A highly sensitive two-site sandwich ELISA measuring total proinsulin immunoactive material in serum or plasma was developed. The assay was based on two monoclonal antibodies, an anti-C-peptide antibody bound to a microtest plate and a biotin-labeled anti-insulin antibody. The detection limit (3 SD above zero value) in buffer was 0.05 pmol/L, corresponding to 0.25 pmol/L in human serum (diluted 1:5). The linear calibrator range was 0.05–20 pmol/L. Interassay CVs were 4.7% at a median (range) of 2.3 pmol/L (1.4–2.8 pmol/L), n = 8), 6.7% at 5.1 pmol/L (3.3–8.0 pmol/L, n = 8), and 8.7% at 10.0 pmol/L (8–12 pmol/L, n = 10). Mean analytical recovery of added human proinsulin (hPl) (2, 5, and 10 pmol/L) to serum was 94% (range 86–125%, n = 9). Human insulin and human C-peptide did not cross-react at 5000 and 10,000 pmol/L, respectively. The four major proinsulin conversion intermediates reacted 65–99%: split(32–33)hPl 74%, des(31,32)hPl 65%, split(65–66)hPl 78%, and des(64,65)hPl 99%. All serum values from 38 fasting healthy subjects were above the detection limit: median (range) 4.0 (2.1–12.6) pmol/L.

Indexing Terms: biotin–streptavidin interaction • C-peptide • insulin • enzyme-linked immunoasorbent assay

Interest in proinsulin and its possible physiological importance has been increasing ([1, 2]). An altered secretion of this beta-cell peptide might have pathophysiological significance (3, 4) and may be a prognostic marker in the study of treatment of diabetes mellitus (5, 6).

The detection limit of most proinsulin immunoassays is >1 pmol/L ([7–10]). In two recently described assays ([11, 12]), the detection limits were 0.1 and 0.8 pmol/L, respectively. However, enzyme amplification is needed in both assays. For analysis in patients with limited residual beta-cell function, as in insulin-dependent diabetes mellitus (IDDM), highly sensitive and easily performed assays are needed.

We describe such an assay, based on the ELISA technique for proinsulin immunoactive material (PIM). Its

1 Steno Diabetes Center, Niels Steensens Vej 2, DK-2820 Gentofte, Denmark.
2 Novo Nordisk A/S DK-2880 Bagsvaerd, Denmark.
3 Author for correspondence. Present address: Steno Diabetes Center, Fax Int +45-31-88-23-22.
4 Present address: Veterans Affairs Medical Center, Research and Development (181L), 1680 South Columbian Way, Seattle, WA 98108.
5 Received January 5, 1993; accepted May 12, 1993.
Materials and Methods

Antibodies. Available monoclonal antibodies at Novo Nordisk (Bagsvaerd, Denmark) raised against human proinsulin, insulin, or C-peptide were screened for specificity to proinsulin and proinsulin conversion intermediates. PEP-001 and HUI-001 were chosen for this assay because of their ability to cooperate in a sandwich assay, their almost equal reactivity with proinsulin and its four conversion intermediates, and the resulting limit of detection for proinsulin. PEP-001 has the affinity $10^{10}$ L/mol to human C-peptide, and its target epitope is within the C-terminal half of this molecule. HUI-001 has the affinity $10^{9}$ L/mol to human insulin, and its target epitope is within the C-terminal third of the insulin B-chain. The monoclonal antibodies were from BALB/c mice immunized with human C-peptide (PEP-001) or human insulin (HUI-001) and developed with the hybridoma technique (13). The antibodies were produced in cell culture in serum-free medium and purified by affinity chromatography on Protein A-Sepharose columns (Pharmacia, Bromma, Sweden).

Biotinylation of HUI-001 was performed essentially as described by Berger et al. (14), modified to allow use of PD-10 columns (Pharmacia) rather than dialysis for buffer exchange. A 100-fold molar excess of biotinyl-$\epsilon$-aminocaproic acid $N$-hydroxysuccinimide ester (Calbiochem, La Jolla, CA) relative to antibody molecules was used.

Reagents, buffers, and equipment. Buffer A (coating buffer) was sodium carbonate buffer, 0.1 mol/L, pH 9.8. Buffer B (washing solution) was 0.15 mol/L sodium chloride solution containing 1 mL of Tween 20 (Merck, Darmstadt, Germany) per liter. Buffer C (for labeled antibody incubation and dilution of the streptavidin–peroxidase conjugate) was 0.04 mol/L sodium phosphate buffer supplemented with 0.1 mol/L sodium chloride, 5 g/L human serum albumin (HSA; Hoechst-Behringwerke, Marburg, Germany), and 1 mL/L Tween 20, pH 7.4. Buffer D (antigen incubation buffer) was the same as buffer C, but with 1.0 mol/L sodium chloride, 30 g/L HSA, and 1 g/L bovine gammaglobulin (Sigma, St. Louis, MO).

The streptavidin–peroxidase conjugate (Kirkegaard and Perry Labs., Gaithersburg, MD), was diluted in 500 mL/L glycerol, 0.5 mg in 1 mL, and further diluted to 25 \mu L/L in buffer C before use. The enzyme substrate solution was 3, 3',5,5'-tetramethylbenzidine/H$_2$O$_2$ solution (Kirkegaard and Perry Labs.).

The washing equipment was a Well Wash 4 (Denley Instruments, Billingshurst, UK); the microtest plates (Immunoplate, Maxisorp with certificate) came from Nunc (Roskilde, Denmark). The enzymatically formed color was read with an Immunoreader NJ-2000 (Nippon Intermed, Tokyo, Japan) at 450 nm, corrected for absorbance at 620 nm.

Standards and controls. Human proinsulin (hPI) used to prepare calibrators, along with semisynthetic human insulin, human C-peptide, bovine- and porcine proinsulin, was obtained from Novo Nordisk. The proinsulin conversion intermediates split(32-33)-, des(31,32)-, split(65-66)-, and des(64,65) hPI were kind gifts from Bruce Frank (Eli Lilly Research Labs., Indianapolis, IN). Insulin-like growth factor I (IGF-I) was from Calbiochem, and insulin-like growth factor II (IGF-II) was kindly given by Peter Zenobi, University Hospital, Zurich, Switzerland. Control materials, selected human sera, were stored at $-20^\circ$ C.

Serum Samples

All serum samples were routinely analyzed after dilution 1:5. On each assay plate we included two control materials in at least two dilutions. Both serum and lithium heparinate plasma from 38 fasting healthy subjects (12 of them men) were analyzed to establish a normal range. Median (range) age was 35.5 (19-60) years, body mass index (BMI) 21.8 (17.9-27.7) kg/m$^2$. Serum and plasma from 23 fasting patients (14 men) with non-insulin-dependent diabetes mellitus (NIDDM) were analyzed; the median (range) age of these patients was 56 (33-70) years, and their BMI was 27.8 (18.7-43.8) kg/m$^2$. All samples were collected from fasting subjects. Serum from one patient with total pancreatectomy was analyzed to give information about a possible nonspecific serum interference in the assay.

Assay Procedure

Coating antibody (PEP-001) was diluted to 5 mg/L in buffer A and 125 \mu L was pipetted into each well on the microtest plate. The plate was covered with tape and incubated at 4 $^\circ$C for at least 3 days before use (stability, 2 months). Before antigen incubation, the plate was washed four times with 350 \mu L of washing solution for not $<$65 s each time. We then pipetted 100 \mu L of calibrators, controls, or appropriately diluted samples (in buffer D) in duplicate or triplicate (calibrators) into the wells of the plate. Considering dilution, this corresponds to a required volume of 40 \mu L of serum per assay. The tape-covered plate was incubated at 4 $^\circ$C for 16-24 h, after which we repeated the washing procedure and pipetted into each well 100 \mu L of biotin-labeled HUI-001 (10 \mu g/L) diluted in buffer C. The plate was incubated at 4 $^\circ$C for 24 h and the washing procedure was repeated. Freshly prepared streptavidin–peroxidase conjugate (100 \mu L) was then pipetted into each well and the plate was incubated in the dark at room temperature for 1 h. The washing procedure was repeated and 125 \mu L of freshly prepared enzyme substrate was pipetted into each well. The plate was incubated in the dark at room temperature for 30 min, and then the enzymatic reaction was stopped with 125 \mu L of 2.0 mol/L phosphoric acid per well. The color was read at 450 nm, corrected
for absorbance at 620 nm. Controls and unknown samples were read on the calibrator curve.

For method comparison, we analyzed 49 consecutive sera with the present ELISA and compared the results with those obtained with our formerly described ELISA of hPI (7).

Results and Discussion
Assay Optimization

Signal-to-noise ratios, defined as the signal obtained at 0.1 pmol/L hPI divided by the signal at zero dose, was used in optimization experiments. Ratios <2 were considered insufficient.

Coating procedure. Using 5 mg/L PEP-001 yielded the optimal number of functional binding sites. Ordinarily, 2–10 mg/L coating concentration has been used (11, 15).

Antigen incubation. The influence of incubation time (1–24 h) and incubation temperature (4, 22, and 37 °C) was systematically examined. An equilibrium was reached after 16 h at 4 °C (Figure 1). Higher incubation temperatures resulted in increased background noise. We examined whether the two immunochemical reactions could be performed simultaneously as in our recently described ELISA of insulin (16), but in the present assay we found it impossible to avoid nonspecific serum effects. To eliminate the nonspecific serum effect, we had to supplement the antigen incubation buffer with sodium chloride to 60 g/L, bovine gammaglobulin to 1 g/L, and HSA to 30 g/L. The use of high salt and (or) protein concentration to reduce serum effects has been shown previously (7, 12, 16).

Labeled antibody incubation. The efficiency of the assay with different lengths of incubation with the detection antibody (biotin-labeled HUI-001) is shown in Figure 2. The incubation time and temperature are both critical. As Figure 2 shows, this assay could not be optimized with incubation at 37 °C in a 1-day procedure. At 4 °C equilibrium was achieved in <20 h. Incubation for 1–5 h at 37 °C gave signal-to-noise ratios <2 for hPI at 0.1 pmol/L. A concentration of 10 μg/L HUI-001 is optimal. Both higher (20 μg/L) and lower concentrations (5 μg/L) resulted in relatively higher zero values and therefore lower signal-to-noise ratios.

On the basis of these optimization experiments, we chose the following routine steps: antigen incubation (100 μL) for 16–24 h at 4 °C and detection antibody incubation (100 μL) for 20–24 h at 4 °C.

Assay Characteristics

We studied the binding capacity of the coating antibody in the presence of increasing C-peptide concentration. As shown in Figure 3, C-peptide did not interfere in concentrations <1000 pmol/L, corresponding to a serum concentration of 5000 pmol/L (diluted 1:5); at 2000 pmol/L (serum concentration 10 000 pmol/L), the absorbance at 10 pmol/L hPI was slightly influenced. These results indicate a very high binding capacity of the antibody-coated solid phase.
There was no cross-reactivity of human insulin at 5000 pmol/L, human C-peptide at 10 000 pmol/L, or the structurally related bovine and porcine proinsulin (1000 pmol/L). Neither IGF-I nor IGF-II reacted at 10^6 pmol/L. The four proinsulin conversion intermediates cross-reacted 65–99% relative to intact proinsulin on a molar basis; split(32–33)hPI cross-reacted 74%, des(31,32)hPI 65%, split(65–66)hPI 78%, and des(64,65)hPI 99%. Calibration curves based on hPI or the four conversion intermediates were parallel throughout the measuring range (data not shown).

The detection limit in buffer—3SD above the zero value, highest value (not mean) of 19 experiments—was 0.05 pmol/L, corresponding to 0.25 pmol/L in undiluted serum. The unlikelihood of a nonspecific serum effect was supported by the facts that serum from one totally pancreatectomized patient showed absorbance values indistinguishable from background absorbance and that serum dilution curves from three healthy subjects were linear in all cases from 1:5 to 1:30.

The intraplate coefficient of variation (CV) was 6.2% at 2.8 pmol/L (n = 20), 2.8% at 7.8 pmol/L (n = 20), and 3.1% at 18 pmol/L (n = 38). Interassay CV was 4.7% at a median (range) of 2.3 pmol/L (1.4–2.8 pmol/L, n = 8), 6.7% at 5.1 pmol/L (3.3–8.0 pmol/L, n = 8), and 8.7% at 10 pmol/L (8.0–12.0 pmol/L, n = 10).

Analytical recovery of added hPI (2, 5, and 10 pmol/L) to serum was 84% (range 65–128%, n = 9) at basal serum concentrations of 0–4.6 pmol/L.

Serum and plasma from 38 fasting normal individuals were analyzed; values for both sample types were comparable. The median PIM concentration was 4.0 pmol/L in serum and 3.8 pmol/L in plasma, with respective ranges of 2.1–12.6 and 1.5–16.1 pmol/L. This is in agreement with previous studies (7–12). All healthy fasting subjects had PIM concentrations ≥1.5 pmol/L, which we conclude is a minimal limit for fasting PIM.

Further, we analyzed sera and plasma from 23 fasting patients with NIDDM. The median (range) for these patients was 30 (5.5–140) pmol/L in serum and 31 (5.2–150) pmol/L in plasma, respectively, significantly higher than the normal population (P <0.001, Mann–Whitney rank sum test), as was also found in previous studies (2).

The assay based on monoclonal antibodies (y) correlated with our previous proinsulin ELISA based on polyclonal antibodies (x) (7) in the clinically most relevant range (0–25 pmol/L): r = 0.87, P <0.001, y = 0.84 x + 1.25 pmol/L (n = 45) (Figure 4). For all 49 consecutive patients’ samples, the correlation between the previous and the new assay was r = 0.98, P <0.001, y = 0.99 x + 0.11 pmol/L (Figure 4). One further sample showed discrepant results by the two proinsulin ELISAs: Basal results with the old and the new assay, respectively, were 124 and 9.5 pmol/L. However, we excluded this sample from the correlation analysis because the fasting value for this subject was >6 SD above the upper limit for normal in the old assay. The subject presenting this sample had undergone a euglycemic hyperinsulinemic clamp (blood glucose = 5.5 mmol/L) and an oral glucose tolerance test (with 75 g of glucose). The suppression of the beta cells in the clamp study was not apparent in the C-peptide data or in the hPI data obtained with the new and old assays. During the oral glucose tolerance test, C-peptide and hPI (by new and old assays) increased as expected. To look for nonspecific effects, we analyzed the serum sample in four dilutions (1:5 to 1:30) with both proinsulin ELISAs. However, we found a linear dilution in both assays. Currently, therefore, we have no explanation to offer regarding this single discrepancy between the two methods.

In conclusion, by combining two high-affinity monoclonal antibodies against human C-peptide and human insulin in a simple sandwich ELISA, we obtained a sufficiently low detection limit for measurement of serum PIM. Furthermore, the working range, 0.25–100 pmol/L in serum or plasma, is large enough to monitor PIM concentrations in sera from all fasting normal subjects as well as estimate pathologically increased concentrations. A minimum PIM concentration in serum from fasting normals is demonstrated. The assay seems therefore useful for studying circulating PIM concentrations in diabetic patients who have remaining beta-cell capacity. Compared with our previously described assay...
(7), the major advances are the use of monoclonal antibodies and a fivefold increase in sensitivity. Recently described sensitive assays (11, 12) are based on the alkaline phosphatase amplification system (17); compared with these assays, the present assay is very simple to perform on a large scale. The method described by Alpha et al. (12) also depends on monoclonal antibodies. The use of monoclonal antibodies makes these assays widely applicable because of the availability of the antibodies on a large scale.

We thank Jane Falk Brønnum and Susanne Kjellberg Ibsen for their technical assistance. Helpful discussion and suggestions from René Djurup are gratefully acknowledged.

References

CLIN. CHEM. 39/10, 2150–2154 (1993)

Modified Assay of Prostate-Specific Antigen with a Detection Limit <0.01 μg/L
Raymond J. Liedtke,1 Gery Kroon, and John D. Batjer

We modified the Hybritech Tandem®-E prostate-specific antigen (PSA) assay by increasing the sample volume, increasing enzyme–substrate incubation time, and using diethanolamine buffer. Our modified method has a detection limit of 0.009 μg/L (P < 0.01). The assay curve is linear from 0.01 to 1.0 μg/L and has an overall assay time of about 4 h. Linear plots are obtained when the 1.0 μg/L standard is diluted with either matrix buffer or serum from men containing PSA <0.01 μg/L. Recovery of PSA (0.10 μg/L) added to serum from men averaged 94%. Interassay CVs were 13%, 7%, and 4% at PSA concentrations of 0.04, 0.07, and 0.30 μg/L, respectively (n = 33). This assay should be useful in the detection of early recurrence of prostate cancer after radical prostatectomy.

Indexing terms: immunoassay · detection limits · prostatectomy

Although the clinical significance of ultrasensitive assays for prostate-specific antigen (PSA) is unclear, recent reports (1–3) have discussed the need for reliable assays of PSA at concentrations <0.1 μg/L for earlier detection of disease recurrence after radical prostatectomy. To achieve this goal, Graves et al. (1, 3) converted the Yang Prog-Check® RIA into a more sensitive 4-day procedure with a working range of 0.06–0.88 μg/L and a lower limit of detection (LLD) equal to 0.06 or 0.12 μg/L, depending on sample matrix.2 Vessella et al. (2) evaluated the performance of the Abbott IMx assay at similarly low PSA concentrations and reported an analytical LLD of 0.03 μg/L, a "biological detection limit" of 0.06 μg/L, and interassay CVs of 25% at PSA concentrations ~0.05 μg/L. The LLD of the IMx PSA assay was realistically estimated as 0.08 μg/L, whereas the LLD for the

2 All PSA concentrations in this report were referenced to the Hybritech Calibrator. A concentration of 1.0 μg/L in the Prog-Check® assay is equivalent to ~0.6 μg/L by the Hybritech Tandem®-E assay.