Fig. 2. Electrophoretic separation of bone and liver AP fractions after the pretreatment of serum with sialidase

A pooled specimen of serum (AP activity, 795 U/L) was obtained from patients with bone and liver diseases. Samples were incubated for 30 min with sialidase from Vibrio cholerae obtained from Serva Feinbiochemicals (lanes 2–5) or from Clostridium perfringens obtained from Boehringer Mannheim (lanes 6–10) of different activities (final activity in the incubation mixture, as measured with sialyl lactose), then electrophoresed. 1: without sialidase pretreatment; 2: 8.7 U/L; 3: 5.8 U/L; 4: 2.9 U/L; 5: 1.45 U/L; 6: 145 U/L; 7: 36.4 U/L; 8: 18.2 U/L; 9: 3.6 U/L; 10: 2.3 U/L

not adequately resolve the two AP fractions (not shown). We investigated a broad series of final concentrations of sialidase from this source (0.6 to 139 U/L; substrate, sialyl lactose) in the incubation mixture, but found no improved separation as compared with non-pretreated samples.

Discussion

We have confirmed that the electrophoretic resolution of bone and liver AP fractions can be specifically improved by pretreating serum samples with sialidase (1). This method was introduced with sialidase from Vibrio cholerae (1). Later, sialidases from other sources (e.g., from Clostridium perfringens) were used for this purpose, but resulted in some disappointments (2). Our findings show that the electrophoretic separation is decisively determined by the type of sialidase used in the preincubation with serum sample. This could explain the divergent results described in the literature. We obtained the best resolution of both AP fractions with sialidase from Vibrio cholerae. In addition, different activity concentrations must be used with the different sialidases. This and the fact that sialidase from Arthrobacter ureafaciens is not suited for this method suggest that the different types of this enzyme vary in their ability to catalyze digestion of the terminal sialic acid residues in AP fractions, but that their different digestion capacities cannot be inferred from the usual activity measurements with mucin or sialyl lactose. However, a standardized method of sialidase activity determination as we applied it would simplify the matter, avoiding an inconvenient number of incubation experiments with different concentrations of sialidase to determine the best activity concentration for use in electrophoretic separation.

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References


Performance and Diagnostic Application of a Two-Site Immunoradiometric Assay for Parathyroid in Serum

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The "N-tact" immunoradiometric assay (IRMA) from INCSTAR for parathyroid (PTH) in serum involves a 125I-labeled affinity-purified antiserum to PTH 1–84 and an affinity-purified antiserum to PTH 36–84, the latter bound to a polystyrene bead. The mean detection limit, determined in six consecutive assays, was 4 ng/L. The within-batch CV was <7% in the range 15 to 2135 ng/L. The between-batch CV was 11.7% and 5.3% at 30 and 371 ng/L, respectively.

Serum PTH in 14 proven cases of primary hyperparathyroidism was 49–808 (median 111) ng/L, undetectable (<5 ng/L) in 10 cases of primary hypoparathyroidism and in 10 cases of hypercalcemia associated with malignancy, compared with 7–39 ng/L in 45 normal subjects. PTH was 9 to 19 ng/L in 4 patients with familial benign hypercalcemia. In 39 patients with renal failure, apparent concentrations were 14 to 857 (median 133) ng/L, but sera from these patients pre-diluted with zero standard did not parallel dilutions of the standard, PTH 1–84. PTH concentrations were not significantly decreased in blood or serum kept at 20 °C for up to 6 h. After successful removal of a parathyroid adenoma, the mean half-time for disappearance of PTH in vivo in five hyperparathyroid patients was 3.3 min.


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The development of two-site immunometric assays for parathyrin (PTH) represents a new generation of immunoassays for use in investigating disorders of calcium metabolism. These assays may offer significant advantages over conventional RIA methods in terms of analytical performance, laboratory practice, and clinical discrimination (1-3). The design of immunometric assays confers a lower detection limit than is currently achieved by most RIAs (4, 5), which should improve the discrimination of hormone concentrations in normal and hyperparathyroid patients, and the identification of patients with suppressed or subnormal PTH concentrations. Specificity is improved by the use of two antisera directed to different regions of the molecule, allowing measurement of "intact" PTH in the presence of fragments, which constitute most of the circulating PTH-related peptides in many pathological conditions (6, 7).

We report here the analytical performance of an immunoradiometric assay (IRMA) of PTH in routine practice, together with the discrimination achieved in defined groups of patients with disorders of calcium metabolism. We examined the stability of PTH in serum and blood to identify suitable conditions for handling specimens before assay. We also studied the rate of disappearance of PTH from serum in vivo after removal of a parathyroid adenoma, to determine the half-life of intact PTH.

Materials and Methods

PTH Assay

"N-tact" PTH IRMA kits were obtained from INCSTAR Ltd., Berkshire, RG11 5AB, U.K. In this direct, two-site IRMA, 200 μL of unknown serum or human-serum-based standard is incubated with 100 μL of 125I-labeled, affinity-purified antiserum specific for PTH 1-34, and with a polystyrene bead coated with affinity-purified antiserum specific for PTH 39-84. The recommended incubation conditions are 22 h at 20-25 °C without agitation (option A) or 4 h at 20-25 °C with agitation (option B). The bead is then washed three times with 1-mL portions of wash solution, and the radioactivity bound to the bead is counted. In the present study we used polystyrene assay tubes, rather than the borosilicate glass recommended by the manufacturers, and we followed option A for all assay incubations. We used a "Star 700" sample processor (IDS Ltd., Tyne and Wear, NE37 3HS, U.K.) to dispense the serum samples and automatically to wash the bead with a wash solution containing, per liter, 5 mmol of potassium phosphate, 76 mmol of sodium chloride, 2.5 mL of Tween 80 surfactant, and 0.1 g of sodium azide. PTH 44-68 and PTH 39-84 were determined from Peninsula Laboratories, St. Helens, WA9 3AJ, U.K.

Radioactivity bound to the beads was counted for 2 min in a Model 1261 gamma counter (Pharmacia, Milton Keynes, MK9 3HP, U.K.). "RiaCalc" software was used for data reduction. Curve fitting of standards was by spline function, and imprecision profiles were calculated from the error of duplicate determinations, with imprecision related to the concentration of the standards. The minimum detection limit calculated by RiaCalc is based on the 95% confidence limit of the zero standard.

Subjects

Blood was placed in polypropylene centrifuge tubes, containing plastic granules to aid separation (Sarstedt Ltd., Leicester, LE4 1AW, U.K.). Specimens from control subjects and patients were collected between 0900 and 1500 hours and the serum was separated within 1 and 2-3 h, respectively, then stored at -20 °C until assay. Blood collected from patients with primary hyperparathyroidism, used for studies on the stability of endogenous PTH, was separated within 10 min of collection. Specimens of blood were collected from a peripheral vein via an indwelling cannula before and at timed intervals after surgical removal of a parathyroid adenoma.

Control subjects (n = 45; 22 male, 23 female) were normocalcemic laboratory staff and patients (mean age 34, range 12-50 y). Fourteen patients (three male, 11 female) had hypercalcaemia (mean serum calcium 2.88, range 2.63-3.57 mmol/L) attributed to primary hyperparathyroidism, as was later confirmed by surgical removal of a parathyroid adenoma followed by normalization of values for serum calcium. Another 38 patients (five male, 33 female) had hypercalcaemia (mean serum calcium 2.89, range 2.65-3.57 mmol/L) attributed to primary hyperparathyroidism, although neck surgery had not been performed. Ten patients (seven male, three female) had hypercalcaemia (mean serum calcium 3.23, range 2.65-3.86 mmol/L) associated with malignancy. The primary sites were lung (3), breast (1), cervix (2), and kidney (1); in two patients the site of the primary tumor was unknown; the remaining patient had myeloma. Ten patients (three male, seven female) had hypoparathyroidism, and nine were receiving treatment with vitamin D or calcium. The mean value for serum calcium was 2.03, range 1.51-2.40 mmol/L. Four patients had hypercalcaemia (mean serum calcium 2.77, range 2.67-2.88 mmol/L), which was attributed to familial benign hypercalcaemia after investigations had shown the familial nature of the hypercalcaemia and had excluded other causes. Thirty-nine patients (27 male, 12 female) with renal failure were receiving treatment by hemodialysis or chronic ambulatory peritoneal dialysis; their mean value for serum calcium was 2.31 (range 1.73-2.59) mmol/L.

Other Procedures

Stability of PTH. We studied the stability of endogenous PTH in blood and serum at ambient temperature by allowing aliquots of whole blood or serum (separated within 10 min of venepuncture) to stand at 20 °C for as long as 30 h before storage at -20 °C, then assaying.

The effect of repeated freezing and thawing of serum (one, two, four, or six times) on measured concentrations of PTH was studied by using serum from six patients with proven or suspected hyperparathyroidism. In each treatment cycle, serum was thawed at 20 °C for 1 h, then refrozen at -20 °C for at least 4 h.

Parallelism of dilutions of endogenous PTH. Immediately before assay, serum from four patients with primary hyperparathyroidism and five patients with chronic renal failure was diluted two-, four-, or eightfold with the zero standard in a matrix of human serum.

Statistics. Data were log-transformed before two- or three-way analysis of variance. The error variance generated was used to compare individual means by Student's t-test, adjusted according to the Bonferroni method for multiple comparisons (8).
bound for the zero and 2135 ng/L standard were 338 and 23 260 counts/min, respectively; the mean minimum detection limit was 4.0 ng/L (range 1.4–10). Imprecision data are summarized in Table 1.

Sera from four patients with primary hyperparathyroidism were diluted two-, four-, and eightfold with zero standard. The mean PTH concentrations were 50%, 23%, and 10%, respectively, of the PTH concentration in undiluted serum. Sera from five patients with renal failure diluted in the same way gave mean PTH concentrations of 62%, 34%, and 16%, respectively. The deviation from linearity was greatest (Figure 2) for the two patients with renal failure who had the highest apparent PTH concentrations (549 and 564 ng/L).

We investigated the cross-reaction by C-terminal fragments of PTH by adding PTH 44–68 or PTH 39–84 to serum from a patient with primary hyperparathyroidism and a serum PTH concentration of 135 ng/L. After addition of 12.5, 25, or 50 μg of PTH 44–68 per liter, the PTH concentrations measured in serum were 139, 136, and 124 ng/L, respectively; after addition of 12.5, 25, or 50 μg/L of PTH 39–84, the PTH concentrations measured in serum were 128, 124, and 117 ng/L, respectively.

PTH concentrations were not significantly decreased when blood or serum was left standing at 20 °C for up to 6 h (Table 2). Analysis of the overall data revealed significantly higher PTH concentrations for specimens of blood rather than serum (P < 0.001), although this was not significant at individual time points. Sera from six patients with proven or suspected primary hyperparathyroidism were frozen and thawed one, two, four, and six times; the respective geometric mean (and 95% confidence interval) PTH concentrations were 88 (84–91), 89 (85–93), 88 (84–92), and 81 (78–85) ng/L. The PTH concentration was significantly lower (P < 0.01) after six cycles of freezing and thawing than for the other treatment groups.

The mean serum PTH in 45 normal subjects was 19.5 (SD 8.6) ng/L, the range 7–39 (Figure 3). PTH concentrations in the 14 proven cases of primary hyperparathyroidism were 49–506 (median 111) ng/L; for the 38 with suspected primary hyperparathyroidism they were 32–790 (median

**Table 1. Precision of the N-tact PTH IRMA**

<table>
<thead>
<tr>
<th>Within-batch</th>
<th>Between-batch</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTH, ng/L</td>
<td>PTH, ng/L</td>
</tr>
<tr>
<td>15</td>
<td>4.7</td>
</tr>
<tr>
<td>160</td>
<td>3.7</td>
</tr>
<tr>
<td>2135</td>
<td>4.2</td>
</tr>
</tbody>
</table>

* Calculated for six consecutive assays by RiaCalc, each assay consisting of at least 70 determinations in duplicate. All assay data relating to standards and patients' samples were included in the analysis.

* Based on 15–18 observations for N-tact PTH QC pools supplied with the kit.

**Table 2. Stability of Intact PTH in Blood and Serum**

<table>
<thead>
<tr>
<th>Time, h, at 20 °C</th>
<th>geometric mean (95% confidence interval)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>105 (100–111)</td>
</tr>
<tr>
<td>1</td>
<td>106 (100–112)</td>
</tr>
<tr>
<td>2</td>
<td>106 (100–112)</td>
</tr>
<tr>
<td>4</td>
<td>107 (102–114)</td>
</tr>
<tr>
<td>6</td>
<td>102 (96–107)</td>
</tr>
<tr>
<td>24</td>
<td>84 (79–88)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>30</td>
<td>76 (72–81)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

* Time before separation of blood and freezing of serum.

<sup>c</sup> Measured by N-tact PTH in six patients with hyperparathyroidism. Specimens from individual subjects were assayed in one batch.

<sup>c</sup> Significantly lower than results for zero time group (P < 0.05).
80) ng/L. In the four patients with familial benign hypercalcemia, PTH concentrations ranged from 9 to 19 ng/L. Ten patients with hypoparathyroidism and 10 patients with hypercalcemia of malignancy had undetectable (<5.0 ng/L) PTH concentrations. PTH concentrations measured in the 39 patients with renal failure ranged from 14 to 857 (median 133) ng/L.

Figure 4 depicts the disappearance of PTH from peripheral serum after successful removal of a parathyroid adenoma. The mean half-time for disappearance of PTH was 3.3 (range 2–5) min in the five subjects studied. PTH was unchanged in one patient in whom no adenoma was found at surgery.

Discussion

We found the mean detection limit of the N-tact PTH IRMA to be 4 ng/L, higher than that (1.2 ng/L) quoted by the manufacturer. Variations in the detection limit (from 1.4 to 10 ng/L) mainly reflected assay imprecision and affected the discrimination achieved between normal and low or suppressed PTH concentrations in different assays. Good within- and between-batch imprecision was achieved in routine practice, superior to that for most RIA methods (4, 9) or for a chemiluminometric assay (10).

Although dilution studies on unknown samples were found by the manufacturers to be satisfactory, we found non-parallelism on dilution of serum from renal patients, which appeared to be greatest in those patients with the highest serum PTH concentrations. The increase in apparent PTH measured on dilution of serum suggested that interference in the assay might relate to the high concentrations of C-terminal fragments in these patients (7). This was confirmed by demonstrating that the addition of PTH 44–68 and PTH 39–84 to serum in concentrations similar to those found in renal failure (9) gave a significantly lower value for measured PTH concentration. These findings suggest that use of the N-tact PTH IRMA for renal patients may not be valid, depending on the relative concentrations of intact PTH and C-terminal fragments. A substantial pre-dilution of the serum sample with zero-standard matrix may provide more nearly accurate results in these patients.

PTH was stable in both blood and serum for at least 6 h, with 80% and 64%, respectively, remaining after 24 h. In contrast, in a previous study in which the "Allegro" IRMA (Nichols Institute, San Juan Capistrano, CA) was used, PTH was found to be less stable in blood than in serum, with 68% and 88%, respectively, remaining after 18 h at 23 °C; the losses could be partly prevented by aprotinin (3). Compared with endogenous hormone, PTH 1–84 added to serum or present in serum-based standards has been found to be less stable at ambient temperature (3, 11), suggesting that endogenous PTH is preferable for quality-control pools. The stability of intact PTH to freezing and thawing up to four times allows specimens to be assayed on more than one occasion if required.

The range of PTH concentrations we found for normal subjects (7–39 ng/L) is similar to that found by INCSTAR when we used option A, 7–33 ng/L (12), although lower than the suggested range in the product insert (10–55 ng/L). Others have found intact PTH reference intervals of 10–55 ng/L by Allegro IRMA (3), or 10–100 ng/L by a chemiluminometric method (2). In primary hyperparathyroidism, none of the proven cases and three of the suspected cases had serum PTH concentrations within the range found in control subjects. Other groups using two-site assays have also reported a small overlap in PTH concentrations in controls and proven and (or) suspected hyperparathyroidism (1, 3), although the discrimination achieved is nevertheless superior to that provided by most RIA methods (5, 8, 13). One patient with hyperparathyroidism excluded from the results of the present study had a history of renal stones, and several months previously had increased values for serum calcium (2.75 mmol/L) and PTH (by RIA). However, on the day before surgery, values for serum calcium (2.47 mmol/L) and intact PTH (26 ng/L) were not above normal, although a typical parathyroid adenoma was removed at surgery and confirmed by histology. Intermittent hypercalcemia occasionally occurs in hyperparathyroidism (14) and would appear,
at least in this case, to be due to intermittent oversecretion of PTH.

Serum PTH by other two-site immunomassays has been found to be undetectable, subnormal, or normal in hypercalcemic patients with malignancy or PTH-independent causes of hypercalcemia (1-3). We found undetectable serum PTH (<5 ng/L) in 10 patients with various malignancies, which may reflect differences in the patients studied and/or differences in the specificity of the assays. It now seems likely that the hypercalcemia of malignancy may result from the production by the tumor of the recently identified protein, PTH-related protein (15); this should in turn lead to suppressed values for PTH, as we found in the present study. Although there is a high degree of homology in the first 13 amino acids of PTH and PTH-related protein, which might lead to both peptides being recognized by an antibody raised to PTH 1-34, there is little C-terminal homology. Thus the antibody to PTH 39-84 used to coat the polystyrene bead would not be expected to cross-react with PTH-related protein.

Patients with the rare condition, familial benign hypercalcemia, had normal values for PTH, whereas conventional RIA methods have consistently failed to discriminate reliably between PTH concentrations in familial benign hypercalcemia and primary hyperparathyroidism (16). Of our 39 patients with renal failure, 37 had increased PTH in serum, suggesting that many continue to have secondary hyperparathyroidism despite the frequent use of measures to suppress this. However, the parallelism studies on serum from renal failure patients suggested that the PTH concentration measured in undiluted serum may underestimate the true concentration in these patients.

The half-time for disappearance of PTH after removal of a parathyroid adenoma was 3.3 min, which agrees well with a previous estimate of rate of disappearance of endogenous or exogenous PTH as determined by an RIA with NH2-terminal specificity (17). This suggests that PTH concentrations as measured by the two-site IRMA reflect closely the bioactive forms of PTH. After successful removal of the adenoma, PTH concentrations in peripheral blood declined to normal within 30 min. This confirms recent studies in which PTH was shown to decline to less than 40% of baseline values 15 min after successful parathyroidectomy (18). The availability of rapid, reliable assays with short incubation times (18) offers the opportunity for intra-operative monitoring of PTH at various sites in the neck to assist in predicting the success of surgery or in the localization of parathyroid tumors, particularly in patients who have already undergone unsuccessful neck surgery.

We conclude that the N-tact PTH IRMA offers reliable diagnostic accuracy in distinguishing PTH-dependent and -independent causes of hypercalcemia and appears to measure the bioactive form(s) of the hormone in primary hyperparathyroidism.

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