C-Terminal Parathyrin (Parathyroid Hormone) Radioimmunoassay in Serum with Commercially Available Reagents

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We describe a sequential double-antibody assay for measuring C-terminal parathyrin in serum with commercially available reagents. Intact bovine hormone, used as a working standard, is iodinated by conventional Chloramine T procedures. The antibody affinity is characteristic of high affinity binding (1.4 to 1.6 X 10^10 L/mol of intact parathyrin). The antibody also cross reacts with a C-terminal parathyrin fragment (amino acids 53-84) but not with a synthetic N-terminal parathyrin fragment (amino acids 1-34). The assay thus also measures both a C-terminal fragment of parathyrin and the intact hormone. The detection limit (250 ng/L; 500 int. units/L) is below the reference interval for healthy adults (430-1880 ng/L; 860-3720 int. units/L). Several commonly recognized problems with iodinated parathyrin are eliminated and accuracy and precision of the procedures for standard preparation and calibration are improved. Overall CV (between-run imprecision) is 10-17%. Analytical recovery is 80-90%, and C-terminal parathyrin measured in fresh sera and in sera stored for seven days at 30 °C is equivalent.

Additional Keyphrases: reference intervals • parathyroid disorders • hormones • disorders of calcium regulation

Parathyrin (parathyroid hormone, PTH) is an 84-amino-acid polypeptide (relative molecular mass 9500) normally produced and released by the parathyroid glands in response to changing serum calcium status (1). Pro-PTH and prepro-PTH are also formed in the glands but cannot be detected in the circulation (2). Intact PTH (amino acids 1-84) is cleaved both within the gland and in the periphery (3) to form N-terminal (amino acids 1-34; N-PTH) and C-terminal fragments (4). The biological half-lives of these fragments are less than 5 min and 40 min, respectively, before further metabolism and excretion by the kidney (5). Only the N-PTH fragment and the intact hormone are biologically active (6). Measurement of N-PTH reportedly is of value in observing acute responses to serum calcium changes and after venous catheterization of the neck in an attempt to localize the site of hyperfunctioning glands (7). Serum or plasma C-PTH activity has been used to diagnose primary hyperparathyroidism, to follow parathyroid response in chronic renal failure, and to aid in differential diagnosis of other hypercalcemic and hypocalcemic disorders (8, 9).

A controversy regarding the appropriate use of N-PTH and C-PTH assays in patient management has not been fully resolved, partly because of the overlap observed in PTH results obtained for groups of patients believed to be clinically distinct (8, 10, 11). Thus, accurate and precise assays are required clinically. In developing the present assay to meet these requirements, we have used only reagents available through common commercial sources.

Materials and Methods

Reagent and Standard Preparation Procedures

Buffer. Dissolve 5.89 g of sodium barbital, 3.89 g of sodium acetate, 6.8 g of sodium chloride, and 110.0 g of polyethylene glycol (no. 6103, average M, 7500; Polysciences, Inc., Warrington, PA 18976) in 950 mL of distilled water in a 1-L volumetric flask. Add 25 mL of aprotinin (Trasylol, 10,000 Kallikrein Inactivator Units/mL; Mobay Chemical Co., New York, NY 10022). Mix well. Adjust to pH 7.4 with 6 mol/L HCl. Dilute to 1000 mL with distilled water and check the pH.

Serum buffer. Mix 50 mL of human serum (obtained from a community blood bank and containing, per liter, less than 800 ng or 1600 int. units of PTH) with 450 mL of the buffer described above. Adjust the pH to 7.4.

Assay standards. Prepare a 1 mg/L (1200 int. units/L by bioassay) stock solution of intact (1-84) bovine PTH (highly purified, lot no. 1615-B001; Inolex Corp., Park Forest South, IL 60466) by dissolving the contents of a 200-μg vial in 200 mL of serum buffer. Determine the appropriate dilution by calibration to the reference preparation (described below) at least every six months and dilute to 5000, 2500, 1250, 625, and 312 ng/L (10,000, 5000, 2500, 1250, 625 int. units/L) working concentrations with serum buffer. Prepare five independent dilutions of assay standards (see above) and calibration standards and assay in a single batch. Adjust the dilutions of the working assay standards and repeat as necessary until the standard curves are exactly congruent.

N-terminal PTH 1-34 fragment. Synthetic bovine (cat. no. 337700; Beckman Biologicals, Palo Alto, CA 94304).

C-terminal PTH (amino acids 53-84) fragment. This was received (from Dr. L. Deftos, University of California, La Jolla, CA) as a 5 μg/L solution in 0.1 mol/L acetic acid (14).

C-terminal PTH (53-84) fragment. This was synthesized in the human amino acid sequence (by Peninsula Labs., San Carlos, CA 94070; cat. no. 6103).

Goat anti-rabbit gamma-globulin. This was from Scantibodies Inc., Lakeside, CA 92040.

Rabbit gamma-globulin. This was from Schwarz/Mann, Div. Becton Dickinson, Orangeburg, NY 10962. Dilute 50 mg to 100 mL with serum buffer.

Rabbit anti-PTH antibody (CIS Radioimmunochemicals, Bedford, MA 01730). Reconstitute one vial with 2 mL of distilled water and dilute 45-fold with the rabbit gamma-globulin solution.

Normal pool. Normal human serum, obtained from a community blood bank (should contain 1000-1500 ng/L or 2000-3000 int. units PTH per liter by assay), is stored in aliquots, frozen.
**High-concentration pool.** Add about 0.4 mL of assay standard stock to 250 mL of normal human serum (see above) to give a solution containing 2800–3200 ng/L or 5600 int. units of PTH per liter. Store frozen, in aliquots.

**Low-concentration pool.** Prepare PTH-free normal human serum by reacting one vial of PTH complex [cat. no. CNR 540, lot “40C x LA Anti-PTH,” conjugated to agarose (Sepharose); Cambridge Nuclear, Billerica, MA 01865] with 50 mL of serum. Mix by rotation for three days at 4°C. Centrifuge at 7000 × g and freeze aliquots of the supernatant serum.

**Iodinated PTH stock solution.** Iodinate 5 μg of bovine PTH (purified, lot 1508; Inexel Corp.) with 2 mCi of Na\(^{125}\)I (Amersham Searle, Arlington Heights, IL 60005) by a modified Hunter and Greenwood procedure (12). Purify the iodinated PTH with “microfine” precipitated silica (QUISO, G22; Calbiochem, La Jolla, CA 92112) according to described procedures, and follow by column gel filtration with BioRad p-10 (13). Freeze aliquots (−20°C) in 0.1 mol/L acetic acid containing 6 g of albumin (Bovine Fraction V, cat. no. 81-003; Miles Labs, Elkhart, IN 46515) per liter.

**Purification of \(^{125}\)I-labeled PTH.** Repurify the iodinated PTH (see above) immediately before use by vortex-mixing 0.2 mL of the stock frozen \(^{125}\)I-labeled PTH with 1 mL of human serum. When this has stood for 10 min, vortex-mix with 5 mL of the silica, centrifuge (2000 × g, 5 min), and discard the supernatant. Wash the pellet with 1 mL of freshly mixed acetone/acetate acid (2 mL/mL of acetone is added to 8 mL of a 10 mL/L solution of acetic acid). With a gamma counter, count the radioactivity of a 0.3-mL aliquot of a 100-fold dilution of the \(^{125}\)I-labeled PTH with serum buffer, and calculate the appropriate dilution to provide 60 pg of PTH per 0.3 mL of serum buffer as follows:

\[
\text{DDPM} = (0.5/E) \times C
\]

\[
\text{RDPD} = \frac{S \times A \times 2.2 \times 60 \times F}{\text{DDPM/RDPM} \times 100} = \text{working dilution of purified tracer}
\]

where

- DDPM = disintegrations per minute (dpm) determined on the day of repurification
- E = efficiency (as a decimal fraction) of the gamma counter
- C = counts per 2 min of 0.3 mL of diluted (100-fold) purified tracer
- RDPD = disintegrations per minute required in the assay to maintain the same mass of labeled PTH in each batch
- SA = specific activity of the tracer on the day of iodination, in microcuries per microgram
- 2.2 = factor to convert μCi/μg to DPM per pg, assuming 2.2 × 10\(^{6}\) DPM per microcurie and 10\(^{-6}\) μg/pg
- 60 = picograms per tube
- F = decay factor

**Radioimmunoassay Procedure.**

Each standard curve point is assayed in triplicate and each sample or pool is assayed in duplicate, plus individual nonspecific-count measurements. The incubation mixture includes 0.3 mL of working standard (or unknown or pool sera) and 0.1 mL of gamma-globulin for nonspecific binding tubes or 0.1 mL of noniodinated PTH antibody. To balance the effect of the serum buffer used to prepare the standards, add 50 μL of dilute serum (10 mL of the same serum used to prepare the serum buffer plus 6.6 mL of serum buffer) to unknowns and pools and add 50 μL of buffer alone to the standard curve tubes to compensate volume. Vortex-mix the contents of all tubes and incubate at 4–6°C for 20–24 h. Add 0.3 mL of repurified \(^{125}\)I-labeled PTH to all tubes, mix, and incubate for 60–72 h at 4–6°C. Incubate for an additional 3–4 h with 0.1 mL of second antibody and centrifuge (4°C, 2000 × g) after mixing with 1.0 mL of buffer. Aspirate the supernatant fluids and count the radioactivity of the pellets for 2 min each.

Average each triplicate set of standard concentrations, subtract the nonspecific counts, and divide each by the zero binding counts (B\(_0\)) to obtain B/B\(_0\). Plot logit-log of B/B\(_0\) vs concentration. Calculate B/B\(_0\) of samples and pools by averaging the duplicate bound counts, subtracting the nonspecific counts determined for each sample, and dividing by the zero binding. Obtain PTH concentration in samples and pools by comparing B/B\(_0\) with the plotted standard curve.

**Serum Calcium**

We assayed calcium by atomic absorption spectroscopy (15).

**Results**

Figure 1 shows the molar cross-reactivity of the antibody with intact bovine hormone (amino acids 1–84), C-terminal fragment (amino acids 53–84), and N-terminal fragment (amino acids 1–34). Intact hormone competes most effectively for antibody binding with the iodinated hormone because the tracer is prepared from intact bovine hormone. The intact hormone (expressed on a molar basis as the ratio of concentrations found to give 50% B/B\(_0\)) cross reacts about 65% with the synthetic C-PTH fragment and 13% with the native preparation. Parallel curves are obtained with human PTH preparations (nos. 79/500 and 75/549; World Health Organization Reference Preparations). No specific inhibition of binding is observed with the N-fragments up to 5000 pmol (equivalent to 15 000 pg of N-PTH per milliliter of serum).

The antibody affinity constant calculated by Scatchard analysis is 1.4 × 10\(^{10}\) L/mol; calculated by the Michaelis-Menten saturation analysis, it is 1.6 × 10\(^{10}\) L/mol. These results are consistent with a high-affinity antibody system.

Table 1 lists typical characteristics of a standard curve and nonspecific counts. Zero-binding and nonspecific counts depend somewhat on the age of the \(^{125}\)I-labeled PTH. Nonspecific counts in the samples and the pools are always less than those in the standard curve because the diluent in the standards contains only 0.1 volume of serum. Assay performance depends greatly on the presence of unidentified factors in serum (16).

Our attempts to replace the serum component of the assay buffer with albumin have been unsuccessful; each described component of the assay buffer is required for optimum assay performance. Increasing pH from 6.4 to 8.4 causes a shift.
Table 1. Typical C-PTH Assay Characteristics

<table>
<thead>
<tr>
<th></th>
<th>Routine batch</th>
<th>Batch with impure tracer</th>
</tr>
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<tbody>
<tr>
<td>Total counts (per 2 min)</td>
<td>40 300</td>
<td>30 790</td>
</tr>
<tr>
<td>Zero binding (net %)</td>
<td>25–30</td>
<td>25</td>
</tr>
<tr>
<td>Standard curve</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonspecific counts (%)</td>
<td>5</td>
<td>13.8</td>
</tr>
<tr>
<td>Slope</td>
<td>-1.15</td>
<td>-0.84</td>
</tr>
<tr>
<td>50% B/B₀ (pg/mL)</td>
<td>1700</td>
<td>780</td>
</tr>
<tr>
<td>(int. units/L)</td>
<td>3400</td>
<td>1560</td>
</tr>
<tr>
<td>Sample nonspecific counts (%)</td>
<td>4</td>
<td>6–11</td>
</tr>
</tbody>
</table>

upward and to the right in the standard curve (Figure 2) without substantially affecting zero binding (B₀). The present assay requires a one-day preincubation before tracer is added, followed by a three-day incubation with tracer. As shown in Figure 3, shortening the second incubation to one day reduces the zero binding from 26% to 15% and results in a relatively flat standard curve. Omitting the preincubation step (without tracer) decreases label displacement by the standards or samples and thus causes a loss in sensitivity.

The detection limit of the present assay is 250 ng/L (500 int. units/L), as defined by the standard concentration that inhibits tracer binding by more than 10%. The definition assumes that most nonspecific depression of tracer binding will be <10%, and less than 250 ng of PTH (500 int. units) per liter cannot be distinguished reliably from such nonspecific effects.

In spite of our vigorous efforts to control quality of iodinations, occasional aliquots show high nonspecific binding. These “impure” tracers produce severe deviation of sample and pool results from the standard curve and require a repeat analysis with a new aliquot of tracer. The effect of such a tracer on the assay characteristics is shown in Table 1. These problems are overcome by using the daily tracer re-purification procedure as described in Methods and Materials. Figure 4 shows the results of cellulose acetate electrophoresis of an “impure” tracer before and after re-purification with silica. The impurity showing radioactivity in fraction 7 contributes about 6% of the total counts and is removed by the purification.

Table 2 shows the within-run and overall precision of the C-PTH assay, as determined by including two to four aliquots of each pool (in duplicate) in seven independent batches over a four-month period. Within-run variation (CV) ranged from

Fig. 2. Effect of assay buffer pH on the binding of intact bovine parathyroid hormone in the standard curve. Incubation conditions as described in the text.

Fig. 3. Effect of incubation timing on zero binding and standard PTH displacement.

Preincubation of antibody with unlabeled PTH in samples and standards is followed by postincubation with iodinated tracer before precipitation by the second antibody. Other conditions as described in the text.

Fig. 4. Electrophoresis of ¹²⁵I-labeled PTH at pH 8.4 on cellulose acetate before and after repurification with QUSO silica.

3.6 to 8.5%; overall CV was less than 10% except in the low-concentration pool, for which it was 17.2%.

Analytical recovery of PTH in the assay is 80–90%, in pools fortified with the same intact bovine hormone used as a standard.

Storage of the quality-control pools (including some fortified pools) either for two months at 4 °C or frozen for at least one year resulted in no systematic drift in results, indicating that C-PTH stability was not compromised.

Figure 5 illustrates the stability of patients' samples, comparing results for fresh samples with results obtained after storage of the serum for seven days at 30 °C. The correlation coefficient was 0.92, the slope 1.03.

Serum is apparently the most appropriate matrix for this particular C-PTH assay. Table 3 shows results for serum and heparin- and EDTA-treated plasma samples, each obtained

Table 2. Precision of C-PTH Assay Results in Seven Independent Batches

<table>
<thead>
<tr>
<th>Pool</th>
<th>C-PTH</th>
<th>CV, %</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>pg/mL</td>
<td>Int. units/L</td>
</tr>
<tr>
<td>High</td>
<td>2610</td>
<td>5220</td>
</tr>
<tr>
<td>Medium</td>
<td>2065</td>
<td>4130</td>
</tr>
<tr>
<td>Normal</td>
<td>1120</td>
<td>2240</td>
</tr>
<tr>
<td>Low</td>
<td>540</td>
<td>1080</td>
</tr>
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from five subjects in a single venipuncture. PTH results from EDTA-treated plasma are uniformly very low; heparin-treated plasma gives results that are low for three of the five subjects. Addition of heparin or EDTA to the standard tubes does not affect the resulting standard curve directly; the mechanism of this assay interference remains unresolved.

A histogram of C-PTH concentrations in healthy adults is plotted in Figure 6. All subjects used for the reference range study had serum calcium values within the normal range (2.2–2.6 mmol/L; 88–104 mg/L). The C-PTH reference values form a skewed (non-gaussian) distribution, with a range estimate (17) of 430–1860 ng/L or 860–3720 int. units/L (95% confidence limits).

Discussion

Well-defined criteria for evaluating the performance of C-terminal PTH assays have not yet been generally accepted. The following observations may be used to characterize and evaluate C-PTH assays (18):

1. There should be no antibody cross-reactivity with N-PTH fragments. This characteristic limits the PTH species measured to the intact hormone and one or more C-terminal fragments. A straightforward demonstration of this point is possible because of the commercial availability of synthetic N-PTH fragments in the bovine amino acid sequence (Figure 1). An absence of N-PTH cross-reactivity with the present antibody is not unexpected because the antibody used is reported to be raised to bovine PTH conjugated at the N-terminus (D. J. Sakelaris, CIS Radiopharmaceuticals, personal communication).

2. The antibody should demonstrate cross-reactivity to C-PTH fragments. Figure 1 shows good displacement of labeled PTH by a C-terminal fragment preparation. Because the proportions of the several circulating C-terminal fragments are unknown, displacement of intact iodinated hormone by the fragment used indicates qualitatively that the antibody contains C-terminal-specific determinant sites.

3. The PTH concentrations in sera of normal, healthy adults measured with intact hormone as standard and label should be higher by the C-assay than by an N-assay. This characteristic is based on the reported longer half-life of the C-PTH species. As shown in Figure 6, the adult reference range of the present assay is higher than N-PTH ranges reported (11).

4. An as yet unpublished characteristic of C-PTH suggested recently (D. C. Arnaud, Veterans Administration, San Francisco, CA 94121) is its increased stability over that of N-PTH or intact PTH. Stability of C-PTH has been demonstrated in normal sera for at least seven days at 30°C with the present C-PTH assay. In contrast, assays for measuring N-PTH or intact PTH require frozen storage or fresh samples.

5. Finally, the most relevant criterion for any PTH assay is its clinical utility. Because screening PTH antibodies with patients’ samples is impractical, investigators have attempted to relate cross-reactivity results to the ability of PTH antibodies to discriminate among clinical groups. Recently, the validity of this practice has been questioned (10). In an accompanying paper (this issue), we present results of a small clinical study involving the antibody described in this paper; this assay, combined with serum calcium determination, shows good discrimination between hypoparathyroid patients, healthy adults, patients with primary and secondary hyperparathyroidism, and patients with non-parathyroid malignancy.

We used intact bovine PTH to prepare the tracer and standard curve in the present assay. Although various standard materials have been used in other PTH assays, the intact bovine hormone has both theoretical and practical advantages. Two heterogeneities exist in the present assay: (a) in the use of bovine label/standard in measuring human samples (het-
erology in assay technique) and (b) in the proven heterogeneity of circulating molecules with C-terminal sequences. At least two different C-terminal fragments (M, 7000 and 4500) have been reported in addition to the intact molecule (M, 9500), which also contains the C-terminal sequence (4). The proportions of these species differ markedly in different clinical situations and also among patients with the same diagnosis (3, 4, 19, 20). An extreme point of view would demand that four to six separate assays be offered for PTH, each with its own human-derived standards (and iodinated tracers) for each clinical group, but no practical necessity for such extremes has yet been demonstrated. Buckle (21) has suggested that the best compromise is to use a standard that is common to all groups, i.e., the intact hormone as in the present assay. He also states that an assay involving hyperparathyroidism serum standards should not be applied to samples from patients with malignancy or renal failure because of the differing proportions of circulating PTH fragments.

C-PTH assays have been described in which dilutions of a hyperparathyroid serum or plasma pool are used as the working standard (8, 10, 18). In these assays, a patient's results are reported in microliter equivalent units, i.e., the microliter amount of the "standard" serum or plasma found to give the same B/B₀ as the patient's sample. In addition to the theoretical objections to using hyperparathyroid sera as assay standards, several practical problems arise—such as (a) the influence on the assay of changing serum sources (inter-patient heterogeneity), (b) stability of raw serum over long periods, (c) difficulty in obtaining a representative source, and (d) lack of calibration—which preclude any interlaboratory comparison. We used purified bovine intact hormone in the present assay because human hormones have not yet been reliably produced in sufficient quantity and because the cross-reactivity between human and bovine intact PTH has been found by several different investigators to be high (10, 14). The present assay is maintained in calibration with the World Health Organization First International Reference Preparation.

The optimal timing of incubation with the present antibody from CIS Radiopharmaceuticals is the sequential system described in Methods and Materials. Most C-PTH assays reported (4, 8, 10, 16, 21) use similar non-equilibrium conditions and require relatively long incubation times for good binding of the tracer and maximum sensitivity (i.e., displacement of label by an appropriate range of standards). A nonsequential incubation at 25 °C for 24 h with the present antibody is not useful, requiring threefold more antibody and displacing only the most concentrated standards. Adequate sensitivity in the standard curve could be retained during a one-day preincubation, but eliminating the preincubation entirely produces an unacceptable standard curve. Reducing postincubation to one day produces very low binding; the best zero binding (26% B/T) requires three days at 4 °C, as shown in Figure 3.

The effect of incubation pH on the assay is important, not only because of the potential effect of laboratory errors but also because of reports that PTH changes conformation at alkaline pH (22) and that binding of N-PTH by antibodies may be increased at acid pH (23). Several other C-PTH assays are reported at pH 8.4 (4, 10, 12, 16, 18), whereas the present method is pH 7.4. The standard curves shown in Figure 2 are markedly affected by pH, making routine control of the buffer pH imperative. Because possible conformational changes at pH 8.4 may be different in the various circulating species measured, we compared results for clinical samples at different incubation pH; discrimination among groups was better at pH 7.4 than at 8.4. Therefore, pH 7.4 appears to be optimal for the present antibody.

After questions of antibody characterization, standard-


19. Yalow, R. S., Significance of the heterogeneity of parathyroid hormone. In Endocrinology of Calcium Metabolism (see ref. 3), pp 308–312.


