Two Homologous Radioimmunoassays for Parathyrin Compared and Applied to Disorders of Calcium Metabolism

Heinrich Schmidt-Gayk, Markus Schmitt-Flebig, Walter Hitzler, Franz Paul Armbruster, and Eberhard Mayer

These 48-h homologous equilibrium radioimmunoassays for human parathyrin (hPTH) are based on the use of two antisera, MS 6 and MS 7, raised in guinea pigs against human parathyroid adenoma extract. For the assay with MS 6, Tyr\(^{53}\)hPTH(44-68) and hPTH(44-68) were radiiodinated for use as the assay tracer. Labeled peptides were separated from free iodine by passage through Sep-Pak C\(_{18}\) cartridges. This RIA appears to be mid-region specific: hPTH(28-48) and hPTH(64-84) were not recognized, whereas hPTH(53-84) was 100% cross reactive with hPTH(44-68). Thus, the PTH recognition site of antisera MS 6 must be between amino-acid residues 53 and 63. With antisera MS 7, which recognized the PTH molecule between amino-acid residues 69 and 84, we used hPTH(53-84) for preparing the standard curve and \(^{125}\)I-labeled Tyr\(^{52}\)hPTH(53-84) as the assay tracer. Because this RIA recognized PTH fragments containing residues 69-84 of hPTH, we termed it a C-terminal assay. Both assays were useful for diagnosis of primary and secondary hyperparathyroidism.

Additional Keyphrases: hyperparathyroidism · variation, source of · reference interval

Along with the steroid hormone 1,25-dihydroxyvitamin \(D_3\) and the peptide hormone calcitonin, the polypeptide parathyrin (PTH) is responsible for maintaining calcium and phosphorus homeostasis in vertebrates. A decrease in the concentration of calcium in serum is followed by the release of the 84-amino-acid polypeptide PTH, and some of its fragments, from the secretory granules of the parathyroid glands into the blood stream. These peptides undergo rapid proteolytic cleavage in the liver and kidneys to yield the N- and C-terminal fragments of PTH. The half-life of the C-terminal fragment, believed to be biochemically inert, is about 20–40 min, whereas the N-terminal fragment and the intact hormone disappear much faster from the circulation \((t_{1/2} < 10\text{ min})\) (1–3). Thus, N-terminal assays reflect the secretory state of the parathyroid glands at the time of blood sampling. For the diagnosis of hyperparathyroidism, however, these results might be misleading if PTH secretion has temporarily been suppressed (3). In contrast, assays for both the middle region (residues 44-68) and the C-terminal region (residues 53-84) of PTH will give an integrated value for iPTH (immunoreactive PTH), reflecting the chronic state of parathyroid gland function (3). Because circulating PTH is heterogeneous, both assays will probably detect several fragments of PTH, each of which contains the respective amino acid sequence; these fragments are mainly derived from the peripheral metabolism of native PTH, but they also appear to be, in part, secretory products of the parathyroid glands (4, 5).

Several heterologous (6–9) and homologous (9–11) RIAs for hPTH(44-68) have been developed. Here we describe a homologous sensitive RIA system for the mid-region of PTH, in which we use a guinea-pig antiserum raised against a partly purified extract from human parathyroid adenomas, radiiodinated tyrosinylated hPTH(44–68) as tracer, and hPTH(44–68) as standard. We have compared the clinical applicability of this RIA with that of our homologous RIA for hPTH(53–84) reported previously (12); to our knowledge, this is the first comparison of two homologous RIA systems for PTH. Also, we describe a new tracer-purification technique, in which we use Sep-Pak C\(_{18}\) cartridges to purify the iodinated form of hPTH(44–68) and hPTH(53–84).

Materials and Methods

Specimens

Blood was sampled, and the serum was stored at \(-30^\circ\text{C}\). Control samples from apparently healthy volunteers and from patients with primary hyperparathyroidism were divided into 1-mL aliquots and stored at \(-30^\circ\text{C}\) until use. Storage of the samples for 18 months did not measurably change the iPTH concentration, nor did repeated freezing and thawing as many as three times affect the iPTH concentration measured. Thawing the samples and storing them for 48 h at room temperature also did not alter the concentration of iPTH.

Reagents

To prepare 1 L of PPPNE buffer (pH 7.4) we combined 800 mL of 67 mmol/L \(\text{Na}_2\text{HPO}_4\) and 200 mL of 67 mmol/L \(\text{KH}_2\text{PO}_4\) (Merck AG, Darmstadt, F.R.G.), then added 1 g of sodium azide (Merck AG), 400 mg of EDTA, and 1 g of human serum albumin (Behringwerke AG, Marburg, F.R.G.).

Antisera (MS 6 and MS 7) were obtained by immunizing guinea pigs with partly purified hPTH that had been extracted from adenomas and passed through a column of Sephadex G50 (12). The specificity of the antisera obtained differed substantially. We diluted 200 \(\mu\text{L}\) of each antisera with 995 mL of PPPNE buffer, then added 4.8 mL of normal guinea pig serum to each preparation.

Standards

Standards were prepared by diluting the synthetic fragments hPTH(44-68), hPTH(1–34), hPTH(28–48), hPTH(53-84), hPTH(64-84) and hPTH(1–84) in PPPNE buffer in which the human serum albumin had been increased to 10 g/L. hPTH(1–84) was purchased from Peptide Institute Inc., Osaka, Japan; the other PTH peptides were from Bachem Co., Bubendorf, Switzerland.

1 Chirurgische Universitätsklinik, Klinisches Labor, Im Neuenheimer Feld 110, D-6900 Heidelberg, F.R.G.
2 Medizinische Poliklinik, Innere Medizin VI, Endokrinologie, Luisenstraße 5, D-6900 Heidelberg, F.R.G.
3 Nonstandard abbreviations: hPTH, human parathyrin (parathyroid hormone); iPTH, immunoreactive PTH; PPPNE, phosphate buffer containing human serum albumin, sodium azide, and EDTA, as described in Materials and Methods.
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Antiserum to guinea pig IgG was obtained by immunizing a goat with guinea pig IgG. A 31-fold dilution of this antiserum in PPPNE completely precipitated the first antibody (antiserum MS 6 or MS 7) and was therefore a suitable second antibody in the RIA.

Radioiodination Procedure

We radio-labeled 1 μg of either Tyr°32-hPTH(44-68), hPTH(44-68), Tyr°32-hPTH(53-84), or hPTH(53-84) with 0.5 mCi of Na°125I (Amersham Buchler, Frankfurt/M., F.R.G.) by a modification of the procedure described by Hunter and Greenwood (13), as follows. Dissolve 1 μg of the respective peptide in 10 μL of phosphate buffer (pH 7.4), then add 5 μL (0.5 mCi) of Na°125I solution and 10 μL (1 μg) of Chloramine T. After 60 s at room temperature, stop the reaction by adding 10 μL (2.5 μg) of sodium metabisulfite. Next add 1 mL of PPPNE buffer and 1 mL of a 50 mmol/L solution of trifluoroacetic acid. Transfer this mixture onto a Sep-Pak C18 cartridge (Waters Associates, Eschborn, F.R.G.) and elute free °125I with 2 mL of the trifluoroacetic acid solution. Elute the labeled tyrosylated peptide with 2 mL of a 50/50 (vol) mixture of the trifluoroacetic acid and acetone (14). Add to the tracer solution 5 mL of PPPNE buffer in which the human serum albumin content has been increased to 20 g/L. Further dilute the tracer in PPPNE buffer (to which 19 g of human serum albumin and 60 g of polyethylene glycol had been added per liter) to about 30 000 counts/min per 100 μL.

Radioimmunoassays

For hPTH(44-68). Place 100 μL of synthetic hPTH(44-68), the PTH peptide standard, or the patient's sample, 100 μL of tracer (30 000 counts/min of Tyr°32-hPTH(44-68) or radioiodinated native hPTH(44-68) per 100 μL of PPPNE buffer) to which 60 g of polyethylene glycol (M, 6000; Serva Chemicals, Heidelberg, F.R.G.) and 19 g of human serum albumin have been added per liter, and 100 μL of first antibody (antiserum MS 6, diluted 10 000-fold in PPPNE buffer containing normal guinea pig serum, 5 mL/L) into "Radioimmunoassay-special" tubes (Fa. Sarstedt, Nürmburg, F.R.G.), mix, and incubate at room temperature (22°C) for 48 h. Then, after adding 100 μL of second antibody (goat anti-guinea-pig IgG), again mix the tubes' contents, and incubate for another 60 min at 4°C. Centrifuge the tubes (2000 × g, 10 min) to separate bound from free peptide. Discard the supernates and count the radioactivity of the pellets. For this we used a Searle 1285 gamma counter (Fa. Zinsser, Frankfurt/M., F.R.G.) and performed determinations in triplicates.

For hPTH(53-84). The assay was performed as described previously (12), with a slight modification: the incubation was at 4°C for 48 h and °125I-labeled Tyr°32-hPTH(53-84) was used instead of radioiodinated hPTH(44-68) as a tracer. hPTH(53-84) served as a standard and guinea pig antiserum MS 7 (5000-fold diluted) was the binding protein.

Calculations

To construct the assay standard curves, we plotted the concentration of the respective hPTH-peptide, in picomoles per liter, vs. specifically bound tracer in counts per minute. Alternatively, decays per second (bequerels, Bq) can be calculated (e.g., see Figures 1, 2, and 4). The sensitivity of the system was defined as the value 3 SD less than the Bq value (determined, in turn, from 12 triplicate measurements of the zero-hPTH sample). Relative binding affinity was determined as the ratio of the molar concentrations of the various hPTH fragments to that of hPTH(44-68) or hPTH(53-84) at 50% displacement of the respective tracer.

Results

Radioiodination of hPTH(44-68) vs Tyr°32-hPTH(44-68)

We used Sep-Pak C18 cartridges to separate the labeled peptides from a °125I. The specific radioactivity for the labeled peptides obtained was 450 Ci/g for Tyr°32-hPTH(44-68) and 67 Ci/g for hPTH(44-68). Figure 1 depicts a typical standard curve, showing the binding of °125I-labeled Tyr°32-hPTH(44-68) and its displacement by increasing concentrations of hPTH(44-68). Analogously, °125I-labeled hPTH(44-68) was applied as a tracer in the RIA for hPTH(44-68). As Figure 1 shows, in our mid-region assay for hPTH the radioiodinated tyrosylated fragment is the only suitable tracer. °125I-labeled hPTH(44-68) bound only weakly to the antiserum and was not displaced by hPTH(44-68); the binding, therefore, was nonspecific. When we radioiodinated Tyr°32-hPTH(53-84) and the corresponding non-tyrosylated peptide, the specific radioactivities obtained were 420 and 50 Ci/g respectively. Again, the tyrosylated fragment was the superior tracer (Figure 2).

Characteristics of Assay of hPTH(44-68) RIA

Sensitivity and specificity of hPTH(44-68) RIA. Figure 1 depicts a typical displacement curve of the homologous RIA for hPTH(44-68). The smallest detectable concentration in the assay, defined as the point 3 SD below the Bq values 6 fmol per tube, corresponding to a detection limit of 15 pmol/L. The range of hPTH(44-68) concentrations covered by the displacement curve was from 6 fmol per tube to 660 fmol per tube.

Synthetic fragments of PTH, representing other regions of the intact hormone—such as hPTH(1-34), hPTH(28-48), and hPTH(64-84)—were not recognized by antiserum MS 6 (Figure 3). In contrast, hPTH(53-84) was 100% cross reac-

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parathyroid adenomas paralleled the standard curves for hPTH(1-84) and hPTH(44-68).

Precision of hPTH(44–68) RIA. The between- and within-run CV's, estimated by the repeated measurement of both a pool of normal (iPTH concentration 37.1 pmol/L) and hyperparathyroid human serum (iPTH concentration 202 pmol/L), were respectively 12% (n = 11) and 10% (n = 12) for the latter, 21% (n = 11) and 13% (n = 12) for the former.

Assay sensitivity and specificity of hPTH(53–84) RIA. For additional validation of the assay reported previously (12), we did experiments to assess the sensitivity and specificity of the hPTH(53–84) RIA. The sensitivity of this assay was 4 fmol per tube and the range of the hPTH(53–84) standard curve was from 4 fmol per tube to 330 fmol per tube. hPTH(1–34), hPTH(28–48), and hPTH(44–68) showed no cross reactivity in this assay. In contrast, hPTH(64–84) was equicompetitive to hPTH(53–84). Finally, the affinity of intact hPTH(1–84) to antiserum MS 7 was threefold lower than that of hPTH(53–84).

Temperature-dependency of the binding of Tyr43-hPTH(44–68) to antiserum MS 6. We performed the assay at both 4 and 22 °C, all other incubation conditions being identical (see Materials and Methods). The binding of tracer at standard zero and the sensitivity of the assay carried out at 22 °C was superior to the assay performed at 4 °C (Figure 4). As expected, a prolonged incubation (96 h) of the 4 °C assay mixture resulted in a standard curve identical to the assay performed with incubation at 22 °C for 48 h (data not shown). The reason for this finding is that, in the assays at 22 °C and 4 °C, equilibrium was reached after 48 and 96 h,

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**Fig. 2.** Standard curves for hPTH(53–84) radioimmunoassay, prepared with either 125I-labeled Tyr43-hPTH(53–84) (●) or 125I-labeled hPTH(53–84) (Δ—Δ) as the assay tracer.

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**Fig. 3.** Cross reaction of antiserum MS 6 with various PTH peptides and with extract of human parathyroid gland in hPTH(44–68) RIA.

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**Fig. 4.** Effect of temperature on the RIA for hPTH(44–68).
respectively, as evaluated by equilibrium kinetic experiments (data not shown).

Clinical Results

We evaluated all serum samples by both mid-region-specific and C-terminal assay. The normal intervals in the hPTH(44–68) and hPTH(53–84) assays were <15 up to 40 pmol/L and <10 up to 40 pmol/L, respectively, based on data for 124 normal adults.

In patients with primary hyperparathyroidism, iPTH values were above normal in both assays; there was no overlap with the upper limit of the normal range (Figure 5). The mean iPTH concentrations in the hPTH(44–68) and hPTH(53–84) assays were 225 (SD 130) pmol/L and 158 (SD 92) pmol/L (n = 34), respectively. Results for iPTH obtained by the two assays correlated significantly (r = 0.68). For 12 patients with malignancy-associated hypercalcemia the iPTH values were within the low-normal range as measured by both assays. In a group of patients with renal failure not requiring dialysis, iPTH values were increased in both assays. The mean iPTH values in the hPTH(44–68) and hPTH(53–84) assay were 408 (SD 368) and 301 (SD 238) pmol/L (n = 146), respectively, with r = 0.79 for the relationship between results by the two assay systems.

iPTH values for patients on chronic hemodialysis treatment were even higher than for the former group: the means for hPTH(44–68) and hPTH(53–84) were 732 (SD 628) pmol/L and 485 (SD 394) pmol/L, respectively (n = 36) with r = 0.91 (Figure 6).

Serum samples obtained by selective neck-vein catheterization from patients with primary hyperparathyroidism after previously unsuccessful neck surgery showed iPTH gradients in both the hPTH(44–68) and hPTH(53–84) assays. Generally, higher concentrations of iPTH near the adenoma were observed by the hPTH(44–68) assay than with the C-terminal assay.

Figure 7 shows that iPTH was increased in all samples obtained during venous catheterization of patient E.P. in both the mid-region specific and C-terminal assay. At the junction of the left inferior thyroid vein into the jugular vein there was a three- to 10-fold iPTH gradient in the C-terminal and mid-region specific RIA, respectively. A parathyroid adenoma, located at the left caudal site of the thyroid gland, was found at surgery.

Discussion

Sep-Pak C₁₈-cartridges were used by Schönhöfer et al. (14) in purification of the radiolabeled peptides human gastrin, porcine insulin, and human corticotropin(1–34). Here we report the adaptation of this method to preparation of radiolabeled PTH peptides for use as RIA tracers. We found that Sep-Pak C₁₈ cartridges are well suited for use in the purification of ¹²⁵I-labeled Tyrᵣ⁻¹₄-hPTH(44–68) and Tyr⁻⁴₆-hPTH(53–84). This method is less time consuming than the conventional Sephadex G-10 column chromatography applied for tracer purification.

We describe here a homologous RIA for hPTH(44–68), in which ¹²⁵I-labeled Tyrᵣ⁻¹₄-hPTH(44–68) is used as tracer and guinea pig antiserum MS 6 as binding protein. This system was sensitive to 6 fmol of hPTH(44–68) per tube. hPTH(1–34) and hPTH(28–48) showed no cross reactivity up to a concentration of 660 fmol per tube, indicating no N-terminal

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**Fig. 5.** iPTH concentrations in patients with surgically proven primary hyperparathyroidism (1°HPT) and in normal controls as assessed by hPTH(44–68) and hPTH(53–84) RIAs.

**Fig. 6.** Correlation of iPTH concentrations in serum of patients receiving chronic hemodialysis treatment, as measured by the hPTH(44–68) and hPTH(53–84) RIAs.
Fig. 7. IPTh concentrations (pmol/L) in samples obtained by selective venous catheterization of the neck from a patient (E.P.) with parathyroid adenoma (stippled circle). All samples were evaluated by hPTH(44-68) and hPTH(53-84) RIAs; the respective values are shown.

PTH-reactivity of the system. Also, hPTH(64-84) was not recognized by the antibody. Therefore, this RIA is selective for the mid-region of PTH. Further, hPTH(53-84) was almost equipotent to hPTH(44-68), indicating that the amino acid sequence of PTH recognized by antiserum MS 6 is located between amino-acid residues 53 and 63. The standard curve for hPTH(1-84) paralleled the curve for hPTH(44-68), but was about 2.4 times less sensitive.

Using a sheep antiserum raised against partly purified extract from human parathyroid adenomas, Jüppner et al. (10, 11) developed a mid-region assay for PTH, which recognized intact hPTH about 10 times less well than hPTH(44-68). These authors concluded that conformational differences must exist that result in the lower affinity for hPTH(1-84). In our study, this difference in affinity between the intact hormone and the hPTH(44-68) fragment was much (2.4 times) less pronounced than in their study, indicating possible species differences. As already pointed out, our antiserum was raised in a guinea pig; that applied by Jüppner et al. was derived from sheep.

In this communication we describe the thorough characterization of our C-terminal RIA for PTH described elsewhere (12). Guinea pig antiserum MS 7 did not cross react with hPTH(44-68), hPTH(28-48), or hPTH(1-34), whereas hPTH(64-84) was equipotent to hPTH(53-84), indicating that the sequence recognized by antiserum MS 7 is located somewhere between amino-acid residues 69 and 84. These findings support our previous assumption that the RIA established for hPTH(53-84) is truly a C-terminal assay.

The normal range for IPTh was found to be identical by both the hPTH(44-68) RIA and the hPTH(53-84) RIA. In primary hyperparathyroidism much higher hormone concentrations were detected when the mid-region RIA was compared with the C-terminal assay. This might be explained in part by the difference in binding affinity of intact PTH to the antisera, because the hPTH(44-68) RIA detects intact PTH 2.4 times less well, and the hPTH(53-84) RIA three times less well, than the respective standard peptide. Moreover, Roos et al. (7) showed that, for sera from normal persons and from patients with primary hyperparathyroidism, the predominant PTH species cross react both in the C-terminal RIA and the mid-region RIA. However, plasma from persons with primary and secondary hyperparathyroidism contained an additional PTH peptide (M, 4400), which was well recognized by a mid-region RIA but not by a C-terminal assay. This fragment was also detected in extracts from human adenomas. Therefore, the higher basal values and steeper gradients detected in our mid-region RIA as compared with our C-terminal RIA in samples obtained by selective catheterization of a vein draining the parathyroid adenomas might be due to the secretion of the M, 4400 mid-region fragment of PTH described by Roos et al. (7).

Patients with renal failure showed markedly high values for IPTh in both assays. This well-known fact poses a substantial problem in the diagnosis of primary hyperparathyroidism. Because hypercalcemia of non-parathyroid origin (e.g., in sarcoidosis or in malignancy-associated hypercalcemia) might be accompanied by renal failure, or impaired renal function might even be the consequence of hypercalcemia, the interpretation of results for PTH should take kidney function into account.

In summary, mid-region and C-terminal PTH assays are both suitable for use in clinical studies. Each gives a similar normal range. In primary as well as in secondary hyperparathyroidism higher IPTh concentrations were detected by the mid-region assay. As a disadvantage, this assay is more influenced by kidney function than is the C-terminal RIA. In samples obtained by venous catheterization, IPTh gradients were recognized much better by the mid-region assay than by the C-terminal assay.

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References

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