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Enzyme-linked sandwich immunoassay for insulin using laser fluorimetric detection

(insulin detection in serum samples/enzyme amplification/laser fluorimetry)

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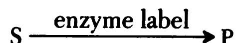
ABSTRACT **Human serum samples are assayed for insulin by an enzyme-linked sandwich immunoassay. Horseradish peroxidase is used as an enzyme label for antibody, and enzyme activity is measured by means of the fluorogenic substrate, *p*-hydroxyphenylacetic acid. The product is detected by excitation of fluorescence with the 325-nm line of a continuous-wave helium/cadmium ion laser on line with reverse-phase high-pressure liquid chromatography. The incubation period is 90 min and the limit of detection of insulin is 30 pM, corresponding to 5 microunits/ml. This method correlates highly with radioimmunoassay, with coefficient of correlation $r = 0.95$.**

Since its development in the late nineteen fifties by Yalow, Berson, and their colleagues, radioimmunoassay (RIA) has proven to be a powerful tool for the analysis of a broad spectrum of substances of biologic interest (1). Despite its advantages of high sensitivity and specificity, there are a number of drawbacks associated with this technique. These include difficulties in storage, handling, and disposal of the radioisotopic tag and its inherently limited shelf life. As a result, a number of alternative approaches to RIA have been considered. A notable success has been in the development of enzyme immunoassay, in which an enzyme replaces the radioisotopic label, and label content is determined by measuring enzyme activity (2).

The sensitivity and rapidity of enzyme immunoassays can be limited by the sensitivity of the method used to determine enzyme activity. Typically one measures activity by means of a substrate, S, which is converted upon enzyme catalysis to a product, P, whose spectroscopic properties differ from those of S:



$$S \xrightarrow{\text{enzyme label}} P.$$

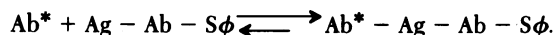
In the presence of excess S, the amount of P formed is proportional to the amount of enzyme label present and to the duration of enzyme catalysis. Thus, if a more sensitive method for product quantitation is employed, it should prove possible to detect lower levels of enzyme label and to shorten the duration of the enzyme catalysis step of the assay.

Commonly, colorimetric or fluorimetric methods are used to measure enzyme activity. Work in our laboratory has demonstrated that the use of a laser as a fluorescence excitation source makes possible the detection of enzyme reaction products at much lower levels than can be detected using colorimetric or conventional fluorimetric techniques (3). We report here results of a study wherein laser fluorimetric methods are applied to enzyme immunoassay.

Evaluation of a commercial sandwich enzyme immunoassay for insulin has been reported by Yoshioka *et al.* (4). In this method horseradish peroxidase (HRPase) serves as the antibody label, and enzyme activity is measured by absorbance through a chromogenic substrate. The limit of detection for insulin (30 pM) is comparable to that of RIA, and the total incubation time [3 hr (5)] is shorter than most assay durations in RIA. Moreover, measurements of insulin concentrations in human serum correlate quite well with those performed by RIA. The method can be schematized in the following way. The antigen Ag (insulin in our work) in the solution to be analyzed binds to antibody Ab that is linked to solid phase Sϕ, (Ab − Sϕ):

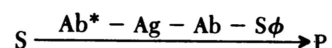
$$\text{Ag} + \text{Ab} - \text{S}\phi \rightleftharpoons \text{Ag} - \text{Ab} - \text{S}\phi.$$

Enzyme-labeled antibody, Ab*, is added to the mixture and binds to antigen:



$$\text{Ab}^* + \text{Ag} - \text{Ab} - \text{S}\phi \rightleftharpoons \text{Ab}^* - \text{Ag} - \text{Ab} - \text{S}\phi.$$

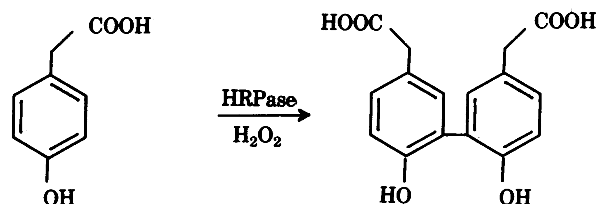
Ab* unbound to the solid phase is removed, and the remaining enzyme activity is measured by means of a colorless substrate that is converted to a colored product:



$$S \xrightarrow{\text{Ab}^* - \text{Ag} - \text{Ab} - \text{S}\phi} P.$$

Our strategy is to combine this enzyme immunoassay for insulin with laser fluorimetric detection of an enzyme reaction product and, thereby, reduce the time requirements of the assay.

There are a number of fluorogenic substrates for HRPase (6). In this work, we use the nonfluorescent substrate, *p*-hydroxyphenylacetic acid (HPA), which in the presence of HRPase and H_2O_2 is converted into the fluorescent product 6,6′-dihydroxy-(1,1′-biphenyl)-3,3′-diacetic acid (DBDA) as shown below (7):



DBDA exhibits an emission maximum at 414 nm and is excited maximally at 317 nm (6); the 325-nm line of a helium/cadmium ion laser is thus a nearly ideal excitation source. By utilizing HPA as a substrate for HRPase and detecting its product by laser

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Abbreviations: RIA, radioimmunoassay; HRPase, horseradish peroxidase; HPA, *p*-hydroxyphenylacetic acid; DBDA, 6,6′-dihydroxy-(1,1′-biphenyl)-3,3′-diacetic acid; HPLC, high-pressure liquid chromatography.

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fluorimetry in conjunction with reverse-phase high-pressure liquid chromatography (HPLC), we demonstrate that the measurement of insulin in human serum can be performed significantly more rapidly than by existing methods.

MATERIALS AND METHODS

Detection of DBDA. Fig. 1 shows a schematic of the experimental apparatus, which is a modification of a laser fluorimeter previously described (8). To optimize detection of DBDA, which fluoresces most intensely under alkaline conditions, an alkalinization scheme is used after chromatography. The eluant from the HPLC column exits into a stainless steel tee connector (low dead volume). Into another part of the tee is pumped 0.075 M NaOH. The effluent passes from the tee to a mixing coil and then exits into the flowing droplet detector through a low dead-volume union.

The 325-nm output (2 mW) of a continuous-wave feedback-stabilized helium/cadmium ion laser (model 4050 UV, Liconix) is focused into the droplet by lens L_1. Plasma superradiance is removed by an interference filter (Corion) centered around the laser line. The droplet is imaged onto the cathode of the photomultiplier (Q4249B, Centronic) by lens L_2, L_3, and the aperture. The spectral region between 410 nm and 490 nm is isolated by the liquid filters F_1 ($NaNO_2$, 133 g/l) and F_2 ($CuSO_4$, saturated solution). In this way Rayleigh and Raman scattering from the solvent are largely suppressed (3). The output of the photomultiplier is displayed on a stripchart recorder that has a 0.5-sec time constant.

All chromatographic equipment was obtained from Waters Associates. The elution solvent, which consists of methanol/aqueous acetic acid (0.07 M), 70:30 (vol/vol) is delivered to the HPLC column (μBondapak C_{18}) by a model 6000 A pump. Samples are introduced onto the column through a model U6K injector. The 0.075 M NaOH, prepared with CO_2-free water (simultaneously sonicated and flushed with helium), is protected from atmospheric CO_2 by a drying tube packed with soda lime and is delivered to the tee by a model M-45 pump. The NaOH solution must be kept CO_2-free to prevent bubble formation at the tee, where the solution becomes more acidic. The flow rates of both the elution solvent and the NaOH were 1.0 ml/min.

Materials. Methanol and acetic acid were reagent grade and were distilled before use. Water was deionized and distilled. HPA was obtained from Aldrich and purified by sublimation and reverse-phase medium-pressure liquid chromatography (2×) with a Lobar RP8 (EM Associates, Elmsford, NY) column and methanol/water (acidified with acetic acid to pH 2.5), 70:30 (vol/vol), as elution solvent. HRPase (250 units/mg) was obtained from Boehringer Mannheim. The reagents for immunoassay (Insulotec Kit) were obtained from Mochida Pharmaceutical, Tokyo, Japan. These included solid phase antibody, which consisted of a plastic disc (1-cm diameter, 0.5 cm thick) coated with anti-insulin antiserum, standard solutions of insulin, HRPase-labeled anti-insulin antiserum, assay buffer [0.076 M phosphate buffer (pH 6.4) containing animal serum], and Tween 20 surfactant. Three lyophilized serum samples were obtained from Nuclear Medical Systems, Newport Beach, CA, and six fresh serum samples were obtained from the Stanford University Medical Center.

Determination of HRPase Activity. Serial dilutions of HRPase were prepared in 0.01 M phosphate buffer, (pH 7.0). A 12 mM solution of HPA was prepared by dissolving the substrate in the same buffer; the pH of the resulting solution was 4.3. Fifty microliters of HRPase solution was incubated with 100 μl of H_2O_2 solution (0.53 mM in the pH 7 phosphate buffer) and 100 μl of HPA solution for 15 min at 25°C (the pH of the final incubation mixture was 5.5). The reaction was halted by the addition of 10 μl of 2% (wt/vol) aqueous NaN_3; 200-μl aliquots from each reaction mixture were mixed with 200 μl of methanol. Ten microliters of each resulting solution was injected onto the HPLC column.

Immunoassay. The procedure was adapted from that described previously (4). To initiate the first reaction, a disc was added to each insulin-containing sample (0.1 ml of serum plus 0.3 ml of assay buffer). After 1 hr at 25°C, 0.1 ml of HRPase-labeled antibody solution was added to each tube. This second reaction proceeded for 15 min at 25°C and was halted by the addition of 5 ml of a solution of Tween 20 surfactant. Fluid was withdrawn by aspiration. This washing procedure was repeated three additional times (total, 4 × 5 ml). Two hundred microliters of HPA solution (12 mM, prepared as described above) and 200 μl of H_2O_2 solution [0.53 mM in 0.01 M phosphate buffer

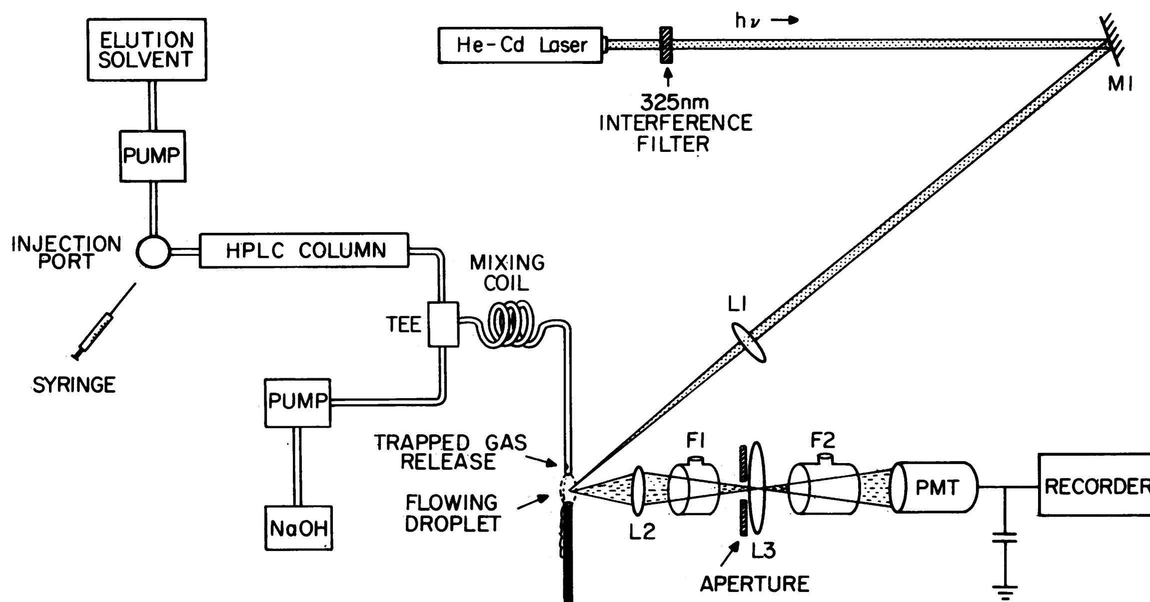

FIG. 1. Schematic of the laser fluorimeter.

(pH 7.0) were added to each tube. The third reaction proceeded for 15 min at 25°C and was halted by the addition of 10 μl of 2% aqueous NaN_3. Two hundred microliters of each sample was mixed with 200 μl of methanol. Aliquots (10 μl) from each resulting solution were injected onto the HPLC column.

RIA was performed independently by Jo Moore at the Palo Alto Veterans Administration Hospital.

RESULTS

Limits of Detection for HRPase. Fig. 2 shows a sample chromatogram. One peak, which corresponds to DBDA, is present. When DBDA peak height was plotted against enzyme concentration in the reaction mixture, the relation was found to be linear over the range 1–100 pM. The limit of detection for HRPase was 1 pM. This limit is set by the condition that the peak height-to-noise ratio is 2 for the sample containing the minimum concentration.

Immunoassay. Fig. 3 shows two chromatograms from immunoassay samples. Each is identical in form to the one shown in Fig. 2. The peak height-to-noise ratio is ≥100. Fig. 3*A* shows a sample that contained no insulin. The peak represents enzyme activity from nonspecifically bound HRPase-labeled antibody. Fig. 3*B* shows a sample that contained 20 microunits (120 pmol) of insulin per ml. The DBDA peak is larger than that in Fig. 3*A*, as expected.

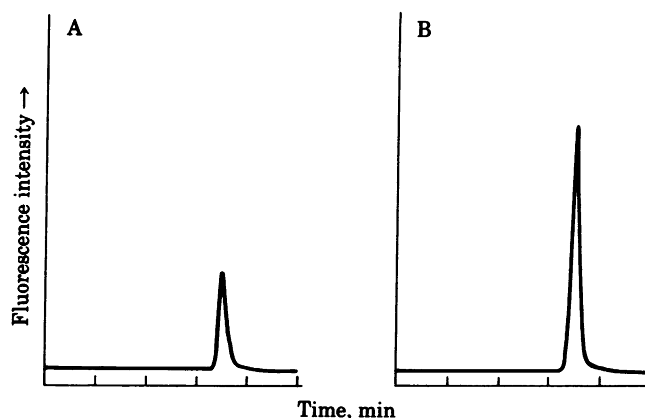

FIG. 3. Chromatograms of immunoassay samples. (*A*) No insulin. (*B*) Twenty microunits of insulin per ml.

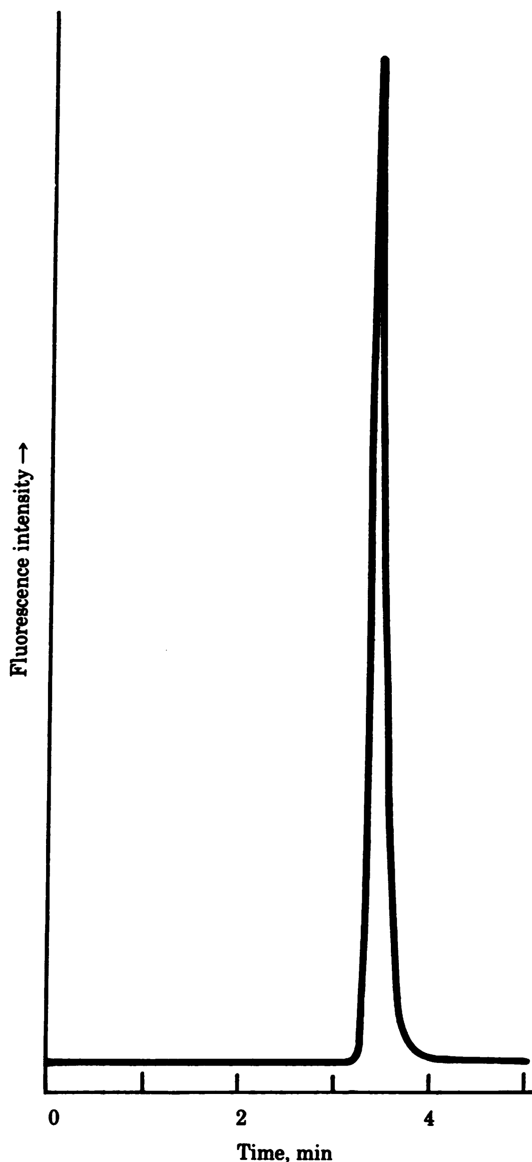

FIG. 2. Chromatogram of sample containing HRPase, HPA, and H_2O_2 in assay buffer. The peak corresponds to the product DBDA.

Fig. 4 shows the standard curve for insulin immunoassay obtained under the experimental conditions described above. DBDA peak height is plotted against insulin concentration. The limit of detection for insulin is 5 microunits/ml (30 pM). This limit corresponds to the condition $h - h_b = 2\sigma_b$ where h is the mean DBDA peak height for a series of samples of a given insulin concentration, σ is the standard deviation in peak height measurements, and the subscript b denotes blank samples (i.e., samples devoid of insulin).

When colorimetric methods are employed, the enzyme immunoassay requires (at a minimum) 60 min for each of the three incubations (4, 5)—a total of 180 min. The effect of incubation time on the sensitivity of the immunoassay with laser fluorimetric detection was examined. When the duration of each of the latter two incubations was decreased from 1 hr to 15 min, the initial slope of the standard curve, (hence, the sensitivity) remained the same. When the duration of the first incubation step was shortened from 1 hr to 30 min, the initial slope decreased by a factor of 1.4. This corresponded to a detection limit for insulin of 7 microunits/ml. When the duration of the first incubation step was increased from 1 to 2 hr, no change in the initial value of the slope was observed.

Insulin concentrations were determined in nine serum samples (six fresh, three lyophilized). The measured insulin values were then correlated with those obtained independently by RIA. The results of this double-blind procedure are plotted in Fig. 5. The data fit a line of the form $y = 0.9x + 7.9$.[‡] The coefficient of correlation, r, is 0.95.

DISCUSSION

This report describes a successful measurement of insulin in serum by an immunoassay that employs laser fluorimetry. We have reported (8) the sensitive detection of insulin in aqueous buffers. In that work, a fluorescent antigen label was employed, and bound and free insulin were separated on-line by gel filtration HPLC prior to laser fluorimetric detection of the chro-

‡ The existence of a nonzero intercept is quite common and represents the difference in response of the two immunoassays. In the present method, it does not result from failure to correct for background enzyme activity.

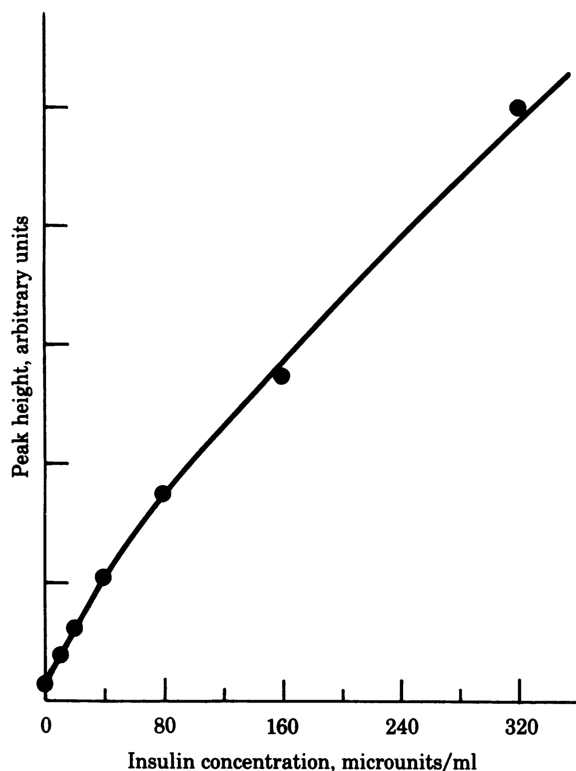

FIG. 4. Standard curve for sandwich immunoassay. The data points represent the average of duplicate determinations.

matographic eluant. Measurements of insulin in human serum were seriously hampered by interfering fluorescent components.

In subsequent work (9) we used, in analogy to Kato *et al.* (10), the enzyme β-galactosidase as a label for insulin. In this case, bound and free insulin were separated by utilizing a solid-phase antibody. Enzyme activity was detected by means of a fluorogenic substrate and laser fluorimetric detection of its product. Our limit of detection in the assay buffer was a factor of five lower than that of Kato *et al.* (10) and a factor of six lower than that of a similar enzyme immunoassay for insulin developed by

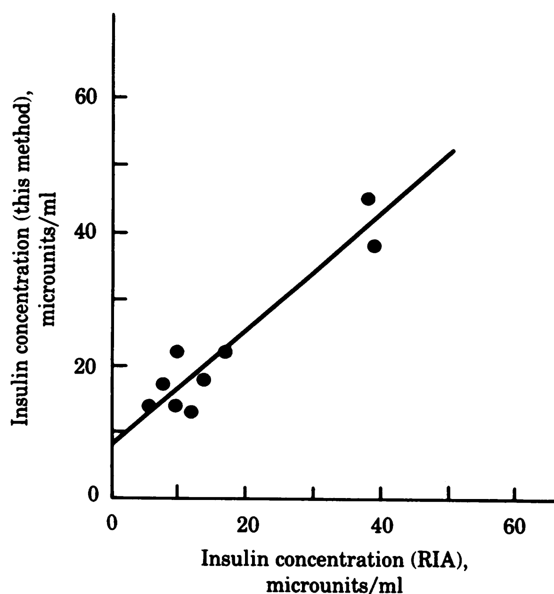

FIG. 5. Correlation between sandwich immunoassay (this work) and RIA. The straight line is a least squares fit to the data points, which represent the average of duplicate determinations.

Kitagawa and Aikawa which employed β-galactosidase as an antigen label (11). Thus, our work clearly demonstrated that laser fluorimetry can enhance the sensitivity of enzyme immunoassay. When we attempted to extend our studies to human serum, our measurements of insulin were obscured by components of serum which interfered with β-galactosidase activity on the solid phase. At about the same time as these studies, Kato *et al.* (12) reported encountering a similar difficulty in their experience with β-galactosidase as an enzyme label.

The limit of insulin detection in the present work, 5 microunits/ml (30 pM), is comparable to that of RIA (13). Moreover, the correlation with RIA measurements is quite good. In work by Yoshioka *et al.* (4), 216 serum samples were analyzed with the same immunoassay reagents we used. They reported a high degree of correlation with RIA; the data fit a line of the form $y = 1.172x - 4.960$, with a coefficient of correlation equal to 0.938. We have replaced the chromogenic substrate with a fluorogenic substitute, and we have verified that the correlation is essentially unaffected by this substitution.

Laser fluorimetry makes possible the detection of the enzyme reaction product DBDA with a high degree of sensitivity. As a result, the immunoassay may be carried out with significant gains in rapidity. This work represents a significant improvement in speed of immunoassay capable of detecting insulin at the 5 microunits/ml level. The total incubation time, 90 min, is at least a factor of two shorter than that of the method of Yoshioka *et al.* (4) which uses the same immunoassay reagents but employs chromogenic detection; moreover, it is shorter than that of commercially available insulin RIAs of comparable sensitivity.[§] In addition, our method displays the potential for further gains in sensitivity and speed of analysis.

Because this method is a sandwich immunoassay, one need not allow the incubation steps to reach a state of near equilibrium as one would in competitive-binding assays. At short incubation times, the concentration of product is linearly proportional to both the original concentration of insulin and the product of all three incubation times (9). In principle, then, this enzyme immunoassay may be carried out in some arbitrarily short time and at some arbitrarily high sensitivity for insulin, both to be determined by the sensitivity with which product can be detected. At present, however, the assay is not limited by our ability to detect DBDA; the signal/noise ratio on our chromatograms is at least 100. The major constraint in both the sensitivity and rapidity of our assay is the background activity caused by nonspecific binding of HRPase-labeled material to the solid phase.

In order to attain the full potential of the method, this nonspecific binding must be decreased. Preliminary experiments indicate that the background activity may be substantially reduced by reagent pretreatment and simple modifications of the assay protocol. Purification of the HRPase-labeled antibody, [for example by affinity chromatography (14, 15)] may further diminish this unwanted effect. The use of high-affinity monoclonal antibodies and modifications of the solid phase are other promising routes.

In conclusion, this work demonstrates that laser fluorimetry may be successfully incorporated into an enzyme immunoassay method. The resulting technique is clinically applicable, and

§ Commercial insulin RIA kits typically call for 3- to 4-hr incubation times to reach sensitivities comparable to the present method. Examples are: the Phadebas Insulin Test RIA Kit (Pharmacia) whose protocol calls for a minimum of 3 hr of incubation time; the Insulin ^{125}I RIA Kit (Serono Laboratories, Braintree, MA), which calls for a 3- to 18-hr incubation; and the Insulin RIA Diagnostic Kit (Immuno Nuclear, Stillwater, MN), which specifies a 4-hr to overnight incubation time.

its high sensitivity allows one to carry out analyses with significant gains in rapidity.

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