Highly Sensitive Two-Site Immunoradiometric Assay of Parathyrin, and Its Clinical Utility in Evaluating Patients with Hypercalcemia


We have developed a highly sensitive, two-site immunoradiometric assay (IRMA) for human parathyrin (PTH) that is specific for the intact, secreted, biologically active 84-amino-acid peptide. This assay has several technical advantages: it does not detect even high concentrations of inactive carboxyl-terminal fragments, results are available within 24 h, and the detection limit for intact hormone is low (1 ng/L). The assay readily measures concentrations of PTH in all healthy subjects and distinguishes these values from low or undetectable PTH values observed in clinical situations in which PTH secretion is expected to be suppressed. We found complete separation of results from 37 patients with surgically proven hyperparathyroidism and those from 23 patients with hypercalcemia associated with malignancy, the latter having PTH values at or below the lower limits of normal for this assay. The sensitivity, specificity, and rapid turnaround time of this two-site IRMA should advance the laboratory evaluation of patients with disorders of calcium metabolism.

Additional Keyphrases: cancer • metabolism of calcium • hyperparathyroidism • reference interval • biologically inactive fragments

The complexities involved in measurement of parathyrin (PTH, parathyroid hormone) are related not only to the very low (10^{-12} mol/L) concentrations of biologically active hormone in the blood but also to the heterogeneity of circulating hormone, owing to metabolism and secretion of fragments of PTH. Current radioimmunoassays of PTH generally require prolonged incubation or sample extraction to detect the hormone concentrations present in normal, healthy subjects, and results often are spurious, owing to nonspecific effects of plasma or serum or interference by fragments. Existing assays of PTH, even those that identify the more abundant mid-hormone and carboxyl-terminal fragments, are insufficiently sensitive to distinguish subnormal from normal values or reliably to differentiate patients with hypercalcemia of malignancy from those with hyperparathyroidism (3).

Immunometric assays involving excess antibody have many advantages over competitive-binding radioimmunoassays: increased sensitivity, extended standard range, shortened incubation time, enhanced precision, improved specificity, and technical simplicity (4, 5). Nonetheless, development of new PTH assays continues to involve traditional competitive radioimmunoassay approaches, in part, because of inadequate quantities of suitable polyclonal antisera and the limited success thus far reported with developing monoclonal antibodies of requisite sensitivity for PTH (6). We have circumvented these practical limitations by harvesting large volumes of antisera from goats and by preparing immunological reagents having specificity restricted to the desired epitopes on the PTH molecule, using affinity purification with selected hormone-fragment ligands immobilized to a solid phase. The quantities of antisera harvested are sufficient to assure the stability of this assay for at least several decades.

Here we describe the development of a two-site immunoradiometric assay (IRMA) for PTH, based on affinity-purified polyclonal antisera: one group of which are specific for the amino-terminal 1–34 portion of the PTH molecule, the other for the 39–84 amino acid sequence. With this assay, we detect only biologically active intact PTH, with no interference from circulating PTH fragments. The assay provides all of the advantages of IRMA methodology and, most importantly, it has completely differentiated patients with surgically proven hyperparathyroidism from patients with hypercalcemia of malignancy.

Materials and Methods

Reagents

We raised antisera in goats immunized with partly purified human PTH from human parathyroid adenomatous glands. After preparing two separate immunoaffinity columns, by binding 2 mg of either human PTH(1–34) or PTH(39–84) (Peninsula Laboratories, Inc., Belmont, CA) to 1 g of CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden), we immunoadsorbed portions of the antisera to one or the other of the affinity columns. Unbound serum components were removed by washing with pH 7.4 phosphate buffer (10 mmol/L) containing 9.0 g of sodium chloride per liter. Bound antisera was then eluted with glycine buffer (0.2 mol/L, pH 2.3).

The antisera that had been immunoaffinity-purified to bind human PTH(39–84) was diluted in pH 7.4 phosphate buffer to 1.5 μg/mL, then immobilized by adsorption onto 8-mm-diameter polystyrene beads (Precision Plastic Ball Co., Chicago, IL). The immunoaffinity-purified antibody to human PTH(1–34) was radiiodinated with 125I by the Chloramine T procedure (7) to a specific activity of 30 Ci/g, and diluted in phosphate-buffered saline (0.6 mol/L phosphate buffer containing 9.0 g of sodium chloride, 1.0 g of bovine serum albumin, and 1.0 g of sodium azide per liter, pH 7.4) to give a concentration of 66 μg/L.

To prepare standards, we diluted synthetic human PTH(1–84) (Peninsula Laboratories) in PTH-free human serum, to give concentrations of 1, 2, 4, 8, 15, 25, 32, 40, 45, 50, 150, 450, and 1500 ng of synthetic human PTH(1–84) per liter. Human serum free of PTH was used as a zero standard. Standards must be diluted in volumes of serum.
equal to the sample volume for appropriate calibration. All standards were stored frozen at -20 °C or lyophilized.

Assay Procedure

Combine 200 µL of standard or serum sample in a test tube containing a bead coated with anti-PTH(39–84) (the capture antibody) and 100 µL of radiolabeled anti-PTH(1–34), and incubate at room temperature for 22 h. Wash the beads twice with 2 mL of the phosphate-buffered saline containing 1 mL of Triton X-100 per liter, and count the radioactivity of each bead for 1 min in a gamma spectrometer.

Subjects

On the day before surgical neck exploration, we obtained serum samples from 37 consecutive patients with hypercalcemia who, by clinical and laboratory evaluation, were thought to have hyperparathyroidism that required surgical cure. All of these patients, who were surgically explored for hyperparathyroidism by our Endocrine Surgical Service, are included in our series. All had operative findings, confirmed histologically, of parathyroid adenoma or four-gland parathyroid hyperplasia, and all had normalization of their calcium concentrations in serum after successful neck surgery.

We also measured PTH in serum from 23 patients with hypercalcemia and various cancers, evident clinically and proven pathologically, from either our inpatient Oncology Service or from the office practices of physicians at this hospital. Approximately half of these patients had metastases to the skeleton associated with breast and lung cancer; the others had hypophosphatemia with no evidence of skeletal metastases, and represent humoral hypercalcemia of malignancy (8, 9). To establish the normal reference interval, we used serum samples obtained from 72 healthy blood donors in Boston, none of whom were clinically suspected of having disorders of calcium metabolism.

Results

Assay Characteristics

For convenience we plotted the assay dose–response, the corrected counts/min (counts/min for each standard minus the counts/min for the zero standard) vs concentrations of PTH 1–84, on log-log paper for standard concentrations between 15 and 1500 ng/L; for lower concentrations (between zero and 45 ng/L) a linear plot is more convenient (Figure 1) and permits accurate determination of PTH values over that range. Values of unknowns were read directly from the plots. In practice, the detection limit of the assay is 1 ng/L, based on precision in counting small amounts of bound radioactivity.

To determine whether fragments of PTH react or otherwise interfere with measurement of intact human PTH by our two-site rMAA, we used it to assay the synthetic peptides PTH 1–34, PTH 39–68, PTH 53–84, PTH 44–68, and PTH 39–84. Human PTH 1–34 at a concentration of 300 ng/L, and each of the middle and carboxyl-terminal fragments at concentrations up to 100 000 ng/L (values substantially exceeding the concentrations of these fragments found in conditions such as renal failure), were introduced into zero and mid-range standards with no consequent change in assay results. This confirmed that the assay measures only intact human PTH 1–84.

We calculated the precision (intra-assay variance) of the assay from 20 replicate determinations of two quality-control sera in a single assay. The reproducibility (inter-assay variance) was calculated from data obtained over four weeks, during which we made an additional 20 determinations of these two control sera. The inter-assay CV ranged from 1.8% to 3.4%, the inter-assay CV from 5.6% to 6.1%.

We further assessed the utility of the assay by studying analytical recovery and parallelism. Various amounts of exogenous PTH 1–84 were added to a series of patients' samples, then measured by the rMAA; analytical recovery ranged from 93% to 105% (Table 1). Parallelism was assessed by use of a series of dilutions in zero standard for three patients' samples. These results show excellent agreement with predicted values (Table 2).

Clinical Studies

We determined the normal reference values for this assay as 12–65 ng/L, using nonparametric analysis of the 72 blood donors' sera by calculating +2 SD from their geometric mean of 28 ng/L.

Assay results for 37 individuals with surgically proven hyperparathyroidism were completely resolved from those for 24 patients with hypercalcemia of malignancy (Figure 2). All patients with surgically proven hyperparathyroidism had increased PTH concentrations except for two persons whose PTH was at the upper limit of normal. Ten patients

![Graph](image-url)
Table 2. Parallelism of PTH Assay

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<th>Sample</th>
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Three patients' sera were diluted with zero PTH standard.

Fig. 2. Intact PTH 1–84 measured by IRMA in sera from 72 normal individuals, 37 patients with surgically proven hyperparathyroidism (1st HPT), and 24 patients with hypercalcemia associated with malignancy. The normal reference interval is 12–65 pg/mL. Mean PTH in patients with hyperparathyroidism was 206 (±43 SEM) pg/mL and 3.35 (±0.8 SEM) pg/mL in individuals with hypercalcemia associated with malignancy.

Discussion

Biologically active intact PTH is secreted as an 84-amino-acid peptide; peripheral metabolism and intraglandular proteolytic modification of the hormone result in circulating peptide fragments that are principally middle and carboxyl-terminal biologically inactive fragments (1, 10–12). Biologically active intact PTH 1–84 has a half-life of <5 min, whereas the inactive middle and carboxyl-terminal fragments, which are cleared by the kidneys, have half-lives that are at least five- to 10-fold longer than the native 1–84 peptide (13). Thus the concentration of biologically inactive fragments is high relative to the concentration of active, intact hormone, especially during renal insufficiency, when the half-life of carboxyl-terminal PTH may approach 24 h (14).

Therefore, the ideal PTH assay for clinical situations involving assessment of calcium metabolism is one that is highly sensitive, specific for intact hormone, and free of nonspecific serum effects. These goals are satisfied by the IRMA for PTH that we describe. Sensitivity for intact hormone is high, and low values can be distinguished from those in normal subjects. The intrinsic affinity of the antisera for PTH is high, but less than needed for conventional radioimmunoassays, which suggests cooperative interaction among multiple antibodies (15). Specificity for intact hormone is accomplished by selecting antisera that recognize epitopes in widely divergent regions of the PTH peptide. Although both intact hormone and middle-carboxyl terminal fragments are bound to the "capture" antisera, the 125I-labeled amino-terminal antisera will bind only intact hormone and not middle-carboxyl terminal fragments; increasing counts bound correlate directly with the amount of intact hormone captured by the first antisera. The wide range of concentration over which the assay can be used without loss of precision (approximately 500-fold), and the one-day turnaround time are important technical advantages that also make patient care more timely by expediting correct diagnosis and institution of appropriate therapy.

It is vital for the clinician to establish the cause for hypercalcemia. Although the majority of clinically asymptomatic individuals found to have hypercalcemia have hyperparathyroidism as their underlying diagnosis (16, 17), humoral hypercalcemia of malignancy associated with a clinically occult and potentially curable cancer may be present. If clinical evidence of malignancy is not present and
the patient does not provide the evidence of chronic hypercalcemia as seen in hyperparathyroidism, the clinician must rely heavily on laboratory testing to establish a diagnosis. Unfortunately, many laboratory features of the humoral hypercalcemia of malignancy resemble findings in hyperparathyroidism: measurement of phosphate and cyclic AMP will not differentiate these two entities, for example (8, 9). Currently available radioimmunoassays of PTH fail to completely distinguish humoral hypercalcemia of malignancy from primary hyperparathyroidism. From a physiological standpoint, in situations other than hyperparathyroidism that cause hypercalcemia, PTH secretion should be suppressed to undetectable concentrations. Yet radioimmunoassays give measurements of PTH within the normal range in 25–50% of patients with malignancy, limiting the usefulness of the PTH immunoassay as a definitive tool in differential diagnosis (3, 18, 19).

The explanation for the observations of apparent immunoreactive PTH in the blood of patients with hypercalcemia associated with malignancy is still unresolved. Chromatographic and mRNA analyses of tumor extracts have provided no evidence that tumors associated with hypercalcemia produce PTH (20), and the biological activity of factors that appear to bind to the PTH receptor is not blocked by antisera to PTH (21). Most likely, the apparent increase of PTH seen in conventional competitive radioimmunoassays are nonspecific effects from factors in plasma from cancer patients, which interfere with binding of radiolabeled PTH to its antibody; alternatively, there could be sequence homology between PTH and factors responsible for hormonal hypercalcemia of malignancy.

The unique approach in our two-site immunoradiometric assay—the use of two distinct antisera pools, each recognizing a sequence within PTH—seems to eliminate the false “signals” from nonspecific effects that appear in competitive radioimmunoassays.

We are continuing an extensive evaluation of this assay in a wide spectrum of clinical conditions, such as hypoparathyroidism, pseudohypoparathyroidism, renal failure, and other forms of secondary hyperparathyroidism; dynamic testing with alterations in calcium is also being explored. Preliminary results in these other areas are encouraging. For example, individuals with idiopathic hypoparathyroidism most often have undetectable concentrations of PTH.

From a clinical standpoint, the rapid determination of PTH by this two-site IRMA within 24 h, coupled with the complete separation of patients with surgically proven hyperparathyroidism from those with hypercalcemia associated with malignancy, could help reduce or eliminate the need for the additional laboratory or radiographic procedures that are sometimes required to search for occult malignancy in patients with recently discovered hypercalcemia.

References