# SPECIFIC AND DIRECT PROINSULIN RADIOIMMUNOASSAY FOR THE EVALUATION OF INSULINOMAS

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## Abstract

SPECIFIC AND DIRECT PROINSULIN RADIOIMMUNOASSAY FOR THE EVALUATION OF INSULINOMAS.

The paper describes a highly specific human proinsulin (hPI) radioimmunoassay (RIA) developed by using biosynthetic hPI as immunogen, standard and tracer. Proinsulin was radioiodinated by the iodogen method and purified on QAE-Sephadex A-25 to a specific activity of 6.5 MBq/$\mu$g. The antiserum was raised in a guinea pig and then adsorbed against insulin and C-peptide conjugate to Sepharose to improve its specificity. The range of the standard curve extended from 0.004 to 16 pmol/mL, with a 50% displacement between 0.19–0.26 pmol/mL and the minimal detectable concentration between 0.01–0.04 pmol/mL. This sensitive and specific RIA proved suitable for measurements of serum hPI concentrations in patients with insulinomas, which levels ranged from 0.11 to 1.80 pmol/mL (n = 8).

## 1. INTRODUCTION

Using recombinant DNA technologies it is now possible to prepare large quantities of human proinsulin (hPI), which exhibits the same physico-chemical and biological properties as the pancreatic hormone [1].

The availability of this hormone enabled the development of specific and sensitive radioimmunoassays for hPI using the biosynthetic preparation as immunogen, standard and tracer [2–5] allowing a wide variety of physiological and clinical studies.

This paper describes a sensitive and specific hPI radioimmunoassay (RIA) employing a carefully prepared tracer, with great stability after QAE-Sephadex purification [6] and an antiserum produced in guinea pig [7] that does not cross-react with insulin and C-peptide.

This specific assay was used to study circulating proinsulin from patients with insulinoma.

## 2. MATERIALS AND METHODS

Human biosynthetic proinsulin (lot No. 509-EM4), insulin (lot No. 615-7U7-208) and C-peptide (lot No. A18-TU8-44D) were kindly supplied by Eli Lilly and Company, USA. Porcine insulin type III (lot No. MLS3.142) was kindly supplied by Biobrás Bioquímica do Brasil, Brazil. Second antibody, goat anti-guinea pig immunoglobulin G (IgG) was from Pel-Freez, USA (lot No. 12890-3CC). Bovine serum albumin (BSA) and bovine g-globulin (BgG) were from Sigma Chemical Co., St. Louis, MO, USA. Materials for chromatography were from Pharmacia, Sweden.

### 2.1. Preparation of $^{125}$I labelled proinsulin

Iodine labelling was carried out using a modification of the iodination method of Fraker and Speck [8]. Polypropylene iodination vials (tubes 12 × 75 mm) were coated with 16 μg of Iodo-gen (Sigma) dissolved in dichloromethane and evaporated to dryness at room temperature under a nitrogen atmosphere, in a fume hood. The dried tubes were stored in a desiccator at −20°C and used within nine months. They were rinsed with the iodination buffer immediately before use, in order to remove any loose flakes of chloroglycoluril. Proinsulin (5 μg in 100 μL of 0.05M sodium phosphate buffer, pH7.4) was incubated with Na$^{125}$I (37 MBq) for 5 min at 4°C in the iodination tube. The iodination yield was determined as the percentage of radioactivity precipitated by the 10% trichloroacetic acid (TCA) [9].

The reaction mixture was chromatographed on a column (30 × 1.0 cm) of QAE Sephadex A-25 equilibrated with 0.08M Tris-HCl, 0.08M NaCl pH8.6 and containing 1% BSA (RIA grade, fraction V). The column was eluted at 4°C and at a flow rate of 15 mL/h with a linear gradient formed from starting buffer (150 mL) and starting buffer containing 0.3M NaCl (150 mL). Fractions (2.5 mL) were collected and radioactivity counted. The main peak related fractions from the $^{125}$I-hPI purification were evaluated by their reactivity to excess of the antiserum (not purified) according to the RIA procedure reported by Deacon and Conlon [3]. Fractions which showed maximum binding to the antiserum were pooled, stored at −20°C and used as the RIA tracer. Its purity was also estimated by the TCA precipitability.

The efficiency of the purification as well as the stability of the tracer were analysed with 7% polyacrylamide gel electrophoresis (PAGE), according to the modified method of Davis [10] as previously described [11]. Tracer integrity was accomplished by calculating the percentage of the surface area under the peak of the electrophoretic profile of $^{125}$I-hPI and free iodide.

### 2.2. Development of the anti-proinsulin antiserum

A hPI antiserum (OP) was raised by immunizing a male albino guinea pig (220 g) with repeated subcutaneous injections of an emulsion (2 mL) formed from equal volumes of proinsulin (50 $\mu$g in saline) and Freund's adjuvant (complete for the primary immunization and incomplete for subsequent injections) in multiple sites according to the method of Vaitukaitis et al. [12]. Booster injections were administered four weeks after the primary immunization and then at three-week intervals. Water containing 5% glucose was given throughout the experiment in order to prevent hypoglycaemia. Total blood (25 mL) was taken by cardiac puncture two weeks after the third booster and the antiserum was evaluated for cross-reactivity with insulin and C-peptide. As the antiserum was not sufficiently specific for proinsulin it was adsorbed against porcine insulin–Sepharose and human C-peptide–Sepharose. The gel was activated at our laboratory [13] (150 mg CNBr (Sigma) per g Sepharose 4B) and the conjugation was carried out by the procedure reported by Cohen et al. [2].

### 2.3. Radioimmunoassay procedure

All RIA reagents (label, antiserum, standard and normal guinea pig serum (NGPS)) were diluted in 0.04M sodium phosphate buffer pH7.4, containing 0.1% BSA and 0.02% thiomersal (assay buffer). A stock hPI solution (1065 pmol/mL) was prepared from the hPI solution surplus in the iodination, aliquoted into fractions (100 $\mu$L) and stored at $-20$°C in polypropylene tubes. The standard curves (ranging from 0.004 to 16 pmol/mL) were prepared diluting the stock hPI solution with blood bank plasma. The assay separation reagents (NGPS, second antibody and polyethylene glycol (PEG)) were diluted in 0.025M sodium phosphate — 0.02M ethylenediaminetetraacetate (EDTA) pH7.5, containing 0.1% BSA and 0.02% thiomersal.

The RIA was carried out using a non-equilibrium system. Antiserum OP or NGPS for the blanks (100 $\mu$L, final dilution 1:15 000) and hPI standards or serum (100 $\mu$L) were incubated at 4°C for 24 hours. Afterwards, the $^{125}$I-hPI (100 $\mu$L, approximately 10 000 counts/min) was added and incubated for another 3 hours. This was followed by addition of NGPS (100 $\mu$L, 1:400), second antibody (100 $\mu$L, 1:9) and PEG 8000 from Sigma (900 $\mu$L, 10% wt/vol.) and a further 1 hour incubation at 23°C. Precipitates were collected by centrifugation at 3500 g for 30 min at

4°C (Incibras Centrifuge, Brazil) and the pellets counted for 5 min in an ANSR automatic gamma counter (Abbott Laboratories, USA) with an efficiency of 65%. Samples were read from the standard curve using a four parameter logistic model algorithm [14].

Fasting levels of serum hPI were measured in six patients with insulinoma, aged from 17 to 52 years, three females and three males. Three of them had received diazoxide.

## 3. RESULTS

### 3.1. Preparation of $^{125}$I labelled proinsulin

The iodination yield revealed a value in the range 83–90% for six preparations corresponding to an average specific activity of 6.45 ± 0.17 MBq/$\mu$g (±SD) and a degree of iodination lower than 1.0 atom of $^{125}$I per hPI molecule. The elution pattern of radioactivity from the anion exchange chromatography is shown in Fig. 1. The hatched area represents the most immunoreactive fractions pooled. The pools from the six preparations presented a purity in the range 91–97% by the trichloroacetic acid (TCA) test.

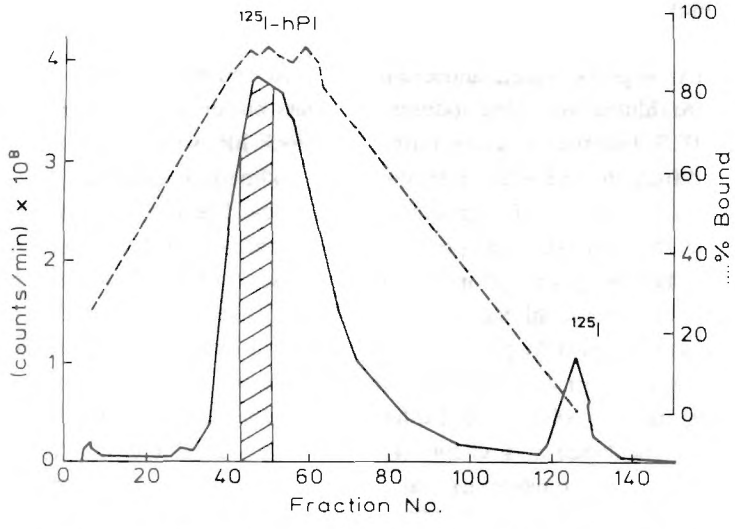


*FIG. 1. Purification of $^{125}$I-hPI on QAE-Sephadex A-25 showing radioactivity (—) and immunoreactivity (---) for each fraction.*

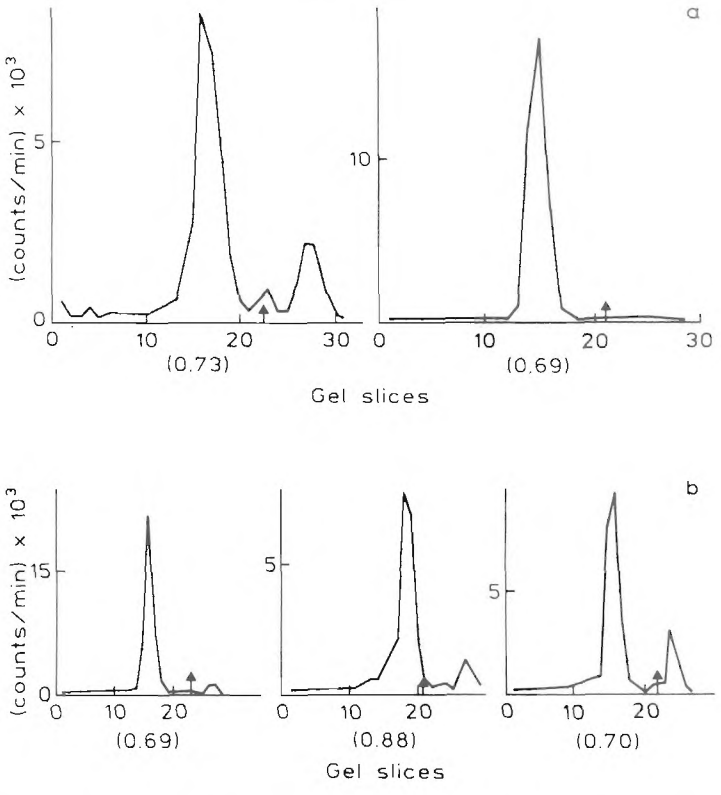


*FIG. 2. (a) Distribution of radioactivity from PAGE of the hPI immediately after radioiodination (left) and after purification (right). (b) Distribution of radioactivity from PAGE of the $^{125}$I-hPI after one, two and three months after purification (from left to right). The RM values of the main $^{125}$I-hPI component are indicated in parenthesis and the arrows indicate the position of the TD.*

Figure 2 presents the electrophoretograms revealed in the analysis of the $^{125}$I-hPI just before and after purification and in the analysis of the tracer throughout storage. The ratio of migration (RM) of the $^{125}$I-hPI was determined in relation to the tracking dye (TD) bromophenol blue. After labelling, the radioactivity in the $^{125}$I-hPI peak was found to contain 73% of the total activity in the gel, increasing to 98% after purification, when the free iodide was completely absent (Fig. 2(a)). After one, two and three months from the purification the value of the $^{125}$I-hPI peak decreased to 82, 71 and 62% respectively, whereas the free iodide increased (Fig. 2(b)).

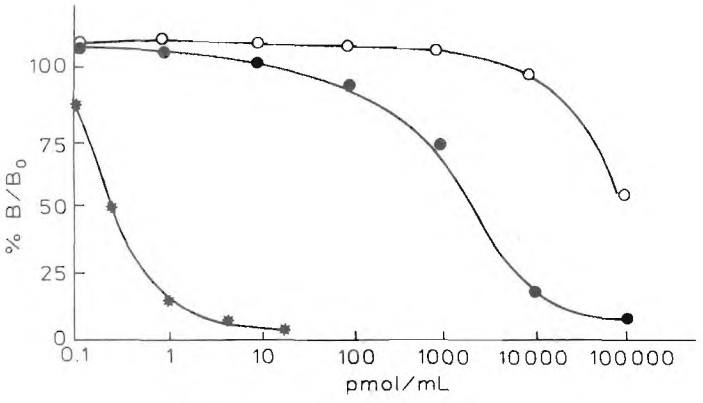


*FIG. 3. Displacement of $^{125}$I-hPI by human proinsulin (* — *), human insulin ( o — o ) and human C-peptide ( ● — ● ). Each displacement experiment was performed according to the RIA procedure described in Materials and Methods.*

### 3.2. Characterization of the anti-proinsulin antiserum

Within 13 weeks the guinea pig produced antibody to hPI of sufficient titre (1:5000) and avidity (Ka value of $1.00 \times 10^{10}$ L/mol) and highly specific after purification. The effect of increasing concentrations of biosynthetic hPI, insulin and C-peptide on the binding of $^{125}$I-hPI to the antiserum is shown in Fig. 3. As shown in the figure, hPI produced 50% displacement of tracer ($ED_{50}$) at a concentration of 0.25 pmol/mL, whereas C-peptide and insulin failed to displace tracer at concentrations as high as 10 and 1000 pmol/mL, respectively.

### 3.3. Radioimmunoassay procedure

Equilibrium binding studies involving the antiserum OP demonstrated that 24 hours are required for achieving maximum binding with the tracer at 4°C (Fig. 4). The delayed addition of the tracer by 24 hours with a three hour incubation improved significantly the sensitivity of the assay, with $ED_{50}$ values decreasing from 0.78 to 0.22 pmol/mL and the minimum detectable concentration (MDC) (2.5 SD from the mean zero-dose, $B_0$) decreasing from 0.07 to 0.01 pmol/mL; despite the lower $B_0$ value (30 against 53%). The same delayed addition of the tracer followed by a six hour incubation presented a higher $B_0$ value (38%) but the assay sensitivity was not so improved ($ED_{50}$ value of 0.33 and MDC of 0.07 pmol/mL).

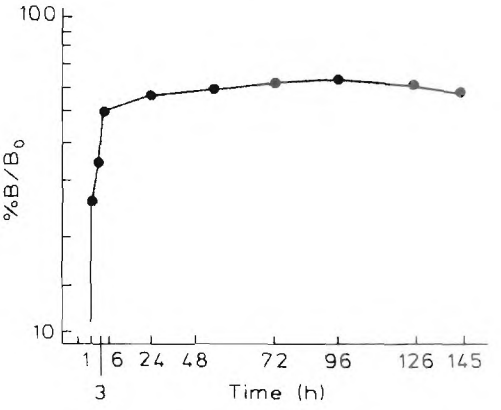


*FIG. 4. Effect of incubation time on the antiserum binding with the $^{125}$I-hPI at 4°C. The RIA reagents were the same as described in Materials and Methods but the assay was separated with PEG 20%.*

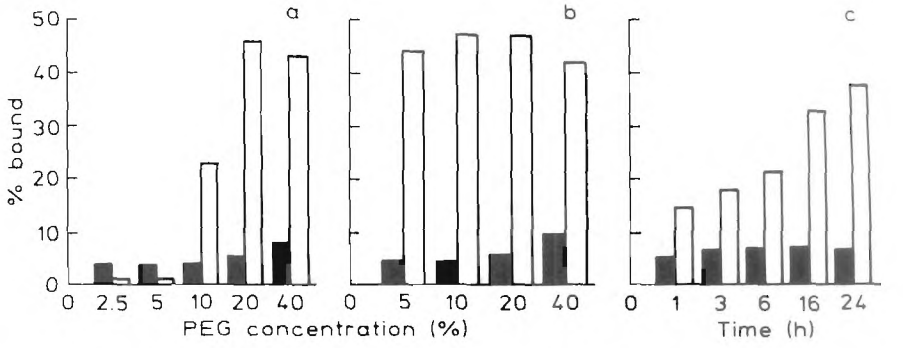


*FIG. 5. Specific ($B_0$) and non-specific (hatched area) values obtained by the employment of various separation techniques: (a) PEG 6000, Atlas, Brazil (1 mL) and BgG 1% (100 μL) followed by centrifugation, dissolution of the pellets with 0.4M NaCl (300 μL) and by a further PEG precipitation; (b) PEG assisted second antibody as described in Materials and Methods; and (c) second antibody (100 μL, 1:9).*

Blood bank plasma added to the assay standards instead of the assay buffer did not result in a shift in the standard curve, indicating that there was no interference from the plasma in which hPI concentration was below the assay sensitivity (data not shown).

Figure 5 shows the result of the investigation into the optimal reagents for the RIA separation. Very similar results (maximum $B_0$ and minimum non-specific

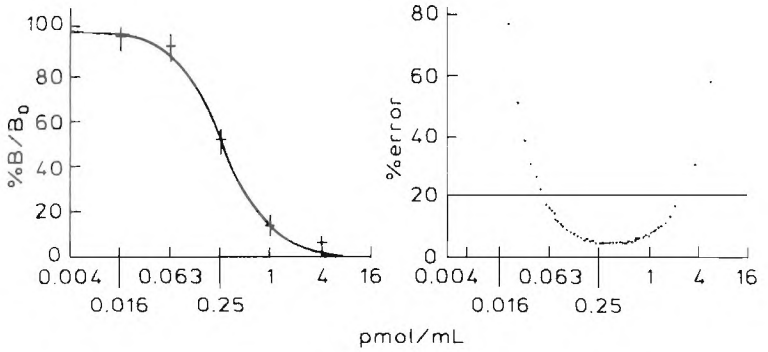


*FIG. 6. RIA standard curve (mean of triplicate determinations, left) and its respective precision profile estimated by the QC RIA program [14] (right).*

TABLE I. SERUM PROINSULIN CONCENTRATIONS IN PATIENTS WITH INSULINOMA

| Subject | hPI concentration (pmol/mL) | | |
|---|---|---|---|
| 1 | 0.11 | | |
| 2 | 0.14 | | |
| 3 | 0.17 | | |
| 4 | 0.31 | 0.20 | |
| 5 | 0.30 | 0.22* | 0.17* |
| 6 | 1.80* | | |

Serum samples from subject 5 were obtained at one week intervals and those from subject 4 at a one month interval. Asterisks indicate the samples from subjects who had received diazoxide.

binding (NSB) values) were obtained by the use of PEG 20% (Fig. 5(a)) and second antibody assisted by PEG 10% after a one hour incubation (Fig. 5(b)). However, the use of the second antibody alone could not achieve the complete separation of bound and free hPI even after a 24 hour incubation (Fig. 5(c)). Accordingly, the RIA displacement curves separated by PEG assisting the second antibody or not were superimposable (values of 0.19 and 0.21 pmol/mL for $ED_{50}$ and 1.54 and 1.45 for slope, respectively) in contrast to that separated by the second antibody ($ED_{50}$ of 0.33 pmol/mL and slope of 1.06).

A typical RIA displacement curve and its precision profile are illustrated in Fig. 6. This curve was obtained according to the established procedure described in the journal Materials and Methods. The $B_0$ values ranged from 30 to 24% throughout 3 months, at the same time the level of NSB remained constant and lower than 4%. Assay sensitivity (MDC) ranged from 0.01 to 0.04 pmol/mL while slope and $ED_{50}$ values were 1.43 ± 0.09 and 0.22 ± 0.02 pmol/mL, respectively, for 6 assays (mean ± SD). The usual working range of the curves was of the order of 0.06 to 1 pmol/mL (error lower than 20%). Fasting levels of serum hPI determined through these assays in the patients with insulinoma are presented in Table I.

## 4. DISCUSSION

The sensitivity of the RIA is critically dependent on the quality of the tracer. Although the preparation and purification of monoido-$^{125}$I-tyr(A14)-hPI by high pressure liquid chromatography (HPLC) has been described [15], this apparatus is not available at our laboratory and this method has the disadvantage that the apparatus becomes highly contaminated with radioactivity [3]. However, the use of the Iodo-gen, a gentle, easy, inexpensive and reproducible method for labelling proteins followed by purification on QAE Sephadex A-25 yielded hPI tracers with high specific activity, purity and long shelf-life suitable for RIA. Besides the tracer, the sensitivity and specificity of the RIA depend predominantly on the avidity and specificity of the antiserum used. Only a few laboratories have developed antisera for biosynthetic hPI and the homologous radioimmunoassays reported in the literature were performed using them [2–5]. The antiserum OP prepared at our laboratory [7] became highly specific after adsorption against insulin and C-peptide, presenting characteristics very close to those antisera prepared in guinea pig [2, 3]. Thus, the use of this antiserum enables direct measurement of hPI in biological samples in one incubation step, without the need for prior separation of insulin and C-peptide by chromatographic methods [16].

Regarding the RIA separation technique, the addition of PEG to the second antibody not only reduces significantly the incubation time but also seems to be critical for the complete separation of bound and free hPI. Although the use of PEG 20% has produced equivalent levels of maximal $B_0$ and minimal NSB in relation to those produced by PEG 10% assisted second antibody (Fig. 5), the need for an additional precipitation diminishes the assay practicability and can affect its accuracy.

The sensitivity of the hPI RIA described here is extremely close to that reported in the published methods [2–5] although it is inferior to that achieved by Bowsher [17] with a MDC as low as 0.0034 pmol/mL. Consequently, like most of those methods our assay is not sufficiently sensitive to measure fasting proinsulin levels in healthy subjects without the use of concentration techniques. However, its sensitivity and its wide working range proved suitable for the measurement of

serum circulating proinsulin in patients with insulinoma, which levels are in good agreement with a previous report [4].

This technically simple RIA can also be used to confirm the diagnosis of hyperproinsulinaemic states other than insulinomas, e.g. familial hyperproinsulinaemia [4], and to measure hPI after stimuli [18], without the need for prior concentration.

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