First direct assay for intact human proinsulin

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We describe a sensitive two-site sandwich enzyme-linked immunosorbent assay for the measurement of intact human proinsulin in 100 μL of serum or plasma. The assay is based on the use of two monoclonal antibodies specific for epitopes at the C-peptide/insulin A chain junction and at the insulin B chain/C-peptide junction, respectively. Cross-reactivities with insulin, C-peptide, and the four proinsulin conversion intermediates were negligible. The detection limit in buffer was 0.2 pmol/L (3 standard deviations from zero). The working range was 0.2–100 pmol/L. The mean intra- and interassay coefficients of variation were 2.4% and 8.9%, respectively. The mean recovery of added proinsulin was 103%. Dilution curves of 40 serum samples are parallel to the proinsulin calibration curve. Proinsulin concentrations in 20 fasting healthy subjects were all above the limit of detection: median (range), 2.7 pmol/L (1.1–6.9 pmol/L). Six fasting non-insulin-dependent diabetes mellitus and five insulinoma patients had proinsulin concentrations significantly higher than healthy subjects: median (range), 7.7 pmol/L (3.2–18 pmol/L) and 153 pmol/L (98–320 pmol/L), respectively.

Proinsulin (PI)5 conversion to insulin usually is almost completed before secretion, and proinsulin-like material thus represents only 10–20% of serum immunoreactive insulin in fasting healthy subjects (1,2). Intact human proinsulin (hPI) and des (31,32)-hPI, usually in almost equimolar amounts (3), are the major circulating forms of insulin precursors, probably because the PC2 type endopeptidase specific for the C-peptide/insulin A chain junction is present in the pancreatic beta cell in lower amounts than the PC1 type specific for the C-peptide/insulin B chain junction (4) and has a lower affinity for hPI than for des (31,32)-hPI (5).

Proinsulin-like material is increased in clinical conditions such as insulinoma (6–9), familial hyperproinsulinemia (10–13), and non-insulin-dependent diabetes mellitus (NIDDM) (3,9,14–17). This could be the consequence of a primary beta cell anomaly of PI processing and/or secretion (18,19), or it could be secondary, in NIDDM, to hyperglycemia and increased demand on the beta cells (20,21). Moreover, the liver uptake, biological activity, and plasma half-life of each precursor differ markedly from those of insulin (22–25). It is therefore of primary interest to distinguish these precursor molecules in patients at risk of impairment of glucose tolerance, and a serum hPI assay should fulfill four requirements: (a) specificity for intact hPI without interference of any conversion intermediates; (b) sensitivity sufficient to determine fasting serum hPI concentrations in nondiabetic controls; (c) ability to handle unprocessed serum samples; and (d) high assay capacity.

Thus far, these four requirements have not been reached in any one assay. The earlier methods for hPI determination, based on gel filtration (26) or using degrading enzymes (27), were laborious and lacked specificity. High performance liquid chromatography allows separation of the precursor peptides of insulin (28,29) but requires large volumes of serum, at the same time being time-consuming and of low capacity. Indirect RIAs (2,30) based on the separation of insulin or C-peptide before PI assay or direct RIAs (9,31,32) that use polyclonal antibodies to PI do not distinguish intact PI from its intermediate forms and often fail to detect fasting serum concentrations. Three ELISAs detected PI at concentrations ≤1.2 pmol/L, but included conversion intermediates (33–35). Three IRMAs (36–38), one immunoenzymometric assay (39), and one immunofluorometric assay (40) measured PI with good sensitivity and improved specificity but still cross-reacted with des (64,65)-PI and split (65–66)-PI.

In this study, we describe the first sensitive two-site sandwich ELISA specific for intact hPI alone. The assay is based on the use of two monoclonal antibodies (MoAbs)

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5 Nonstandard abbreviations: PI, proinsulin; hPI, human proinsulin; and NIDDM, non-insulin-dependent diabetes mellitus.

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specific for the prohormone C-peptide/A chain and C-peptide/B chain junctions, respectively. High sensitivity is achieved without the use of an amplification system. This assay can reproducibly handle several hundreds samples per run.

**Materials and Methods**

This study is in accordance with the Ethical Committee of the University of Liège.

**Peptides, Buffers, and Equipment**

*Peptides.* hPI, used for mice immunization and preparation of the calibration curve, PI conversion intermediates des (31,32)-PI, split (32–33)-PI, des (64,65)-PI, split (65–66)-PI, and C-peptide were from Eli Lilly Research Laboratories. Human insulin and the reference standard of hPI (lot no. Eno 3B4033) were from Novo Nordisk, the latter being standardized to Human Proinsulin First Reference Reagent 1986 (WHO 84/611), using the total hPI assay (35).

**Buffers and Reagents.** Buffers A, B, C, and D and the streptavidin-peroxidase conjugate were slightly modified from Kjems et al. (35). Briefly, buffer A, the coating buffer, was 0.1 mol/L NaHCO₃, pH 9.8. Solution B, the washing solution, was a solution of 1.5 mmol/L NaCl, 5 mL/L Tween 20, pH 7.0. Buffer C, for labeled antibody incubation and dilution of the streptavidin-peroxidase conjugate, was composed of 0.03 mol/L Na₂HPO₄, pH 7.0. Buffer D, for antigen incubation, was the same as Buffer C, but with 1.0 mol/L NaCl, 30 g/L human serum albumin, and 1 g/L bovine gamma globulin (Sigma). The enzyme substrate solution was TMB Microwell Peroxidase Substrate System (KPL).

*Equipment.* The microtest plates were immunoplates Maxisorp™ with Certificate (Nunc). Buffers and all reagents were prepared with ultrapure water produced from a Millipore MilliQ RG System (Millipore), because we observed a marked effect of water purity on the hPI ELISA in preliminary work. The washing equipment was a Well Wash 4 (Welltech Laboratories). The enzymatically formed color was read with a LP200 microplate reader (Vel) at 450 nm, corrected for absorbance at 620 nm.

**Antibodies**

The MoAbs S2 and S53 were developed by hybridoma technology (41). Briefly, hybridomas were produced from spleen lymphocytes of BALB/c mice immunized with hPI. The splenocytes were fused with SP2/O myeloma cells. The antibody-producing hybridomas were cloned by limiting dilutions. Both antibodies were produced in cell culture [culture medium: Hybridoma High Protein (Gibco), 5 × 10⁻³ mol/L 2-mercaptoethanol, 2 × 10⁻² mol/L glutamine, 100 000 units/L penicillin, 100 mg/L streptomycin, 10⁻⁴ mol/L hypoxanthine, and 1.6 × 10⁻³ mol/L thymidine], purified on Hi-Trap™ Protein G columns (Pharmacia) and stored at −20 °C in phosphate-buffered saline containing 0.01 mol/L phosphate and 0.145 mol/L NaCl, pH 7.3.

Antibodies characteristics are described in Deberg et al. (42). Briefly, MoAb S2 and S53 subclasses, determined by ELISA (Mouse-Hybridoma Subtyping kit, Boehringer Mannheim), were IgG1. MoAb affinity constants for hPI measured using the method of Scatchard (43) were 1.8 × 10¹⁰ and 1.5 × 10¹⁰ L/mol, respectively. S2 and S53 epitopes, defined by competition with insulin, C-peptide, and the two des-conversion intermediates, were the C-peptide/insulin A chain junction and the insulin B chain/C-peptide junction, respectively. MoAb S2 had the ability to bind to intact PI, des (31,32)-PI, and split (32–33)-PI but not to insulin, C-peptide, and the other des and split forms. MoAb S53 was able to bind to intact, des (64,65)-PI, and split (65–66)-PI but not to insulin, C-peptide, and the other des and split forms. Together, MoAb S2 and S53 had the ability to form a sandwich in ELISA specific for intact hPI.

**Biotinylation of Antibody**

The biotinylation of MoAb S53 was performed according to Berger et al. (44) with some modifications. Briefly, 100 µL of a 60-fold molar excess of biotinyl-e-aminocaproic acid-N-hydroxysuccinimiderester (bixin-N-XHS, Calbiochem) in dimethyl sulfoxide was added per mL of IgG-solution (1 g/L in phosphate-buffered saline, containing 0.01 mol/L phosphate, 0.145 mol/L NaCl, pH 7.3) under shaking and incubated overnight at 4 °C. After the incubation, 900 µL of imidazole buffer (0.5 mol/L imidazole, 0.15 mol/L NaCl, pH 7.3) was added to the mixture to bind the excess of bixin-N-XHS. Biotin-labeled S53 was stored in 500 mL/L glycerol at −20 °C.

**Serum Samples**

Samples of blood were collected from 20 overnight fasted healthy subjects (6 men, 14 women; age: mean, 31 years; range, 23–47 years; fasting plasma glucose: mean, 4.7 mmol/L; range, 3.9–6.1 mmol/L), from six overnight fasted patients with NIDDM (one man, five women; age: mean, 72 years; range, 67–77 years; body mass index: mean, 29.3 kg/m²; range: 23.3–36.5 kg/m²; fasting plasma glucose: mean, 7.2 mmol/L; range, 4.6–10.1 mmol/L) before and 180 min after a meal, and from five patients suffering from recurrent episodes of hypoglycemia and with surgically proven insulinomas. The NIDDM patients had not been treated with insulin but were on oral hypoglycemic treatment at the time of the study. The serum samples were centrifuged at 4 °C and 1800 g for 5 min and stored at −20 °C until assayed for hPI. Plasma samples gave similar results but were not used in this study.

Preliminary studies showed that lipids (Intralipid 20%, Pharmacia), hemoglobin, and bilirubin (concentrations up to 5 g/L, 200 µmol/L, and 500 µmol/L, respectively) did not interfere with PI measurement in serum.
ASSAY PROCEDURE
The hPI ELISA was performed as follows: immunoplates were coated for at least 3 days at 4°C with 125 μL per well of 2 mg/L S2 diluted in buffer A. The plates were washed four times with 350 μL per well of solution B. One hundred microliters of calibrators (in buffer D), reference standard (in buffer D), and samples were then pipetted in duplicate or triplicate (calibrators) into the wells. The plates were covered with tape and incubated at 4°C for 24 h. The washing procedure was repeated, and 100 μL of biotin-labeled S53 diluted to a concentration of 50 μg/L in buffer C was pipetted into each well. The tape-covered plates were incubated at 4°C for 4 h. The washing procedure was repeated; 100 μL of 1.25 × 10⁻⁵ g/L streptavidin-peroxidase conjugate (buffer C) was pipetted into each well, and the tape-covered plates were incubated at room temperature for 1 h in the dark. The washing step was repeated, and 100 μL of freshly prepared enzyme substrate was added into each well. The tape-covered plates were incubated at room temperature for 30 min in the dark, and the enzymatic reaction was stopped with 100 μL per well of 4 mol/L phosphoric acid. The color was read at 450 nm, corrected for absorbance at 620 nm. Reference standards and samples were read on the calibration curve.

STATISTICAL ANALYSIS
The nonparametric test of Wilcoxon was used to estimate differences between groups of patients.

ASSAY OPTIMIZATION
The signal and signal-to-noise ratio—defined as the signal measured at 1 and 10 pmol/L of PI divided by the signal at zero dose—were used for optimizing each of the following conditions.

Coating procedure. One, 2, 5, or 10 mg/L of monoclonal S2 were tested in the coating procedure, as described in Materials and Methods. Antigen and labeled antibody incubations were carried out at 4°C for 24 h and 4 h, respectively. An optimal signal-to-noise ratio was obtained at 2 mg/L (data not shown).

Labeled antibody concentration. S53 antibody concentration was optimized after each biotinylation. In our hands, optimal concentration varied between 25 and 50 μg/L.

Antigen and labeled antibody incubation. Incubation time (1, 4, 24, and 48 h) and temperature (4, 20, or 37°C) of the antigen and of the labeled antibody were systematically analyzed at a coating concentration of 2 mg/L S2 (data not shown).

(a) Antigen incubation. A 1-h incubation was not practical because of the time required to pipet all the samples into the plate. At 4°C and 20°C, a 48-h incubation gave no better signal than a shorter incubation. At 37°C, a 24-h and a 48-h incubation produced a lower signal than a 4-h incubation. Each of the other combinations of time and temperature produced good signal and signal-to-noise ratios for antigen in buffer D. However, hPI measurements in most serum samples incubated at 20°C or at 37°C were lower than those obtained at 4°C, suggesting degradation of hPI in these conditions.

An incubation temperature of 4°C and an incubation time of 4 h to 24 h gave optimal signal-to-noise ratios. The 24-h incubation was selected for practical reasons only.

(b) Labeled antibody incubation. Incubation time and temperature of biotin-labeled S53 were optimized under the best coating and antigen incubation conditions. A 1-h incubation never led to an optimal signal, regardless of temperature. The longer the incubation was, the higher the nonspecific binding was, regardless of temperature. A short incubation time of 4 h was thus selected. An incubation temperature of 4°C was chosen because higher temperatures seemed to lead to degradation of hPI in serum (see results of antigen incubation in this section).

In conclusion, combining the optimal conditions of the above tested assay conditions led to the following 3-step procedure: (a) coating antibody S2: 2 mg/L for 3 days at 4°C; (b) antigen (samples and calibrators): 4 to 24 h at 4°C; and (c) biotin-labeled antibody S53: 4 h at 4°C.

ASSAY CHARACTERISTICS
The calibration curve of the 3-step assay procedure is shown in Fig. 1.

Specificity. The cross-reactivities of the sandwich S2-S53 with insulin, C-peptide, des (31,32)-PI, and des (64,65)-PI
are shown in Fig. 1. Insulin and C-peptide did not interfere at concentrations <10 000 and 50 000 pmol/L respectively. Des (31,32)-PI, split (32–33)-PI, des (64,65)-PI, and split (65–66)-PI did not cross-react with the antibodies at concentrations <200, 5000, 200, and 1000 pmol/L, respectively (results of split molecules are not shown). Because the dilution curves of cross-reacting antigens were never parallel to the PI calibration curve, regardless of concentration, cross-reactivity could not be expressed in percentage of PI binding.

The cross-reactivities of des (31,32)-PI and des (64,65)-PI were also studied in the presence of PI. PI calibrators measured in the presence or absence of 100 pmol/L of des (31,32)-PI or des (64,65)-PI gave superimposable calibration curves.

Limit of detection/limit of quantitation. The detection limit of the assay in buffer was 0.2 pmol/L of PI, as assessed by the value corresponding to 3 SD above the mean of the zero response measured in five independent assays.

The working range of the assay was established by calculating the CV of each calibrator in five independent calibration curves. The CV obtained for each calibrator from 0.2 to 100 pmol/L was <10%.

Reproducibility. The mean intraassay CV, calculated from five replicate measurements on five plates, covering a concentration range 2.3–49 pmol/L of PI, was 2.4% (range, 1.6–3.2%; Table 1).

The mean interassay CV, determined from the mean of two replicate measurements in five independent assays over the concentration range 2.3–49 pmol/L of PI, was 8.9% (range, 4.9–13%; Table 1).

Recovery. The mean recovery of PI added to a human serum sample containing 2.7 pmol/L of PI was 103% (range, 83–124%) as determined at four different concentrations (5–49 pmol/L; Table 1).

Linearity. Human serum samples were serially diluted to ensure that their dilution curves were parallel to the calibration curve.

Forty samples over the concentration range 2.3–75 pmol/L were assayed undiluted and diluted 1:2 and 1:4 in buffer D (Table 2). Results obtained from samples diluted 1:2 and 1:4 were highly correlated with those of the undiluted samples (respectively, \( y = 0.94x - 0.35, r^2 = 0.99; y = 0.99x - 0.28, r^2 = 0.99 \)), and the CV calculated from the three dilutions averaged 7.9%. These data indicated parallelism between samples and calibrators as well as the absence of serum matrix effects.

**CLINICAL DATA**

The data presented correspond to small groups of subjects. They have essentially a value of example and will require validation by larger clinical studies.

Fig. 2 shows hPI measurements in sera from 20 fasting healthy subjects, 6 NIDDM patients before and 180 min after a meal test, and 5 patients with proven insulinoma. In fasting healthy subjects, the median PI concentration was 2.7 pmol/L (range, 1.1–6.9 pmol/L). The median PI concentration was significantly higher in sera from fasting patients with NIDDM (\( 2\alpha \leq 0.05 \)): 7.7 pmol/L (range, 3.2–18 pmol/L). One hundred and eighty minutes after a meal, the median PI concentration in these NIDDM subjects was 15.2 pmol/L (range, 9.5–23 pmol/L), significantly higher than in the fasting state (\( 2\alpha \leq 0.01 \)). In five patients with proven insulinoma, the median PI concentration was 153 pmol/L (range, 98–320 pmol/L). This value is 57- and 20-fold higher than those from fasting healthy subjects and NIDDM patients, respectively.

**Discussion**

To date, many attempts to measure intact and partially processed PI have been made. Nevertheless, most methods lacked specificity because they did not distinguish the individual proinsulin-like molecules in serum or plasma samples (33–35). However five assays, three IRMAs (36–38), one immunoenzymetric assay (39), and one immunofluorometric assay (40), showed narrower specificities, although they were still not selective for a single molecule. These assays detected intact PI and the two conversion intermediates: split (65–66)-PI and des (64,65)-

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**Table 1. Intra- and interassay CVs and recovery of the hPI ELISA assay.**

<table>
<thead>
<tr>
<th>Calibrator addition, pmol/L</th>
<th>Intraassay CV, %</th>
<th>Interassay CV, %</th>
<th>Recovery % (mean ± SD)</th>
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</thead>
<tbody>
<tr>
<td>+ 0.0</td>
<td>3.2</td>
<td>4.9</td>
<td>104 ± 6.5</td>
</tr>
<tr>
<td>+ 5.2</td>
<td>2.3</td>
<td>5.3</td>
<td>103 ± 14.2</td>
</tr>
<tr>
<td>+ 9.2</td>
<td>2.5</td>
<td>12.8</td>
<td>111 ± 16.3</td>
</tr>
<tr>
<td>+ 18.2</td>
<td>1.6</td>
<td>13.0</td>
<td>96 ± 5.6</td>
</tr>
<tr>
<td>Average</td>
<td>2.4</td>
<td>8.7</td>
<td>103</td>
</tr>
</tbody>
</table>

*Added to human serum containing 2.7 pmol/L PI.

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**Table 2. Linearity of PI from 40 serially diluted human serum samples.**

<table>
<thead>
<tr>
<th>PI concentration range, pmol/L</th>
<th>Sample dilution</th>
<th>Mean</th>
<th>Minimum</th>
<th>Expected PI concentration, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total range: 2.3–75</td>
<td>1:2</td>
<td>107</td>
<td>79</td>
<td>124</td>
</tr>
<tr>
<td>(n = 40)</td>
<td>1:4</td>
<td>106</td>
<td>70</td>
<td>139</td>
</tr>
<tr>
<td>Low range: 2.3–5.0</td>
<td>1:2</td>
<td>98</td>
<td>79</td>
<td>115</td>
</tr>
<tr>
<td>(n = 12)</td>
<td>1:4</td>
<td>92</td>
<td>70</td>
<td>122</td>
</tr>
<tr>
<td>High range: &gt;5.0–75</td>
<td>1:2</td>
<td>111</td>
<td>87</td>
<td>124</td>
</tr>
<tr>
<td>(n = 28)</td>
<td>1:4</td>
<td>113</td>
<td>87</td>
<td>139</td>
</tr>
</tbody>
</table>

*Undiluted sample = 100.0%.

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**Table 3. Linearity of PI from 40 serially diluted human serum samples.**

<table>
<thead>
<tr>
<th>PI concentration range, pmol/L</th>
<th>Sample dilution</th>
<th>Mean</th>
<th>Minimum</th>
<th>Expected PI concentration, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total range: 2.3–75</td>
<td>1:2</td>
<td>107</td>
<td>79</td>
<td>124</td>
</tr>
<tr>
<td>(n = 40)</td>
<td>1:4</td>
<td>106</td>
<td>70</td>
<td>139</td>
</tr>
<tr>
<td>Low range: 2.3–5.0</td>
<td>1:2</td>
<td>98</td>
<td>79</td>
<td>115</td>
</tr>
<tr>
<td>(n = 12)</td>
<td>1:4</td>
<td>92</td>
<td>70</td>
<td>122</td>
</tr>
<tr>
<td>High range: &gt;5.0–75</td>
<td>1:2</td>
<td>111</td>
<td>87</td>
<td>124</td>
</tr>
<tr>
<td>(n = 28)</td>
<td>1:4</td>
<td>113</td>
<td>87</td>
<td>139</td>
</tr>
</tbody>
</table>

*Undiluted sample = 100.0%.
PI. This degree of specificity was found acceptable because these two intermediate forms are usually the least abundant within the PI family in the circulation (37). Nevertheless, in some patients, these two conversion intermediates represent up to 30% of the proinsulin-like immunoreactivity (3). The method we describe in this study seems to be the first direct assay specific for intact hPI. Interferences in this assay by C-peptide, insulin, and the four PI conversion intermediates, even in supraphysiological concentrations, are negligible. The present assay detects intact PI concentrations of 0.2 pmol/L in serum or plasma samples. Because such a concentration is well below average basal concentration in healthy subjects, this method provides an accurate mean of assessing basal PI concentrations. This ELISA is easy to perform. Its demand for sample volume is low: 200 μL of sample for duplicate analysis. It has a practical broad range and a high capacity for routine applications as well as for large scale studies.

The current method was further characterized by analyzing sera from three groups of subjects. The results of intact PI obtained in fasting healthy subjects (median, 2.7 pmol/L; range, 1.1–6.9 pmol/L) were lower than those reported for total PI [6.7 ± 1.7 pmol/L (9), 4.7 ± 2.9 pmol/L (34), and 4.0 pmol/L (range, 2.1–12.6 pmol/L) (35)] or for intact plus des (31,32)-PI and split (32–33)-PI [5.2 ± 2.4 pmol/L (8) and 3.6 ± 0.1 pmol/L (32)] but were close to those reported for intact plus des (64,65)-PI and split (65–66)-PI: 2.7 ± 1.5 pmol/L (8); 3.4 pmol/L (range, 1.0–9.1 pmol/L) (38); and 2.1 pmol/L (range, 1.1–3.8 pmol/L) (45). This is in agreement with previous reports (8,37) that demonstrated that proinsulin-like immunoreactivity was heterogeneous and consisted mainly of intact and des (31,32)-PI. The PI concentration was significantly increased in fasting NIDDM patients, compared with fasting healthy patients. These results correlated well with previous studies (3,9,35). In agreement with the findings of Rainbow et al. (7), Cohen et al. (8), and Hampton et al. (9), fasting serum PI concentrations in the insulinoma patients were very high and varied widely. The lowest value observed in the present study was 36-fold higher than the PI concentration in healthy fasting subjects and ~13-fold higher than the concentration found in fasting NIDDM subjects.

In conclusion, this new method enables the direct specific measurement of intact circulating PI in fasting healthy humans as well as in various pathological conditions. It will hopefully be useful to elucidate PI secretion and processing in physiological conditions as well as pathological conditions such as beta-cell dysfunction, islet cell tumors, and grafted patients.

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