Parathyrin (Parathyroid Hormone): Radioimmunoassays for Intact and Carboxyl-Terminal Moieties

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Introduction

Parathyrin (parathyroid hormone, PTH), a single-chain peptide of 84 amino acids secreted by the parathyroid glands, is one of the three hormones that regulate the homeostatic mechanisms for calcium (the other two are calcitonin and vitamin D). Although physicians have been interested in the diagnosis and treatment of disorders of parathyroid gland function and mineral metabolism for much of this century, the development in the 1960s of reliable methods for the direct measurement of PTH in the clinical laboratory has ushered in a new era in the clinical evaluation of these diseases (1).

Calcium is one of the most important substances in the body, and its concentration in blood is closely regulated, largely by PTH. Derangements in this regulation can have profound pathologic consequences resulting from involvement of the skeletal, neuromuscular, renal, gastrointestinal, or other systems. Radioimmunoassay (RIA) is presently the method of choice for measuring PTH. Although conventional bioassays in whole animals lack the sensitivity required, there are two new methods on the horizon: a cytochemical bioassay (2) and a radioreceptor assay. However, these methods, while deserving close attention as they develop, are still a long way from universal applicability.

The first RIA for PTH was described by Berson et al. in 1963 (3). An explosion of interest followed, and within the next 10 years many other such RIAs were described. Disillusionment soon set in, however, when it became evident that the measurement of PTH and the interpretation of assay results were not as simple as for other peptide hormones being measured by RIA. Confusion and disenchantment followed, prompted by conflicting reports of the clinical performance of PTH assays developed in different laboratories.

The classic paper by Berson and Yalow on the immunoheterogeneity of PTH was a landmark in the field of RIA measurement of peptide hormones (4). It gradually became apparent why this hormone was so difficult to assay: PTH in blood circulates as a complex mixture of many peptide forms, and different assays can measure only a portion of the total immunoreactivity. In the early 1970s, the important studies of Arnaud and others clarified the role of PTH heterogeneity and helped dispel the myths about its measurement by RIA (5-7). It is now almost universally agreed that one of the most useful and commonly ordered RIA tests is that for PTH (8).

We will have more to say about the clinical applications of PTH assays in the Discussion section. We will first describe the procedures for measurement by RIA of the two major forms of PTH in blood: intact hormone and its carboxyl (C)-terminal fragments.

Note: These assays use reagents (antisera, PTH standards, etc.) and procedures developed in our own laboratory. We have no experience with other methods and reagents, some of which are commercially available. However, it should be possible to use these in RIA procedures similar to ours, provided extensive studies of specificity, clinical validation, etc., are done, as discussed later. On the other hand, we are aware that these commercially available reagents vary considerably in quality and potential utility. The reader would be well advised to evaluate such reagents critically. We have recently described in detail the criteria we believe important in evaluating PTH assay reagents (or laboratory services) (9).

Principle

The principle of RIA is so well known that it need not be described here. The interested reader may consult any of a number of books and articles on this general subject.

Materials and Methods

Reagents

1. Antisera

The fundamental difference between the two PTH assays described is the antisera used.

(a) Antiserum against intact PTH. This antiserum was produced by subcutaneous immunization of a guinea pig with a preparation of bovine PTH (see 2a, below) conjugated to bovine serum albumin. Use of the conjugate in primary or booster immunizations did not lead to useful antiserum, but subsequent booster immunizations with unconjugated hormone resulted in rapid development of high-titer, high-affinity
antiserum to PTH. Complete details of the immunization protocol have been reported elsewhere (10).

(b) Antiserum against C-terminal PTH fragments. This antiserum was produced in a guinea pig by intradermal immunization with the C-terminal human PTH fragment preparation described in 2b. Details of the immunization protocol have been described (11).

2. Parathyroid hormone preparations

(a) Bovine hormone purified from parathyroid glands by the methods of Hawker et al. (12). The product of the G-100 gel filtration step possessed a specific biological activity of 300-500 kilo-USP units/g (13) and was used as the immunogen for the intact PTH antiserum without further purification. For use as primary RIA standard and for preparation of the radiiodinated hormone (see below), this material was purified by gel filtration and ion-exchange chromatography (12). The final product is salt-free and has a specific biological activity of 3000 kilo-USP units/g.

Note: Some commercial PTH preparations may not be salt-free.

(b) A human C-terminal PTH fragment preparation, for use as the immunogen for the C-terminal PTH antiserum, was obtained from pooled human parathyroid adenoma tissue, as described (11).

3. Reagents for radioiodination

(a) Cellulose powder: CF-1; Whatman, H. Reeve Angel and Co., Inc., Clifton, NJ 07014.

(b) Chloramine T (p-CH2CH2SO3NC1Na • 3H2O); Eastman Kodak Co., Rochester, NY 14650.

(c) Sodium [125I]iodide: IMS-300; Amersham Corp., Arlington Heights, IL 60005.

4. Other reagents for RIA procedures

(a) Charcoal: Norit A, neutral activated charcoal, pharmaceutical grade; Amend Drug and Chemical Co., Irvington, NJ 07111.

(b) Dextran T-70; Pharmacia Fine Chemicals, Inc., Piscataway, NJ 08854.

(c) Normal horse and guinea pig sera; Pel-Freez Biologicals, Rogers, AR 72756.

(d) Thimerosal, sodium salt; Sigma Chemical Co., St. Louis, MO 63178.

(e) Aprotinin (10^7 inhibitor units/L): Trasylol®, FBA Pharmaceuticals, Mobay Chemical Corp., New York, NY 10022.

(f) Normal human sera for quality-control pools and diluent. Outdated blood-bank plasma, previously tested for low PTH content and minimal ability to produce "damaged" [125I]-labeled PTH (see below for details) is first converted to serum, as follows:

To 1 L of plasma, add 6 mL of thrombin (Fibrinex®, Ortho Pharmaceutical Corp., Raritan, NJ 08869) and 6 mL of prothamine sulfate, USP (Eli Lilly & Co., Indianapolis, IN 46206). Stir. Let mixture stand in 37 °C water bath for 1 h. Centrifuge for 20 min at 10,000 x g. After filtering the supernate through glass wool, apportion it into capped 12 x 75 mm polystyrene tubes. Store at -70 °C.

(g) Hyperparathyroid serum, for quality-control pools. Previously assayed sera from patients with surgically proven primary hyperparathyroidism, first tested for hepatitis B surface antigen. Pool those found to be negative, filter through glass wool, and store as for normal human serum (above).

5. Common reagents

All other chemicals are of reagent grade. Glass-distilled, de-ionized water is used throughout.

Solutions

1. Buffers

(a) Sodium barbital buffer, 0.1 mol/L, pH 8.6. Dissolve 44.39 g of barbital and 197.71 g of sodium barbital in about 3 L of H2O by stirring magnetically overnight. Titrate to pH 8.6 with NaOH solution (5 mol/L). Add H2O to a final volume of 12 L; check the pH. Store in a polyethylene container at 4 °C.

Note: Do not store this solution in glass.

(b) Sodium barbital buffer, 0.02 mol/L, pH 8.6. Make from the above by diluting fivefold. Check pH.

(c) Sodium phosphate buffer, 0.5 mol/L, pH 7.45. Dissolve 17.28 g of sodium phosphate (monobasic, Na2HPO4 • H2O) in 200 mL of H2O. Titrate to pH 7.45 with NaOH solution (5 mol/L); dilute to 250 mL. Check pH. Store at room temperature.

(d) Sodium phosphate buffer, 0.05 mol/L, pH 7.45. Make from the above by diluting 10-fold. Check the pH. Store at 4 °C.

2. For radioiodination

(a) Chloramine T. Just before use, weigh 35 mg of Chloramine T into a large test tube. Add 10 mL of 0.05 mol/L sodium phosphate buffer, pH 7.45, to dissolve the solid.

(b) Sodium metabisulfite. Prepare freshly daily. Dissolve 24 mg in 10 mL of 0.05 mol/L sodium phosphate buffer, pH 7.45.

(c) Parathyrin. Weigh the purified bovine hormone on a Cahn Electrobalance (or equivalent) and dissolve in 0.01 mol/L acetic acid to a concentration of 0.5 g/L. Accurately dispense 5-µL aliquots with an automatic pipettor into the tips of 0.4 mL polyethylene conical centrifuge tubes; cap the tubes and store at -70 °C for radioiodination at a later date. Generally, 50 µg of purified PTH, when diluted as described, will supply enough hormone for many hundreds of assays.

3. For RIA procedures

(a) Dextran-coated charcoal suspension. To 13 g of charcoal and 1.3 g of Dextran T-70 in an Erlenmeyer flask add 78 mL of 0.1 mol/L barbital buffer, pH 8.6; 7.8 mL of normal human serum; and 304 mL of water. Stir the suspension at 4 °C overnight (with magnetic stirrer). Centrifuge the suspension (10 min, 4 °C, 275 x g) and decant to remove fine charcoal particles and uncoated dextran. Resuspend the precipitate in 400 mL of 0.02 mol/L sodium barbital buffer, pH 8.6, containing 20 mL of normal horse serum per liter. Stir magnetically at 4 °C overnight; use within three days.

(b) Diluents

For intact PTH assay. Use a diluent solution of 0.02 mol/L sodium barbital buffer, pH 8.6, containing, per liter, 250 x 10^6 inhibitor units of Trasylol, 20 mL of normal guinea pig serum, and 0.2 g of thimerosal, to dilute standard PTH, antiserum, labeled PTH, and assay tube contents to final volume.

For C-terminal PTH assay. Use a diluent solution of 85 mmol/L sodium barbital buffer, pH 8.6, containing, per liter, 500 x 10^6 inhibitor units of Trasylol, 100 mL of normal human serum (see above), and 0.2 g of thimerosal, to dilute standard PTH, antiserum, labeled PTH, and assay tube contents to final volume.

Note: The diluents for the two assays must not be interchanged. Use only the appropriate diluent. Interchanging the diluents seriously hampers the performance of each assay. It is apparently a characteristic of each antiserum that certain constituents or concentrations of buffer may work successfully for one antiserum, but may inhibit the performance of the other (15).

(c) Parathyrin RIA standards

For intact PTH assay: Dilute 5-µL of the PTH solution (2.5 µg of PTH) for radioiodination to a concentration of 1 µg/L with intact-assy diluent. Dispense diluted solution in 400-µL aliquots into 12 x 75 mm polystyrene tubes. Cap tubes and store at -70 °C.

For C-terminal PTH assay: Dilute 2 µL of the PTH solution (1 µg of PTH) for radioiodination to a concentration of 1 µg/L with acetate acid (10 mmol/L). Dispense in 200-µL aliquots. Freeze and store as above.
(d) Antisera
For the intact PTH assay. Dilute antiserum (GP204) 40 000-fold with intact-assay diluent; final dilution in the assay itself is 400 000-fold, because the final assay volume is 1 mL.

For the C-terminal PTH assay. Dilute the antiserum (GP75U) 1500-fold; add 0.1 mL of this solution to each assay tube requiring antiserum. The final assay volume is 0.5 mL, so the final dilution is 7500-fold. Prepare this just before use in C-terminal diluent from aliquots of stock antiserum stored at $-70 \, ^\circ \text{C}$.

Special Apparatus
1. For radioiodination
   (a) Chromatofocusing apparatus: Model 3-1200CE; Buchler Instruments, Inc., Fort Lee, NJ 07024.
   (b) Power supply (for the above), adjustable to 1000 V, with constant-voltage regulation: Buchler Instruments, Model 3-1014A.
   (c) Chromatography paper: Toyo No. 21-689; Nuclear Associates, Inc., Carle Place, NY 11514. This paper is tested by supplier's consultant for ability to adsorb PTH and other peptide hormones.
   (d) Radiochromatogram scanner: Model 7201; Packard Instrument Co., Inc., Downers Grove, IL 60515.
   (e) Micro-columns. Columns for purification of $^{125}$I-labeled PTH by adsorption to cellulose are made from 10-mL polystyrene disposable pipets (Falcon) cut in half with a sharp knife. The tip of the lower half is fitted with a small glass wool plug and filled with dry cellulose powder, which, when taped firmly with a small rod, occupies a volume of 2 mL.

2. For RIA procedures
   (a) Automatic pipetter: Model 25004 with Model I 200-$\mu$L pump and Model B 1000-$\mu$L pump; Micromedic Systems, Inc., Hosham, PA 19044.
   (b) Freezer, ultracold (to $-70 \, ^\circ \text{C}$): Model UC-105; Kelviniator, Inc., Manitowoc, WI 54220.
   (c) Reciprocating shaker: Model 6000; Eberbach Corp., Ann Arbor, MI 48106.
   (d) Automatic gamma-scintillation spectrometer: Model 1285, 1008-tube capacity; Tracor Analytic, Inc., Chicago, IL 60636.
   (e) Refrigerated centrifuge: Beckman J-6; Beckman Instruments, Inc., Fullerton, CA 92634. Must be able to generate a centrifugal force of 2000 $\times g$ and hold at least 200 10 $\times 75$ mm tubes.
   (f) Incubation tubes, soda-lime glass: Neutrex$^\text{a}$; Chase Instruments Corp., Poultny, VT 05764.

Note: Avoid use of tubes made of borosilicate glass or plastic. PTH is notorious for its ability to bind to almost any surface. This binding has been found to be lowest in soda-lime (or flint) glass.

Preparation of $^{125}\text{I}$-Labeled PTH
Highly purified bovine PTH is labeled with $^{125}$I every two weeks by modification of the procedures of Hunter and Greenwood (14, 15).
   1. Thaw one small tube containing 2.5 $\mu$g of pure bovine PTH in 5 $\mu$L of acetic acid (10 mm/L).
   2. Add to this 1.2 mCi of $^{125}$I (diluted to 20 $\mu$L with sodium phosphate buffer, 0.5 mm/L).
   3. At time zero add 24 $\mu$L of freshly prepared Chloramine T solution; vortex-mix.
   4. After 25 s stop the reaction by adding 96 $\mu$L of sodium metabisulfite solution; vortex-mix.
   5. Add 600 $\mu$L of normal human serum.
   6. Transfer the contents of reaction tube to the top of a dry column of cellulose CF-1.
   7. Wash column five times with 1.5 mL of sodium barbital buffer (20 mmol/L, pH 8.6).

8. Elute with five 1.0-mL volumes of acetone/acetic acid/water (20/0.8/79.2, by vol). Collect separate 1.0-mL fractions.

9. Divide the most highly radioactive fraction of the acetone/acetic acid/water eluate into several smaller aliquots. Store at $-70 \, ^\circ \text{C}$.

10. Analyze droplets of the acetone/acetic acid/water eluate and the original reaction mixture by paper chromatofocusing and radiochromatogram scanning (16) to determine the purity and specific activity of the labeled PTH preparation.

Note: The $^{125}$I-labeled PTH prepared as described should be essentially completely free of unreacted Na$^{251}$I and "damaged" $^{125}$I-labeled PTH components; its specific activity should range from 250 to 360 Ci/g.

Collection and Handling of Specimens
Obtain blood samples between 0700 and 0900 hours from patients after an overnight fast$^3$ and allow to clot at room temperature for 1 h. Separate the serum in the usual manner and store frozen ($-20 \, ^\circ \text{C}$) until assay. Although we have no conclusive data on this, it seems best to avoid assaying sera more than 12 months old. Plasma samples are acceptable for assay; we prefer serum, however, because it seems to produce fewer problems in the RIA. Avoid specimens collected in Corvac$^b$ or similar tubes, which may adsorb some of the PTH. Severely lipemic or badly hemolyzed specimens will produce unacceptable interference and are best avoided.

Procedure
The basic procedures for assay of intact or C-terminal PTH are nearly identical. Both RIAs use the same bovine PTH for standards and radioiodination, the same dextran-coated charcoal preparation (for separation of antibody-bound and free $^{125}$I-labeled PTH), and the same types of equipment, such as soft glass incubation tubes, pipettors, etc.: The only major difference between the assays is the antiserum. We will first describe the C-terminal PTH assay in detail, then the assay for the intact hormone. For the latter we will point out only the significant areas of difference from the C-terminal assay.

Procedure for C-terminal PTH assay. The day before the assay, consecutively number disposable 12 $\times$ 75 mm glass culture tubes and place all frozen serum specimens to be assayed and an appropriate number of standards (see below) in the refrigerator (4 $\, ^\circ \text{C}$) to thaw overnight.

Note: The size of the run is governed by the number of tubes that can be centrifuged at one time.

On the day assay is started (day 0), place all assay tubes and solutions in an ice bath (keep reagents cold throughout all procedures).

Note: Wear disposable gloves at all times when patients' sera are being handled.

Using the automatic pipettor, deliver the appropriate amount of diluent into the assay tubes shown in the protocol (Table 1). Mix each serum specimen thoroughly before pipetting, in succession, the 50-, 100-, and 200-$\mu$L aliquots into the tubes shown on the protocol. Note that two 100-$\mu$L and 200-$\mu$L aliquots of each specimen are pipetted. Only one of each volume will receive antisera; the other will become a "serum damage" tube (see Calculations) for that patient.

Pipet the quality-control serum pools the same as the samples.

For every seven assay runs (approximately), use one 200-$\mu$L
tube of the 100 μg/L PTH standard. Add 0.8 mL of diluent directly to the tube and vortex-mix. This is now a 20 μg/L solution of PTH. For the firstRIA standard, dilute 0.2 mL of this solution to 2.0 mL by adding to 1.8 mL of diluent in a tube numbered standard 1; vortex-mix. Prepare six other consecutively numbered tubes and add 0.6 mL of diluent to each. Then carefully remove 0.9 mL of standard no. 1 and add to standard tube no. 2. Vortex-mix, then remove 0.9 mL of tube no. 2 and add to standard tube no. 3. Continue this procedure of adding 0.9 mL of diluted standard to the next numbered tube until all seven standards are prepared. Each successive tube will have 0.6 the concentration of PTH of the previous tube (i.e., 2000 ng/L for standard no. 1, 1200 ng/L for no. 2, etc. for the seven tubes). This entire procedure must be done with utmost care to ensure the greatest possible accuracy and precision in the standard curve. Dispense 200-μL aliquots of each standard PTH concentration, as shown in the protocol (Table 1). Use a Hamilton repeating syringe or equivalent.

When all diluent, standards, patients' sera, and quality-control pools have been pipetted, the final step is addition of 100 μL of diluted antisera to the appropriate tubes; use an automated pipettor for the additions. Vortex-mix all tubes and cover with clear plastic wrap secured with a rubber band. Place all racks in shakers in a cold (4°C) room for 72 h.

**Note:** In our experience, shaking increases the binding of labeled PTH to antibody by 10–20%. Thus a bit less antibody can be used to achieve a satisfactory degree of binding of the tracer, which, in turn, enhances the sensitivity of the assay.

On day 3, add the 125I-labeled PTH. First prepare the solution of labeled hormone, as follows: Allow 0.2 mL of C-terminal diluent per assay tube plus at least 20% extra. Add to this diluent thawed 125I-labeled PTH stock (stored at −70°C); dilute to a count rate of 3000–3250 cpm/200 μL. The amount of labeled hormone stock to be added varies somewhat with each iodination lot. Mix the 125I-labeled PTH solution well by gentle swirling. Keep the solution on ice. Add the label (200 μL of correctly diluted 125I-labeled PTH), using a Micromedic or other similar device, to all tubes in the run while they are in an ice bath. Vortex-mix the contents of the tubes and return them to the shaker in the refrigerated (4°C) room for an additional 72 h.

On day 6, separate "bound" and "free" 125I-labeled PTH as follows: Remove the charcoal–dextran suspension from the refrigerator and place it in an ice bath, on a magnetic mixer. Mix for at least 10 min to ensure that all of the sedimented charcoal is resuspended. Keep mixing the suspension throughout the pipetting step. Prepare a second set of numbered tubes for the supernates. Number these tubes consecutively. Add 1.5 mL of buffered horse serum to tubes 1–4 of the run. Vortex-mix, cap, and let stand. These will be the "total counts" tubes for that run. Add 1.5 mL of dextran-coated charcoal to the rest of the tubes of the run, beginning with no. 5, and vortex-mix. Centrifuge all tubes from no. 5 to end of run (see Table 1) at 2000 × g for 10 min.

**Note:** Each centrifuge run must contain a complete set of controls and standards because of inter-assay variation in timing of charcoal addition and centrifugation. Therefore, use the centrifuge that can hold the largest number of tubes.

Pour the supernate carefully from the assay tube into the corresponding numbered tube in second set. Cap tubes, then count the radioactivity of the supernates for 5 min.

**Procedure for intact PTH assay.** The procedure for this assay (Table 2) includes tubes for later calculation of the following parameters: binding of 125I-labeled PTH to glass, 125I-labeled PTH "damaged" by incubation, and interference of serum (50-, 100-, and 200-μL volumes) with adsorption of 125I-labeled PTH to dextran-coated charcoal.

For every two assays runs, thaw one 400-μL tube of the 100 μg/L PTH standard. Dilute each 400-μL aliquot with 1.6 mL of diluent to produce a 20 μg/L solution of PTH; mix thoroughly. Into each of nine 15-μL vials, pipet 1 mL of diluent per tube of standard thawed. With a serological pipet withdraw that same volume of 20 μg/L standard from the vial of diluted standard and transfer quantitatively to the first of the series of vials; mix thoroughly. Withdraw the same volume from this second vial and transfer quantitatively to the third vial, using the same pipet. Mix thoroughly. By repeating this sequence through all of the vials, a series of 10 concentrations of PTH standard ranging from 20 μg/L to 39 ng/L will have been prepared, each concentration being exactly half of that in the preceding vial. This entire procedure should be done with maximum care to ensure the greatest accuracy and precision in preparing the standard curve.

Diluent (for intact assay), standards, patient sera, and quality-control serum pools are pipetted as was described for the C-terminal assay. Note that three tubes (containing 50, 100, or 200 μL of serum) are run for each patient. After adding antisera, incubate the gently mixed tubes on a shaker at 4
Table 2. Protocol for Assay of Intact PTH

<table>
<thead>
<tr>
<th>Tube no.</th>
<th>Diluent</th>
<th>Antibody</th>
<th>Standard</th>
<th>Serum</th>
<th>Vol. added, μL</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1–4</td>
<td>300</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>200</td>
<td>Serum interference with charcoal</td>
</tr>
<tr>
<td>5–8</td>
<td>(These tubes receive the contents of tubes 9–12.)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>200</td>
<td>Glass binding tubes</td>
</tr>
<tr>
<td>9–12</td>
<td>300</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>200</td>
<td>Buffer instead of charcoal</td>
</tr>
<tr>
<td>13–16</td>
<td>(These tubes receive the supernates from tubes 17–20 after addition of dextran-coated charcoal.)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>200</td>
<td>Buffer damage controls</td>
</tr>
<tr>
<td>17–20</td>
<td>300</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>200</td>
<td>Serum interference with charcoal</td>
</tr>
<tr>
<td>21–24</td>
<td>(These tubes receive the supernates from tubes 25–28 after addition of dextran-coated charcoal.)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>200</td>
<td>Serum interference with charcoal</td>
</tr>
<tr>
<td>25–28</td>
<td>250</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>200</td>
<td>Serum interference with charcoal</td>
</tr>
<tr>
<td>30–32</td>
<td>(These tubes receive the supernates from tubes 33–36 after addition of dextran-coated charcoal.)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>200</td>
<td>Serum interference with charcoal</td>
</tr>
<tr>
<td>33–36</td>
<td>200</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>200</td>
<td>Serum interference with charcoal</td>
</tr>
<tr>
<td>37–40</td>
<td>(These tubes receive the supernates from 41–44 after addition of dextran-coated charcoal.)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>200</td>
<td>Serum interference with charcoal</td>
</tr>
<tr>
<td>41–44</td>
<td>100</td>
<td>100</td>
<td>200</td>
<td>—</td>
<td>200</td>
<td>Zero standards</td>
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<td>45–48</td>
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<td>100</td>
<td>200</td>
<td>—</td>
<td>200</td>
<td>4000-pg standard</td>
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<td>49–50</td>
<td>—</td>
<td>100</td>
<td>200</td>
<td>—</td>
<td>200</td>
<td>2000-pg standard</td>
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<td>51–52</td>
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<td>100</td>
<td>200</td>
<td>—</td>
<td>200</td>
<td>1000-pg standard</td>
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<td>53–54</td>
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<td>55–56</td>
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<td>100</td>
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<td>—</td>
<td>200</td>
<td>250-pg standard</td>
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<td>57–58</td>
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<td>100</td>
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<td>59–60</td>
<td>—</td>
<td>100</td>
<td>200</td>
<td>—</td>
<td>200</td>
<td>62.5-pg standard</td>
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<td>61–62</td>
<td>—</td>
<td>100</td>
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<td>—</td>
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<td>—</td>
<td>200</td>
<td>7.8-pg standard</td>
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<td>67–68</td>
<td>—</td>
<td>100</td>
<td>200</td>
<td>—</td>
<td>200</td>
<td>Patient’s serum</td>
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<tr>
<td>69*</td>
<td>150</td>
<td>100</td>
<td>—</td>
<td>—</td>
<td>50</td>
<td>Patient’s serum</td>
</tr>
<tr>
<td>70*</td>
<td>100</td>
<td>100</td>
<td>—</td>
<td>—</td>
<td>100</td>
<td>Patient’s serum</td>
</tr>
<tr>
<td>71*</td>
<td>0</td>
<td>100</td>
<td>—</td>
<td>—</td>
<td>200</td>
<td>Patient’s serum</td>
</tr>
</tbody>
</table>

* Repeat this tube sequence for each unknown serum or quality-control pool.

9°C for two days. Then add the 125I-labeled PTH, prepared as for the C-terminal assay, except that intact PTH assay diluent is used and a count rate of 2500–2750 dpm/200 μL of diluted tracer is required. After addition of the labeled PTH, add an additional 300 μL of diluent to each tube. Vortex-mix the tubes’ contents and return them to the cold room to shake for another 72 h.

Add 1.5 mL of well-mixed charcoal suspension to each tube in the protocol after tube no. 12 (see Table 2). Add 1.5 mL of 0.02 mol/L barbital buffer, pH 8.6, containing 20 mL of horse serum per liter, to tubes 9–12. After vortex-mixing, centrifuge all tubes after no. 12 at 2000 × g for 10 min. Transfer the supernates from tubes 9–12 to tubes 5–8 with a Pasteur pipette. Wash tubes 9–12 with 1.5 mL of water, vortex-mix, and discard the supernates. Save other supernates as indicated on the protocol. Remove the supernates from all other tubes by aspiration.

Note: Take care not to disturb the charcoal pellets.

Add 2.5 mL of water to each tube. Vortex-mix again to resuspend charcoal pellets. Centrifuge as before. Aspirate supernates. Cap the tubes and count the radioactivity of the pellets for 5 min.

Calculation of Results

In the C-terminal PTH assay, the values for antibody-bound counts (supernates) are counted directly rather than being computed as in the assay of intact PTH (see below). However, these counts must be corrected for incubation damage and serum interference with the binding of 125I-labeled PTH to charcoal as follows: the average counts for the incubation damage controls (tubes 5–8, Table 1) are subtracted from the counts for each standard or zero standard tube, from the counts for serum tubes in which the serum specimen was diluted before assay, and from the counts for all tubes containing 50 μL of serum. The counts for the individual serum specimen controls containing 100 or 200 μL of serum specimen are subtracted from the count values for the corresponding tubes that also contained antisem (see Table 1).

In the intact PTH assay, the average number of counts in the glass-binding controls is subtracted from the counts for each tube containing charcoal, because this portion of the 125I-labeled PTH is adsorbed to glass rather than charcoal. The counts for each standard or zero standard tube and the average incubation damage counts are subtracted from the average total counts to obtain the values for antibody-bound counts for the standards. The counts for all tubes containing unknown or control serum and the counts for the serum interference controls for the corresponding serum volumes are subtracted from the average total counts to obtain the values for antibody-bound counts for all of the serum tubes.

The rest of the calculation procedure is the same for both assays. The average antibody-bound counts in the zero standards are divided by the average total counts to determine "asay binding" for each assay run. All of the antibody-bound counts for standards and unknown or control serum samples are divided by the antibody-bound counts for the zero stan-
standards and multiplied by 100 to obtain the percentage of 125I-labeled PTH bound for each standard or serum tube. The standards are plotted by the logit procedure (17), in which the logit of the percent bound is plotted as a function of the log of the picograms of standard PTH in each tube. The amount of PTH in each unknown or control serum tube is then obtained from the standard curve by using the logit of the percent bound for each serum tube. This amount of PTH is then divided by the volume of serum sample assayed to give the final value. All of the necessary calculations can be readily done by computer program, using any of a variety of data-reduction methods.

Note: PTH values for both assays are expressed as ng Eq/L (pg Eq/mL). The term "equivalent" is used because the PTH assay standard is bovine hormone, not human.

Results

Performance Characteristics

Standardization. Unlike the situation for many other laboratory tests, there is no universal standard for PTH. Of major significance in this regard is the fact that PTH in serum is heterogeneous. Moreover, this heterogeneity varies considerably among patients with clinical conditions affecting calcium metabolism and parathyroid-gland function (18). Thus, it is impossible to have a PTH reference standard that would be identical to the forms of the hormone being measured in all patients. Some laboratories have used a carefully calibrated serum as the RIA standard. The use of a gravimetric standard of pure PTH as described here has an important theoretical advantage: a major PTH form probably present in nearly all types of patients is the biologically active, intact hormone.

Quality control. Three quality-control serum pools representing one high and two normal PTH concentrations are used in every assay. One normal pool is run in triplicate at different locations throughout the run to monitor "drift" (see note below). Only freshly thawed aliquots of these serum pools are used; all pools are stored at −70 °C and thawed only once. Thus any variations observed in the PTH values for these pools should be due to test factors only and not to deterioration. At least 100 determinations are required on each pool to establish the acceptable limits for results. These limits are determined by computer for each run, and the overall means and 95% confidence intervals (mean ± 2 SD) are continually updated as more values for each serum pool are accumulated. If no more than one pool in a run has values outside these 95% confidence intervals, the run may be considered acceptable.

The following performance characteristics should be met for every assay run to verify consistent performance:

1. Percent binding for the zero standard tubes (counts bound corrected for nonspecific binding) should be between 25% and 35% of the counts added, for both assays.
2. Nonspecific binding (i.e., "damage" counts in the supernates of tubes containing no antibody, representing the 125I-labeled PTH not bound to the dextran-coated charcoal) should be <15% of the total counts.
3. Poor agreement (CV >30%) in the PTH values determined for the multiple dilutions of any patient's serum requires that such specimens be reassayed. A large number (>15%) of specimens with such characteristics in any one run may indicate problems in the assay itself.

Note: Each patient's serum must be run at multiple dilutions to verify "parallelism" between the PTH in the assay standard and the PTH in the specimen. A few patients' sera (perhaps as many as 5%) produce nonspecific interference in the assay; PTH cannot be validly quantitated in such specimens, of course (9).

4. Consistent bias in quality-control pools in a series of runs (i.e., all high or all low) may reflect deterioration of the assay standard or of the 125I-labeled PTH.

5. Any assay is valid only if it provides results consistent with other clinical information on the patients tested.

Sensitivity. Figure 1 shows typical standard logit curves (17) for pure bovine PTH obtained with each of the two assays. The lower limit of detection in the assay for intact PTH is about 75 ng Eq of bovine intact PTH per liter (96% of antibody-bound 125I-labeled PTH); for the C-terminal PTH assay, about 150 ng Eq/L (90% of antibody-bound 125I-labeled PTH). Measurement of PTH in many thousands of patients' sera with both assays has indicated parallelism between dilution curves of sera and the bovine intact PTH standard in all but a very few cases. This parallelism confirms the validity of using bovine PTH as the standard for each assay.

Specificity. As previously reported, the two assays have different reactivities toward pure peptide fragments of bovine PTH (15). The antisera used for the intact PTH assay (GP204) does not react with a fragment from the C-terminal region (amino acids 53 to 84) of intact PTH, nor with a fragment from the middle region (27 to 44) of intact PTH. Reactivity with a fragment from the N-terminal region (1 to 34) of intact PTH is only about 0.007% of the reactivity with intact PTH (15). In sharp contrast, antisera GP75U, used in the C-terminal PTH assay, reacts with the 53 to 84 C-terminal fragment on an equimolar basis with intact PTH; however, it does not recognize large excesses of either the 1 to 34 or 27 to 44 fragments (15).

Results of further investigation of the specificity of each assay are shown in Figure 2. Serum from a patient with surgically proven hyperparathyroidism was fractionated by high-resolution gel filtration on a column of Bio-Gel P-150 (19). This technique separates the forms of PTH in serum on the basis of their molecular sizes, thus permitting direct evaluation of the reactivity of each antisera with these various forms. The assay for intact PTH reacts primarily with intact hormone, which is the first peak detected by either assay. It has weak recognition for a large peak of C-terminal PTH fragments known to be present in the serum of hyperparathyroid patients; these fragments are recognized well by
the C-terminal assay (29). Thus, the two PTH assays have widely different specificities.

**Precision.** Both intra- and inter-assay coefficients of variation for the three serum pools used in the intact PTH assay range between 7 and 12% (15). In the C-terminal assay the corresponding values range from 10 to 16%.

**Accuracy.** In analytical-recovery studies done for the C-terminal antisera used here, recovery of PTH ranged from 85 to 107% (mean, 96.7%). The correlation coefficient (picograms of pure human PTH added to hypoparathyroid serum vs picograms of PTH measured by RIA) was 0.98 (20).

**Clinical Validation**

The diagnostic performance and reliability of any laboratory test can be no better than its clinical validation. In the case of a PTH assay we and many others believe that the following must be done for such validation (9): (a) identification of the region of the PTH molecule recognized by the PTH assay in question, through use of pure PTH fragments (commercially available) and by gel-filtration studies; (b) examination of at least 300 serum specimens from normal subjects and patients with disorders of parathyroid gland function and calcium homeostasis.

**C-terminal PTH Assay**

Normal subjects: The 183 normal subjects were approximately equally divided between men and women. These age-matched individuals all had physical examinations and normal results for several conventional laboratory tests. The mean C-terminal PTH (± SD) in this group was 256 (± 59.5) ng Eq/L, and the mean ± 2 SD range would therefore be 137 to 375 ng Eq/L. Because the lower limit of detection in this assay is considered to be 150 ng Eq/L, the normal range is expressed as <150 to 375 ng Eq/L. Only seven (4%) of the normal subjects had C-terminal PTH values <150 ng Eq/L. There was no statistically significant correlation between concentrations of C-terminal PTH and calcium in this group of normal subjects.

Patients with calcium–parathyroid disorders: With this C-terminal PTH assay, approximately 95% of patients with surgically confirmed primary hyperparathyroidism have above-normal values (>375 ng Eq/L), as do about 20% of hypercalcemic patients with cancer (21). All patients with chronic renal failure have increases in C-terminal PTH concentration. In hypoparathyroid patients, C-terminal PTH is generally nondetectable (21).

**Note:** Correct interpretation of a patient's PTH value (whether measured with the C-terminal or the intact assay) can only be made by also considering the serum calcium value (as measured by atomic absorption spectroscopy).

**Intact PTH assay**

Normal subjects: Mean intact PTH (± SD) in 93 normal subjects was 255 (±46) ng Eq/L. The mean ± 2 SD range is 163–347 ng Eq/L. This assay demonstrates an inverse relationship between PTH and serum calcium concentrations in normal individuals (10): a plot of intact PTH vs calcium shows a highly significant negative correlation (r = −0.412, p < 0.001).

Patients with calcium–parathyroid disorders: In hyperparathyroid patients, only about 55% have intact PTH values >347 ng Eq/L. However, as previously reported, many of the apparently normal values in these patients are, in fact, inappropriately high for the serum calcium values; 97% of these patients are separated from normals and other hypercalcemic individuals on the basis of computer-assisted formal discriminant analysis of calcium and intact PTH. In a group of 160 patients with chronic renal failure, 86% had increased concentrations of intact PTH.

**Note:** The interested reader is referred to recent articles for detailed discussions of the clinical interpretation of results for both of these PTH assays in several large groups of patients (15, 21).

**Discussion**

Several important factors must be controlled if an RIA procedure for PTH is to be useful and reliable in routine

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Clinical diagnosis. The preparations of $^{125}$I-labeled PTH used must have relatively uniform specific activities and high purity, to prevent large variations in assay binding and other problems. Key reagents such as antisera, PTH standards, $^{125}$I-labeled PTH, and quality-control serum pools should be stored in aliquots at $-70$ °C to protect against losses of activity.

The PTH assays described above have achieved a consistent record of performance, reliability, and clinical usefulness. Both assays have demonstrated precision comparable with or better than that for other published PTH assays. Both assays can differentiate more than 90% of patients with primary hyperparathyroidism from normal subjects, although the assay for intact PTH requires the use of a simultaneous determination of serum calcium and formal discriminant analysis of serum PTH and calcium values to achieve that high degree of differentiation. With the C-terminal assay, about 95% of hyperparathyroid patients have values distinctly increased above the normal mean ±2 SD; formal discriminant analysis of calcium vs C-terminal PTH gives an essentially complete differentiation between the two groups.\(^5\)

Clinical Applications

Much has been written about how the widespread availability of RIA methods for measuring PTH has greatly improved the diagnosis and treatment of disorders of parathyroid-gland function and calcium homeostasis. Our purpose here is to summarize some of this information and place it in perspective. The interested reader may consult a number of recent reports on this subject (1, 8, 22).

Hypercalcemia. The principal application for measuring PTH is in the differential diagnosis of the cause of a patient's hypercalcemia (Table 3). Of these, primary hyperparathyroidism is probably the most important. Recently published, detailed epidemiological studies have shown that the frequency of detection of this disease, which once was thought to be rare, has increased since the mid-1970s with the introduction of routine screening methods for hypercalcemia (as part of use of multi-channel analyzers) (23). It is now estimated that at least 50,000 new cases of primary hyperparathyroidism will be diagnosed in the United States each year. Because PTH measurements are such an important part of the diagnosis of this disease, the role of PTH assays in the clinical laboratory seems secure.

It is widely agreed that PTH assays with specificity for the C-terminal region of the PTH molecule give more clearcut diagnostic information to the clinician investigating the cause of a patient's hypercalcemia than do other types of PTH assays (8). The reason for this superiority of C-terminal assays is not known for certain but is probably related to the presence of much greater absolute quantities of C-terminal PTH fragments in hyperparathyroid sera (due to their longer half-lives) than in normal sera (7).

With regard to the other causes of hypercalcemia listed in Table 3, almost all other conditions except "tertiary" and "ectopic" hyperparathyroidism are generally associated with low or undetectable concentrations of serum PTH. The term "tertiary" is sometimes applied to the hypercalcemic hyperparathyroidism that may develop in patients with secondary hyperparathyroidism resulting from chronic renal failure. "Ectopic" secretion of PTH is associated with many types of nonparathyroid cancer. Many cancer patients become hypercalcemic at some time or other during the course of their disease, and as many as 20% of these patients have increased concentrations of PTH, from tumor-elaborated hormone (24). At least some of this hormone is thought to be biologically active, even though there is evidence that the relative quantities of intact PTH and fragments in the serum of patients with hyperparathyroidism or cancer may differ and that there may be different PTH forms in cancer patients (25). On the other hand, several other factors may be involved in the hypercalcemia of malignancy besides ectopic secretion of PTH (21, 22).

Hypocalcemia. In hypocalcemic states, which are far less common, measurement of PTH is useful in the detection and management of secondary hyperparathyroidism resulting from chronic renal failure (see below). Serum PTH is generally increased in such conditions as pseudohyperparathyroidism, vitamin D deficiency, and certain gastrointestinal diseases that result in malabsorption. Such an increase is the appropriate physiologic response to hypocalcemia, which results in increased secretion of PTH. Patients who are physiologically or iatrogenically hypoparathyroid generally have low or undetectable PTH concentrations despite the hypocalcemia.

Venous catheterization. A final application of PTH measurements is in preoperative studies to localize hyperfunctioning parathyroid tissue, particularly in patients who have already undergone unsuccessful parathyroid exploration (26). Blood samples are collected by selective catheterization of small veins draining that patient's neck and mediastinum (27). Increased concentrations of PTH in such samples are invariably more difficult to detect with the C-terminal PTH assay because of high background readings. An assay that measures the N-terminal or intact (secreted) form of the hormone is more likely to yield clinically meaningful information.\(^6\)


Chronic renal failure. Secondary hyperparathyroidism is an almost universal consequence of chronic impairment of renal function. Diagnosis of such impairment is, of course, based on results of various other laboratory tests; however, the need for PTH measurements in patients with chronic renal disease is increasing. The prolongation of life in these patients, the result of better access to dialysis procedures, has in turn allowed more time for the development of a complex group of metabolic bone diseases, collectively termed renal osteodystrophy. The role of PTH measurements in these patients is largely one of monitoring their clinical condition over time during a program of dialysis or other therapeutic measures (such as renal transplantation). Reversal of secondary hyperparathyroidism should be reflected by a decrease in PTH values. Measurements of intact (biologically active) hormone correlate with clinical improvement in many, but not all, patients with chronic renal failure better than do C-terminal PTH values; the latter may remain anomalously high, despite clinical improvements (28–30), presumably because C-terminal PTH fragments require renal function for clearance from the circulation (31).

In predicting the onset of secondary hyperparathyroidism in patients with deteriorating renal function, the assays measuring C-terminal PTH fragments may show increases in concentration with only marginal decreases in the glomerular filtration rate (GFR) (32). Histomorphometric analysis of bone biopsies did not show secondary hyperparathyroidism until the GFR fell below 50 mL/min per 1.73 m² (33). These increased C-terminal PTH concentrations may not reflect secondary hyperparathyroidism at all, but rather an accumulation of C-terminal PTH fragments, owing to their reduced clearance by the kidney (the sole site for their removal from blood).

If values for C-terminal PTH concentrations predict secondary hyperparathyroidism "too early," intact PTH values predict it "too late." Data from our laboratory show that PTH concentrations measured with the intact PTH assay described here invariably do not exceed the normal range until the GFR is less than 20 mL/min per 1.73 m², probably well after secondary hyperparathyroidism (by histomorphometric criteria) has developed (33).

Current thinking favors the idea that both assays should be used to monitor PTH concentrations in patients with renal failure (34). To assess a patient's response to a dialysis program, for example, determination of baseline PTH values with both assays has been suggested, with periodic monitoring with one or both assays, depending upon the degree of correlation of these results with the patient's clinical condition. Although there are only limited data on these points, we believe that measurement of intact PTH provides an assessment of the response to therapy, in terms of shutdown of secretion of biologically active (or potentially active) PTH, and that measurement of C-terminal PTH provides an assessment of chronic PTH secretion and of the mass of parathyroid tissue (34). Much work must yet be done to determine the ultimate value and role of specific PTH assays in assessing and monitoring renal osteodystrophy patients; PTH assay may become as useful to the clinician in assessing parathyroid secretory status as quantitative histomorphometry is in assessing the nature and extent of the bone disease.

Table 4 summarizes our experience with both PTH assays by indicating which assay has shown the best clinical usefulness in a particular diagnostic setting. We also give our suggestions for possible alternative tests for equivocal situations.

Note: Because the most commonly recognized application for PTH measurement is the evaluation of hypercalcemic disorders (i.e., endocrine applications), a C-terminal PTH assay is most frequently recommended in most situations. On the other hand, no single type of assay can be expected to serve the clinician's needs for all diagnostic applications.

The availability of two different PTH assays to measure the two major forms of the hormone in peripheral blood has added a new dimension to the diagnostic evaluation of hyper- and hypercalcemic patients. Now it is not only possible but also highly desirable to select a particular assay for a specific diagnostic application.

References
11. Di Bella, F. P., Giklinski, J. B., Flueck, J., and Arnaud, C. D., Carboxyl-terminal fragments of human parathyroid hormone in

| Table 4. Suggested Clinical Applications of PTH Assays |
|---------------------------------|-------------|-----------|
| **Hypercaldemic disorders**     | **Recommended initial test** | **Alternative test (if necessary)** |
| Primary or ectopic              | C-terminal  | Intact    |
| hyperparathyroidism             |             |           |
| Nonhyperparathyroid hypercalicia| C-terminal  | Intact    |
| Chronic renal failure           |             |           |
| Screening for secondary or      | Both assays |           |
| tertiary hyperparathyroidism    |             |           |
| Monitoring patient therapy and  | Both assays |           |
| disease course                  |             |           |
| Hypocalcemic disorders          |             |           |
| Hypoparathyroidism              | C-terminal  | Intact    |
| Vitamin D deficiency disorders  | C-terminal  | Intact    |
| Acute renal failure             | C-terminal  | Intact    |
| Miscellaneous                   |             |           |
| Nornormalcalic hypercalciuria   | C-terminal  | Intact    |
| Other disorders                 | C-terminal  | Intact    |
| Selective venous catheterization| Intact      |           |
Methods


Editor's note: The reader is reminded that Selected Methods do not bear the official imprimatur of the Association. They are methods that seem durable and generally useful, and that have been checked by several evaluators. As detailed elsewhere (Clin. Chem. 19: 1207, 1973), these methods are offered here for criticism by the world community of users, and will be revised appropriately before being collected into a bound volume, Selected Methods of Clinical Chemistry. The last such volume was published by the Association in 1977.

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