Development and Validation of an Immunoradiometric Assay of Parathyrin-Related Protein in Unextracted Plasma

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This two-site immunoradiometric assay for human parathyrin-related protein 1-86 (PTHRP1-86) in plasma uses a mouse monoclonal antibody to PTHRP1-34 coupled to cellulose particles for immunextraction of N-terminal immunoreactivity, and a rabbit antiserum to PTHRP37-67 that is indirectly labeled with 125I-labeled PTHRP37-67 for quantifying the bound analyte. The detection limit of the assay is 0.23 pmol/L, corresponding to 0.4 pg (0.04 fmol) per tube, for a sample volume of 200 μL. Recovery of PTHRP1-86 added to serum is essentially quantitative, and within- and between-batch precision is 4.4% and 11.1%, respectively. PTH1-84, PTHRP18-34, PTHRP9-34, PTHRP1-34, and PTHRP37-67 do not cross-react in the assay at concentrations up to 2 nmol/L. Plasma concentrations of PTHRP1-86 were below or close to the detection limit of the assay in normal subjects and in patients with primary hyperparathyroidism, hypoparathyroidism, chronic renal failure, and normocalcemic malignancy. In 37 hypercalcemic patients with various malignancies, we found detectable PTHRP1-86 concentrations in 35 (95%, mean 7.4 pmol/L, range 0.46-24.7). The data support the proposed humoral role of PTHRP in cancer-associated hypercalcemia and suggest that the assay has clinical utility in the differential diagnosis of hypercalcemia.

Additional Keyphrases: immunoextraction with monoclonal antibodies · hypercalcemia of malignancy · cancer · hyperparathyroidism · chronic renal failure

Hypercalcemia, the most common metabolic abnormality in malignancy, arises either by production of humoral factors by the tumor, which stimulate generalized bone resorption, or by metastases in bone, which stimulate local bone resorption (1, 2). Current evidence suggests that the recently identified hormone, parathyrin-related protein (PTHRP), isolated from cancer cell lines and tumors associated with hypercalcemia, may be an important mediator of humoral hypercalcemia of malignancy (HHM) (3). It may also play a role in hypercalcemia commonly associated with metastatic disease (e.g., in breast cancer) and also in certain hematological malignancies (4).

The biochemical abnormalities in HHM resemble those in primary hyperparathyroidism: hypercalcemia, reflecting increased bone resorption and reduced urinary excretion of calcium; increased urinary phosphate excretion; and increased excretion of nephrogenous cAMP (5). Because of overwhelming evidence that PTH itself is rarely responsible for HHM (3, 6), peptides with PTH-like activity were implicated as possible mediators of HHM. In 1987, the search for the factor(s) involved led to the isolation and identification of PTHRP from two cancer cell lines and a tumor associated with hypercalcemia (7-9). Cloning of cDNA for PTHRP predicted a protein of 141 amino acids having significant homology of its amino terminus to that of PTH (10). Subsequent studies have predicted three native forms of the protein, of 139, 141, and 173 amino acids, which differ only at the carboxyl terminus and which arise by alternative splicing of mRNA (10-12). The amino terminus and mid-region of PTHRP are highly conserved between species (13), and the protein is relatively rich in lysine and arginine residues, which may be possible sites of proteolytic cleavage. Little is known regarding the post-translational processing or metabolism of PTHRP, and the major circulating forms are not yet identified.

Sequence homology with PTH is greatest at the amino terminus, where eight of the first 13 amino acids for each molecule are identical (10). The PTH-like bioactivity of PTHRP, like that of PTH, is contained within the first 34 amino acids, and evidence suggests that this activity is expressed via PTH receptors (14). Although some differences in the relative potencies of PTH and PTHRP have been reported, in vitro studies show that PTHRP is at least as potent as PTH in stimulating cAMP by osteogenic sarcoma cells and renal membranes (15), promoting resorption of bone (16), and inhibiting phosphate transport (17). In vivo studies also provide convincing evidence that the biochemical features of HHM may be explained by excess production of PTHRP (18, 19).

Although PTHRP in biological fluids (e.g., cell culture fluid, milk, and tissue extracts) has been assayed by in vitro bioassays involving stimulation of cAMP production by osteosarcoma cell lines and renal membranes, these methods generally lack the sensitivity required to assay circulating PTHRP. Bioactive PTHRP has been assayed in plasma by highly sensitive cytochemical bioassays, but these methods are complex and impractical for clinical studies (20). A further disadvantage of the bioassays is their potential for cross-reaction with PTH.

To date, immunoassays described for plasma PTHRP include a direct radioimmunoassay for PTHRP1-34 (21),

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3 Nonstandard abbreviations: PTH, parathyroid; BMA, immunoradiometric assay; PTHRP, PTH-related protein; PBS, phosphate-buffered saline; HHM, humoral hypercalcemia of malignancy; MAb, monoclonal antibody; and BSA, bovine serum albumin.
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Materials and Methods

Materials

Bovine serum albumin (BSA; Fraction V), fatty acid-, nuclelease-, and protease-free, was from Cambridge Bioscience, Cambridge, U.K. PTHRP 37-67, PTHRP 18-34, and PTHRP 18-34 were prepared by Alta Bioscience, University of Birmingham, Birmingham, U.K. (24). PTHRP 31-34 and PTHRP 38-64 amides were from Peninsula Labs., St. Helens, Merseyside, U.K. PTH 1-84 was from Scientific Marketing Assoc., London, U.K. PTHRP 1-86 was from Bachem, Saffron Walden, Essex, U.K. PTHRP 141, prepared by Hammond et al. (25), was a kind gift from Prof. A.D. Care, University of Leyds, Leeds, U.K. Pre-packed PD-10 chromatography columns and Protein A-Sepharose CL-4B were from Pharmacia, Milton Keynes, Bucks, U.K. Polyep (low-viscosity peptides from digested protein), microcrystalline cellulose (type 20), 4,4'-dithiodipyridine, L-cysteine, 1,1'-carbonyldiimidazole, S-acetylmercaptosuccinic anhydride, and 4-(N-maleimidomethyl)cyclohexane-1-carboxylic acid N-hydroxysuccinimide ester were from Sigma Chemical Co., Poole, Dorset, U.K. [\(^{125}\)I]NaI (0.1 kCi/L) was from Amersham International, Amersham, Bucks., U.K. C\(^{18}\) Sep-Pak cartridges were from Waters, Watford, Herts., U.K. Sac-Cel (anti-rabbit immunoglobulin) was from IDS, Washington, Tyne and Wear, U.K. RPMI 1640, Dulbecco's modified Eagles medium essential medium, and horse serum were from Gibco, Paisley, Strathclyde, U.K. Bovine fetal serum and mouse serum were from Sera-Lab, Crawley Down, Sussex, U.K. PTH 1-86 was assayed by "N-tact" IRMA (Incstar, Wokingham, Berkshire, U.K.). All other chemicals were of the highest purity available.

Standards. Peptides were initially dissolved in acetic acid (10 mmol/L) before dilution in Buffer A and stored as aliquots at \(-70^\circ\)C. We prepared standards from these stock solutions by dilution in Buffer A for RIA, or in Buffer B for the IRMA. Buffer A consisted of phosphate-buffered saline (PBS), pH 7.4, containing, per liter, 2.5 g of Polyep, 0.1 g of sodium azide, and 1 mL of Triton X-100. Buffer B consisted of PBS, pH 7.4, containing, per liter, 20 mL of heat-inactivated horse serum, 10 mL of mouse serum, 0.1 g of sodium azide, and 1 mL of Triton X-100.

Pools for precision, recovery, and stability studies. PTHRP 1-86 was added at three concentrations to donor human serum from the Blood Transfusion Service to increase PTHRP immunoreactivity to within the range found in patients with cancer-related hypercalcemia. We stored aliquot of these pools at \(-70^\circ\)C before assay.

Subjects studied. Control subjects (38) were laboratory staff (23 men, 15 women) and 16 normocalcemic patients (six men, 10 women) with a range of malignancies, including lung (three), ovary (three), bladder (two), epiglottis (one), larynx (one), anal canal (one), testis (one), cervix (one), and unknown primary tumor (two). Mean serum calcium and PTH 1-84 concentrations in the cancer controls were 2.45 mmol/L (range 2.25-2.63) and 1.56 pmol/L (range <0.5-3.0), respectively. The laboratory reference range for PTH 1-84 is 0.9-4.0 pmol/L.

Ten patients had hypoparathyroidism (two men, eight women), two of whom had vitamin D intoxication at the time of sampling. Their mean serum calcium concentration was 2.40 mmol/L (range 1.67-3.30). PTH 1-84 was undetectable (<0.5 pmol/L) in eight patients; it was 0.73 and 0.59 pmol/L in the remaining two patients. Twenty-three patients had chronic renal failure (12 men, 11 women), 18 of whom were receiving hemodialysis; their mean serum calcium and PTH 1-84 concentrations were 2.22 mmol/L (range 1.33-2.73) and 22.1 pmol/L (range 3.1-86), respectively. In 23 additional patients (six men, 17 women), hypercalcemia was attributed to primary hyperparathyroidism; in 13 of these, this was later confirmed by surgical removal of a parathyroid adenoma, followed by normalization of serum calcium. In one patient, parathyroidectomy had been unsuccessful, and the serum calcium concentration remained abnormal. Mean serum calcium and PTH 1-84 concentrations in this group were 2.94 mmol/L (range 2.70-3.63) and 26.1 pmol/L (range 2.7-179), respectively. Three patients (two men, one woman) had hyperparathyroidism due to parathyroid malignancy; their mean serum calcium and PTH 1-84 concentrations were 3.70 mmol/L (range 3.12-4.35) and 29.5 pmol/L (range 5.9-39.4), respectively.

Thirty-seven patients (28 men, nine women) had hypercalcemia associated with malignancy. Serum PTH 1-84 was undetectable (<0.5 pmol/L) in 36 patients, but was 1.19 pmol/L in one patient who had been treated with a bisphosphonate and whose serum calcium was normal at the time of sampling. None of the patients had evidence of significantly impaired renal function. The primary tumor sites were lung (12), pancreas (four; including one somatostatinoma and two pancreatic islet tumors), breast (two), renal cortex (three), prostate (two), esophagus (two), bladder (one), ovary (one), malignant pheochromocytoma (one), and liver (one). Three patients had an unidentified abdominal mass, and in five, the site of the primary tumor was uncertain.
majority of these patients were studied as they presented in local hospital practice. Samples from patients with rare neuroendocrine malignancies (somatostatinoma, pancreatic islet cell tumors, and pheochromocytoma) were referred from hospitals elsewhere in the U.K. Tumor histology was not available in all cases and is therefore not described. Most patients had advanced malignancy; metastatic disease was clinically apparent in 20, although this may be an underestimate because a systematic search for metastases was not feasible in all patients. Seven patients had received intravenous bisphosphonates to inhibit bone resorption and, although serum calcium concentrations had fallen at the time of sampling, corrected calcium remained increased in six and became normal in one. The serum calcium concentrations were corrected for the effect of low serum albumin concentrations: corrected calcium (mmol/L) = total calcium (mmol/L) + 0.02 [40 – albumin (g/L)].

Methods

Preparation of PTHRP37-67 immunogen. We coupled PTHRP37-67 to BSA by using heterobifunctional reagents. We reacted 2 μmol of PTHRP37-67 with 20 μmol of 4-(N-maleimidomethyl)cyclohexane-1-carboxylic acid N-hydroxysuccinimide ester in phosphate buffer (100 mmol/L, pH 6.5) for 30 min at 30 °C. 125I-labeled PTHRP37-67 (100 000 counts/min) was included in the reaction to monitor recovery and coupling of the peptide. Centrifugation of the reaction solution sedimented the insoluble material. The supernate was desalted on a PD-10 gel-filtration column in phosphate buffer (100 mmol/L, pH 6.0) containing EDTA (5 mmol/L). Incubation with a known concentration of L-cysteine determined the incorporation of maleimide groups. Remaining thiol groups were measured by using 4,4′-dithiodipyridine (26). The molar ratio of maleimide groups coupled to PTHRP was 1:2. We reacted BSA (0.12 μmol) with a 10-fold molar excess of S-acetylmercaptosuccinimide anhydride in phosphate buffer (100 mmol/L, pH 6.0) for 30 min at 22 °C. After addition of 20 μL of EDTA (100 mmol/L), 100 μL of Tris (100 mmol/L, pH 7.0), and 100 μL of hydroxylamine (1 mol/L, pH 7.0), we desalted the reaction solution as described above. The molar ratio of thiol groups coupled to BSA, determined by using 4,4′-dithiodipyridine, was 25:1. We then coupled PTHRP37-67 and BSA overnight at 4 °C at a molar ratio of approximately 25:1. We desalted the conjugate by dialysis against phosphate-buffered saline (PBS), pH 7.4, and stored it at −20 °C.

Production and characterization of antisera to PTHRP37-67. We immunized five rabbits with 150 μg of PTHRP37-67–BSA conjugate emulsified in Freund’s complete adjuvant. We gave subsequent boosts of 25 μg of conjugate in Freund’s incomplete adjuvant at four to six-week intervals, and bled the rabbits seven days later. Titer was determined by incubation with 125I-labeled PTHRP37-67 (15 000 counts/min), in Buffer A. The specificity of the selected antisera was examined by RIA by incubating PTHRP37-67, PTHRP38-64 amide, PTH1-84, PTHRP1-86, or dilutions of culture fluid from the BEN lung cancer cell line overnight at 4 °C with 100 μL each of 125I-labeled PTHRP37-67 (15 000 counts/min) and 3000-fold-diluted anti-PTHRP37-67, in a total volume of 0.5 mL. We used Sac-Cel to separate antibody-bound and free fractions.

Preparation of anti-PTHRP1-34 solid phase. The production and characterization of monoclonal antibodies to PTHRP1-34 has been described elsewhere (24). We used Protein A–Sepharose 4B affinity chromatography to purify MAb ID5 from ascitic fluid and coupled the purified MAb to cellulose (5 mg/g cellulose) activated by 1,1′-carbonyldimidazole (27); the cellulose suspension (50 g/L) was stored at 4 °C in Buffer A.

Iodination of PTHRP37-67. We iodinated PTHRP37-67 (2 μg) in the presence of [125I]NaI (1.0 mCi) and Chloramine T (10 μg) and purified the product by passing it through a C18 Sep-Pak cartridge as previously described (24). The incorporation of radiiodine was between 85% and 95%; the specific activity of the tracer ranged from 400 to 450 Ci/g. The shelf life of the tracer was four weeks at 4 °C.

Immunoradiometric assay of PTHRP1-86. We diluted standard PTHRP1-86 through a range of dilutions, from 505 to 0.23 pmol/L. Buffer B as assay diluent gave essentially quantitative recovery of PTHRP1-86 added to donor serum (see Results). Inclusion of bovine and mouse serum in the diluent effectively abolished high results in a normal subject that were attributed to heterophilic anti-mouse antibodies. We incubated in duplicate 200 μL of standards, four quality-control pool samples, or patients’ specimens with 100 μL of anti-PTHRP1-34–cellulose suspension diluted in Buffer B in 75 × 12 mm polystyrene tubes for 1 h at 22 °C with shaking. Then we added 3 mL of wash solution (Buffer A), centrifuged the tubes at 3000 × g at 4 °C for 10 min, and aspirated the supernate to waste. Rabbit anti-PTHRP37-67 (50 μL, diluted 200-fold in Buffer B) was added and incubated at 4 °C for 16–20 h. We washed the cellulose solid phase with 3 mL of Buffer A as described above. 125I-labeled PTHRP37-67 (100 000–120 000 counts/min) in 50 μL of Buffer A was added and incubated for 5 h at 4 °C. Then we washed the cellulose solid phase twice with 3 mL of Buffer A as described above, and counted the bound radioactivity for 2 min in a Model 1261 MultiGamma counter (LKB, Bromma, Sweden), with data reduction by RIA Calc II software.

Cell culture. The BEN lung cancer cell line, which secretes PTHRP (10), was maintained in monolayer culture in RPMI 1640 medium supplemented with bovine fetal serum (100 mL/L), penicillin (100 units/mL), streptomycin (50 units/mL), L-glutamine (2 mmol/L), and sodium pyruvate (1 mmol/L). A clonal rat parathyroid cell line (PT-r, clone 4) donated by Dr G.D. Aurbach, National Institutes of Health, Bethesda, MD, was maintained in an equivalent mixture of Coon’s modified Hams F12 and Dulbecco’s modified Eagles minimum essential medium, supplemented with bovine fetal serum (100 mL/L), penicillin (100 units/mL), and streptomycin (50 units/mL). This parathyroid cell line se-
cretes PTHRP and not PTH (28). Ms. A. Blight, Birmingham Accident Hospital, Birmingham, U.K., kindly provided culture medium from human keratinocytes.

**Optimization and validation of the IRMA for PTHRP1-86.** The following reagents and assay conditions were optimized in the development of the IRMA. The dilution of the anti-PTHRP1-34 cellulose suspension selected corresponded to 0.5–1.0 mg/tube and gave maximum binding of PTHRP1-86. In the case of rabbit anti-PTHRP37-67, 50 μL of a 200-fold dilution gave maximum binding and economical use of this antiserum. Addition of increasing amounts of 125I-labeled PTHRP37-67 increased both specific and nonspecific binding of tracer. Because the signal-to-noise ratio remained relatively constant for different amounts of the label, we selected 100 000–120 000 counts/min per tube arbitrarily. The incubation conditions selected gave optimal specific binding in the assay, and also allowed the assay to be completed within one and one-half working days. A sample volume of 200 μL gave satisfactory assay sensitivity. Preliminary studies showed the response was linearly related to sample volume (up to 500 μL), suggesting that assay sensitivity may be further improved by increasing the sample volume.

**Sample collection and handling.** Blood was collected from patients and volunteers either by syringe or into evacuated tubes with use of lithium heparin as anticoagulant. Ideally, plasma was separated from cells within 30 min of collection and stored at −20°C until assay.

**Statistics.** We used a two-way analysis of variance for statistical analysis.

**Results**

**Characterization of antiserum to PTHRP37-67.** All five rabbits produced antisera that bound 125I-labeled PTHRP37-67 in titers ranging from <1:5000 to 1:500 000 after two booster immunizations. Antisera from three of the five rabbits cross-reacted with PTHRP in human milk, culture medium from the BEN lung cancer cell line, and human keratinocytes. One of these antisera (no. 3592) was compatible with MAb 1D5 in a two-site assay. In an RIA for PTHRP37-67, the detection limit corresponding to 95% of specific binding was 30 pg/tube (17 pmol/L). PTHRP1-86 and PTHRP38-64 amide cross-reacted in the assay but produced dilution curves that were nonparallel to that of the standard, whereas culture medium from the BEN cell line and keratinocytes and human milk yielded dilution curves that approximately paralleled that of PTHRP37-67. We found no cross-reaction with PTH1-84 (up to 100 ng/tube, 20.2 nmol/L).

**Characterization of MAb 1D5 to PTHRP1-34.** MAb 1D5 was of high titer in ascitic fluid (>1:10⁶) and high affinity (Kₐ 8.4 × 10¹⁰ L/mol), and the detection limit in an RIA was 25 pg of PTHRP1-34 per tube (12.5 pmol/L). The binding site of MAb 1D5 extended from residue 18 to between residues 23 and 34, as assessed by inhibition studies with various PTHRP fragments (24). PTHRP1-86 and native forms of PTHRP present in culture fluid from keratinocytes and in human and bovine milk cross-reacted with MAb 1D5 and yielded dilution curves parallel to those of standard PTHRP1-34. No cross-reaction was found with PTH1-84 up to 100 ng/tube (20 nmol/L).

**Characteristics of IRMA standard curve.** The standard curve for PTHRP1-86 is shown in Figure 1. There is a linear response between counts bound and low concentrations of PTHRP1-86. No reduction in binding was found at concentrations of PTHRP ≤505 pmol/L. Nonspecific binding was <0.2% of the total activity added, and was typically between 180 and 240 counts/min. The detection limit of the assay, taken as 3 SD above the mean counts of the zero standard, was 0.23 pmol/L, corresponding to 0.4 pg of PTHRP1-86 per tube (0.04 fmol).

**Cross-reaction.** Molar cross-reaction of recombinant PTHRP1-141 was 61%; culture fluid from the BEN lung cancer cell line, human keratinocytes, and a rat parathyroid cell line, and plasma from two patients with cancer-associated hypercalcemia yield dilution curves approximately parallel to the PTHRP1-86 standard curve (Figure 2).

**Interference of fragments of PTHRP.** We examined for potential interference in the IRMA various sub-fragments of PTHRP. PTHRP1-34 (8 nmol/L), PTHRP9-34 (10 nmol/L), PTHRP18-34 (17 nmol/L), and PTHRP37-67 (9 nmol/L) were assayed in Buffer B and gave no significant increase in the counts bound over that of the zero standard. Similarly, when donor serum containing PTHRP1-86 (20 pmol/L) was supplemented with PTHRP1-34 (2 nmol/L), PTHRP9-34 (2.5 nmol/L), PTHRP18-34 (4.3 nmol/L), or PTHRP37-67 (2.3 nmol/L), the concentration of PTHRP1-86 measured in the IRMA was unaffected.

**Linearity.** The relationship between PTHRP1-86 concentration and sample volume is shown in Figure 3. The five specimens studied were from patients with cancer-associated hypercalcemia. Nonlinearity was apparent at the highest PTHRP1-86 concentrations, particularly at those >10–15 pmol/L, and indicated that such spec-
Concentrations of PTHRP1-86 (O) and recombinant PTHRP1-141 (C) are given on the x-axis. Also shown is the cross-reactivity of PTHRP in culture fluid from the BEN cell line (M), human keratinocytes (Δ), and the P-r rat parathyroid cell line (C). The dilutions of culture fluid tested ranged from neat (N) to 729-fold. The cross-reactivity of PTHRP in plasma from two patients (A and E) with cancer-associated hypercalcemia was tested in neat plasma (N) and in plasma diluted two-, four-, and eightfold.

![Image](https://example.com/image.png)

**Table 1.** Analytical Recovery of PTHRP1-86 Added to Serum, and Within-Batch Precision

<table>
<thead>
<tr>
<th>Control serum</th>
<th>Added (pmol/L)</th>
<th>Measured mean (SD)</th>
<th>CV, %</th>
<th>Theoretical recovery, %</th>
</tr>
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<tbody>
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<td>1 (basal)</td>
<td>—</td>
<td>&lt;0.23</td>
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<tr>
<td>2</td>
<td>10.1</td>
<td>11.0 (0.41)</td>
<td>3.7</td>
<td>109</td>
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<tr>
<td>3</td>
<td>20.2</td>
<td>19.8 (1.05)</td>
<td>5.3</td>
<td>98</td>
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<tr>
<td>4</td>
<td>40.4</td>
<td>40.0 (1.70)</td>
<td>4.3</td>
<td>99</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td></td>
<td>4.4</td>
<td>102</td>
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PTHRP1-86 in assay diluent was added to donor serum, which was then assayed five times in duplicate by the two-site IRMA.
pmol/L were assayed at a range of dilutions to ensure a linear response in the assay. Two patients with undetectable PTHRP1-86 concentrations had plasma calcium concentrations of 3.06 and 3.31 mmol/L associated with breast and lung cancer, respectively. The correlation of plasma PTHRP1-86 (y) and corrected calcium concentrations (x) was y = 5.52x - 11.9 (n = 37, r = 0.414, P = 0.011).

Discussion

We have developed and validated a direct two-site IRMA of PTHRP in plasma. Both antibodies used were produced by using nonoverlapping synthetic fragments of PTHRP, and both antibodies cross-react with native and recombinant forms of PTHRP. A MAb of high specificity and avidity selected from a large panel of MAbs specific for PTHRP1-34 (24) was coupled to cellulose for immunoextraction of amino-terminal PTHRP activity from plasma. A polyclonal antiserum, the first to be produced to the 37-67 region of PTHRP, binds to a compatible epitope on the protein and, when subsequently labeled indirectly with 125I-labeled PTHRP37-67, can be used to quantify the molecular forms of PTHRP that contain both epitopes.

The IRMA of PTHRP1-86 is similar in design to an IRMA previously described for PTH1-84 (29). This assay format offers several advantages. The initial immunoextraction step allows extraction and concentration of molecular species with amino-terminal immunoreactivity. Non-cross-reacting species and potential interferents are selectively removed by washing the solid phase. Indirect labeling of the polyclonal antiserum makes unnecessary affinity purification of the antiserum, with its associated losses, so that the available antiserum can be used as economically as possible. This assay design provides excellent analytical performance, with a low detection limit and good precision. For PTH1-84, Blind et al. (29) reported comparable performance with that of conventional two-site assays involving a single incubation step with antibodies labeled directly, with or without a radioisotope (30, 31).

The minor degree of nonlinearity reported at high concentrations in both the PTH assay and the present PTHRP assay may reflect the assay design. Alternatively, unidentified circulating fragments of PTHRP may cross-react and deplete one of the antibodies, resulting in an underestimated value. The extraction of amino-terminal immunoreactivity, followed by a separate incubation with labeled antibody of mid- or C-terminal specificity, is the most appropriate assay design when mid- or C-terminal fragments are in high molar excess relative to the sequence of interest, as with PTH. The optimal immunoassay design for PTHRP can be determined only when more information is available on its metabolism in tissues and on the relative concentrations of circulating molecular forms in normal and pathological conditions. The cross-reactivity of rat PTHRP in the IRMA probably reflects the high degree of homology that exists within the 1–86 sequence of rat and human PTHRP (13).

So far, few immunoassays for PTHRP have been validated for clinical use. A direct RIA for PTHRP1-34 (21) reported serum concentrations much higher than those found with either an extraction RIA of PTHRP1-34 or IRMAs of PTHRP1-74 or 1-86. That is, in normal subjects, mean PTHRP1-34 concentrations were 30 pmol/L; in patients with cancer and hypercalcemia, mean concentrations were 190 pmol/L (21). These discrepancies may indicate interference in direct assays for PTHRP1-34 by amino-terminal subfragments or structurally unrelated substances. Furthermore, diagnostic discrimination between primary hyperparathyroidism and malignancy-associated hypercalcemia was poor. An affinity-extraction RIA of PTHRP1-34 (22), with a detection limit of 2.5 pmol/L, gave undetectable concentrations in 68% of normal subjects, and increased concentrations in 55% of patients with cancer-associated hypercalcemia (mean 6.1 pmol/L).

A recent two-site IRMA of PTHRP1-74 (23) reported undetectable concentrations (<1 pmol/L) in 53% of normal subjects, whereas PTHRP1-74 was detected in all the patients with cancer-associated hypercalcemia. Most of the latter group (83%) had concentrations >5 pmol/L, but in only 57% were the concentrations clearly above the absolute upper reference limit of normal (11 pmol/L). In the present study, PTHRP1-86 concentrations were detectable and increased (>0.23 pmol/L) in 35 of 37 (95%) patients with cancer-associated hypercalcemia. Differences in the prevalence of above-normal PTHRP concentrations between studies based on either
affinity extraction or two-site IRMA are probably due, in part, to differences in patient selection, given the absence of generally agreed-upon criteria for classifying cancer-associated hypercalcemia.

The absolute PTHRP1-86 concentrations reported here are similar to previous estimates of PTH-like bioactivity in HHM, as measured by cytochemical bioassay (20). The positive correlation between PTHRP and calcium concentrations was also reported in an earlier study (22). In our study, PTHRP1-86 was detectable in only one of 16 patients with cancer who had normal calcium concentrations in plasma. Overall, these results provide convincing evidence that PTHRP may have a humoral role in the majority of patients with malignancy-associated hypercalcemia with suppressed PTH concentrations, although they do not exclude the possibility that other tumor factors may be involved.

Many of the patients with above-normal PTHRP1-86 concentrations had tumors commonly associated with HHM (3), e.g., lung (11), esophagus (2), renal cortex (3), liver (1), ovary (1), whereas others had rare tumors that have only recently been associated with HHM and increased secretion of PTHRP, e.g., pancreatic islet cell tumors (two), (32) and pheochromocytoma (one) (33). The finding of increased PTHRP1-86 concentrations in a patient with breast cancer suggests that humoral mechanisms involving PTHRP may have a role in malignancies in which hypercalcemia is usually attributed to local osteolytic mechanisms. Further work is required to examine PTHRP concentrations systematically in a range of malignancies in which the histological and metastatic status is more rigorously assessed than was possible in the present study.

The role of PTHRP in primary hyperparathyroidism remains to be resolved. Although there is good evidence of increased mRNA for PTHRP in parathyroid adenomas (34), and positive staining for PTHRP in adenomas by immunohistochemistry (35), immunoassay of circulating PTHRP concentrations has not provided convincing evidence of increased PTHRP secretion in this condition (22, 23). In 23 patients with chronic renal failure, PTHRP1-86 concentrations were slightly increased in five. Others have reported detectable concentrations in chronic renal failure, using a two-site IRMA of PTHRP1-74; they also found uniformly increased concentrations when using an RIA of PTHRP109-138 (23). It is not clear whether these observations reflect impaired clearance or increased production of PTHRP and its fragments.

The relationship between immuno- and bioactivity of circulating PTHRP also needs further investigation. As with most proteins of similar molecular size, circulating fragments may retain immunological activity, even when bioactivity, which requires an intact amino-terminal region (e.g., PTHRP1-30) (36), is lost. Nevertheless, the excellent clinical discrimination found in hypercalcemic states in the present study and the demonstrated cross-reaction of native forms, but not of the amino-terminal subfragments of PTHRP, suggests that immunoactive PTHRP1-86 concentrations are related to those molecular species in plasma with biological activity.

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References