Rapid Equilibrium Radioimmunoassay for the Amino-Terminal Propeptide of Human Type III Procollagen

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This is an equilibrium-type radioimmunoassay for the amino-terminal propeptide of type III procollagen (PIIIINP), which overcomes the problem of nonparallelism between the standard and human serum samples encountered with earlier assays. Proper selection of antisera and reaction conditions diminishes interference from degradation products of the propeptide in serum. Because a rapid solid-phase-bound second-antibody step is included, the assay takes only 3 h. The intra-assay and the interassay CVs are both about 5%. In infants and children the concentration of PIIIINP in serum closely parallels the growth-velocity curve. For 88 presumably healthy adults, the PIIIINP concentration was 1.7–4.2 μg/L, about a third that measured with the previously available commercial assay. This is because of lack of inhibition by small Col 1 domain-related degradation products.

Additional Keyphrases: pediatric chemistry • fibrotic disorders • cancer • collagen • reference interval • age-related differences

Type III collagen is present in dense and loose connective tissues throughout the body. It is usually found with type I collagen, but during a fibroproliferative response, such as seen in wound healing or during development of a fibrotic disease, deposition of type III precedes that of type I (1).

By use of several different radioimmunoassays (2), it has been shown that the concentration of antigens related to the amino-terminal propeptide of type III procollagen (PIIIINP) increases in human serum in many pathological conditions where there is excessive deposition of collagen, such as liver fibrosis and cirrhosis or myelofibrosis, as well as during normal growth (3). Accurate assay of these antigens could have great value in follow-up of patients with fibrotic disorders, for whom currently the only means of detecting ongoing connective-tissue accumulation is histological evaluation of a biopsy. Such procedures cannot be repeated often, and they provide only a static view of the state of the connective tissue at the time of the biopsy.

The commonly available assay for the propeptide antigens in serum presents some practical problems (4). The assay recognizes the major antigenic determinant in the propeptide, located in the amino-terminal Col 1 domain (5), in at least three forms, of different sizes (5, 6). The smallest antigenic form is related to the monomeric Col 1 domain of the propeptide. These monomeric degradation products are present in greater concentrations than is the trimeric propeptide in normal serum, and they react with the propeptide antibodies with less affinity than does the complete propeptide. Thus the inhibition curves obtained with serum samples are less steep than that for the trimeric propeptide standard. Because this makes the assay nontitratable (i.e., the more the sample is diluted, the higher are the apparent concentrations obtained), a 50% intercept method, based on at least three different dilutions, has been recommended for evaluating the results for serum samples (7). To overcome this problem, modifications of the assay have been introduced to measure mainly the Col 1 domain-related degradation products (8, 9).

In the present study our strategy has been to minimize the interference from the low-Mr antigens in serum, by proper selection of the specific antisera and assay conditions. Here we describe a rapid assay for the PIIIINP. With this method, most serum samples give an inhibition curve that parallels that of the propeptide standard.

Materials and Methods

Materials

The tracer and the antiserum were diluted with phosphate buffer (0.1 mol/L, pH 7.2) containing 9 g of NaCl, 5 g of bovine serum albumin, and 0.5 g of NaN3 per liter. Human serum used for the preparation of standards was obtained from Laboratory Serum Inc., Davie, FL. The separation agent (second antibody) was a suspension of anti-rabbit antibody covalently bound to solid particles in 0.1 mol/L Tris HCl buffer, pH 7.4, containing 9 g of NaCl, 2 g of bovine serum albumin, 1 mL of Tween 20, and 1 g of NaN3 per liter. We obtained this suspension from Farmos Diagnostica, Turku, Finland.

Procedures

Purification of the propeptide and production of antiserum. We first purified PIIIINP from ascitic fluid from cancer patients as described previously (10). The isolated propeptide was finally subjected to reversed-phase HPLC (column: Vydac 201TP; mobile phase: 1 mL/L trifluoroacetic acid reagent containing 100 mL/L of 2-iso propanol per liter). The bound propeptide was eluted with a linear gradient of 2-iso propanol (from 100 to 700 mL/L in 0 to 45 min) in 1 mL/L aqueous trifluoroacetic acid solution, emerging as a sharp peak in the first half of the gradient. We verified the purity of the peptide by sodium dodecyl sulfate/polyacrylamide electrophoresis in 125 g/L acrylamide gels, obtaining a single band with a relative molecular mass of 42 000. The concentration of the final propeptide preparation was determined by quantitative analysis for amino acids after acid hydrolysis (10).

Polyclonal antibodies against the propeptide antigen were raised in New Zealand White rabbits by injecting purified propeptide, essentially as described previously (9, 10).

Iodination of the tracer and preparation of the standards. We labeled the purified antigen with 125I by the Chloramine-T method (2), separating the labeled antigen from free iodide by gel filtration on a 1.5 cm × 45 cm column of Sephadryl S-300 (Pharmacia, Uppsala, Sweden) equilibrated at room temperature in the eluent, which consisted of
phosphate buffer (0.1 mol/L, pH 7.2) containing 2 g of bovine serum albumin and 1 g of NaN₃ per liter. The calculated specific activity of the labeled antigen was about 50 Ci/g.

The standards were prepared by adding known amounts of the purified propeptide to human serum that had first been treated batchwise with DEAE-Sephacel (Pharmacia; 200 mL of pre-swollen gel per liter of serum) for 1 h at room temperature. That high-M₄ propeptide antigens had been removed was tested with the PIINP radioimmunoassay. After filtering the treated serum through 0.2-μm (pore-size) filters, we added NaN₃ (final concentration 1 g/L) as a preservative. The standards were stable for at least two months at 4 °C.

**Assay procedure.** Incubate 100- or 200-μL aliquots of standards or serum samples with 200 μL of the tracer solution (about 50000 counts/min) and 200 μL of diluted antiserum for 2 h at 37 °C. Then add 500 μL of the solid-phase second-antibody suspension to each tube and vortex mix. After 15 min at room temperature, separate the bound fraction by centrifugation (2000 × g, 15 min, 4 °C). Decant the supernate containing the unbound tracer, and count the radioactivity of the precipitate containing the bound tracer (we used a Multigamma counter, LKB-Wallac, Turku, Finland).

**Comparison method.** We also analyzed serum samples from infants and children with an "RIA-gnost Procollagen-III-Peptid" kit (Behringwerke AG, Marburg, F.R.G.) according to the instructions of the manufacturer (7).

**Gel filtration of serum.** To test the antigen-form specificity of the developed assay, we separated the serum antigens by gel filtration, using a 1.5 × 110 cm column of Sephacryl S-300 (Pharmacia) equilibrated in phosphate-buffered saline containing 0.4 mL of Tween 20 per liter (6). The column was standardized with the purified PIINP and with its Col 1 domain, the latter obtained after digestion with bacterial collagenase (10).

**Computer analysis of radioimmunoassay data.** The slopes of the inhibition curves obtained with the reference preparation of PIINP and with serum samples were calculated with an MS-DOS-based Kaypro PC20 microcomputer (functionally compatible with IBM PC), with use of a program written in BASIC (11, see 2). We used logit and log transformations to obtain a linear dose–response curve, followed by iterated weighted-least-squares regression analysis.

**Statistical methods.** We compared values calculated for healthy men and women by Student's t-test. The regression between the results obtained with our equilibrium assay and the RIA-gnost assay was analyzed by linear regression analysis, and the correlation between them by Spearman's rank correlation test.

**Control Subjects**

Reference values for healthy adults were obtained by analyzing serum samples from 88 healthy Finnish blood donors (44 men and 44 women; mean age 39 years, range 21-65 years).

Reference values for infants and children were obtained by analyzing serum samples from 271 healthy, non-hospitalized white children, 125 girls and 146 boys (ages one month to 18 years). The subjects, recruited in Southeast England from the local population, were tested for normal liver and kidney functions. Informed consent was obtained from each child and his or her parents before taking the blood sample. (This aspect of the study had the approval of the Ethical Committee of King’s College Hospital, London.) The samples were collected between 1985 and 1987 and stored at -20 °C until assayed. Most of the samples were previously assayed with the Behringwerke RIA-gnost kit and had therefore been frozen/thawed at least once (12, 13).

**Patients**

Sera from several patients whose PIINP concentrations were increased for various reasons were used for linearity tests and for gel-filtration experiments. Individual results are shown below for a patient with stage IV primary biliary cirrhosis (Figure 1B) and a patient with stage IV ovarian carcinoma (Figure 2).

**Results**

**Antigen specificity.** This PIINP assay, with the specific antisera we prepared, did not detect the small degradation products of the amino-terminal propeptide in human serum samples (Figure 1). Two peaks of antigenicity, which were

![Fig. 1. Gel filtration analysis of two serum samples on a column of Sephacryl S-300](image)

**Fig. 1.** Gel filtration analysis of two serum samples on a column of Sephacryl S-300. The fractions from 200-μL samples were measured with the PIINP equilibrium assay (6). The arrows indicate the elution positions of isolated human PIINP and its Col 1 domain (13). (—), total protein; A: a control serum (PIINP 3.2 μg/L); B: a sample from a patient with stage IV primary biliary cirrhosis (PIINP 16.5 μg/L). Peak 1, early-eluting reactive peak; peak 2, PIINP.

![Fig. 2. Assay curves for PIINP standard (---): slope 0.925, 50% intercept 6.3 μg/L; and serum sample from a patient with stage IV ovarian carcinoma (---): slope 0.932, 50% intercept 68.8 μg/L (PIINP 19.5 μg/L)](image)
eluted earlier than the Col I domain-related degradation products, were recognized (peaks 1 and 2 in Figure 1 corresponding to pools I and II in reference 6).

Parallelism of the standard curves. The radioactivity of the standards was expressed as percentage of the maximum binding (zero standard) vs the concentration on a semilogarithmic scale ( spline-function fitting), and the concentrations of the serum samples were read from the standard curve. The linearity of the standard curves was also evaluated with a logit-log transformation. Typically, a concentration of 6.3 μg/L gave 50% inhibition (Figure 2).

We serially diluted, with serum-based zero standard, those patients’ serum samples that contained increased concentrations of the propeptide. The inhibition curves obtained with pathological serum samples were similar to that of the standard (one example is given in Figure 2), demonstrating parallelism. In another experiment, we determined that the mean slope of five serum samples from children of different ages and thus with different concentrations of PIINP (slope -1.008 ± -0.069) did not differ from that of the standard (slope -1.018).

Precision. The intra- and interassay variations (n = 10) were tested with 200-μL serum samples containing three different concentrations of the PIINP antigen (Table 1). Both coefficients of variation (CVs) were near 5% at all the antigen concentrations tested. Also, with 100-μL sample volumes (PIINP 5.4 μg/L), which we used for analyzing samples from infants and children, intra- and interassay CVs of 4.0% and 4.9%, respectively, were obtained.

Sensitivity. The sensitivity of the PIINP assay, defined as the detectable mass equivalent to twice the standard deviation of the zero binding value, was 0.2 μg/L.

Analytical recovery. Known amounts of the purified propeptide were added to a human serum sample containing a normal propeptide concentration (3.4 μg/L). On assay, the mean recovery was 94.0% (SD 6.0%, n = 7) within the assay concentration range 6.0 to 44.1 μg/L. We also added two known concentrations of the purified PIINP to hemolyzed, lipemic, or icteric sera and assayed, finding no interference with the recovery of PIINP.

Reference values. Table 2 gives the reference values for serum from healthy adults, based on samples from the 88 blood donors. There was no statistically significant sex-related difference (men 3.04 ± 0.68 μg/L; women 2.88 ± 0.55 μg/L).

Mean PIINP concentrations for infants and children are given in Figure 3. The high values seen for infants decrease within the first two years. Between the age of two years and puberty the reference interval is 3.5–12.1 μg/L, with no sex-related difference. At puberty the PIINP concentration increases by 50% to 100%, and this occurs two to three years earlier in girls than in boys. After the pubertal growth period the PIINP concentration decreases to the value for adults.

The difference between serum and plasma samples was tested by measuring eight samples from the same healthy volunteers. The mean PIINP concentrations obtained were 2.64 (SD 0.88) μg/L for serum and 2.22 (SD 0.70) μg/L for plasma. The Pearson correlation coefficient was 0.985 (P <0.001).

Comparison with RIA-gnost assay. The concentrations obtained with the PIINP equilibrium assay were on the average 2.8-fold lower than those given by the RIA-gnost assay. However, the Spearman’s correlation coefficient between the assays was highly significant (0.747; P < 0.001; Figure 4).

Discussion

This is a rapid radioimmunoassay, based on equilibrium conditions, for the amino-terminal propeptide of type III procollagen. It offers several advantages over the previously available RIA-gnost assay (7,14). Our assay is based on the use of a highly purified human antigen, whereas the other assay depends on the immunological cross reaction between the human propeptide and the corresponding bovine protein. However, the problem of non-parallelism between the standard curves and the inhibition curves of the serum samples is not abolished solely by changing the antigen (9). This is only achieved by selection of the specific antiserum

**Table 1. Intra- and Interassay Variations of PIINP Assay**

<table>
<thead>
<tr>
<th>Mean PIINP, μg/L</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intra-assay</td>
<td></td>
</tr>
<tr>
<td>2.6</td>
<td>4.3</td>
</tr>
<tr>
<td>5.1</td>
<td>2.5</td>
</tr>
<tr>
<td>27.4</td>
<td>1.7</td>
</tr>
<tr>
<td>Interassay</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>4.0</td>
</tr>
<tr>
<td>4.7</td>
<td>3.2</td>
</tr>
<tr>
<td>26.8</td>
<td>5.3</td>
</tr>
<tr>
<td>n = 10 each</td>
<td></td>
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</tbody>
</table>

**Table 2. Reference Values for Healthy Finnish Blood Donors**

<table>
<thead>
<tr>
<th>Range of PIINP, μg/L</th>
<th>No. of persons</th>
<th>Frequency</th>
<th>Cumulative frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.50–1.99</td>
<td>3</td>
<td>0.03</td>
<td>0.03</td>
</tr>
<tr>
<td>2.00–2.49</td>
<td>20</td>
<td>0.23</td>
<td>0.26</td>
</tr>
<tr>
<td>2.50–2.99</td>
<td>24</td>
<td>0.27</td>
<td>0.53</td>
</tr>
<tr>
<td>3.00–3.49</td>
<td>22</td>
<td>0.25</td>
<td>0.78</td>
</tr>
<tr>
<td>3.50–3.99</td>
<td>16</td>
<td>0.18</td>
<td>0.97</td>
</tr>
<tr>
<td>4.00–4.50</td>
<td>3</td>
<td>0.03</td>
<td>1.00</td>
</tr>
</tbody>
</table>

44 women and 44 men, mean age 39 years, range 21–65 years.

![Fig. 3. PIINP concentrations in the serum of infants and children](image-url)
and by proper reaction conditions. Although the variation of the slopes seems to be greater with serum samples, they do not vary by more than 10% from the mean slope of the standard; thus this presents no practical problems.

An advantage for the routine handling of the assay in the clinical laboratory is the fact that the present assay can be performed relatively fast—in 3 to 4 h. The pipetting is especially fast, simpler, and more economical, because no serial dilution of the serum samples is necessary. Results of the present assay can thus be directly calculated with automatic gamma counters at the same time that the radioactivity is being counted. Because the range of the standard curve is often broader in the equilibrium assay than in the sequential (e.g., RIA-gnost) assay, fewer samples must be diluted and re-analyzed. To conserve sample, the analysis can be done with half of the ordinary volumes with no loss of precision. This is an advantage when serum from infants and children is being assayed; they often have high concentrations of PIINP, but the sample volume may be limited.

Because no serial dilution of the sample is necessary, inaccuracy from repeated pipetting is eliminated. Also, the small degradation products are no longer inhibitory, and this leads to the low variations observed with the present PIINP assay. The intra- and interassay CVs can be considered excellent relative to protein antigen radioimmunoassays in general.

The mean PIINP concentration in serum measured with the equilibrium assay is on the average 2.8-fold lower than that obtained with the RIA-gnost assay (14). This is obviously due to the fact that the small Col I domain-related degradation products of the propeptide (14) do not inhibit in the present equilibrium assay. With this new assay we could expect larger changes in the serum PIINP concentration in pathological states, because the concentration of the larger antigens usually increases preferentially. However, this is not necessarily always the case.

From the clinical point of view, the new assay allows more accurate follow-up of patients, because a change exceeding 10% (i.e., only twice the interassay variation) can be regarded as significant. In liver fibrosis without inflammation, such as is seen in methotrexate-induced liver damage in peptic patients (J Risteli, H Sögärd, A Oikarinen, L Risteli, J Karvonen, H Zachariae, ms. submitted for publication), changes in the PIINP concentration in serum are relatively small. Both in organ fibroses and in other situations where the assay has been used, its value is mainly associated with the changes observed with time. Recent new applications include response evaluation of treatment of bone metastases in patients with breast carcinoma (15). In ovarian carcinoma the PIINP seems to be an indicator of clinical behavior (16).

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The healthy infants and children tested were recruited through the Medway and Gillingham Branch of the Michael McGough Foundation against Liver Disease in Children. We are very grateful to all the children and their parents who participated in this study.

References