Development and Validation of a New Radioimmunoassay for Parathyrin (PTH)

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A radioimmunoassay for human parathyrin has been developed and characterized with whole-molecule (residues 1–84) human parathyrin and with the 1–34, 44–68, and 53–84 amino acid residue fragments of it. The antiserum used reacted with the whole molecule and with the 44–68 and 53–84 fragments, but not with the 1–34 fragment. Parathyrin concentrations in the serum of 118 normal subjects and of 112 patients with surgically proved primary hyperparathyroidism were determined with this assay. The mean results were 39 (SD 13) μL-Eq/mL for the normals and 111 (SD 77) μL-Eq/mL for the patients with primary hyperparathyroidism (p < 0.0005). The upper 95% confidence limit of the normal range was 60 μL-Eq/mL. For 54 patients with primary hyperparathyroidism, the preoperative values for serum parathyrin, calcium, and phosphate—but not creatinine—were statistically different from the postoperative values (paired t-test, p < 0.0005). Normal subjects showed significant (p < 0.0005) differences in serum calcium concentrations but not in parathyrin concentrations, compared with concentrations found in cancer patients and patients who had thiazide-induced hypercalcemia. Phosphate concentration in serum, although not a specific indicator of disease, is a valuable clue to the diagnosis of primary hyperparathyroidism.

Additional Keyphrases: charcoal and double-antibody separation compared • hyperparathyroidism • cancer • hypercalcemia • calcium • hormones • inorganic phosphate

Pioneer works on the development and validation of parathyrin (PTH) radioimmunoassay (1–4) have significantly contributed to the diagnosis and understanding of disorders involving calcium metabolism. The assay for PTH and the antiserum used for it, designated as GP-1M, developed by Arnaud et al. (3), have been easily accessible for clinical diagnosis and have contributed substantially to the medical care of patients with diseases of calcium metabolism. However, these earlier PTH assays involve prolonged incubation. An assay might take a week or more, although important clinical decisions frequently must be made more quickly than that.

A second drawback of some of the assays is the use of dextran-coated charcoal to separate free and bound hormone. We find that charcoal separation causes considerable variability and instability of the assay, especially when an antibody of relatively low affinity is used.

Here we characterize and report our clinical experience with a new PTH assay based on a new antiserum (GP-235), with which a shorter incubation can be used. We also compare the specificity of GP-235 with two other widely used antisera: GP-1M and a sheep anti-PTH developed in Europe.

Materials and Methods

Sample Preparation

Between 0800 and 1100 hours, 15 mL of blood is drawn from the patient, who has fasted overnight. The blood is allowed to clot at room temperature for 30 min, then centrifuged in a refrigerated centrifuge at 3000 rpm. Three milliliters of the resulting serum is placed in each of two vials and sent to the assay laboratory packed in ice; for long-distance shipment, serum is frozen and packed in solid CO₂. On arrival, the serum is stored at −20 °C until assay. One vial of serum is used for PTH determination, the second for determinations of calcium (5), phosphate (6), and creatinine (7).

Normal Subjects and Patients

The 118 normal subjects in this study were healthy and not pregnant, had normal serum calcium concentrations, and had no disease affecting bone or calcium metabolism. The ages of these men and women ranged from 20 to 55 years.

For patients with disorders of calcium metabolism, we reviewed their histories and classified patients with surgically proven primary hyperparathyroidism according to their surgical pathology report. We studied 112 patients with surgically proven primary hyperparathyroidism, 28 with malignant disease, six with hypoparathyroidism, 31 with chronic renal failure, 16 with thiazide-induced hypercalcemia, two long immobilized, and one with hypernephroma.

Reagents and Solutions

Thimerosal was purchased from Eastman Kodak Co., Rochester, NY 14650, and aprotinin (Trasylol), 10 000 kio-int. units/L, from FBA Pharmaceutical, New York, NY 10022. Antiserum GP-235 was obtained from one of the five guinea pigs injected intradermally with crude porcine PTH (3) of variable dose and at an irregular schedule. Blood was drawn seven to 10 days after injection. Several bleedings with appropriate titers were pooled and used for assay. Precipitation antiserum of burro antiserum to guinea pig immunoglobulin G (IgG) was prepared by immunizing a burro with IgG from a guinea pig. Blood from individual bleedings was pooled and titrated to achieve maximum precipitation. The details of immunization were similar to those described by Chase (8). Antiserum of sheep anti-PTH was a gift from Dr. R. Hehrmann, Hannover, F.R.G.

Human 1–84 PTH was a gift from Dr. Claude D. Arnaud, San Francisco, CA.

Synthetic human 1–34 PTH, 44–68 PTH, and 53–84 PTH were gifts from Dr. Henry T. Keutmann, Boston, MA.

Bovine PTH for radioiodination (lot no. 1515C001) was purchased from Inolex, Inc., Park Forest South, IL 60466.

Other reagents, and their preparation, were as follows: Sodium barbital buffer, pH 8.6, 50 mmol/L. Dissolve 10.3 g of sodium barbital, 6.8 g of sodium acetate, and 40 mg of

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1 Nonstandard abbreviations: PTH, bPTH, parathyrin (parathyroid hormone) and bovine parathyrin; PEG, polyethylene glycol; IgG, immunoglobulin G.

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thimerosal in 900 mL of distilled water. Adjust the pH to 8.6, dilute with water to 1 L, and recheck the pH.

**Hypoparathyroid patients' plasma.** Obtain plasma by plasmapheresis from three selected donors who have either postoperative hypoparathyroidism or familial hypoparathyroidism. Centrifuge the plasma at 4800 × g for 20 min at 4 °C. Measure PTH, calcium, phosphate, creatinine, and total protein concentrations, and freeze the plasma in 60-mL aliquots. For some unexplained reason, not all hypoparathyroid patients' plasma can be used in the assay.

**Diluent.** Mix 85 mL of barbital buffer, 10 mL of hypoparathyroid plasma, and 5 mL of aprotinin.

**Assay buffer.** Dissolve 3.3 g of polyethylene glycol 6000 (PEG; Fisher Scientific Co., Fair Lawn, NJ 07410) in 100 mL of diluent.

**Antibody dilution.** To prepare stock solution, dilute pooled antiserum GP-235 100-fold with diluent, divide into aliquots, and store at −70 °C.

To prepare working solution, on the day of assay further dilute the stock solution 900-fold with assay buffer. This 90 000-fold diluted antiserum should be used on the same day that it is prepared.

**Normal guinea pig serum solution.** Dilute normal guinea pig serum 20-fold with barbital buffer. This solution is used as carrier protein for the second-stage separation.

**Polyethylene glycol (PEG) solution.** 30 g/L. Dissolve 3 g of PEG 6000 in 100 mL of water.

**Standards.** Prepare a series of standard solutions with PTH concentrations ranging from 10 to 1000 μg/L by diluting with diluent a crude preparation of human PTH from trichloroacetic acid extract of human parathyroid adenomas (9). Calibrate this standard against human 1–84 PTH standard and against the serum of a patient with primary hyperparathyroidism, such as was used as the standard by Arnaud et al. (3). One nanogram of the crude preparation of human PTH is equal to 5.34 pg of human 1–84 PTH and 0.48 μL-Eq of Arnaud standard (3).

**Quality-control samples.** Prepare three concentrations of quality-control samples by pooling sera from patients with primary hyperparathyroidism at ranges between 50 and 70, 90 and 130, and 200 and 220 μL-Eq/mL, respectively. Check for hepatitis antigen; if negative, freeze the samples and use them for quality control. In addition, use serum from hypoparathyroid donors to monitor background; its PTH concentration will be about 20 μL-Eq/mL.

**Procedure.**

**Iodination of bovine PTH (bPTH).** We iodinated bPTH by the modified Hunter and Greenwood method (10), as follows.

Dissolve 5 μg of bPTH in 70 μL of phosphate buffer (0.5 mol/L, pH 7.5). Add 2.5 mCi of Na125I and 10 μL of Chloramine T (2 g/L in phosphate buffer); let react for 30 s by gently shaking. Add 50 μL of sodium metabisulfite (48 g/L) and 50 μL of hypoparathyroid plasma. Transfer the reaction mixture quantitatively to a 7 × 15 cm Bio-Rad P-6 column, elute with ammonium acetate buffer (0.2 mol/L, pH 4.8), and collect 0.5-mL fractions. Pool the fractions corresponding to the first radioactive peak (generally fractions 4 and 5), then apply to a 2.5 × 30 cm Bio-Rad P-60 column, which is equilibrated and eluted with the same ammonium acetate buffer. Collect 1-mL fractions, and pool those fractions corresponding to the second or third radioactive peak of the P-60 column, to be used as label.

**Assay procedure.** Thaw samples at room temperature. Assay each sample at two dilutions, using a duplicate set of tubes for each dilution. Most of the samples will be assayed both undiluted and diluted twofold with diluent. For samples with a high concentration of PTH and for samples from patients with renal failure, dilute the sample two- and 10-fold with diluent. Dilute each sample on the same day it is assayed, and keep both diluted and undiluted samples in an ice bath before and during assay.

Set a limit of 250 tubes in each assay (including the quality-control samples). To reduce the possibility of misleading results, include four quality-control specimens in duplicate at the beginning, middle, and end of the assays. The four controls consist of three pools with PTH concentrations of 50–70, 90–130, and 200–220 μL-Eq/mL, and an aliquot of serum from a hypoparathyroid donor, which is used to monitor background (~20 μL-Eq/mL). In addition to these controls, nonspecific-binding (blank) and total-binding tubes also are run at the beginning, middle, and end of each assay to give further assurance of the integrity of the assay.

The following procedure is designed so that the assay can be set up by manual equipment or by automatic pipette, such as the Micromedic pipetting station. Routinely, we use the Micromedic pipetting station to set up our assay.

Add a 100-μL sample or diluted sample to a 12 × 75 mm tube and 300 μL of 90 000-fold diluted working antisera solution. For the blank tube, add 300 μL of assay buffer instead of antisera; for the standard curve, replace the sample with 100 μL of standard solutions containing 0, 1, 2, 3, 5, 10, 20, 30, 50, and 100 ng of the crude preparation of human PTH. Run all standards and blank in duplicate tubes. Mix each well and incubate the mixture for 24 h at 4 °C. Then add 10 000 cpm of 125I-labeled bPTH dissolved in 100 μL of diluent; mix well and incubate for another 24 h at 4 °C. Add 50 μL of 20-fold diluted normal guinea pig serum and 100 μL of burro anti-guinea pig serum; mix well and incubate for 60 min at 4 °C. Add 400 μL of cold PEG solution; mix, incubate for an additional 30 min at 4 °C, and centrifuge at 3000 rpm for 20 min at 4 °C. Decant the supernate and count the radioactivity of the precipitate.

Calculate the net percentage binding of standard or sample tubes vs zero tube (B/Bo) by subtracting the average radioactivity counts of the blank tubes. Plot a standard curve of B/B0 vs dose of PTH on a semilog graph paper. Read sample
results from the standard curve. In routine assay, this process is done by an online computer. Report the average values obtained from two dilutions; if the difference of values from two dilutions of a sample exceeds 40%, re-assay the sample.

At the end of each month, enter the quality-control values into an analysis of variance program to determine the within-assay, among-assay, and total-assay variabilities.

Results

Purification of Labeled PTH

We used a two-column system to purify the labeled PTH. The first column, Bio-Rad P-6, separates the protein-bound and free iodide; the second column, Bio-Rad P-60, further purifies the label for assay. When Inoxel bPTH was iodinated, three peaks eluted from the P-60 column (Figure 1A). The blank values were 4.9, 4.4, and 2.6%, and net bindings were 9.5, 29, and 31% for the first, second, and third peaks, respectively. Because the difference in net binding between the second and third peaks was not significant, we pooled the second and third peaks and used that as labeled bPTH. When bPTH prepared by Dr. Keutmann (11) was iodinated with the same procedure to compare the quality of labeled bPTH, only two peaks were obtained (Figure 1B); the blank values were 10 and 5.5%, and net bindings were 13 and 28% for the first and second peaks, respectively. Obviously, both bPTH preparations are heterogeneous; however, when purified by P-60 chromatography, either can be used in the assay. We routinely use Inoxel bPTH to prepare labeled PTH.

Assay Conditions

To shorten the incubation period (from the seven days of the assay we previously used), we tested a number of conditions. Increasing the incubation temperature and adding PEG to the incubation mixture to a final concentration of 20 g/L speeded up the reaction. After 24 h of incubation at 4 °C, the binding of the zero tube reached 20%; at room temperature, it reached 30%; at 4 °C with PEG included (20 g/L), it also reached 30% (Figure 2). The fastest reaction was at room temperature with PEG: >40% binding in 24 h. We chose 4 °C with PEG as our assay incubation condition because the assay was more reproducible under these conditions.

Second-stage antibody vs charcoal separation. When dextran-coated charcoal was used to separate free and bound fractions, the blank values of most of the samples were between 8 and 10% of the total radioactivity added. Occasionally, however, the blanks of some samples had much higher values. Because the value of a blank for an individual sample cannot be predicted, a blank control must be assayed with every sample to safeguard against underestimating PTH for samples with high blank values. One advantage of the precipitation-antibody separation was the fact that the blank value was always between 5 and 6% of the total counts, even on samples that showed a high blank value by the charcoal method.

The values for 14 sample blanks with high values (12–34% B/T, mean 19.2%, SD 7.7%) by the charcoal separation method (3) were redetermined by our second-antibody separation method. By the second-antibody method, the range decreased to between 4.7 and 6.0%, and the mean was 5.4% (SD 0.4%).

Fig. 3. Characterization of three antisera with whole-molecule human PTH and amino acid fragments 1–34, 44–68, and 53–84 Guine pig-235 (O), sheep (△), and guinea pig-1M (△). Guine pig-1M reacts with whole-molecule human PTH assayed by C. Arnaud's laboratory (△)
We therefore selected the second-antibody method to separate the free and bound phase. Because the value of each individual sample blank was near that of the assay blank, we could omit the individual sample blanks.

Characterization of the antisera and comparison with other antisera. The antisera was characterized by reaction with whole-molecule human PTH (1-84 amino acid residues), N-terminal human PTH (1-34), and C-terminal human PTH fragments of 44-68 and 53-84 amino-acid residues, and compared with GP-1M and another clinically useful antisera from sheep (all three antisera were obtained with porcine PTH as immunogen). The three antisera all recognized the 1-84 whole molecule and the 44-68 portion of the C-terminal; none of them reacted with the 1-34 N-terminal; and only the new antisera GP-235 reacted with the 53-84 portion of the C-terminal of human PTH (Figure 3 and Table 1).

Quality control. A combination of Levey-Jennings plots, professional judgment, and analysis-of-variance calculations was used to monitor quality control. With 12 controls in each assay, the probability that at least one control would be outside the mean ±2 SD limits approaches 50%; therefore, we examined the controls to determine whether more than one control in a given portion of the assay was shifted in the same direction or whether there was evidence of drift or changes in binding from the beginning to the end of the assay. Based on approximately 40 assays per month, the monthly within-assay CV averaged 5 to 10%, the monthly among-assay CV averaged 8 to 13%, and the monthly total-assay CV averaged 10 to 15% over the past two years.

Clinical Results

The PTH values of normal subjects (Figure 4) were significantly (p < 0.0005) lower than those of patients with primary hyperparathyroidism, as compared by Student’s t-test (Figure 5). There were no significant differences in PTH values among normals, patients with cancer, and patients taking thiazide (Table 2); however, the calcium values of normal subjects were significantly (p < 0.0005) lower than those of patients with hyperparathyroidism or cancer or of patients taking thiazide. Calcium values in postoperative patients with hyperparathyroidism were slightly lower than in normal subjects (p < 0.001) (Tables 2 and 3).

Comparison of preoperative and postoperative results in primary hyperparathyroidism. In 54 of the 112 patients with surgically proven primary hyperparathyroidism, the postoperative assays for PTH revealed (Table 3) significant de-
creases in PTH and calcium concentrations and a significant increase in phosphate concentration after surgery (p < 0.0005 by paired t-test). The creatinine concentrations showed no significant change before and after parathyroid operation.

In addition, the PTH values of patients with primary hyperparathyroidism were significantly different from those of patients with cancer, and the calcium and phosphate values of patients with primary hyperparathyroidism also were significantly different from those of patients with cancer and patients taking thiazide (p < 0.0005) (Table 2).

Serum phosphate concentrations of normal subjects and patients. Serum phosphate concentrations were lower in patients with primary hyperparathyroidism than in normal subjects (p < 0.0005) (Table 2). Serum phosphate concentrations of patients after surgery for primary hyperparathyroidism were no different from those of normal subjects and patients taking thiazides (Tables 2 and 3, Figure 6).

Tumor weight and PTH concentration. For 92 of the 112 patients with surgically proven primary hyperparathyroidism, tumor weights were determined and their pathologies examined microscopically. Most of the tumors were chief cell adenomas; a few were oxyphilic cell adenomas. The weight of tumors ranged from 50 mg to 3.67 g. In the four patients with multiple gland enlargement, we used the combined tumor weight in our calculations.

We did not find a direct linear correlation between tumor weight and PTH concentration (r = 0.30) or between different types of tumor and PTH concentration. However, the preoperative PTH concentrations of patients with primary hyperparathyroidism still can be used to estimate tumor weight; for example, among the 92 patients we studied, 53 of 72 patients whose tumors weighed less than 1 g had PTH concentrations less than 100 μL-Eq/mL. Moreover, 15 of 20 patients who had tumors weighing more than 1 g also had PTH concentrations exceeding 100 μL-Eq/mL. Thus, if patients had PTH concentrations greater than 100 μL-Eq/mL, there was a 75% chance that they would also have a tumor weighing more than 1 g.

Discussion

The method reported herein is a modification of the PTH assay described by Arnaud et al. (3). The incorporation of PEG (20 g/L final concentration) in the incubation mixture reduced the incubation time from seven days to two days. The use of second-stage antibody precipitation instead of dextran-coated charcoal to separate the free and bound fractions improved the stability of the assay and eliminated the need to perform a blank control on every sample. The PEG increased the rate of the reaction and hence shortened the incubation time (Figure 2).

The problems of nonspecific binding and high sample-blank values generally are poorly understood. Either problem may be caused by some substances in the serum that prevent the charcoal absorption of free PTH in the incubation mixture. The second-stage antibody separation is not influenced by these substances. In addition to the 14 samples reported in Table 1, of thousands of samples analyzed over a long period, not one showed a high blank value by the precipitation antibody technique.

We also introduced the two-column procedure for purifying the labeled bPTH. Although more tedious than the QUISO method used in the original procedure (3), the two-column procedure ensures a consistent quality of labeled bPTH.

We studied the specificity of three PTH antisera with four peptides: whole hPTH 1–84; N-terminal amino acid 1–34; and C-terminal 44–68 and 53–84 fragments. All three antisera did not react with the N-terminal 1–34 fragment but reacted with the 1–84 whole molecule and the 44–68 fragment of human PTH. Among the three antisera, only one, GP-235, reacted slightly with the 53–84 fragment. For all three antisera, the 1–84 whole PTH molecule reacted much better than the 44–68 fragment. The reactivity 44/68:reactivity 53/84 ratios were 8, 10, and 56 for GP-235, GP-1M, and sheep antisera, respectively.

Table 3. Comparison of Preoperative and Postoperative Values in 54 Patients with Primary Hyperparathyroidism

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Preoperative</th>
<th>Postoperative</th>
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<tbody>
<tr>
<td>PTH, μL-Eq/mL</td>
<td>103 ± 68</td>
<td>38 ± 12*</td>
</tr>
<tr>
<td>Ca, mg/L</td>
<td>115 ± 9</td>
<td>89 ± 6*</td>
</tr>
<tr>
<td>PO₄, mg/L</td>
<td>26 ± 4</td>
<td>34 ± 7*</td>
</tr>
<tr>
<td>Creatinine, mg/L</td>
<td>11 ± 5</td>
<td>11 ± 5</td>
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* Significant difference from preoperative value (paired t-test, p < 0.0005).
The GP-1M and the sheep antisera were 1.67-fold more sensitive than the GP-235 antiserum reactions to the 1–84 whole PTH molecule.

Among the PTH assays reported, only that of Reiss and Canterbury (2) could distinguish PTH concentrations of normal subjects from those of patients with primary hyperparathyroidism, without overlap. Other investigators found overlap of PTH concentrations of normal subjects with those of patients with primary or ectopic hyperparathyroidism (1, 3, 4, 12). Arnaud et al. (3) suggested a two-dimensional reporting procedure, plotting PTH concentrations on the ordinate against calcium concentrations on the abscissa. This method performed well in differentiating normal subjects from patients with primary hyperparathyroidism, hypoparathyroidism, or chronic renal failure. However, some overlap existed between primary hyperparathyroidism and cancer-related hypercalcemia in our current assay, as it had in other assays (13). To differentiate these two types of hypercalcemia, at present, is beyond the boundary of a clinical laboratory and depends on the clinician’s judgment and the use of other diagnostic procedures (14, 15).

Patients with primary hyperparathyroidism tend to have low serum phosphate concentrations. In our study, there was a statistically significant difference between normal subjects and patients with primary hyperparathyroidism (p < 0.0005). However, hypophosphatemia, defined as being less than the normal range of serum phosphate (25–45 mg/L), does not help to interpret PTH results, because 67% of the patients with primary hyperparathyroidism in our study had normal concentrations of serum phosphate, >25 mg/L. Twenty years ago, Keating also found that most patients with primary hyperparathyroidism, 41% without azotemia (blood urea <400 mg/L), and 81% with azotemia (blood urea >400 mg/L) had normal concentrations of serum phosphate (16). However, a serum phosphate concentration of 30 mg/L may be used as a guideline in interpreting PTH results. In our experience, 80% of patients with surgically proved primary hyperparathyroidism who had normal renal function had phosphate concentrations less than 30 mg/L, and 97% had concentrations less than 33 mg/L (Figure 6). The phosphate concentrations of primary hyperparathyroidism also were significantly lower than those of patients with cancer and of patients taking thiazide (p < 0.0005, Table 2). The phosphate concentration is not a specific indicator, but may serve as an important clue in the diagnosis of primary hyperparathyroidism. Phosphate results should be interpreted with caution, because many factors, for example, the administration of aluminum hydroxide gel, cause hypophosphatemia.

Patients with chronic renal failure have high concentrations of serum PTH, phosphate, and creatinine, and low concentrations of serum calcium. Typically, a patient with chronic renal failure has an increased concentration of blood creatinine; hence, the creatinine determination should be included with the PTH assay. Arnaud et al. (17) have shown that the concentration of serum PTH begins to increase when the creatinine clearance is less than 60 mL/min per 1.73 m².

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References