Radioimmunoassay of Calcitonin in Human Plasma

Omega L. Silva, Richard H. Snider, and Kenneth L. Becker

A sensitive, specific radioimmunoassay of calcitonin is described, suitable for measuring the hormone in peripheral plasma of normal man. With this assay we have demonstrated that calcitonin is detectable under basal conditions and the concentrations respond to induced and spontaneous hypercalcemia and induced hypocalcemia. The mean value for basal calcitonin of normal men was 182 pg/ml (range 63–450); patients with hypercalcemia of various etiologies had a statistically significant higher mean value of 510 pg/ml (range 110–2700). In calcium-infusion studies, concentrations of both total calcium and ionic calcium correlated well with plasma calcitonin (0.77 and 0.84, respectively). These findings strongly suggest that calcitonin is of physiological importance in man.

Additional Keyphrases: normal values • calcium metabolism • preparation of conjugate with hemocyanin • physiological importance of calcitonin in man

Calcitonin, a hypocalcemic hormone, is secreted by the C-cells of the mammalian thyroid gland. Since its discovery by Copp et al. (1) in 1961, laboratory and clinical studies have underlined its importance in both normal and abnormal calcium metabolism. Unfortunately, studies in man have been hindered greatly by an apparent difficulty in measuring, reproducibly and specifically, sufficiently low concentrations of this hormone.

The first group to develop a radioimmunoassay for calcitonin (CT) in humans could not measure the hormone in the peripheral blood of normal persons, because the assay was too insensitive and there was difficulty with separating free and antibody-bound CT (2). However, these authors were able to detect the very high concentrations of CT in peripheral blood of patients with medullary carcinoma. Subsequently, Tashjian et al. (3) measured immunoassayable CT in the plasmas of some normocalcemic subjects and of some with induced hypercalcemia. In 1971, Deftos et al. (4, 5) reported a radioimmunoassay that measured the concentrations of CT in the plasma of patients with medullary carcinoma, but which was not sensitive enough to detect the hormone in peripheral blood or thyroid venous blood of either normal or hypercalcemic persons. They suggested that the basal concentrations of CT in the peripheral plasma of humans is less than 100 pg/ml. Frolich et al. (6) reported an assay of comparable sensitivity, but gave no data for normal persons. In a recent abstract, Sizemore et al. (7) have reported that plasma CT values increased by 30% in response to intravenous calcium infusions.

This report describes a sensitive and specific radioimmunoassay of CT in man, which is capable of detecting and measuring the hormone in peripheral plasma of normal persons and in those challenged with intravenous calcium. The assay, therefore, lends itself readily to studies of the physiology of calcium metabolism and its disorders.

Materials and Methods

Synthetic human CT (biological activity: 80 MRC units/mg) was obtained through the kind cooperation of Dr. H. M. Greven, Organon Laboratories, Oss, Holland. In addition, reference materials were obtained from the British Medical Research Council, Mill Hill, London, N.W.7. CT was iodinated with Na125I by the method of Hunter and Greenwood (8) and purified on “Quso G-32” (micro-fine precipitated silica; Philadelphia Quartz Co., Philadelphia, Pa. 19106) (9) and further by gel filtration on “Biogel P-10” (Bio-Rad Laboratories, Richmond, Calif. 94804) (10).

The antibodies used in this assay were produced in the rabbit with synthetic CT conjugated to hemocyanin (keyhole limpet; Calbiochem, San Diego, Calif. 92112). The procedure we used to conjugate CT to hemocyanin was derived from the carbodiimide conjugation techniques described by Cuatrecasas et al. (11). During a 15-min period, 1-ethyl-3-(dimethylaminopropyl)carbodiimide (Ott Chemical), 8 mg, in 0.3 ml of 5 mol/liter guanidine hydrochloride (Eastman White Label; Eastman Kodak Co., Rochester, N. Y. 14650) was added dropwise at 25 °C with stirring to 3 mg of CT and 8 mg of hemocyanin, in 1.4 ml of guanidine hydrochloride (5 mol/liter). The pH
was maintained at 4.7 ± 0.1. After stirring at 4 °C for 24 h, the solution was dialyzed against distilled water, and lyophilized. Of this product, 10 mg was used for immunization without further purification, 1.7 mg being injected intradermally with Freund's adjuvant into 15-20 sites on the back of two rabbits together with one 0.1-ml injection of pertussis vaccine (12). Booster injections of 0.5–1 mg of conjugate were given at 3, 4, and 12 weeks. Six months after the initial injection, the antibody which was obtained could be used at a final dilution of 20,000- fold.

In the assay, 0.05–0.2 ml of plasma is incubated with 0.1 ml of antibody and 0.1 ml of labeled hormone, sufficient to provide 1500–2500 cpm. For quality control, pooled plasma of thyroidectomized patients is assayed simultaneously. Each unknown is assayed in triplicate, with and without antibody. The mixture is incubated at pH 7.5 in a buffer containing, per liter, 8.3 g of boric acid, 3.7 g of disodium ethylenediaminetetraacetate and 100 g of human serum albumin for 6–14 days at 4 °C, in a final volume of 0.5 ml. Bound and free hormone are separated by use of polyethylene glycol (13).

Figure 1 shows a typical standard curve, drawn on logit paper (14). The range of this assay is 10–1000 pg of calcitonin, and as little as 50 pg/ml of plasma can be detected.

To determine the stability of the hormone, we performed the following experiments. Of 5 ng of synthetic human calcitonin added to human plasma at room temperature, 95% could be detected after 1 h, whereas 40% remained after 24 h. Calcitonin is stable at 4 °C for at least 4 h. For assay purposes, therefore, plasma is collected in polypropylene syringes and immediately transferred to iced heparinized polypropylene tubes. If the chilled specimens are not to be assayed within 4 h, they are frozen. Immunoreactive calcitonin is relatively stable in frozen plasma for at least 6 months.

When human plasma was treated with charcoal and “Quo,” which adsorb polypeptides, up to 90% of endogenous calcitonin activity by our radioimmunoassay was lost. Endogenous calcitonin in human plasma was eluted from a Biogel P-10 column with phosphate buffer (20 mmol/liter, pH 7.5) at the same eluent volume as labeled calcitonin. Organon and MRC reference materials gave standard curves that were superimposable. Multiple dilutions of plasma gave values that were linearly related to endogenous calcitonin content. Both inter-assay and intra-assay variance were less than 20%.

For the infusion studies, calcium (10 mg/kg body weight, as calcium gluconate) was added to 500 ml of dextrose in water (50 g/liter) and infused during a 1-h period. In one subject, 2 g of trisodium ethylenediaminetetraacetate was added to 500 ml of dextrose in water (50 g/liter) and infused in 1 h. Blood was collected at 10-min intervals from a heparinized indwelling venous catheter.

Results and Discussion

The following hormones, assayed in nanogram quantities, showed no cross-reactivity: porcine calcitonin, salmon calcitonin, bovine parathyroid hormone, porcine insulin, porcine glucagon, and human thyroid-stimulating hormone.

Using this assay, we found the mean value for basal calcitonin in the peripheral blood of 50 fasting normal men to be 182 pg/ml (range, 63–450 pg/ml). Two men (4%) had values of less than 50 pg/ml, and 96% of all fasting values exceeded the mean value for the zero point by two standard deviations. Of normal men, 76% exceeded mean values for the thyroidectomized patients (83 pg/ml; range, 63–110 pg/ml) by two standard deviations. Of 17 normal women, 24% were less than 50 pg/ml, 82% were less than 200 pg/ml, and the highest concentration obtained was 260 pg/ml. A statistically significant difference was noted in hormone concentrations between the thyroid-

![Fig. 1. Standard curve for radioimmunoassay of CT in human plasma](image)

% B/B₀ = 100 (B - N/B₀ - N), where B = number of cpm in the presence of standard or unknown unlabeled hormone; B₀ = number of cpm bound in the absence of unlabeled hormone; and N = mean number of nonspecific cpm bound in the absence of antibody

![Fig. 2. Typical response of plasma calcitonin to calcium infusion](image)

The increase in plasma calcitonin correlates significantly with the increase in ionic and total serum calcium (P <0.001)
ectomized patients and normal persons ($P < 0.05$). The mean CT concentration for 17 patients with hypercalcemia (of various etiologies, including metastatic malignancy, primary hyperparathyroidism, sarcoidosis, and multiple myeloma) was 510 pg/ml (range, 110–2700 pg/ml), significantly ($P < 0.001$) higher than for normocalcemic men.

Plasma CT increased significantly in 12 of 15 men challenged by calcium infusion; Figure 2 shows a typical response to calcium infusion. In this man, peripheral plasma CT increased from 128 pg/ml to 480 pg/ml after an infusion period of 60 min, subsequently decreasing. For the 12 persons who responded to the calcium infusion, the correlation coefficients relating total serum calcium and ionic serum calcium to plasma CT were 0.77 and 0.84, respectively. In another study, infusion of trisodium ethylenediaminetetraacetate caused the value for basal CT to decrease from the initial 300 pg/ml to 150 pg/ml, consistent with the decrease in ionic calcium (Figure 3).

The demonstration of reproducible basal concentrations of calcitonin in the peripheral plasma of man, which change appropriately to perturbations of serum calcium, strongly suggests that this hormone is of physiologic importance in man.

References