Radioimmunoassay of Urinary Calcitonin in Normal Subjects and in Patients with Medullary Carcinoma of the Thyroid

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Abstract

A simple technique for measuring urine calcitonin (CT) was established using a sensitive radioimmunoassay (RIA) system for plasma human calcitonin (hCT). To extract urinary CT, urine samples were fractioned by gel chromatography on a column (0.8 × 20 cm) of Bio Gel P-2. Recovery of synthetic (1-32)hCT was 86.1 ± 6.2% and the intra- and inter-assay coefficients of variation in RIA were 5.9 and 8.2%, respectively. Dilution curves of the urinary CT after gel-filtration were parallel with the standard curve. In 11 patients with medullary carcinoma of the thyroid (MCT), the CT levels of the urine (in ng/mg Cr) were 3.4 to 20.8 times higher than those of the plasma (in ng/ml), and a significant positive correlation (r = 0.93, P<0.001) was obtained between the urinary and plasma levels of CT in these 11 patients. In 32 normal subjects, the CT levels of the urine (in pg/mg Cr) were only 0.41 to 5.1 times of those of the plasma (in pg/ml), and a weak positive correlation (r = 0.408, P<0.05) was obtained between these two levels. However, urinary CT levels in normal subjects apparently reflected a rise in endogenous plasma hCT in response to calcium infusion (4 mg/kg B.W. for 1 min) and that of exogenous plasma CT when synthetic (1-32)hCT (500 µg for 1 min) was injected intravenously.

Following gel filtration on a Bio Gel P-30 1.5 × 80 cm column, larger molecular forms of CT than (1-32)hCT were found in the urine not only in patients with MCT but also in normal subjects after infusion of calcium or synthetic (1-32)hCT.

It is concluded that measurement of urine CT is useful not only for diagnosis of MCT but also for evaluating CT metabolism in normal subjects.

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Abbreviations: The following abbreviations are used: [Asu1,7]-hCT: a synthetic analogue of human calcitonin, in which the N terminal amino group and the disulfide bond of the natural peptide were replaced by a hydrogen atom and by an ethylene linkage, respectively; bPTH: bovine parathyroid hormone; Cr: creatinine; CT: calcitonin; hCT: human calcitonin; (1-32)hCT: synthetic human calcitonin; (11-32)hCT, (17-32)hCT, (21-32)hCT: synthetic fragments of human calcitonin; Kd: distribution coefficient of a solute within a gel; MCT: medullary carcinoma of the thyroid.

Human calcitonin (hCT), composed of 32 amino acids, was discovered to be a calcium-lowering hormone (Copp et al., 1962). Kidney is known to be one of the most important target organs of hCT, and to have a specific receptor for this hormone (Ardaillou, 1975). However, little is known about the concentration or immunological properties of calcitonin (CT) excreted from the kidney in the urine. The present study was designed to establish a technique for measuring urinary CT, and to investigate the size-heterogeneity of CT in the urine.
Materials and Methods

Collection of Samples

Plasma and urine samples: Plasma samples were obtained in the morning after overnight fasting from 32 normal subjects (24 males and 8 females, aged 21–81 yr), and from 11 patients with MCT. Spot urine samples were obtained at the time of blood sampling from these subjects. Usually the first morning urine was discarded, and the second urine sample was collected over a fixed period of time (1–2 h) for assay. Twenty-four hr urine samples, collected at room temperature and mixed with 0.5 g of NaN₃, were also obtained from 20 of these normal subjects the day before blood sampling.

Calcium infusion into normal subjects: Calcium (as 8.5% calcium gluconate solution) was infused intravenously over a period of one minute at a dose of 4 mg/kg of body weight into three male volunteers (aged 19, 27 and 37 yr) after fasting. Plasma samples were obtained before the infusion and 2, 5, 15, 30 and 60 min after the starting of infusion. Urine samples were collected every 15 min from 30 min before to 60 min after the start of infusion.

Infusion of synthetic hCT into normal subjects: Synthetic hCT was infused intravenously over a period of one min at a dose of 0.5 mg in 20 ml of saline into 3 male volunteers (aged 24, 27 and 37 yr) after fasting. Plasma samples were obtained before and 2, 5, 15, 30, 60 and 120 min after the start of infusion. Blood samples were collected in a heparinized syringe and centrifuged immediately.

These samples were stored at −20°C until assay.

Materials

Synthetic (1–32)hCT, [Asu₁,₇]-hCT, and synthetic peptide fragments (11–32)hCT, (17–32)hCT and (21–32)hCT were kindly supplied by Dr. S. Sakakibara, Peptide Institute, Protein Research Foundation, Osaka, Japan. Ammonium bicarbonate, ammonium acetate, urea, creatinine, creatine, caffeine, theophylline, theobromine, guanosine, thymidine, DNA, proline and histidine were obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan. Blue dextran (B-2000, 2,000,000 daltons) and bovine serum albumin were obtained from Sigma Chemical Company, St. Louis, Mo., U.S.A. Bio Gel P-2 (100–200 mesh) and Bio Gel P-30 (100–200 mesh) were obtained from Bio-Rad Laboratories, Richmond, Cal., U.S.A. Bovine parathyroid hormone (bPTH) was obtained from Inorex Co. Ltd., Ill, U.S.A. Synthetic (1–32)hCT for infusion (CIBACALCIN®) was kindly supplied by Ciba-Geigy Co. Ltd., Basel, Switzerland.

Experimental Procedure

Determination of plasma CT concentration: Plasma hCT concentrations were determined by RIA. The procedure and evaluation were reported in detail previously (Okada et al., 1978). Briefly, anti-hCT antiserum was obtained by immunization of rabbits with synthetic (1–32)hCT in Freund’s adjuvant. hCT analogue ([Asu₁,₇]-hCT) was labeled with ¹²⁵I by the glucose oxidase method with lactoperoxidase, yielding a specific activity ranging from 410 to 510 μCi/μg. Synthetic (1–32)hCT was also used as a reference. RIA buffer consisted of a solution of 0.1 M phosphate buffer saline at pH 7.4, 0.037% EDTA-Na₂, 0.5% BSA and 0.1% NaN₃. The assay procedure was based on the double antibody technique. The sensitivity was 25 pg/ml and the recovery was 99.9±17.4% (mean±s.d.). The within assay coefficient of variation was 7.9% and that between assays was 8.3%. Dilution curves of sera of high hCT concentration from patients with MCT were parallel with the standard curve. No inhibitory or enhancing effect of serum protein on the standard curve was observed in this assay system and the sensitivity was 25 pg/ml when samples were diluted with RIA buffer.

Fractionation of immunoreactive CT from urine: Urine samples were fractionated by a modification of the method of Snider et al. (1978). Aliquots of 3 ml of urine for assay were boiled for 3 min, rapidly cooled in an ice-bath, and centrifuged at 20,000 g for 15 min at 4°C. Then 2 ml of the supernatant was mixed with 10 mg of blue dextran in 100 μl of saline, and passed through a Bio Gel P-2, 0.8×20 cm column in 0.1 M ammonium bicarbonate at pH 7.5. The blue colored fraction was collected in a 4 ml poly styrene tube colored fraction was collected in a 4 ml poly styrene tube and lyophilized, and the residue was dissolved in 0.5 ml of RIA buffer and subjected to double antibody RIA as described for plasma samples. These procedures are summarized in Fig. 1.

Antiserum specificity: The sequence-specific antigenic recognition site of the antiserum used for hCT

Fig. 1. Procedures for measurement of urinary CT.
RIA of urinary calcitonin

RIA was determined by comparing the ability of the synthetic (1-32)hCT and its fragments to inhibit the binding of $^{125}$I-labelled [Asu$^{1,7}$]-hCT to each antiserum.

Interference with hCT RIA by various kinds of compounds: The degree of interference with hCT RIA was examined with blue dextran and a series of known constituents of the urine as well as analogues, such as urea, creatinine, creatine, caffeine, theophylline, theobromine, guanosine, thymidine, DNA, proline and histidine. These compounds were dissolved in and diluted with RIA buffer, and 100 µl of solution of each was assayed in the ordinary way for hCT RIA.

Gel filtration: Gel filtration was carried out at 4°C on a 1.5 x 80 cm column of Bio Gel P-30 in 0.1 M ammonium acetate buffer, pH 5.0, supplemented with 0.5% bovine serum albumin and 0.1% NaN₃. The plasma and urine samples were centrifuged at 20,000 g for 30 min and the supernatants were applied to the Bio Gel P-30 column. $^{125}$I-bPTH, $^{125}$I-hCT and $^{125}$INa (10,000 cpm respectively) were used as internal markers of peaks of hormones and salt (Vs), respectively. The void volume (Vo) was determined from the elution position of the largest plasma protein, monitored from the absorption at 277 nm in a spectrophotometer. Appropriate fractions were lyophilized, dissolved in 0.2 to 4 ml of RIA buffer and assayed in duplicate for CT. The elution position of each form of immunoreactive CT, designated as Kd, was calculated in the following way:

$$K_d = \frac{\text{elution volume of each form of CT} - \text{Vo}}{\text{Vs} - \text{Vo}}$$

Urinary creatinine and urea: Creatinine and urea were assayed in aliquots of urine with a Technicon Autoanalizer.

Statistics: Statistical analyses were performed by Student’s t-test.

Results

Specificity of Antiserum for hCT RIA

Figure 2 shows the inhibition of binding of $^{125}$I-labelled [Asu$^{1,7}$]-hCT tracer to antiserum produced by the addition of synthetic (1-32) hCT or its synthetic peptide fragments. The concentrations of [Asu$^{1,7}$]-hCT, the (11-32) hCT fragment and the (17-32)hCT fragment required for 50% inhibition of tracer binding to the antiserum were almost equimolar with that of intact (1-32)hCT. However, about 7.6 times more (21-32)hCT fragment than (1-32)hCT was required to produce 50% inhibition with this antiserum. Consequently the antiserum used for RIA appeared to have a major antigenic site within residues 17-20 of (1-32)hCT and a minor antigenic site within residues 21-32.

Interference with hCT RIA by Various Kinds of Compounds

The degrees of interference with hCT RIA by blue dextran and by a series of known constituents of the urine are shown in Fig. 3. Blue dextrane, creatine, proline and histidine did not interfere with hCT RIA. However, guanosine, DNA, theophylline, thymidine, caffeine, creatine and urea caused interference.

Recovery, Reproducibility and Dilution Curves in Urinary Determination of CT

Seven samples of saline with and without synthetic (1-32)hCT added at concentrations of 100, 400, 800 and 1600 pg/ml were gel-filtrated on a Bio Gel P-2 column and assayed for hCT. The hCT levels of the 7 samples
Fig. 3. Interference with hCT RIA by various compounds. Blue dextran, creatine, proline and histidine did not cause interference. Many constituents of urine, however, and analogues interfered with the RIA system, and the dilution curves of these compounds were not parallel with the standard curve.

without added synthetic (1-32)hCT were all below the detection limit. The degrees of recovery of hCT after filtration on a Bio Gel P-2 column of samples containing synthetic (1-32)hCT at concentrations of 100, 400, 800 and 1600 pg/ml were 92.3±3.1% (mean ±S.D.), 87.3±3.4%, 85.6±2.9% and 81.1±3.1% respectively, the mean±S.D. for all the samples being 86.1±6.2%.

When seven 2 ml aliquots of a single sample of normal urine were gel-filtrated and assayed in a single assay, the mean±S.D. of the CT level was determined as 124.8±8.0 pg/mg creatinine (Cr), and when seven 2 ml of a single urine sample from a patient with MCT were used, the CT level was determined as 1670±90 pg/mg Cr. Thus coefficients of variation (C.V.) within assays were 6.5% and 5.4%, (mean 5.9%). Similarly the C.V. between the 7 assays were 8.4% and 7.9%, respectively (mean 6.2%).

Dilution curves after gel-filtration of urine samples from 3 normal subjects and 3 patients with MCT were parallel with the standard curve for hCT (Fig. 4).

hCT Levels in the Plasma and Urine of Patients with MCT

The hCT levels in the plasma of patients with MCT ranged from 0.24 to 23.8 ng/ml, and those in the urine ranged from 1.7 to 520 ng/mg Cr (Fig. 5). The CT levels in the urine (in ng/mg Cr) were 3.4 to 20.8 times higher than those in the plasma (in ng/ml). The CT levels of urine were correlated with those of plasma (r=0.93, P<0.001).

hCT Levels in the Plasma and Urine of Normal Subjects

The hCT levels in the plasma of 32 normal subjects ranged from less than 25 pg/ml to 115 pg/ml (72.1±33.5 pg/ml, mean±S.D.). The CT levels in the urine ranged from 41 pg/mg Cr, to 214 pg/mg Cr (104.6±39.5 pg/mg Cr, mean±S.D.). The CT levels of the urine were positively correlated with those of the plasma (r=0.408, P<0.05) as shown in Fig. 6.

When the 32 urine samples were assayed directly without gel-filtration, the apparent mean±S.D. of the urine CT level was 524±124 pg/mg Cr. These levels were positively correlated with the urinary creatinine levels in
Fig. 5. Plasma hCT levels and urinary CT levels of 11 patients with MCT. These two levels were positively correlated (*r* = 0.93, *P* < 0.001) and CT levels in the urine (in ng/mg Cr) were 3.4 to 20.8 times higher than those in the plasma (in ng/ml).

mg/ml (*r* = 0.682, *P* < 0.001), and with urinary urea levels in mg/ml (*r* = 0.754, *P* < 0.001). The levels of urinary CT after gel filtration in pg/mg Cr, were not correlated significantly with the urinary creatinine levels (*r* = 0.079, *P* > 0.1), or with the urinary urea levels (*r* = −0.032, *P* > 0.10). The concentrations of creatinine and urea in the blue void volume after purification of the urine of normal subjects were all below the detection limit.

Comparison of CT Levels in Spot Urine Samples and in 24 h Urine Samples from 20 Normal Subjects (Fig. 7)

The levels of CT in spot urine samples from 20 normal subjects ranged from 41 to 170 pg/mg Cr (109.9 ± 34.8 pg/mg Cr, mean ± s.d.). Those in 24 h urine samples from the same 20 subjects ranged from 20 to 220 pg/mg Cr (117.7 ± 36.9 pg/mg Cr, mean ± s.d.). These two levels were positively correlated (*r* = 0.650, *P* < 0.005).
with each other (r = 0.650, P < 0.005), and there was no significant difference between the mean ± S.D. values of the two levels.

Changes in hCT Levels in Plasma and Urine Obtained from Normal Subjects on Calcium Infusion (Fig. 8, a).

As observed in our previous work (Mori-moto et al., 1979), in three normal subjects tested, the plasma hCT levels rose from basal levels of 44, 47 and 52 pg/ml to maximum levels of 280, 560 and 445 pg/ml, respectively, two minutes after the start of calcium infusion, and then dropped, with apparent half-times of 13, 20 and 15 min, respectively. The levels of CT excreted in the urine in 15 min rose from basal levels of 2.8, 2.3 and 2.1 ng/15 min to maximum levels of 22.1, 17.5 and 14.0 ng/15 min, respectively, in the first 15-min period after calcium infusion, and dropped with apparent half-times of 19, 25 and 23 min, respectively.

Changes in hCT Levels in Plasma and Urine of Normal Subjects on Infusion of Synthetic (1-32)hCT (Fig. 8, b).

In the three normal subjects tested, the plasma hCT levels rose from basal levels of 0.100, 0.047 and 0.042 mg/ml to maximum levels of 120, 72 and 53 mg/ml, respectively, after the start of infusion, and then dropped with apparent half-times of 6.5, 7.0 and 8.0 min, respectively, in 30 min after the infusion. The levels of CT excreted in the urine in 15 min rose from basal levels of 2.7, 1.9 and 1.5 ng/15 min to maximum levels of 225, 138 and 149 ng/15 min, respectively, in the first 15 min after the infusion, and then dropped (or fell) with apparent half-times of 27, 34 and 20 min. The total CT immunoreactivities
Fig. 9. Gel filtration profiles of immunoreactive CT in the urine of a patient with MCT (a), in the first 15 min-urine sample from one of the normal subjects after calcium infusion (4 mg/kg) body weight (b), and in the first 15 min-urine sample from one of the normal subjects after infusion of synthetic (1-32)hCT (500 μg) (c). bPTH and hCT indicate the elution positions of 125I-bPTH and 125I-(1-32)hCT, respectively. Urinary CT in all these samples was of larger molecular forms than synthetic (1-32)hCT. *: peaks that seemed to be caused by interference of known constituents of the urine, since they increased in height following the addition of these constituents.

excreted in 2 h after infusion of synthetic (1-32)hCT were only 0.12, 0.10 and 0.07%, respectively, of the infused dose.

Gel Filtration of Urine Samples on Bio Gel P-30 Column.

The elution profiles of 3 urine samples with high CT concentrations are shown in Fig. 9. Of the 3 samples, the first was obtained from a patient with MCT, the second from a normal subject during the first 15 min after calcium infused, and the third from a normal subject after infusion of synthetic (1-32)hCT. The labeled markers, 125I-bPTH and 125I-hCT, were eluted in specific positions. The Kd values of 125I-bPTH and 125I-hCT were 0.27 and 0.53, respectively. The recoveries of immunoreactivity of CT in the 3 samples on gel-filtration were 87.5%, 71.7% and 85.4%, respectively. In the first urine sample two fractions of immunoreactive CT were observed; the major fraction had a Kd value of 0.38, and the minor fraction a Kd value of 0.28. In the second urine sample two fractions of immunoreactive calcitonin were also obtained with molecular sizes corresponding to those in the first urine sample. However, the third urine sample gave only one fraction with a Kd value of 0.38 corresponding to the major fraction in the first and second urine samples. No fraction corresponding in molecular size to 125I-hCT was obtained from any of these 3 urine samples.

Discussion

Measurement of plasma hCT concentration is essential for diagnosis and management of MCT (Foster, 1968). Furthermore its measurement in normal older women appears to be useful for predicting a long-term decrease in skeletal mass, because hCT strongly inhibits osteoclastic bone resorption (Holtrop et al., 1974) and because the basal plasma level of hCT, and that in response to calcium infusion, are reported to rise in old women (Hillyard et al., 1978; Morimoto et al., 1979; Deftos et al., 1980). However, the hCT level in plasma may not always reflect the actual secretion of hCT, first because the plasma hCT levels of normal subjects are known to
be usually below 100 pg/ml, near the limit of detection, not only in our assay (Okada et al., 1978) but also in several other assay systems (Heath and Sizemore, 1977; Parthemore and Deftos, 1978), and second because the plasma hCT level of normal subjects is found to fluctuate (Dymling et al., 1976) like that of patients with MCT (Miyauchi et al., 1980). This is why we measured the urinary CT concentration.

Using gel chromatography by a modification of the method of Snider et al. (1978) and our sensitive hCT RIA reported previously (Okada et al., 1978), we established a simple, reproducible technique for measurement of CT in normal human urine. Many compounds, such as creatinine and urea usually present in normal human urine interfered with our RIA system using midportion antiserum (Fig. 3), but the reasons for this interference are unknown. However, these compounds could be removed by gel filtration, while synthetic (1-32)hCT was recovered well from the column. Blue dextran did not cause interference, so we used it at a concentration of 5 mg/ml. This technique for the measurement of urinary CT showed good recovery and reproducibility and the standard curve was parallel with the dilution curves of purified urines obtained not only from patients with MCT but also from normal human subjects. These results indicate that this assay system is reliable for measurement of the urine CT concentration.

Silva et al. (1979) found high levels of urine CT in patients with MCT, and reported that measurement of urinary CT is valuable in the diagnosis of MCT. In our 11 patients with MCT, the urinary CT levels in ng/mg Cr were 3.4 to 20.8 times higher than the plasma hCT levels in pg/ml, and a good correlation (r=0.93, P<0.001) was noted between these two levels, as shown in Fig. 5. This is compatible with the findings of Silva et al. (1979), and measurement of urinary CT by our method was confirmed to be valuable in diagnosing MCT.

Though only a weak positive correlation was observed between the plasma and urinary CT levels of normal subjects (r=0.408, P<0.05), the urinary CT levels in pg/mg Cr were higher than the plasma hCT levels in pg/ml in 26 (81%) of the 32 normal subjects examined. The CT levels in the morning spot urine (1-2 h duration) and those in 24 h urine samples from the normal subjects were positively correlated with each other (r=0.650, P<0.005), and the mean values of the two levels were similar. These results suggest that the CT level in morning urine reflects that in 24 h urine of normal human subjects. So, for convenience, we used morning spot urine for measurement of urine CT. Moreover the urinary CT level apparently reflected the rise in plasma hCT both on calcium infusion and on infusion of synthetic (1-32)hCT into normal subjects, though the former changed more slowly than the level in plasma in all subjects studied (Fig. 8, a,b). These findings show that the urinary CT levels in normal subjects not only apparently reflect, but also integrate, changes in the plasma hCT level. Measurement of urine CT in normal subjects also seems to be of value as a clinical test for detecting long-term changes in CT secretion.

Only 0.07 to 0.12% of the immunoreactivity of CT was excreted in the urine in 2 h after the infusion of 500 μg of synthetic (1-32)hCT into three normal subjects. One reason for this low recovery in the urine may be that not only kidney but also bone and intestine have a specific receptor for CT (Nagata et al., 1975; Gray et al., 1976).

It is reported that the plasma of patients with MCT gives three to five or more distant peaks of CT-like activity on gel-filtration, which are detected by measuring the immunoreactivity of effluent fraction (Singer and Habener, 1974; Sizemore and Heath, 1975; Snider et al., 1977; Snider et al., 1979; Morimoto et al., 1981). Recent reports showed that several forms of CT-like immunoreactivity are present in the urine of patients with MCT, and that these CT-like compounds are apparently larger.
than the CT monomer (Silva et al., 1979). We also recognized two forms of CT-like immuno-reactivity in the urine of a patient with MCT, both with an apparently larger molecular weight than synthetic (1-32)hCT: the main one was eluted between $^{125}$I-bPTH and $^{125}$I-hCT, and the other near $^{125}$I-bPTH. These findings appear to be compatible with the data of Silva et al. (1979). Similar peaks were obtained from the urine of a normal subject after calcium infusion, and these materials were also larger than synthetic (1-32)hCT. Moreover, the urine sample of another normal subject after infusion of synthetic (1-32)hCT gave only one peak that was larger than synthetic (1-32)hCT and was eluted in the same position as the major peaks in the urine of the patient with MCT and in the urine of the normal subject after calcium infusion. Thus a larger molecular form of CT than synthetic (1-32)hCT was found not only in the urine from patients with MCT, but also in the urine from normal subjects after calcium infusion and after infusion of synthetic (1-32)hCT. It is unknown how this CT-like immunoreactive substance of larger molecular size is excreted even when synthetic (1-32)hCT is injected into normal subjects and further studies are needed on this problem.

It is concluded, however, that measurement of urinary CT levels is valuable not only in detecting a case of MCT, but also for evaluating CT secretion in normal subjects.

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