pa), and the liquid sheath, 2-methoxyethanol (2 μl/min), were introduced at the tip of the ESI needle. The scan speed was 0.5 s/scan. The scan ranges for hCT and rCT were 1140 ± 2 and 1132 ± 2 a.m.u., respectively. These scan ranges switched over at 0.5-s intervals.

This mass spectrometer equips two scanning modes of the mass range. One mode is a normal mode which scans the range from 1 to 2000. The other mode is a high-mass-range scanning mode which scans the mass range from 1 to 4000. The latter mode has a lower mass resolution and sensitivity than the former. Synthetic hCT and rCT were measured in the range 800 to 1800 at 3 s by the normal-mass-range mode for the purpose of examining monitoring ions. Subsequently, the same CTs were determined in the range of 2000 to 4000 at 4 s by the high-mass-range

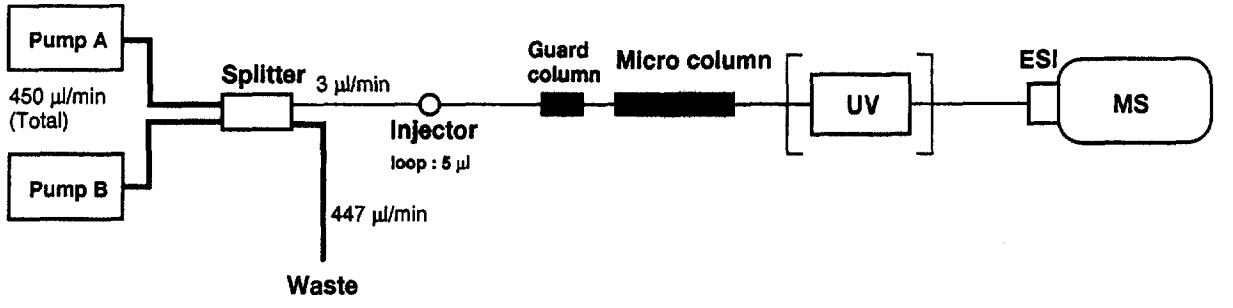

Fig. 2. Schematic diagram of the micro-LC–MS system. Two LC-9A HPLC pumps (Shimadzu), an Acurate microflow splitter (LC packings), C_{18} column, 100 × 0.25 mm I.D., 3 μm particle size (LC packings) were used. Eluent: acetonitrile–water, containing 0.1% TFA. Injection volume: 5 μl. The MS apparatus was a TSQ-700 from Finnigan-Mat in the electrospray ionization positive-ion mode using selected-ion monitoring. Bold lines: stainless-steel or PEEK tube; thin lines: capillary tube (50 μm I.D.); joint: PTFE tube

scanning mode so that $[M+H]^+$ ions and self-adduct ions could be observed. A syringe-type infusion pump (Model 22, Harvard Apparatus, South Natick, MA, USA) was used to introduce synthetic hCT and rCT to the spectrometer directly. The infusion rate was set at 1 μl/min.

3. Results and discussion

3.1. ESI spectrum of synthetic hCT

When quantitative analysis by MS is used for high-molecular-mass compounds, such as peptides, one significant problem is the selection of an I.S. rCT was chosen as the I.S. in the present study, because the amino acid sequence of rCT is very similar to that of hCT, and rCT proved impossible to separate from hCT even by capillary zone electrophoresis. However, the best procedure is to use the stable isotope, which gives a mass number greater than that of the non-labeled hCT by at least 10. We intend to synthesize stable isotope-labeled hCT in a future study.

Samples (10 ng) of hCT and rCT solution were introduced by the infusion pump and analysed. The results are shown in Fig. 3. In the case of hCT, the base peak was m/z 1140, corresponding to $[M+3H]^{3+}$; doubly and quadruply charged ions were also observed in the range from m/z 800 to 1800. The ESI-MS spectrum of rCT was similar to the spectra observed for synthetic hCT. Therefore, hCT and rCT were monitored using their respective $[M+3H]^{3+}$ ions at m/z 1140 and 1134 ions, respectively. The hCT ESI mass spectrum in the mass range from m/z 2000 to 4000 obtained in the high-mass-range scanning mode is shown in Fig. 4. The base peak was found to be m/z 3418, corresponding to $[M+H]^+$, but this is not the preferred ion to monitor from either the resolution or the ion intensity point of view. It is remarkable also to observe the peaks corresponding to self-adducts of hCT ions; i.e., the peaks of m/z 2278, 2564 and 2736 (Fig. 4), which were probably attributable to $[2M+3H]^{3+}$, $[3M+4H]^{4+}$ and $[4M+5H]^{5+}$ ions, respectively.

3.2. Calibration curve of synthetic hCT

A typical LC–MS profile of synthetic hCT is shown in Fig. 5. A 5-μl portion of the sample containing both hCT (30 ng/ml) and I.S. (300 ng/ml) was injected. The calibration curve (not shown) demonstrated good linearity from 5 to 1250 ng/ml, and its correlation coefficient (r) was 0.995. The detection limit was ca. 15 pg (signal-to-noise ratio of 3).

Because the retention time of rCT was the same as that of hCT, rCT was considered to be an appropriate I.S. for hCT quantitation.

3.3. Recovery of synthetic hCT after storage

CTs tend to adsorb onto the containers or to aggregate themselves. The recovery ratios from a polypropylene tube (Eppendorf, Germany) by three different solvents are shown in Fig. 6. Recovery of synthetic hCT decreased rapidly in 0.1% TFA aqueous solution, while good recovery ratios were obtained in the solvents containing methanol. Recovery of hCT from the glass container was similar to that from the polypropylene tube.

It is unclear whether the phenomenon was caused mainly by adsorption or by self-adduct formation, as Fig. 4 might suggest. Careful attention should be paid to the handling of hCT, with due regard for these points, in the future.

3.4. Recovery of synthetic hCT from the immobilized antibody column

The recovery ratio of synthetic hCT from the immobilized antibody column is shown as the percentage of the amount of hCT, once trapped by the column and eluted with methanol, relative to the amount initially charged to the column (see Fig. 7). The amino acid sequence of rCT differs in only two positions from that of hCT, and both CTs have been reported to be immunologically similar to each other [18]. In fact, the antibody of hCT was found to recognize rCT to the same extent as hCT, because the recovery ratio of rCT from the column was same as that of hCT (data not shown). It is preferable to use a

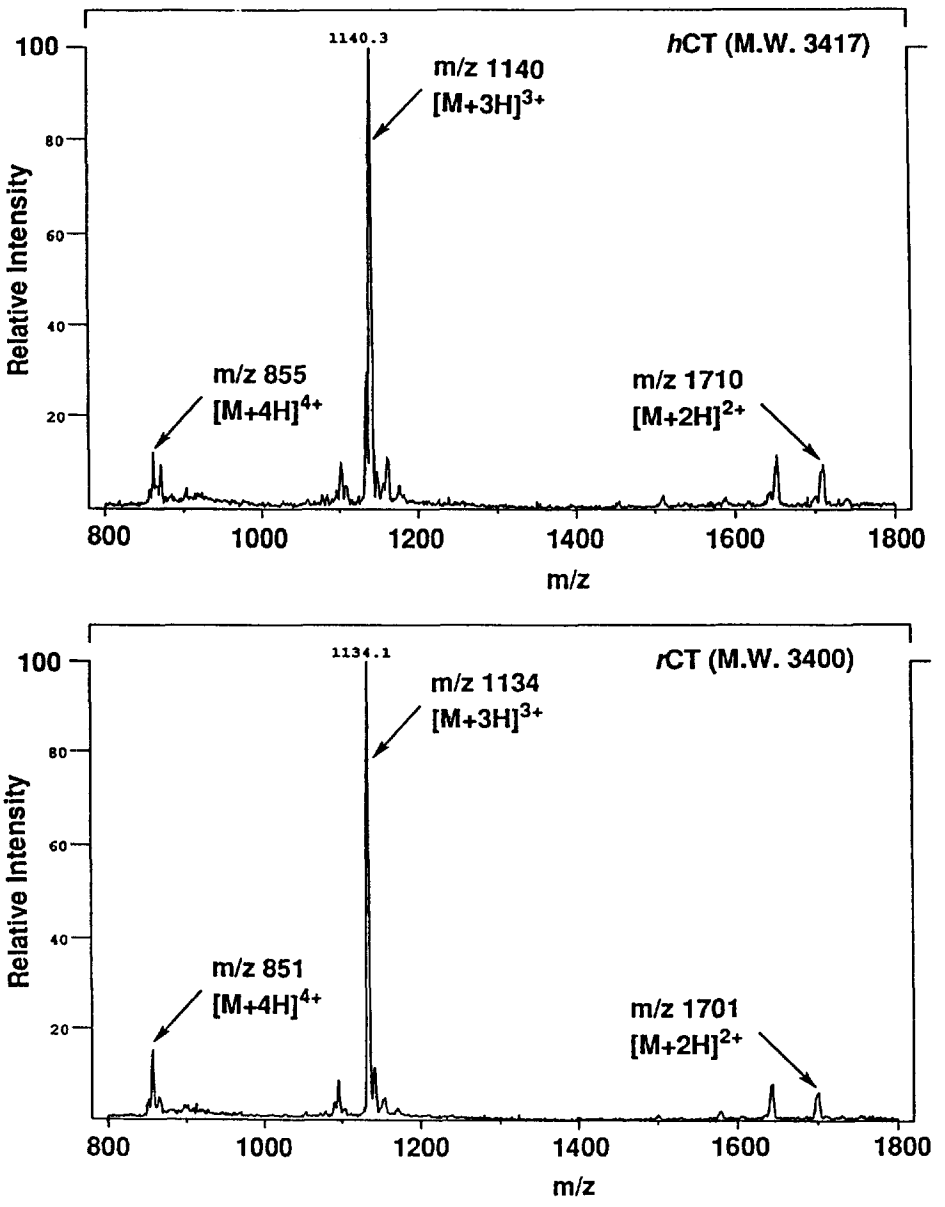

Fig. 3. ESI mass spectra of synthetic hCT (10 ng) and rCT (10 ng).

broadly specific antibody against hCT for simultaneous retention of rCT as I.S. on the column.

The micro-LC system adopted in the present method has the problem that contaminants from the biological sample stick to the column. Therefore, it is necessary to pretreat the sample by a specific clean-up method. The immobilized antibody column served as a highly selective basis for hCT pretreatment.

3.5. Calibration curve of hCT spiked rat plasma

The calibration curve of rat plasma spiked with synthetic hCT is shown in Fig. 7. We did not detect endogenous rCT in the rat control plasma,

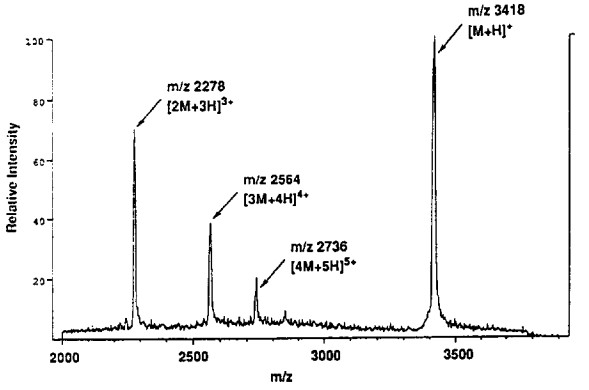

Fig. 4. ESI mass spectrum of synthetic hCT. $[2M + 3H]^{3+}$, $[3M + 4H]^{4+}$ and $[4M + 5H]^{5+}$, corresponding to the dimer, trimer and tetramer of synthetic hCT, were observed.

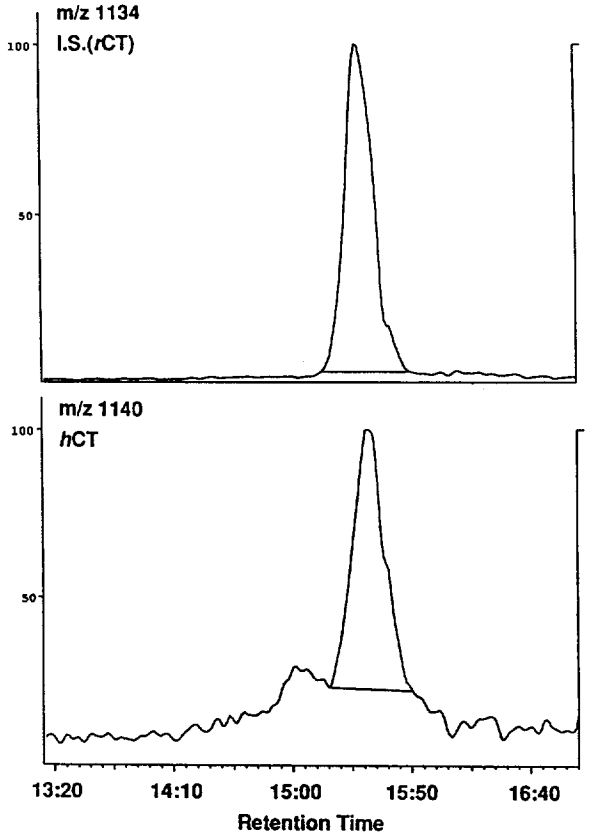

Fig. 5. Typical LC–MS profile in the SIM mode of synthetic hCT. The amounts of hCT and rCT were 0.15 and 1.50 ng, respectively.

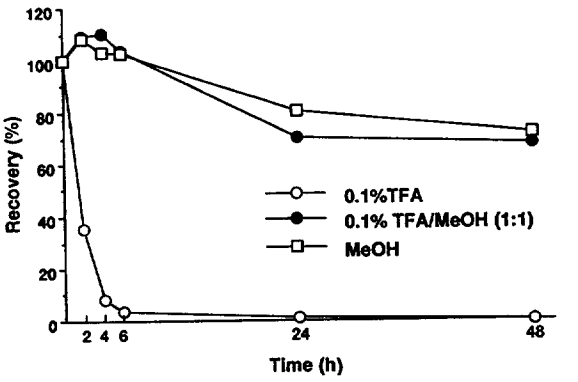

Fig. 6. Decay of synthetic hCT. Synthetic hCT (15 ng) in each solvent was stored at room temperature.

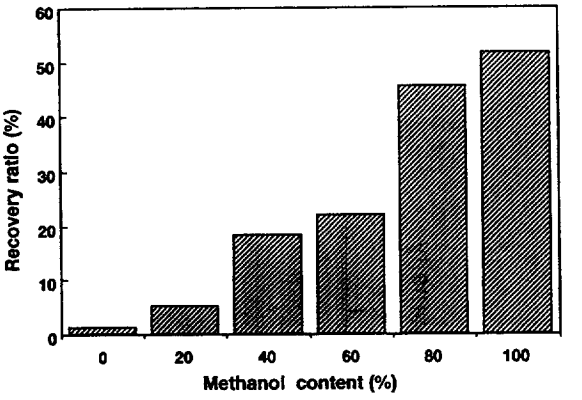

Fig. 7. Recoveries of synthetic hCT from the immobilized antibody column. Amount recovered in eluate relative to amount fed into the column.

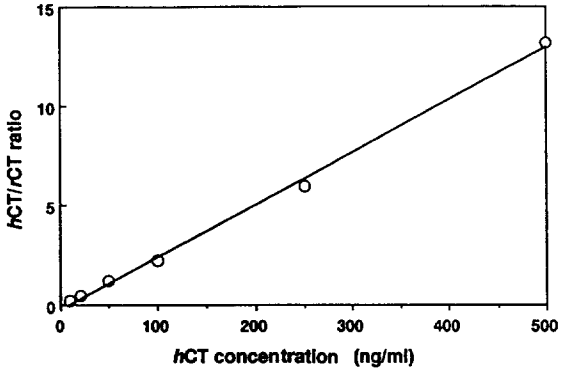

Fig. 8. Calibration curve of rat plasma spiked with synthetic hCT. Values are mean of triplicate experiments. Correlation coefficient: 0.998.

which means that, in the measurement of synthetic hCT, there was no interference from the control levels of rCT.

4. Conclusion

It has proved possible to measure synthetic hCT in rat plasma at ng/ml levels by the LC–ESI-MS method described. An immobilized antibody column provided a selective clean-up of hCT in biological fluid. The column was found also to retain rCT, which was used as an I.S. We believe that, after further improvement, it will be possible to apply the LC–ESI-MS method to the measurement of synthetic hCT in either preclinical or clinical samples.

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