Two-Site Immunochemiluminometric Assay of Intact Human Parathyrin in Serum with Use of a Tracer Peptide Purified by Reversed-Phase High-Performance Liquid Chromatography

Ulrich Böhler, Eberhard Blind, Gisela Vogel, Walter Hitzler, Dagmar Flettje, and Heinrich Schmidt-Gayk

For this two-site immunochemiluminometric assay of intact human parathyrin (hPTH), the luminescent tracer was synthetic hPTH(53-84), conjugated via succinimide linkage to (aminobutyl)ethyl-isoluminol hemisuccinimide (ABEl-H). Purification of the labeled hPTH(53-84) by reversed-phase high-performance liquid chromatography allowed isolation of the conjugate having the highest incorporation of ABEl-H, 1.6 mol per mole of hPTH(53-84). The solid-phase antibody directed against the N-terminal part of hPTH was immobilized by adsorption onto the polystyrene surface of the assay tube and extracted the intact hPTH and N-terminal fragments. Another antibody against synthetic hPTH(53-84), which bound to the C-terminal part of intact hPTH, was indirectly labeled at its second free binding site with the ABEl-H-labeled hPTH(53-84). The assay has a detection limit of 0.5 pmol/L; it is accurate, precise, and reliable; and it shows a linear response for samples containing up to 100 pmol of hPTH per liter. The normal reference range differed from 1.8 to 5.9 pmol/L; 56 patients with primary hyperparathyroidism had concentrations ranging from 5.9 to 113 pmol/L. The concentrations detected in patients with idiopathic hyperparathyroidism were below the normal reference interval.

Additional Keyphrases: hypo- and hyperparathyroidism · reference interval · indirect labeling of antibodies · renal transplant

Measurement of human parathyrin (hPTH) in human serum presents several problems. The metabolism of parathyrin, whether peripherally or within the parathyroid glands, generates several biologically inactive fragments of the hormone. The biologically active intact hormone, however, is present in the circulation in low concentrations and represents only a small proportion of the total PTH immunoreactivity. PTH radioimmunoassays of biologically inactive mid-region and C-terminal fragments are used most widely, because these fragments reach the highest concentrations in the circulation. The usefulness of different types of PTH assays has been reviewed recently. To overcome some limitations of measuring metabolic fragments of PTH, several investigators have attempted to measure the intact hormone directly, e.g., by using region-specific RIAs involving immunochemical extraction steps. Because of the advantages of two-site immunoradiometric assays (IRMAs), especially the short incubation time, high specificity, and high sensitivity, one can now measure intact hPTH sensitively and specifically. These IRMAs have allowed a more direct estimate of the secretory activity of the parathyroid gland than conventional PTH assays and, among other advantages, can reliably differentiate normal from subnormal concentrations of PTH.

Here we describe the development and clinical utility of a two-site immunochemiluminometric assay (ILMA) of intact hPTH, in which the advantages of a non-isotopic tracer peptide are combined with the convenience of a simplified separation procedure, the "coated-tube" technique (9). In addition, this ILMA makes use of a highly purified labeled peptide.

We labeled synthetic hPTH(53-84) with the isoluminol derivative, 6-[N-(4-aminobutyl-N-ethyl)-2,3-dihydrophthalazine-1,4-dione] hemisuccinimide (ABEl-H), coupled via succinimide ester. Reversed-phase high-performance liquid chromatography (HPLC) was used to purify the ABEl-H-labeled hPTH(53-84). This, thus, not only could we separate ABEl-H-labeled hPTH(53-84) from unlabeled peptide and from free ABEl-H, but we also could select for further use the ABEl-H-labeled hPTH(53-84) conjugate with the highest label incorporation ratio.

The two-site ILMA design is based on the method of "indirect antibody labeling," as developed for an IRMA of intact hPTH in our clinical laboratory. The immune complex formed in this assay consists of an immobilized antibody against the N-terminal part of hPTH, intact hPTH, and an antibody against the C-terminal part of hPTH, whose second free binding site has been labeled in a final step with ABEl-H-labeled hPTH(53-84). Finally, we report the application of the present ILMA to the measurement of intact hPTH in specimens of healthy subjects and patients with disorders of calcium metabolism.

Materials and Methods

Reagents

Intact hPTH and hPTH(39-84) were purchased from Peptide Institute Inc., Osaka, Japan; hPTH(1-34), hPTH(1-44), hPTH(28-48), hPTH(44-68), and hPTH(53-84) were from Bachem Co., Bubendorf, Switzerland. ABEl-H was purchased from LKB-Wallac, Turku, Finland. Microperoxidase (MP1; sodium salt, from equine heart cytochrome c) was from Sigma Chemical Co., St. Louis, MO. Human and bovine serum albumin were from Behringwerke AG, Marburg, F.R.G. Water and acetonitrile used for the HPLC were from Baker Co., Deventer, The Netherlands. Trifluoroacetic acid, dimethylformamide, and reagents used for preparing buffer and alkaline peroxide solution were from Merck, Darmstadt, F.R.G. N-Hydroxysuccinimide and dicyclohexylcarbo- diimide were from Serva Feinbiochemica, Heidelberg, F.R.G. Sterile water was obtained from Braun-Melsungen AG, Melsungen, F.R.G. Protein A-agarose gel was from BioRad Laboratories, Richmond, CA.

Assay buffer: This was phosphate buffer (67 mmol/L, pH 7.4) containing 1 g of sodium azide, 400 mg of EDTA, and 1 g of human serum albumin per liter.

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2 Nonstandard abbreviations: hPTH, human parathyrin (parathyroid hormone); ILMA, immunochemiluminometric assay; IRMA, immunoradiometric assay; and ABEl-H, 6-[N-(4-aminobutyl-N-ethyl)-2,3-dihydrophthalazine-1,4-dione] hemisuccinimide.
Reagents for the luminescent reaction: The microperoxidase working solution consisted of 5 μmol of microperoxidase per liter of phosphate buffer (50 mmol/L, pH 8.0) containing 400 mg of bovine serum albumin, 5.84 g of sodium chloride, and 1 g of sodium azide per liter.

The alkaline peroxide solution (final version) contained 2 mol of sodium hydroxide and 625 μL (26.6 mmol) of hydrogen peroxide per liter. We freshly prepared this working solution by adding 100 μL of the 300 mL/L (12.77 mL/mol) hydrogen peroxide stock solution to 48 mL of the 2 mol/L sodium hydroxide stock solution. The first version we had tested contained 330 mmol of sodium hydroxide, 10 mL (425 mmol) of hydrogen peroxide, and 0.1 mol of sodium chloride per liter. Before use, we let the alkaline peroxide working solution stand for 30 min at room temperature.

Reagents for the reversed-phase HPLC: Eluent A was a 50 mmol/L trifluoroacetic acid solution in "HPLC-grade" water; eluent B was acetonitrile.

Antisera

Anti-serum to C-terminal hPTH: The antisera against the C-terminal part of parathyrin, R12, was obtained by immunizing rabbits with synthetic hPTH(53-84) covalently linked to bovine thyroglobulin. This antisera bound specifically to the sequence of amino acids 53–68 (6) and was diluted 150-fold in the assay buffer.

Anti-serum to N-terminal hPTH: The antisera against the N-terminal part of the hormone, Rm, was raised in rabbits by injecting synthetic hPTH(1-34). This antisera recognized the hPTH(1-34) fragment but not fragments hPTH(28-48), hPTH(44-68), or hPTH(53-84).

Standards and Specimens

We prepared the standards by diluting the synthetic fragments hPTH(1-34), hPTH(1-44), hPTH(28-48), hPTH(44-68), hPTH(39-84), hPTH(53-84), and intact hPTH in assay buffer containing hypoparathyroid serum, 100 mL/L. The hypoparathyroid serum, assayed in advance, contained no detectable amounts of intact hPTH (i.e., <0.6 pmol/L). For the routine assay we prepared standards with intact hPTH concentrations of 0, 1, 4, 16, 32, 64, and 125 pmol/L. All standards and sera (or EDTA-treated plasma samples) were divided into 0.5-mL aliquots and stored at −30 °C until use.

Instrumentation

The light-generating reaction was carried out in the automatic luminescence analyzer LB 950 (Laboratorium Berthold, Wildbad, F.R.G.). This computer-assisted analyzer allows fully automated measurement of up to 400 samples.

The reversed-phase HPLC was performed with a 100 × 8 mm (i.d.) Radial-Pak liquid chromatography cartridge, packed with 5-μm particles of C18 material. The column, the radial compression separation system (Model RCM-100), the gradient controller (Model 680), the injection system (Model U6K), and the solvent delivery system (Model 6000A) were all purchased from Waters Associates, Milford, MA.

Preparation of ABEl-H-Labeled hPTH(53-84)

To couple the label ABEl-H with hPTH(53-84) (molecular mass: 3512.4 Da), we first synthesized its succinimide ester by the method reported by Gadow et al. (10). We dissolved 10 mg (27 μmol) of ABEl-H in 800 μL of anhydrous dimethylformamide, with slight warming. As soon as the solution cooled to room temperature, we added 3.1 mg (27 μmol) of N-hydroxysuccinimide, with stirring. We then added 16.7 mg (81 μmol) of dicyclohexylcarbodiimide dissolved in 200 μL of anhydrous dimethylformamide and incubated this mixture in the dark for 20 h at room temperature. This solution contained 27 μmol of activated ABEl-H per milliliter.

To couple the label hPTH(53-84), we chose a molar ratio for hPTH(53-84):ABEl-H (active ester) of 1:2. We prepared a 142.3 μmol/L hPTH(53-84) solution by dissolving 0.25 mg of synthetic hPTH(53-84) in 0.5 mL of 67 mmol/L phosphate buffer, pH 7.4. To this solution (containing 71.1 nmol of hPTH(53-84)) we added 5.35 μL of the ABEl-H active ester solution containing 142.3 nmol of activated ABEl-H and incubated it for 24 h in the dark at 4 °C. Subsequently, we aspirated 250 μL of this reaction mixture into a gas-tight syringe and injected it into the HPLC system.

Purification of Conjugates

To separate the unreacted components and the different ABEl-H-labeled hPTH(53-84) conjugates, we used reversed-phase HPLC, with a concave gradient of eluent B from 50 to 500 mL/L over 30 min at a flow rate of 1 mL/min. In all, 70-0.5-mL fractions were mechanically collected in polypropylene tubes containing 1 mL of assay buffer.

First, to determine the retention time of ABEl-H (active ester), we chromatographed 70 μL of phosphate buffer (67 mmol/L, pH 7.4) containing 54 pmol of ABEl-H (active ester). We pipetted 20 μL of each fraction into "radioimmunoassay-special" tubes (volume 600 μL; Sarstedt, Numbrecht, F.R.G.) (11), and performed the light measurement as described below.

Subsequently, we chromatographed 250 μL of the coupling mixture and diluted 10 μL of each fraction in 10 mL of phosphate buffer (67 mmol/L, pH 7.4) for further investigation. We then tested 50 μL of each diluted fraction for its luminescent activity as described below. The amount of immunoactive hPTH(53-84) of each fraction was determined with a radioimmunoassay specific for hPTH(53-84). This assay was performed as described previously (12).

In all, three HPLC runs were performed as described above. To prevent unsatisfactory separation of the ABEl-H-labeled hPTH(53-84) conjugates, which might be caused by overloading the used column, we did not inject more than 125 μg of hPTH(53-84) for each HPLC run.

Anti-hPTH(1-34) Solid Phase

The anti-hPTH(1-34) solid phase consisted of the N-terminal antibody coated onto the polystyrene surface of the "radioimmunoassay special" tubes.

Purification of antibody: We first purified 40 mL of antisera Rm by affinity chromatography on Protein A agarose as described by Goding (13). The antisera was passed through a column containing 50 mL of Protein A-agarose gel and 2 mg of Protein A per milliliter of the wet gel. After washing the column, we eluted the bound material and diazoyt it against 4 L of pH 9.3 buffer containing 17.3 g of sodium hydrogen carbonate and 8.6 g of sodium carbonate per liter. We obtained 108.5 mL of IgG fraction containing 4.85 g of IgG per liter. This solution was finally diluted 100-fold in sterile water. The pH of this salt-free, non-buffered aqueous IgG-solution was between 9.0 and 9.5.

Immobilization of the N-terminal antibody. We coated the N-terminal antibody by adsorption onto the polystyrene surface of the "radioimmunoassay—special" tubes by incubating 250-μL aliquots of the diluted IgG solution in each
tube for 48 h at 4 °C, with slight shaking. We aspirated and collected the contents, washed the tubes twice by adding 500 μL of isotonic saline, added 250 μL of assay buffer (containing 20 g of bovine serum albumin per liter), and incubated them for 2 h under the same conditions as described above. We washed the tubes twice again and dried them by centrifuging them upside down, mounted on a filter paper. We closed the tubes with adhesive film and stored them at −20 °C until use. Under these conditions, the coated tubes could be used for at least two months.

Immunoluminometric Assay Procedure

Pipet 200-μL aliquots of the intact hPTH standards and samples, in duplicate, into the "radioimmunoassay-special" tubes coated with N-terminal antibody as described above, close the tubes with adhesive film, and incubate them for 24 h at 4 °C. Aspirate the contents, wash the interior of the tubes with 500 μL of isotonic saline, mix, and again aspirate the contents. Add 200 μL of the C-terminal antiserum (diluted 150-fold in assay buffer), close the tubes, and incubate them for 12 h at 4 °C. Perform the same washing procedure and add 200 μL of the ABEI-H-labeled hPTH(53-84) stock solution (diluted 500-fold in assay buffer). This dilution contains ~1.35 nmol of ABEI-H-labeled hPTH(53-84) per liter. Incubate for 8 h at 4 °C, wash three times as described to remove unbound ABEI-H-labeled hPTH(53-84), and perform the light measurement as described below.

Light Measurement

For generating the light output, we added 200 μL of the microperoxidase working solution and fitted the "radioimmunoassay-special" tubes into the 3.5-mL polystyrene tubes (no. 55 484, Sarstedt) in the sample chain of the luminescence analyzer. The light-producing reaction was initiated by automatic injection of 100 μL of the alkaline peroxide working solution (optimized version). The light emission was expressed as total photon counts integrated over 24 s after initiation. At this time, the reaction was at least 91% complete. The light-measuring procedure was performed at room temperature. These measuring conditions, chosen by optimization of the different variables, were used for all light measurements except for the evaluation of the luminescent tracer, for which the non-optimized measuring conditions were as follows: addition of 100 μL of microperoxidase working solution diluted with 200 μL of isotonic saline, injection of 365 μL of alkaline peroxide working solution (first version), and integration of the light signal over 12 s.

Calculations

To construct the assay standard curves, we plotted the concentration of the respective standard, in picomoles per liter, vs. the mean photon counts over a 24-s integral, using linear scales.

IRMA of Intact hPTH

We compared the results of the present assay by measuring the same samples with a previously described IRMA for assay of intact hPTH, used for routine determination in our clinical laboratory (6).

Results

Tracer Evaluation

Figure 1a shows the luminescence chromatogram of 0.2 μL of ABEI-H solution (active ester), prepared as described above, and consisting of at least six different luminescent compounds.

The chromatogram of the immunoactivity for hPTH(53-84) and the luminescence of the reaction mixture are shown in Figure 1b. Four peaks of immunoactive hPTH(53-84) were identified. The first (fractions 41 to 43) showed nearly no luminescence, despite its high amount of hPTH(53-84). The second (fractions 48 and 49), third (fractions 54 and 55), and fourth (fractions 57 and 58) peaks showed good luminescence. Therefore we assumed that fractions 41 to 43 contained the unlabeled hPTH(53-84) and that the other three peaks contained the differently labeled hPTH(53-84). We calculated the total proportion of labeled hPTH(53-84) to be 36.0% by adding the measured amounts of fractions 45 to 64 and comparing it with the amount of hPTH(53-84) applied.

We estimated the incorporation of label into hPTH(53-84) for all three of the peaks obtained containing ABEI-H-labeled hPTH(53-84). The hPTH(53-84) content of each fraction was measured by radioimmunoassay, and the ABEI-H contents of the respective fractions were calculated by comparing the light intensity of each single fraction with the total light intensity of all fractions, which represented the applied 71.1 nmol of activated ABEI-H. To confirm this estimation, we once again determined the ABEI-H concentration in each fraction containing labeled hPTH(53-84) by measuring the intensity of light emission, using different concentrations of activated ABEI-H as standards. Table 1 shows the incorporation ratios, defined as the molar ratio of ABEI-H:hPTH(53-84), as the mean ± SD of both calculations for each peak.

![Fig. 1. (a) Luminescence chromatogram of 5.3 nmol of activated ABEI-H eluted from a C18 reversed-phase HPLC; (b) luminescence chromatogram and immunoactivity of hPTH(53-84) chromatogram.](http://www.clinchem.org/content/35/2/217/F1)

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containing ABEI-H-labeled hPTH(53-84). Three different ABEI-H-labeled hPTH(53-84) conjugates were separated by HPLC.

The conjugate of fractions 54 and 55 appeared to have the highest label incorporation ratio and was therefore selected as the luminescent tracer for further development of the ILMA. We pooled fractions 54, 55, and the respective fractions of three additional HPLC runs, performed in the same manner. The resulting 9 mL of stock solution contained 675 nmol of ABEI-H-labeled hPTH(53-84) per liter, with a label incorporation ratio of 1.63 mol ABEI-H per mole of hPTH(53-84). This extent of label incorporation corresponded to a specific molar activity of 6.31 \times 10^{18} counts per mole of label, when measured with the first version of the measuring procedures described above.

The ABEI-H-labeled hPTH(53-84) stock solution was stored at -20 °C for about one year with no significant loss of either immunological or luminescent activity.

Optimization of Light Measurement

We investigated several variables of the light-measuring procedure to obtain a wide dynamic range of the standard curve (i.e., difference between the response of the 125 pmol/L and the zero standard), and a maximum of sensitivity. Increasing the sodium hydroxide concentration in the alkaline peroxide solution to 2 mol/L led to an increased light emission (Figure 2). To avoid any damage to the injection system by more-concentrated sodium hydroxide solutions, we chose as the working concentration 2 mol/L. Light emission was maximal with a hydrogen peroxide concentration of 625 \mu mol/L (26.6 mmol/L).

We observed the light emission so obtained by testing different volume ratios of the added microperoxidase solution and the injected alkaline peroxide solution. We used a range of both volumes from 50 to 350 \mu L, and the ratio found to generate the highest dynamic range of the standard curve was 200 \mu L of microperoxidase solution to 100 \mu L of alkaline peroxide solution.

Finally, the integration time was chosen. The reaction kinetics of the 125 pmol/L and of the zero standard showed that the maximum was reached 1.33 s after the alkaline peroxide solution was injected, with a half-maximum time of 5.33 s. The whole reaction was almost finished within 1 min. We chose to integrate the light signal from 0 to 24 s. This response represents 91% of the dynamic range obtained by integration from 0 to 60 s.

This optimized measuring procedure led to a 37.7-fold increase of the specific molar activity of the tracer, which was now 2.38 \times 10^{20} counts per mole of ABEI-H-labeled hPTH(53-84), integrated over 24 s.

### Table 1. Characteristics of the ABEI-H-Labeled hPTH(53-84) Conjugates

<table>
<thead>
<tr>
<th>Fraction no.</th>
<th>R0</th>
<th>Incorporation molar ratio, a</th>
<th>Specific molar activity, b counts/</th>
<th>mol of label</th>
</tr>
</thead>
<tbody>
<tr>
<td>48 + 49</td>
<td>24.2</td>
<td>0.44 (0.007)</td>
<td>2.06 \times 10^9</td>
<td></td>
</tr>
<tr>
<td>54 + 55</td>
<td>27.2</td>
<td>1.63 (0.37)</td>
<td>6.31 \times 10^9</td>
<td></td>
</tr>
<tr>
<td>57</td>
<td>26.5</td>
<td>0.76 (0.10)</td>
<td>3.17 \times 10^9</td>
<td></td>
</tr>
</tbody>
</table>

a ABEI-H/PTH(53-84), calculated by two different methods as described in the text.

b Measured by addition of 100 \mu L of microperoxidase working solution, and 200 \mu L of isotonic saline, and injection of 350 \mu L of alkaline peroxide working solution (containing 330 mmol of sodium hydroxide, 0.425 mol of hydrogen peroxide, and 0.1 mol of sodium chloride per liter), and integration over 12 s.

Fig. 2. Light emission of the intact hPTH standards 0 (○), 64 (△), and 125 (●) pmol/L, at different concentrations of sodium hydroxide in the alkaline peroxide solution.

The hydrogen peroxide concentration was constantly 625 \mu L (26.6 mmol/L). The dotted line depicts the pH of the alkaline peroxide solutions.

- The background-count rate was defined as the response obtained by performing the entire light-measuring procedure (including the addition of microperoxidase solution) in a plain tube; it usually ranged from 15,000 to 30,000 counts per 24-s integral. The background markedly depended on the quality of the microperoxidase solution, which remained stable for at least a month when stored at 4 °C.

Assay Development

We chose an incubation temperature of 4 °C for the coating of the N-terminal antibody and for all incubation steps of the assay procedure, except for the light measuring, which was performed at room temperature. Coating at room temperature led neither to a wider dynamic range of the standard curve nor to better sensitivity.

After coating, incubation of the tubes with assay buffer containing 20 g of bovine serum albumin per liter decreased the nonspecific uptake of the label.

Dose–response curves were generated by using various concentrations of IgG solution of the N-terminal antiseraum, C-terminal antiseraum, and the ABEI-H-labeled hPTH(53-84). All final conditions were chosen to achieve a maximum sensitivity and steepness of the standard curve.

Characteristics of ILMA for Intact hPTH

ILMA standard curve: A typical standard curve for intact PTH is shown in Figure 3. The nonspecific uptake of the label, defined as the difference between the response of the zero standard and the background-count rate, was <2% of the range of the standard curve and ~0.1% of the total activity added. No "hook" effect and no saturation of the anti-hPTH(1-34) solid phase were observed up to concentrations of 8000 pmol of intact PTH per liter.

Sensitivity: The lower limit of detection was defined as the minimal concentration of intact hPTH that could be distinguished from a sample containing no intact hPTH. Distinction was based on the confidence limits of the estimate of the zero standard. We calculated the 99.7% confidence limits of the estimate of the zero standard on the basis of 12 replicate determinations and ascertained the point where these confidence limits intersected the standard curve. The minimal detection limit, so determined, was 0.5 pmol (4.75 ng) of intact hPTH per liter.

Precision: We examined variance throughout the ILMA standard curve by assaying each standard six times, in
duplicate, and calculating the coefficient of variation and the standard deviation of the values obtained for each standard. The SD declined to ~0.2 pmol/L at low concentrations of intact hPTH and the CVs ranged from 3.5% to 15.3% (mean 6.8%).

Intra-assay variance was assessed by performing 22 replicate determinations of two serum samples from healthy persons, two serum samples from patients with primary hyperparathyroidism, and one serum sample from a patient with hypoparathyroidism.

Interassay variance was assessed by analyzing aliquots of three serum samples (one healthy person, one patient with hypoparathyroidism, and one patient with primary hyperparathyroidism), in duplicate, in seven consecutive assays.

Table 2 shows the results.

Specificity: To prove that only the intact hormone was being measured, we examined the IRMA for its cross-reactivity with several synthetic hPTH fragments. We assayed different concentrations of hPTH(1-44), hPTH(44-68), hPTH(39-84), and hPTH(53-84) ranging from 64 to 8000 pmol/L. None of these fragments showed an increase of bound activity.

Interference with hPTH fragments: Circulating hPTH fragments are present in specimens, so we investigated the influence of diverse synthetic hPTH fragments on the detection of intact hPTH. We added different known amounts of hPTH(1-44), hPTH(44-68), hPTH(39-84), and hPTH(53-84) to a standard containing 32 pmol of intact hPTH per liter. We saw no influence on the detection of intact hPTH of the C-terminal or the mid-region fragments in concentrations up to 4000 pmol/L, or by the N-terminal fragment at concentrations up to 250 pmol/L per liter.

Linearity: We serially diluted two serum samples from patients with primary hyperparathyroidism with the zero-standard medium. The concentrations of intact hPTH in the undiluted serum samples were 14.4 and 100.5 pmol/L. Linear regression analysis of the expected (x) vs the observed (y) concentrations of intact hPTH yielded the regression equations y = 0.90x + 3.14 pmol/L (n = 10, r = 0.993) for the sample containing 100.5 pmol of intact hPTH per liter and y = 0.87x + 0.98 pmol/L (n = 10, r = 0.961) for the sample containing 14.4 pmol of intact hPTH per liter.

Analytical recovery: We added different known amounts of synthetic intact hPTH to a serum and an EDTA-treated plasma sample, both from patients with primary hyperparathyroidism and then measured the total amount of intact hPTH, in duplicate, to assess the analytical recovery. The percentage recovery was calculated by subtracting the endogenous intact hPTH from the measured total amount, divided by the amount added and multiplied by 100. The mean (n = 11) recovery was 100.88% with SDs up to 25.2% (Table 3).

Correlation with IRMA: We measured intact hPTH concentrations in 42 samples by the present ILMA and by the IRMA for intact hPTH. The relationship was calculated by linear regression analysis and described by the equation ILMA = 1.12 IRMA - 1.36 pmol/L (n = 42, r = 0.96, S_\text{xy} = 938.9, P < 0.001). For samples with intact hPTH values <5.9 pmol/L (representing the normal range) the regression equation for the relation was ILMA = 0.98 IRMA - 0.01 pmol/L (n = 20, r = 0.91, S_\text{xy} = 166.2, P < 0.001).

### Table 2. Precision of the Assay

<table>
<thead>
<tr>
<th>Sample</th>
<th>Intact hPTH, pmol/L</th>
<th>Mean</th>
<th>SD</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inter-assay (n = 7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypoparathyroid serum</td>
<td>&lt;0.5*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal serum</td>
<td>3.5</td>
<td>0.41</td>
<td>11.5</td>
<td></td>
</tr>
<tr>
<td>Hyperparathyroid serum</td>
<td>33.1</td>
<td>3.70</td>
<td>11.1</td>
<td></td>
</tr>
<tr>
<td>Intra-assay (n = 22)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypoparathyroid serum</td>
<td>&lt;0.5^b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal serum 1</td>
<td>3.4</td>
<td>0.39</td>
<td>11.4</td>
<td></td>
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<tr>
<td>Normal serum 2</td>
<td>3.1</td>
<td>0.43</td>
<td>13.8</td>
<td></td>
</tr>
<tr>
<td>Hyperparathyroid serum 1</td>
<td>27.6</td>
<td>1.60</td>
<td>5.7</td>
<td></td>
</tr>
<tr>
<td>Hyperparathyroid serum 2</td>
<td>11.9</td>
<td>1.76</td>
<td>14.7</td>
<td></td>
</tr>
</tbody>
</table>

*For the hypoparathyroid serum sample, three determinations were below the limit of detection; the other four ranged from 0.7 to 1.2 pmol/L (mean 0.8 pmol/L).

**All values were below the limit of detection, except one at 0.9 pmol/L.**

### Table 3. Analytical Recovery of Added Intact hPTH

<table>
<thead>
<tr>
<th>Added (e)</th>
<th>Measured (b)</th>
<th>Recovered (b - endogenous)</th>
<th>Recovery, %</th>
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</thead>
<tbody>
<tr>
<td>Serum</td>
<td>18.5</td>
<td>18.5</td>
<td>100</td>
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<tr>
<td>0.5</td>
<td>19.2</td>
<td>0.7</td>
<td>140</td>
</tr>
<tr>
<td>2.0</td>
<td>20.7</td>
<td>2.2</td>
<td>110</td>
</tr>
<tr>
<td>8.0</td>
<td>26.3</td>
<td>7.8</td>
<td>98</td>
</tr>
<tr>
<td>16.0</td>
<td>36.1</td>
<td>17.6</td>
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Clinical Results

All concentrations of intact hPTH measured in specimens from normal subjects and patients are shown in Figure 4.

Normal reference interval: The concentration of intact hPTH was determined in samples from 37 healthy adults. Intact hPTH was detectable in all samples, the mean being 3.87 pmol/L and the median 3.8 pmol/L. Omitting the highest and lowest detected values, we defined the normal reference interval as ranging from 1.8 to 5.9 pmol of intact hPTH per liter.

Hyperparathyroid subjects: Fifty-six patients with surgically proven primary hyperparathyroidism had above-normal values for intact hPTH in serum, except for one whose value was 5.9 pmol/L, the upper limit of the normal range. The values for intact hPTH ranged from 5.9 to 113 pmol/L (mean 22.0 pmol/L, median 16.1 pmol/L) in this group.

Hypoparathyroid subjects: We assayed serum samples from six patients with idiopathic hypoparathyroidism. All concentrations of intact hPTH measured were below the lower limit of the normal range. Two patients had values of 0.6 and 0.7 pmol/L, the other four had no detectable amounts of intact hPTH.

Patients with transplanted kidneys: We assayed serum samples from patients with kidney transplants, none of them receiving hemodialysis. The values for intact hPTH were widely spread, ranging from 2.9 to 98 pmol/L (mean 17.5 pmol/L, median 8.1 pmol/L). Only seven patients in this group had values for intact hPTH that were within the normal reference interval, whereas the other 22 patients showed above-normal concentrations.

Discussion

RIAs have been widely used for measuring circulating fragments of PTH (2), but except for one two-site RIA in which an acridinium ester-labeled antibody is used (7), all assays of intact hPTH have involved 125I-labeled proteins as tracer (3, 4, 6, 8).

All these assays suffered the well-known disadvantages of 125I-labeled tracers: contact with radioactive material, and short half-life of the tracer and its instability due to radiolytic damage to the labeled protein. Furthermore, long counting times were required to achieve sufficient sensitivity. For these reasons, many non-isotopic labels have been developed and investigated for their use in immunoassays, but most of them have failed to yield assays with the sensitivity attainable by the use of 125I (14).

Luminescent labels, however, have proved to be efficient in terms of both specificity and sensitivity and are therefore suitable to replace radioisotopes as labels (15).

Many chemiluminescent labels, and their use in the labeling of proteins, have been reported in the literature (10, 16, 17), but only a few reports have described the preparation of tracer peptides of low molecular mass (18). We chose ABEl-H as label because it was commercially available, showed good luminescence, and also because its active ester could be relatively easily synthesized (10).

For the synthesis of ABEl-H-labeled hPTH(53-84), we used N-hydroxysuccinimide. This method has proven to be far superior to alternative coupling reagents because the succinimide ester reacts with the amino moieties of the peptide under non-oxidative conditions, and therefore the immunoreactivity of the tracer is mostly retained (10, 18).

The succinimide ester of ABEl-H binds to the lysine amino groups of the polypeptidic chain. The hPTH(53-84) molecule, however, has only six lysine amino groups, thus limiting the number of possibly incorporated luminescent labels. Furthermore, the small size of hPTH(53-84) increases the possibility that incorporation of the luminescent label will affect the immunoreactivity of the hPTH fragment. Therefore we chose a molar ratio of hPTH(53-84) to ABEl-H active ester that was half as much as the corrected substitution ratio recommended by Gadow et al. (10).

Different techniques for the purification of 125I-labeled hPTH fragments and other 125I-labeled peptide hormones have been described, but none of them has been investigated for its practicability for purifying luminescent-labeled hPTH fragments (19-24). An easily performed method is reversed-phase chromatography with use of Sep-Pak C18 cartridges, but even this method does not remove the unlabeled peptide. Reversed-phase HPLC has been recommended, but it best purifies the tracer by separating labeled from unlabeled peptide (21). In our experience this technique proved to be an excellent one for purifying ABEl-H-labeled hPTH(53-84). Reversed-phase HPLC not only separated free ABEl-H(active ester) from ABEl-H-labeled hPTH(53-84), it also resolved ABEl-H-labeled hPTH(53-84) from native hPTH(53-84), and various ABEl-H-labeled hPTH(53-84) conjugates with different label-incorporation ratios could be isolated with no overlap. Thus we obtained a highly purified tracer peptide with high specific activity.

The calculated label-incorporation ratios, however, are only a rough estimate of actual label incorporation. The calculated ratios might be decreased owing to quenching of the luminescence by the peptide. On the other hand, the immunoreactivity of hPTH(53-84) might be affected by the incorporated ABEl-H, thus increasing the calculated ratios.

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Fig. 4. Comparison of the intact hPTH concentrations measured in samples from 37 healthy volunteers, 56 patients with surgically proven primary hyperparathyroidism, six patients with idiopathic hypoparathyroidism, and 29 patients with transplanted kidneys.

The detection limit is indicated by the dotted line. Values >20 pmol/L are identified by their respective concentration.
Therefore, we only used these ratios to identify the separated ABEI-H-labeled hPTH(53-84) conjugates and to choose the one resulting in the highest incorporation of ABEI-H for further use in the development of the ILMA.

The amount of purified ABEI-H-labeled hPTH(53-84) obtained by four HPLC runs was enough to perform ~22 500 single determinations of intact hPTH. Reversed-phase HPLC is therefore also an economical method to produce large amounts of luminescent tracer.

So far it has not been possible to purify directly-labeled antibodies in a manner comparable with that achieved with labeled hPTH(53-84) because of its low molecular mass (3512 Da vs ~150 000 Da). We even succeeded in separating tracer fractions with different molar label incorporations, thus increasing the sensitivity of the assay. In contrast, directly labeled antibodies are a mixture of proteins with various label-incorporation ratios, and our C-terminal antiserum would have required affinity chromatography purification before being labeled, because it had a low titer. Additionally, the attempt to label the C-terminal antibody directly resulted in reduced immunoreactivity, thus making these directly-labeled antibodies unsuitable for further use in the assay. For these reasons we chose the more complicated indirect assay protocol.

Compared with other published assays of intact PTH (6–8), the performance of our assay was simplified by coating the N-terminal antibody at the surface of the "radioimmunoassay-special" tubes. This method was suitable after purification of the N-terminal antiserum by affinity chromatography on Protein A–agarose. Thus, centrifugation steps were replaced by more rapid and cost-effective washing–decoration steps. We used isotonic saline as washing solution instead of non-ionic detergents to avoid removal of the adsorbed antibody from the surface of the polystyrene tube. The small nonspecific uptake of the tracer proved that isotonic saline is a satisfactory washing solution.

Incubation at 25 °C resulted in faster kinetics of the binding reactions, but the steepness of the standard curve and the sensitivity of the assay decreased. Therefore, we compromised, accepting a longer total assay time to achieve a higher sensitivity.

Chemiluminescent reaction conditions were optimized to promote maximum total light output, resulting in increased sensitivity. For this benefit we accepted slower kinetics of the light reaction.

Owing to the particular architecture of the present ILMA, the term "intact hPTH" refers to all PTH molecules that possess both the N-terminal and the C-terminal parts of hPTH; i.e., only hPTH molecules not yet cleaved in the region 34 to 37 (1) will be recognized by the present ILMA.

Accordingly, no cross-reaction was found with synthetic hPTH(1–44), hPTH(44–68), hPTH(39–84), and hPTH(53–84) fragments, which were added in quantities of up to 8000 pmol/L.

The first step of the present ILMA extracts intact hPTH and N-terminal fragments of hPTH. All other circulating substances, including C-terminal and mid-region fragments, are eliminated. Therefore no interference was observed when synthetic hPTH(44–68), hPTH(39–84), and hPTH(53–84) were added in quantities of up to 4000 pmol/L. No interference was found by added synthetic hPTH(1–44) up to 250 pmol/L; at higher concentrations, however, the N-terminal antibody of the solid phase became increasingly saturated. Nevertheless, it is unlikely that circulating N-terminal fragments will interfere with the measurement of intact hPTH by the present ILMA, because these fragments are in relatively low concentrations in the circulation (25, 26).

The use of highly purified ABEI-H-labeled hPTH(53-84) as tracer led to a high signal/noise ratio, which resulted in a high sensitivity and good precision of the ILMA. Another advantage of this tracer was a faster kinetics of the indirect labeling of the C-terminal antibody, which shortened the total incubation time to 48 h, two-thirds that required when 125I-labeled Tyr52-hPTH(53-84) is used as tracer (6).

Because of the linear response of samples containing up to 100 pmol of intact hPTH per liter and the absence of a high-dose hook effect, it was not necessary to repeat measurements of diluted samples that contained large amounts of intact hPTH.

The only other ILMA of intact hPTH (7), mentioned above, involved an acridinium ester-labeled sheep polyclonal antibody against the N-terminal part of hPTH and a mouse monoclonal antibody against the mid-region part of hPTH, which was covalently coupled to diazoo cellulose as solid phase. This ILMA had a shorter total assay time of 5 h, but despite their use of a monoclonal antibody, these workers (7) did not achieve a higher sensitivity than the present ILMA.

The concentrations of intact hPTH in normal subjects are remarkably similar as measured by different assays. On the other hand, normal reference intervals differ slightly in different laboratories, even with the same assay. Suppliers of the commercially available "Allegro Intact hPTH" kit (Nichols Institute Diagnostics, San Juan Capistrano, CA) suggest a normal interval from 1.1 to 5.8 pmol/L, whereas Nusebaum et al. (8) defined a normal interval from 1.3 to 6.9 pmol/L for 72 healthy subjects with the same assay, and Sokoll et al. (27) determined a normal reference interval from 1.5 to 6.8 pmol/L on the basis of data for 245 healthy postmenopausal women. The normal reference interval of the commercially available ILMA of intact hPTH (Immunoagnostik GmbH, Darmstadt, F.R.G.) developed in our clinical laboratory (6) ranged from 2.0 to 6.3 pmol/L, on the basis of data for 60 healthy persons. Thus the normal reference interval of the present ILMA (1.8 to 5.9 pmol/L) is similar to those described above and the mean of the normal range (3.8 pmol/L) is also similar to the values reported by Lindall et al., 4.8 pmol/L (3), and Brown et al., 3.4 pmol/L (7).

The values for intact hPTH in serum of patients with surgically proven primary hyperparathyroidism exceeded the normal reference interval and were completely different from those of normal subjects and patients with idiopathic hypoparathyroidism.

In conclusion: the preparation of ABEI-H-labeled hPTH(53–84) and its purification by reversed-phase HPLC requires expertise and equipment but has the advantage of separating, economically, a highly purified and stable tracer peptide of high specific chemiluminescent activity with preserved immunological activity. In addition, it has all other advantages of chemiluminescent tracers over their 125I-labeled counterparts. In future, the 125I-labeled PTH fragments used in radioimmunoassays might be replaced by ABEI-H-labeled PTH fragments. On the basis of the obtained luminescent-labeled peptide, we have developed an accurate, precise, linear, and reliable ILMA for the measurement of intact hPTH. Its clinical evaluation has shown that intact hPTH determination by our ILMA is a valuable tool in the
assessment of parathyroid secretory activity in patients with disorders of calcium metabolism.

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References