Parathyroid-hormone-related Peptide Immunochemiluminometric Assay

Developed with Polyclonal Antisera Produced from a Single Animal*

TA-JEN WU, M.D.†‡§, ROBERT L. TAYLOR, B.S.†, and PAI C. KAO, Ph.D.†

†Department of Laboratory Medicine and Pathology, Mayo Clinic and Mayo Foundation, Rochester, MN 55905

ABSTRACT

An immunochemiluminometric assay of parathyroid-hormone-related peptide (PTHrP) was developed with purified antisera produced from a single goat immunized with (1-86) PTHrP. One batch of purified antibodies was labeled with acridinium ester used as tracer antibodies; a second batch of the purified antibodies was immobilized onto plastic bead. Sensitivity of the assay was 0.1 pmol/L. The assay had no cross-reactivity with PTH. Seventy-five percent of healthy individuals had undetectable PTHrP (mean ± S.D. were 0.73 pmol/L ± 0.6; n = 110). The 95 percent upper reference limit was 2.0 pmol/L. Five of the seven patients with humoral hypercalcemia associated with malignancy (HHM) of solid-tumor had PTHrP greater than 2.0 pmol/L. The test efficacy in detecting HHM was 71 percent, which is similar to previous extraction radioimmunoassay (RIA) or immunoradiometric assay (IRMA). The new assay lowered the detection limit to 0.1 pmol/L, which is a great improvement from that of RIA at 2.0 pmol/L and IRMA at 1.0 pmol/L; however, it did not improve the test efficacy in detecting patients with HHM. It may indicate the patients with HHM only have elevated plasma PTHrP in 70 percent of them. Technically, the method can be used as a model to develop chemiluminescent esoteric test for research or reference laboratories.

Introduction

Although there are a lot of causes for hypercalcemia, the major causes are cancers and primary hyperparathyroidism.1 According to pathophysiologic mechanisms, cancer-associated hypercalcemia has been classified into local osteolytic hypercalcemia and humoral hypercalcemia of malignancy (HHM).2 Multiple myeloma and breast cancer are the two most common tumors that appear to act on the former mechanism. The tumors associated with HHM are squamous cell carcinomas (lung, esophagus, head, and neck), renal and bladder tumors, and ovarian cancer. Parathyroid-hormone-related peptide (PTHrP), which acts as parathyroid hormone, is now considered as the major factor to HHM.3
The gene of PTHrP was characterized, cDNA was cloned, and messenger ribonucleic acid (mRNA) and protein structure were identified. Parathyroid-hormone-related peptide is a single-chain peptide with isoforms of 139, 141, or 173 amino acid residue; its N-terminal has great similarity to PTH. Within its first 34 amino acid sequences, it has 11 amino acids identical to PTH; it reacts with the PTH receptor and causes hypercalcemia and hypophosphatemia. Measurement of PTHrP either by radioimmunoassay or by immunometric assay showed that 60 to 70 percent of patients with HHM had elevated PTHrP levels in circulation. Measurement of mid-molecule PTHrP did not solve this problem either. On the other hand, greater than 95 percent of patients with HHM had suppressed intact PTH level. A suppressed PTH plus hypercalcemia suggested the patient might have HHM. However, this is not specific; a variety of disorders other than HHM, such as vitamin D intoxication or local osteolytic hypercalcemia, will have suppressed PTH plus hypercalcemia. Currently, an immunochemiluminometric assay was developed which is a nonradioisotope assay that is easy to perform and requires very little specimen (50 μl). Most of the time, the residual specimen left from PTH measurement is enough for the PTHrP assay. This will satisfy the need when physicians receive a suppressed PTH report and then want to order a PTHrP measurement.

Technically, an illustration is shown using polyclonal antisera from a single animal (goat) immunized with a peptide antigen. The same antigen was used to prepare an affinity column for the purification of different batches of antisera. One batch of antibodies was immobilized on the solid-phase, while another batch was labeled with acridinium ester as tracer antibodies. This could set a model for research and reference laboratories to develop their own low test-volume esoteric assays. Those assays, which generally target large test-volume market, will not be available in commercial kit or automatic equipment, in the near future.

**Subjects and Methods**

**Plasma levels of PTHrP were determined from 110 normal subject volunteers (aged 19 to 72 years) with proper consent. The PTHrP was determined from specimens of patients at the Mayo Clinic whose physicians had ordered the test; subsequently, their histories were reviewed. The following patients were determined: 22 patients with chronic renal failure (serum creatinine levels ranging from 433 to 1,547 μmol/L), five patients with surgically proven primary hyperparathyroidism who had PTH levels from 5.0 to 22.0 pmol/L plus a sixth patient with clinically diagnosed primary hyperparathyroidism but without surgical treatment who had an increased PTH level of 6.1 pmol/L, and five patients with sarcoidosis (calcium, 2.5 to 3.25 mmol/L or 10.1 to 13.0 mg/dL. The PTH ranged from suppressed to less than 1 pmol/L in four and 2.3 pmol/L in one). Additionally, seven patients had hypercalcemia associated with solid tumors without bone metastasis (two lung cancer, two reproductive system, one breast, one skin, and one hypernephroma). Four patients had hematologic malignancy (three lymphoma and one multiple myeloma). Their PTHrP levels were determined.

**PTHRP IMMUNOCHEMILUMINOGRAPHIC ASSAY (ICMA)**

A goat was immunized with 100 μg of synthetic human PTHrP(1-86)* homogenized with complete Freund’s adjuvant. After 6 months of monthly immunization, the blood from two different batches of bleeding was immunopurified separately by a PTHrP(1-86) affinity column. One preparation of purified antibody was used for immobilization onto the plastic bead (8 mm) at 1 μg/bead as solid-phase antibody; 50 μg of the affinity purified antibody from the second bleeding was labeled.

*Calbiochem, La Jolla, CA.
with 2 μg acridinium ester and used for signal or tracer antibody. During the assay incubation, a sandwich is formed of PTHrP between the signal antibody and an immobilized antibody on a solid-phase, such as a plastic bead. After a washing step, the unbound signal antibody will be washed off. Only the bound signal antibody via PTHrP and immobilized antibody on the solid-phase will be counted to quantify the amount of PTHrP. Technically, development of the PTHrP ICMA was similar to the development of our C-peptide ICMA except different bleedings of polyclonal antisera from a single goat instead of two goats were immunopurified separately by PTHrP(1-86) affinity column and used for solid-phase immobilized antibody and acridinium ester-labeled signal antibody (figure 1).

For the assay, 50 μL of standard solution containing synthetic human PTHrP(1-86) at concentrations of 0 to 50.5 pmol/L or 50 μL of human EDTA plasma was incubated with 50 μL of acridinium ester-labeled tracer antibody containing 2 million relative light units (RLU) and 150 μL assay buffer (0.1 pmol/L sodium phosphate containing 0.9 percent NaCl, 0.1 percent Triton X-100, 2.0 percent normal goat serum, 1.0 percent trasyol, 0.05 percent azide, and 0.5 percent BSA, pH 7.4) at room temperature overnight. A plastic bead with immobilized antibodies was then added for an additional 3 hours of incubation. The bead was washed three times with washing buffer (0.01 M sodium phosphate containing 0.5 M NaCl, 0.1 percent Tween 20, pH 7.4) and then counted in a luminometer.† Duplicate tubes were required for each specimen.

The 110 healthy individuals had plasma PTHrP levels of 0.73 pmol/L ± 0.61 (mean ± S.D.) (ranging from undetectable to 4.2 pmol/L). Eighty-two of the 110 (75 percent) healthy individuals had undetectable PTHrP levels. The 95 percent of upper limit was 2.0 pmol/L. Provisionally, 2.0 pmol/L was set as the upper reference limit of PTHrP. Six healthy individuals (5 percent) had PTHrP levels greater than 2.0 pmol/L.

Plasma PTH levels were determined with PTH-ICMA, which also had no cross-reactivity with PTHrP. The normal range of the assay was 1-5 pmol/L (30).

**Statistical Methods**

Data are presented in mean ± standard deviation by descriptive statistics. Results between different groups of patients were compared with the two-tail nonpaired Student t-test.

**Results**

**Assay Validations**

The average recovery of added PTHrP at 2.5 to 25 pmol/L levels was 98 percent ± 5.6 (mean ± S.D.). Intra-assay variations of PTHrP controls at 3.2, 5.7, and 1.8 pmol/L in 10 determinations of each level were 3.1 percent, 4.9 percent and 10 percent, respectively; and inter-assay variations at controls of 4.6 and 12.1 pmol/L of PTHrP levels in 20 different assays of each control were 9.1 percent and 8.8 percent, respectively. A standard curve of the assay is shown in figure 2. Elevated PTHrP specimens at levels of 8.1, 8.4, and 9.8 pmol/L from three patients were diluted 2-, 4-, 8-, and 16-fold and then measured by the assay. The results showed good linearity of dilution and parallelism to the standard curve (figure 2, insert). Sensitivity of the assay determined by 2 S.D. above 0 standard tube was 0.1 pmol/L. The assay showed no cross-reactivity with intact PTH (1-84) up to a level of 106 pmol/L (1000 pg/mL) or with PTH(1-44) up to 197 pmol/L (1000 pg/mL).

**Specimen Stability**

For the sample stability study, pooled specimens of ethylenediamine tetraacetic acid (EDTA) plasma from patients were spiked with PTHrP(1-86) to a final concentration of about 10 pmol/L and stored at room tempera-

† Magic Lyte II, Ciba Corning Diagnostics, Boston, MA.
Humoral Hypercalcemia of Malignancy of Other Solid Tumors and Hematologic Cancer

Of the seven patients with HHM of other solid tumors, five had increased PTHrP levels (2.2 to 6.1 pmol/L) and two had normal PTHrP levels (0.6 and 1.1 pmol/L); 71 percent (five of the seven) had increased PTHrP levels (figure 3). The mean ± S.D. of the seven patients were 3.1 pmol/L ± 2.0. They were significantly greater than healthy individuals (p < 0.0001).

Of the four patients with HHM of hematologic cancer, two had increased PTHrP levels (2.2 and 2.6 pmol/L), one had a normal level, and one had an undetectable level (figure 3). The mean ± S.D. of PTHrP was 1.6 pmol/L ± 1.1. They were significantly greater than healthy individuals (p < 0.005).

Primary Hyperparathyroidism and Sarcoidosis

In the six patients with primary hyperparathyroidism (five surgically proven, one medically diagnosed), the PTHrP level (0.2 pmol/L ± 0.3; n = 6) was undetectable in five and low (0.9 pmol/L) in one (figure 3); they were not different (p > 0.05) from healthy individuals. All six had increased PTH levels from 5.0 to 20.0 pmol/L (normal reference, 1.0 to 5.0 pmol/L). Their mean value of PTH was 9.5 pmol/L ± 6.4 (S.D). Two of the five patients with surgically proven primary hyperparathyroidism also had associated malignancy (one with colon cancer and papillary cancer of the thyroid, and the other with renal carcinoma). After parathyroidectomy, however, both serum calcium and PTH levels of these two patients returned to normal.
patients returned to normal. The cause of hypercalcemia in these two patients was due to primary hyperparathyroidism instead of cancer. Five patients with sarcoidosis had undetectable or normal PTHrP levels (0.7 pmol/L ± 0.7), which was not significantly different from healthy individuals (p > 0.05) (figure 3).

Discussion

The new assay is an immunochemiluminometric assay (ICMA) that uses affinity purified antibodies from a goat immunized with PTHrP(1-86). The tracer is acridinium ester, which is a nonradioisotope and environmentally safe. The assay required only 50 μL of specimen instead of 1 mL for RIA and 200 μL for the immunoradiometric assay (IRMA). The sensitivity of the ICMA determined by 2 S.D. above 0 tube was 0.1 pmol/L; the reporting limit was 0.5 pmol/L. The sensitivity of the RIA was 2 pmol/L, and IRMA was 1 pmol/L. Comparatively, the ICMA was more sensitive than the IRMA and RIA. The ICMA required only one-fourth of the sample volume required by IRMA (50 vs 200 μL). Technically, the assay is also easier to perform than our previous extraction radioimmunoassay, which not only required 1.0 mL of plasma specimen for cartridge extraction but also required 3 days to complete. Currently, this ICMA direct assay requires only overnight incubation. The assay is also faster than immunoradiometric assays that require a 2-day incubation period.

In previous reports of extraction RIA or direct immunoradiometric assay in hypercalcemic patients associated with HHM, only 60 to 70 percent of the patients had increased PTHrP. For this reason, the PTHrP assay was not a front-line test for the differential diagnosis of hypercalcemia in our institution. Instead, PTH and serum calcium were used. In a group of 20 patients with HHM, the PTH levels in 19 (or 95 percent) were suppressed below the lower limit of normal (<1 pmol/L). Seven of the 20 PTH levels were undetectable, 12 were between 0.1 and 0.8 pmol/L, and only one patient had PTH 3.5 pmol/L which was within the normal range of 1 to 5 pmol/L. In addition, total serum calcium in this group was highly elevated (12.8 mg/dL ± 1.2; m ± S.D.) and ranged from 11.3 to 17.1 mg/dL. The current assay is easy to perform and requires
only 50 μL of specimen, however, requests for the PTHrP assay were still restricted for the practice of cost-effective diagnosis. Thus, only seven patients with HHM of other solid tumors with PTHrP had been ordered and reported in this study. Five of the seven HHM patients had elevated PTHrP. The prevalence of elevated PTHrP was still about 70 percent. The test efficacy was not substantially improved by ICMA as compared with previous RIA and IRMA. When PTHrP is negative, there is still a 30 percent chance of false-negative. Therefore, the test should never be used to rule out HHM.

Two patients in this study had primary hyperparathyroidism and coexisting solid cancers (one renal cancer and the other colon cancer plus papillary cancer of the thyroid). One of these two patients had increased PTH (5.8 pmol/L); the other had a value at the upper limit of normal (5.0 pmol/L). Both had undetectable PTHrP levels. Surgical treatment of parathyroid lesions solved the hypercalcemia, and pathologic study confirmed the diagnosis of hyperparathyroidism. These data suggest that in cancer patients with hypercalcemia having elevated or inappropriately high PTH levels accompanied with undetectable PTHrP levels, primary hyperparathyroidism should be first considered in the differential diagnosis of hypercalcemia.

In conclusion, in patients with HHM of solid tumors, only 70 percent had elevated PTHrP even when using this sensitive ICMA. Therefore, PTHrP should not be used to rule out hypercalcemia associated with malignancy. Technically, this manuscript can be used as a model to develop other non-radioisotope, sensitive, esoteric assays by using antisera from a single animal immunized with a single antigen.

References