# Measurement of Proinsulin and Intermediates

## Validation of Immunoassay Methods by High-Performance Liquid Chromatography

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**Human proinsulin and 32–33 split proinsulin have been measured in the peripheral circulation by immunoradiometric assays (IRMAs) and have been shown to be elevated in impaired glucose tolerance and non-insulin-dependent diabetes mellitus (NIDDM). The IRMA for 32–33 split proinsulin did not discriminate between this molecule and des-32 or des-31,32 split proinsulin. We describe the comparison of IRMA for human plasma proinsulin and 32–33 split proinsulins with assays combined with high-performance liquid chromatography (HPLC), which can discriminate between 32–33 split, des-32 split, and des-31,32 split proinsulin. Subjects were those with normal glucose tolerance ($n$ = 8) and those with NIDDM ($n$ = 17), who were studied while fasting and 30 min after a glucose load. After collection, blood was centrifuged promptly, and the serum/plasma was stored frozen until assay. Both IRMA and HPLC methods were calibrated against synthetic peptides. Interassay coefficients of variation for the IRMA for proinsulin and 32–33 split proinsulin were <13% over the ranges 3.8–65 pmol/l and 6.4–65 pmol/l, respectively. The following regression lines were obtained: proinsulin IRMA = −0.143 + 1.066 HPLC, $r$ = 0.860; 32–33 split proinsulin IRMA = 0.048 + 1.051 HPLC; and des-31,32 split proinsulin, $r$ = 0.814. For both analytes, there was no significant difference in the relationship of IRMA to HPLC results between the various subject groups and various time points. Thus, the IRMA for proinsulin has been validated by an independent method. The 32–33 split proinsulin IRMA predominately measures des-31,32 split proinsulin, which is the major partially processed proinsulin present in human plasma of both normal and NIDDM subjects. *Diabetes* 44:437–440, 1995**

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BSA, bovine serum albumin; df, degrees of freedom; ELISA, enzyme-linked immunosorbent assay; HPLC, high-performance liquid chromatography; IRMA, immunoradiometric assay; NIDDM, non-insulin-dependent diabetes mellitus; TEAP, triethylammonium phosphate.

Since the biosynthesis of insulin from proinsulin was demonstrated in 1967 (1), a number of studies have shown that abnormalities in the circulating concentrations of these precursors may occur in pathological conditions. Elevations of plasma proinsulin have been described in patients with insulinoma (2) during dexamethasone-induced insulin resistance (3), and elevations of plasma proinsulin and 32–33 split proinsulin have been described in patients with non-insulin-dependent diabetes mellitus (NIDDM) and impaired glucose tolerance (4,5). Knowledge of proinsulin processing in these conditions may lead to an understanding of the pathogenic mechanisms involved.

The majority of the above studies used immunoassays for the measurement of plasma proinsulin and intermediates. These immunological techniques have the advantages of sensitivity and high sample throughput compared with other techniques, such as chromatography. The specificity of the various immunoassays will depend on the antibodies used (6). High-performance liquid chromatography (HPLC) can be used for separating proinsulin, proinsulin intermediates, and insulin from each other. Analysis of the separated components by immunoassay allows quantitation of these components (7). Because the separated components are immunologically similar, a nonspecific immunoassay can be used. However, this technique uses a large volume of sample and is technically demanding. Although biosynthetic preparations of human proinsulin and 32–33 split proinsulin have been produced (8), there are, as yet, no international standards (human proinsulin is available as an International Reference Reagent) nor any suitable quality control materials or external quality assessment schemes. Validation of the immunoassays has relied on the usual experiments demonstrating adequate recovery of added analyte, linearity of signal on dilution of sample, and determination of cross-reactivity. We describe the validation of immunoradiometric assay (IRMA) for plasma intact proinsulin and 32–33 split proinsulin with HPLC followed by immunoassay of separated insulin and proinsulin-like molecules.

### RESEARCH DESIGN AND METHODS

Three groups of subjects were studied: a group of South Asian and Caucasian subjects with normal glucose tolerance according to World Health Organization criteria ($n$ = 8); a group of Caucasian subjects with

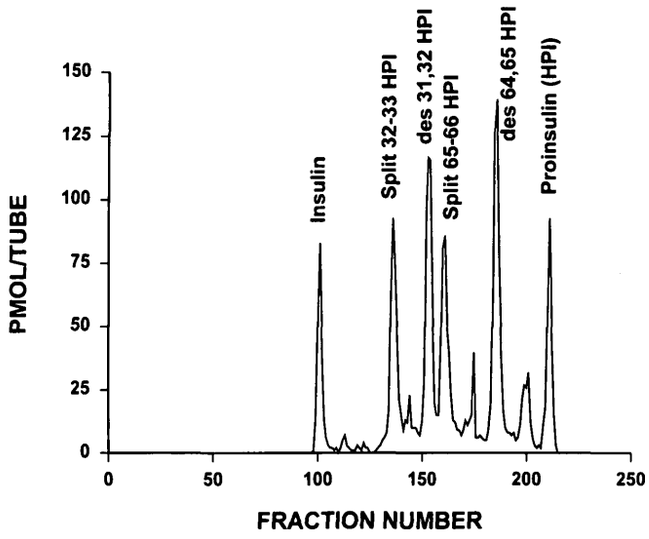


**FIG. 1. HPLC profile of purified insulin, proinsulin, and the proinsulin conversion intermediates.**

NIDDM ($n$ = 8); and a group of South Asian subjects with NIDDM ($n$ = 9). NIDDM subjects were treated by diet alone (Table 1).

Blood samples were collected after a 12-h overnight fast and 30 min after an oral glucose load (75 g). Plasma (lithium heparin) was separated by immediate centrifugation (1,800 *g* for 10 min at 4°C) of whole blood, and serum was similarly isolated after allowing whole blood to clot for ~1 h.

Plasma (10-ml aliquots) and serum (2- to 3-ml aliquots) were stored and transported to Chicago frozen on dry ice and again stored frozen. Plasma samples (2-ml aliquots) were transported to Cambridge frozen on dry ice and stored at −70°C until analyzed.

Approval for this study was obtained from the Ethical Committee of Islington Health Authority.

Plasma glucose was measured by a glucose oxidase method (Beckman, Berkeley, CA) and $HbA_1$ by electroendosmosis (Corning Medical, Halstead, U.K.).

Insulin, proinsulin, and 32–33 split proinsulin were measured by two-site IRMAs (9). The assay for 32–33 split proinsulin does not discriminate between this intermediate and the des-32 split proinsulin or the des-31,32 split proinsulin. The insulin IRMA was calibrated with the first International Reference Preparation 66/304 (National Institute of Biological Standards and Controls, Potters Bar, U.K.) and had an interassay coefficient of variation of <12% over the concentration range 27–341 pmol/l. The intact proinsulin and 32–33 split proinsulin assays were calibrated against synthetic peptides (Lilly, Indianapolis, IN) and had interassay coefficients of variation of <13% over the concentration ranges 3.8–65 and 6.4–65 pmol/l, respectively. The 32–33 split proinsulin results for each patient sample are corrected for the cross-reactivity of intact proinsulin in the assay. The cross-reactivity of proinsulin in the

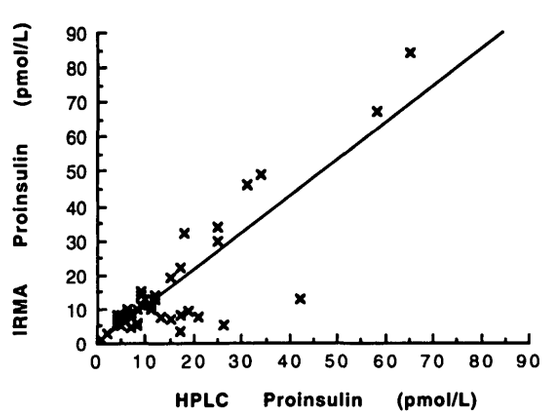


**FIG. 2. Comparison of results for proinsulin as estimated by IRMA and HPLC in all subjects during fasting and postglucose load.**

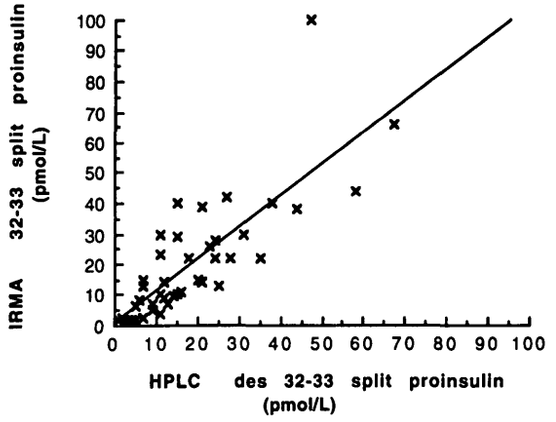


**FIG. 3. Comparison of results for 32–33 split proinsulin (IRMA) and des-31,32 split proinsulin (HPLC) in all subjects during fasting and postglucose load.**

assay is determined by direct analysis for each batch of reagents. The corrected 32–33 split proinsulin is then equivalent to the measured 32–33 split proinsulin minus 84% of the measured proinsulin.

The HPLC separation of circulating insulin, proinsulin, and proinsulin conversion products was conducted as follows (10). To measure the concentration of the individual proinsulin conversion products separate from insulin, serum insulin immunoreactivity was extracted by immunoaffinity chromatography with a nonspecific guinea pig anti-insulin Ig fraction, coupled to Bio-Rad Affi-Gel 10 agarose beads (Bio-Rad, Richmond, CA). Proinsulin and the proinsulin conversion products demonstrate 100% cross-reactivity with these antibodies when compared with insulin. Samples were prepared for extraction by transferring 10 ml of insulin-containing serum and 200 μl of immunobead slurry (a 50:50 vol/vol mixture of immunobead · 0.15 $mol^{-1} \cdot l^{-1}$ phosphate-buffered saline, pH 7.4) to a siliconized Bio-Rad Econocolumn (1.5 × 10 cm). The column was rotated end-over-end at a moderate speed overnight (~16 h) at 4°C on a Scientific Instruments Rotator (Bohemia, NY) set at a speed of 7 rpm. The serum was drained, the beads and column were washed with 30 ml of distilled water, and the insulin was desorbed from the beads by three 0.4-ml sequential elutions (with 1 mol/l acetic acid, 33% vol/vol acetonitrile), separated by 15-min incubations. The eluate was collected into a siliconized 1.5-ml microfuge tube and immediately lyophilized on a Savant Speedvac (Hickoryville, NY).

Before HPLC injection, the resulting lyophilized residue was dissolved in 50 μl of 3 mol/l acetic acid and 150 μl 25% acetonitrile/75% triethylammonium phosphate buffer (TEAP) (0.1 mol/l phosphoric acid, 0.02 mol/l triethylamine, and 0.05 mol/l $NaClO_4$, adjusted to pH 3.0 with NaOH). All reagents were HPLC grade (Fisher, Pittsburgh, PA). Separation of insulin, proinsulin intermediates, and proinsulin was performed by reverse-phase HPLC using a Series 4 liquid chromatograph, ISS-100 column oven, and LC-100 recorder/integrator (Perkin-Elmer/Cetus, Norwalk, CT). Reconstituted immunopurified insulin product (165 μl) was injected onto an Ultrasphere Ion Pair $C_{18}$ column, 4.6 mm × 25 cm, 5-μm particle size (Beckman) at an elution rate of 1 ml/min and a temperature of 45°C. For each proinsulin run, the column was first equilibrated in 30% acetonitrile/70% TEAP (pH 3.0) for 15 min. The sample was applied to the column, and insulin, proinsulin, and proinsulin intermediates were then sequentially eluted with a linear gradient of increasing acetonitrile concentration (up to 33%) over a period of 90 min.

Fractions (0.4 ml) were collected and neutralized with 50 μl 0.5 mol/l borate (pH 9.3) and 1% (wt/vol) bovine serum albumin (BSA) and then lyophilized in a Savant Speedvac. Before the next HPLC run, albumin was washed off the column by increasing the acetonitrile concentration to 55% with a linear gradient, and then the column was re-equilibrated to 30% acetonitrile before the next peptide separation. Each sample was prepared for assay of immunoreactive proinsulin and proinsulin intermediates by dissolving the lyophilized residue to 1.0 ml in 40 mmol/l sodium phosphate (pH 7.4), 0.1 mol/l sodium chloride, 1% wt/vol BSA, and 0.1% Tween-20. The samples were assayed in the proinsulin enzyme-linked immunosorbent assay (ELISA). The peptides used for HPLC standardization were biosynthetic human insulin; proinsulin; 32–33 split proinsulin; des-31,32 proinsulin; 65–66 split proinsulin; and des-64,65 proinsulin (Lilly). The standards were prepared by dissolving the

TABLE 1
Details of subjects participating in the study

| | Normal glucose tolerance Asian and Caucasian | NIDDM Asian | NIDDM Caucasian |
|---|---|---|---|
| *n* | 8 | 9 | 8 |
| Sex | | | |
| Women | 3 | 2 | 2 |
| Men | 5 | 7 | 6 |
| Age (years) | 52.5 (37–66) | 51.2 (39–59) | 61.5 (52–70) |
| Body mass index (kg/m$^2$) | 26.5 (21.5–35.0) | 27.9 (21.7–37.4) | 27.7 (24.7–31.3) |
| Oral glucose tolerance test: plasma glucose (mmol/l) | | | |
| Fasting | 5.2 (4.8–5.9) | 10.5 (7.3–16.8) | 10.7 (6.7–17.5) |
| 30 min | 8.7 (7.0–11.8) | 15.5 (12.4–23.5) | 14.5 (9.1–17.8) |
| 120 min | 5.7 (2.7–8.4) | 18.4 (13.2–27.9) | 18.3 (10.3–27.0) |
| $HbA_{1c}$ (%) | 6.9 (5.4–7.8) | 10.6 (8.4–13.4) | 9.6 (7.6–16.8) |

Data are means (ranges).

peptide in 30% acetonitrile/70% TEAP (pH 3.0) containing 0.5 mg/ml BSA and stored at −20°C until use.

**Proinsulin ELISA.** Insulin and C-peptide do not cross-react in this assay (11,12). The two major proinsulin conversion products, des-31,32 and des-64,65, demonstrate 100% cross-reactivity in the assay in relation to proinsulin. Biosynthetic human proinsulin (Lilly) is used as the assay standard. Crystalline biosynthetic human proinsulin (Lilly) was used as the assay standard. Proinsulin standards of 0.625, 1.25, 2.5, 5, 10, 20, 40, and 80 pmol/l were prepared in 40 mmol/l phosphate (pH 7.4), 0.1 mol/l NaCl, 0.5% human serum albumin, and 0.1% Tween-20 and stored at −20°C. Primary (coating) antibody was isolated from guinea pig anti-insulin antiserum (immunogen was pork insulin, Lilly) by affinity chromatography with protein A. Horseradish peroxidase-labeled-$F(ab')_2$ fragments purified from rabbit anti-C-peptide antiserum (immunogen was biosynthetic human C-peptide [Lilly] coupled to keyhole lympet hemocyanin) were used as the secondary (detecting) antibodies. Purified IgG was prepared from guinea pig anti-human C-peptide antiserum on a protein A column. $F(ab')_2$ fragments were then obtained by pepsin digestion, followed by separation and purification on an Ultrogel AcA44 column (Sepracor, Marlboro, MA). Guinea pig anti-insulin antibody was diluted to a concentration of 2.2 μg/ml in 0.1 mmol/l sodium carbonate (pH 9.8); and 125 μl was adsorbed to a 96-well polystyrene plate (Immuno Plate I, Nunc, Roskilde, Denmark).

After incubation at 4°C for 72 h, the plates were washed five times with 350 μl of a solution of 0.15 mol/l sodium chloride and 0.1% (wt/vol) Tween-20 (Bio-Rad). Then, 100 μl biosynthetic human proinsulin standards or HPLC sample (diluted at least 50% in a solution of 40 mmol/l sodium phosphate [pH 7.4], 0.1 mol/l sodium chloride, 3% vol/vol human serum, 0.005% bovine γ-globulin, and 0.1% Tween-20) were added. After incubation for 24 h at 4°C, the plate was washed. $F(ab')_2$-horseradish peroxidase conjugate was then diluted to a concentration of 0.4 μg/ml in a solution of 40 mmol/l sodium phosphate (pH 7.4), 0.1 mol/l sodium chloride, 0.5% human serum albumin, and 0.1% vol/vol Tween-20, and then 100 μl of the diluted conjugate was added to each well. Plates were incubated at 4°C for 6 days and then washed. Freshly prepared tetramethylbenzidine substrate mixture (KPL, Gaithersburg, MD) (125 μl) was added to each well. The plate was covered and incubated in the dark for 1 h at room temperature. Then, 125 μl 0.1 mol/l phosphoric acid was added to each well to stop the enzyme reaction. The optical density was read at dual wavelengths of 450 and 690 nm. Pipetting and optical density readings were performed with the Biomek 100 Robotic workstation (Beckman). Plates were washed with a Titertek Microplate Washer 120 (ICN, Costa Mesa, CA). Standard curves were constructed, and the results were analyzed with the Beckman Immunofit EIA/RIA Analysis Software. A four-parameter logistic algorithm was used to fit the data and calculate proinsulin concentrations. The sensitivity of the ELISA is 1–2 fmol/ml, as determined by measuring the lowest concentration of added proinsulin that gave a reading that was statistically different from zero. The interassay coefficients of variation are 10.4 and 5.5% for values below and above 10 fmol/ml, respectively. An example of the HPLC results is shown in Fig. 1.

**Statistical analysis.** The relationship between the corresponding measurements obtained by IRMA and HPLC was analyzed by regression analysis. The slopes and *y*-intercepts of the regression equations were tested against unity and 0, respectively, using a special case of Student's *t* test to examine the difference between a sample mean and a single value (13).

## RESULTS

A comparison of the intact proinsulin results obtained by the IRMA with the HPLC results is shown in Fig. 2. There was no significant difference ($P > 0.05$) in the relationship of the results between the different subject groups or time points. For all of the data points, the regression (±SE) line was: IRMA = −0.143 (± 1.93) + 1.066 (± 0.10) HPLC, $r = 0.860$. The slope was not significantly different from 1 ($t = -0.103$,

TABLE 2
Concentrations of circulating proinsulin and intermediates in different subject groups

| | Insulin (IRMA) (pmol/l) | Proinsulin (pmol/l) | | 32–33 Split proinsulin (pmol/l) | |
|---|---|---|---|---|---|
| | | HPLC | IRMA | HPLC | IRMA |
| Normal subjects | | | | | |
| 0 min | 42.4 (15–78) | 4.8 (1.0–8.0)* | 4.5 (1.1–5.7) | 3.0 (1.0–6.0)† | 2.7 (2.0–8.2) |
| 30 min | 278 (92–446) | 9.8 (2.0–19) | 7.6 (3.2–11) | 13 (2.0–24)‡ | 12.4 (2.8–26) |
| Diabetic Asians | | | | | |
| 0 min | 72.3 (35–152) | 15.0 (5.0–25) | 15.1 (7.6–34) | 20.1 (5.0–38) | 22.2 (2.0–40) |
| 30 min | 131 (36–292) | 18.5 (5.0–42) | 19.7 (5.7–49) | 27.6 (11–67) | 18.1 (2.8–66) |
| Diabetic Caucasians | | | | | |
| 0 min | 52.6 (37–70) | 16.8 (4.0–65) | 20.8 (5.6–84) | 17.1 (5.0–58) | 17.9 (5.0–44) |
| 30 min | 87.8 (32–160) | 19.4 (7.0–58) | 20.6 (8.8–67) | 19.4 (11–47)§ | 25.9 (5.0–100) |

Data are means (ranges). The number of patients in each group is as given in METHODS unless otherwise indicated, due to insufficient sample volume. *$n = 5$. †$n = 5$. ‡$n = 7$. §$n = 7$.

degrees of freedom [df] = 44) and the $y$-intercept was not significantly different from zero ($t$ = 0.015, df = 44). When the data were recalculated minus the top two points, $r$ = 0.67 with a slope of 0.79, which was also not significantly different from 1.

The HPLC analysis of split proinsulins demonstrated that in only one subject was 32–33 split proinsulin detected at 1 pmol/l. Thus, des-31,32 split proinsulin was the major circulating form.

In seven subjects, des-64,65 split proinsulin was detected, generally at concentrations of <5 pmol/l. In 11 of the 14 samples, the concentration was <4 pmol/l; therefore, no corrections were made from cross-reactivity in the IRMA results. The measurable values were equally distributed among the subject groups and showed no consistent trend with glucose load.

The comparison of the 32–33 split proinsulin results is shown in Fig. 3. Similarly, there was no significant difference in the relationship of the results between the different subject groups or time points ($P > 0.05$). For all of the data points, the regression line (± SE) was: IRMA 32–33 split proinsulin = 0.048 (± 2.71) + 1.051 (± 0.12) HPLC; des-31,32 split proinsulin, $r$ = 0.814. The slope and the intercept were not different from 1 and 0, respectively (slope, $t$ = −0.049; intercept, $t$ = −0.045; df = 44).

The results for each subject group are shown in Table 2.

## DISCUSSION

These results, which were obtained in samples taken at different times during an oral glucose tolerance test from nondiabetic subjects, diabetic Caucasians, and diabetic South Asians for intact proinsulin using IRMA and HPLC methods, showed good agreement. The HPLC method, unlike the IRMA, is able to distinguish between 32–33, des-32, and des-31,32 split proinsulins. Therefore, most of the material measured as 32–33 split proinsulin by the IRMA appears to be des-31,32 split proinsulin, as previously expected (9).

Some discrepant results were found. Additional investigation is necessary to determine the cause. The procedure for the HPLC analysis is relatively complex, thus allowing the potential for error in occasional samples. Despite all of these possible problems and the need to assay both proinsulin and split proinsulin at very low concentrations, there was no evidence of any systematic discrepancy between the different methods. We conclude that these assays represent alternative and valid methods for the measurement in plasma of these two proinsulin-related molecules during glucose tolerance tests of normal and NIDDM subjects. The greater scatter of the 32–33 split proinsulin results reflects the greater imprecision of the IRMA with correction for cross-reactivity of proinsulin compared with the intact proinsulin assay and the fact that some of the results were less than the detection limit of the assay.

This study also confirms the heterogeneous nature of circulating proinsulin-related peptides in both those with normal and those with diabetic glucose tolerance. It demonstrates that des-31,32 split proinsulin is the predominant proinsulin conversion intermediate.

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