

Insulin Radioimmunoassay by the Charcoal-Dextran Technique*

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Summary. The influence exerted by the inactive support (charcoal-dextran) and the proteins in the medium, on the separation of free from antibody-bound ^{125}I -insulin by the charcoal-dextran insulin radioimmunoassay method was studied. The behavior of both fractions in the presence of different concentrations of albumin, dextran, normal guinea pig serum, and normal human serum was observed, and the conditions were established under which maximum charcoal adsorption of free insulin and minimum adsorption of bound insulin occur. The results obtained were compared with those yielded by the double antibody technique. — Under these working conditions it is possible to determine insulin in an unknown serum at 1/20 or 1/10 dilutions with a sensibility of 0.5 μU .

Dosage radio-immunologique de l'insuline au charbon-dextran

Résumé. Nous avons étudié l'influence exercée par le support inactif (charbon-dextran) et par les protéines dans le milieu sur la séparation de l'insuline libre de l'insuline ^{125}I liée aux anticorps par la méthode de dosage radioimmunologique de l'insuline au charbon-dextran. Nous avons observé le comportement des deux fractions en présence de différentes concentrations d'albumine, de dextran, de sérum normal de cobaye et de sérum humain normal, puis nous avons établi les conditions dans lesquelles se produisent d'une part, le maximum d'absorption d'insuline libre par le charbon, d'autre part, le minimum

d'absorption d'insuline liée. Les résultats obtenus ont été comparés avec ceux qui ont été trouvés par la méthode des anticorps doubles. Dans ces conditions de travail, il est possible de déterminer l'insuline dans un sérum inconnu avec des dilutions de 1/20 et 1/10 et avec une sensibilité de 0.5 μU .

Die radioaktive Insulinbestimmung mit Hilfe der Kohle-Dextran-Methode

Zusammenfassung. Es wurde der Einfluß des inaktiven Trägers (Kohle-Dextran) und des Proteinmilieus auf die Trennung von freiem und an Antikörper gebundenem ^{125}I -Insulin auf die radioaktive Insulinbestimmung mit der Kohle-Dextran-Methode untersucht. Das Verhalten beider Fraktionen in Anwesenheit von verschiedenen Konzentrationen von Albumin, Dextran, normalem Meerschweinchenserum und normalem Humanserum wurde studiert und die Bedingungen für eine maximale Adsorption von freiem Insulin an Kohle bei minimaler Adsorption von gebundenem Insulin aufgestellt. Die erhaltenen Ergebnisse wurden mit denen der doppelten Antikörpermethode verglichen. — Unter diesen Arbeitsbedingungen ist es möglich, Insulin in einem unbekanntem Serum einer Verdünnung von 1/20 oder 1/10 mit einer Empfindlichkeit von 0,5 μU zu bestimmen.

Key words: Insulin radioimmunoassay, general variables, charcoal-dextran, double antibody.

One of the techniques for insulin radioimmunoassay, first described by Herbert *et al.* [1], separates antibody-bound antigen from free antigen by means of a dextran-coated charcoal suspension. These authors found that although charcoal adsorbs 99% of the ^{131}I -insulin, it also adsorbs a high percentage of the antibody. The addition of dextran (charcoal-dextran) minimized antibody adsorption without altering insulin uptake, which is still adsorbed in a range of 90 to 95%.

However, Keane *et al.* [2] believe that there is a 15% adsorption of gamma globulin which always takes place with this method, and which is impossible to avoid. Therefore, values obtained for plasma insulin by means of the charcoal-dextran technique are subject to an error due to the fact that separation of antibody-bound antigen from free antigen is made through a procedure which implies a certain number of inter-

actions between the inactive support and the proteins in the medium.

In the present work, we carried out a study of the variables involved in the technique, in order to achieve maximum adsorption of free insulin and minimum adsorption of bound hormone, both of which imply optimum effectiveness of the technic under study.

Methods

Reagents were prepared according to the original description by Herbert *et al.* [1]. Since it is known that in this method charcoal adsorption varies with the ionic strength of the buffer, pH, temperature, etc., these parameters were kept at constant values (see Appendix).

Interactions of the inactive support (charcoal-dextran) with insulin and with the insulin-antiserum complex were studied while parameters were individually modified.

Radioactive porcine insulin and guinea pig anti-

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porcine insulin serum (AIS) were incubated for 96 h at 4°C. The value found for each parameter resulted from three experimental and three blank tubes¹. Standard error was calculated in order to determine the precision of the method employed. Since all other ingredients remained constant, whatever changes appeared were necessarily due to the parameter altered in each case.

In the present study we were not interested in the mode of interaction of each variable considered, but rather in its final result: the separation of free from bound antigen fractions.

Study of Interactions

Independent studies on the influence exerted by albumin, dextran, and normal guinea pig serum (NGPS) concentrations on the separation of free from bound insulin were performed.

While variations in the albumin concentration (150, 350 or 500 mg%) (Fig. 1) did not alter antigen adsorption by charcoal, the percentage of radioactivity of the insulin plus antibody fraction increased. With growing dextran concentrations, (500, 750 or 1000 mg%) the adsorption of free insulin became higher, and the percentage of radioactivity of the insulin plus antibody fraction increased slightly. The addition of NGPS to the buffer up to a final concentration of 1/100 had little effect on the antigen adsorption, but raised further the radioactivity of the insulin plus anti-insulin serum fraction. Fig. 1 also shows that at a 1/10 dilution of NGPS, insulin uptake by charcoal is reduced, without any concomitant increase of the insulin-AIS complex.

On the basis of these observations, we undertook a study of these variables while the remaining parameters were varied in different combinations. As a result of the findings mentioned above, we decided to keep the albumin concentration at a constant value of 500 mg%.

Dextran variable — The behavior of free and anti-

1 The following formula summarizes the analysis performed:

$$\% \text{ of insulin plus antibody radioactivity in the supernatant} = \frac{\bar{x}E - \bar{x}B}{T - \bar{x}B} \cdot 100 \quad (1)$$

$\bar{x}E$ = c.p.m., experimental

$\bar{x}B$ = c.p.m., blank

T = total counts

As we were working with a quotient, standard error was calculated by the following formula:

$$\widehat{\text{VAR}}\left(\frac{a}{b}\right) = \frac{1}{b^2} \left(\widehat{\text{VAR}}(a) + \frac{a^2}{b^2} \widehat{\text{VAR}}(b) \right) \quad (2)$$

$$\text{S.E.}\left(\frac{a}{b}\right) = \sqrt{\widehat{\text{VAR}}\left(\frac{a}{b}\right)} \quad \text{this result being then multiplied by 100 for percentile expression.}$$

Formula (2) is used to calculate the approximate variance of a quotient of independent random variables. This approximation is highly satisfactory when the coefficient of variation of the denominator is small (as in the present case).

body-bound insulin as the dextran concentration varied in different situations was studied (Fig. 2).

Variations in dextran concentration did not alter the interaction between insulin and charcoal, but they

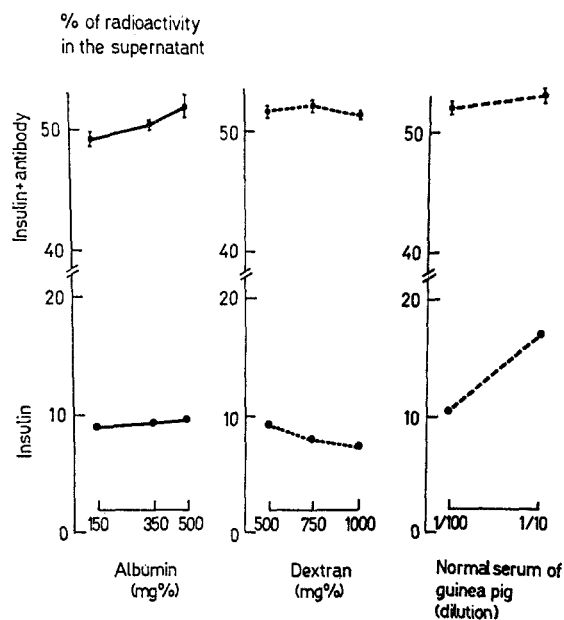


Fig. 1. General study of variables. Individual behavior of albumin, dextran and normal guinea-pig serum. — Albumin; Dextran; - - - - Normal Guinea-Pig Serum; $\bar{x} \pm$ S.E.

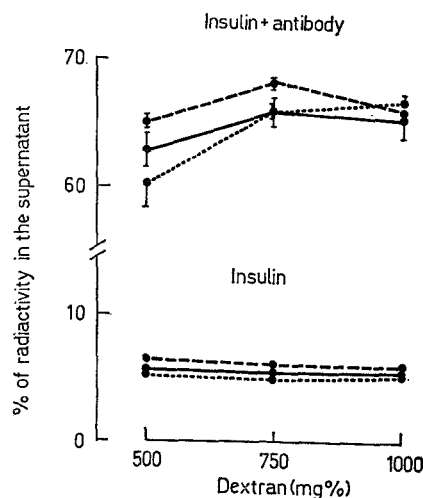


Fig. 2. Dextran variable. Behavior of this variable with different combinations of the remaining variables. — Albumin (500 mg%); Albumin (500 mg%) and Normal Guinea-Pig Serum (1%); - - - - Albumin (500 mg%) and Normal Guinea-Pig Serum (1/100); - · - · Albumin (500 mg%) and Normal Guinea-Pig Serum (1/10); $\bar{x} \pm$ S.E.

raised to a varying degree the percentage of insulin plus antibody radioactivity. The highest point was attained while working with a 1/100 solution of NGPS in buffer containing 500 mg% human albumin, the charcoal being associated with 750 mg% dextran.

Validity of the Method

The standard curve and the sera were determined according to the scheme shown on Table 1. After 96 h incubation at 4°C, 0.5 ml of charcoal-dextran (main-

Table 1. *Preparation of the standard curve*

Reagents	Standard curve ml	Blank ml
Albumin buffer	0.7	0.9
Crystalline insulin ^a	0.1	0.0
¹²⁵ I-insulin ^b	0.1	0.1
Anti-insulin serum ^c	0.1	0.0

Preparation of the unknown

Reagents	Experimental ^d ml	Blank ^d ml
Albumin buffer	0.7	0.8
Plasma or serum	0.1	0.1
¹²⁵ I-insulin ^b	0.1	0.1
Anti-insulin serum ^c	0.1	0.0

^a Insulin concentrations ($\mu\text{U}/0.1$ ml): 0, 2.5, 5.0, 10.0, 20.0, and 40.0.

^b Solution containing 2.0 $\mu\text{U}/0.1$ ml.

^c AIS dilution is 1/15500; therefore its final dilution is 1/155000.

^d All determinations are done in duplicate.

Table 2. *Insulin assays on the same serum — reproducibility with the course of time^a*

Date	29-10-70	2-12-70	6-12-70	28-12-70	30-12-70	6-1-71	7-1-71	$\bar{x} \pm \text{S.E.}$
Insulin $\mu\text{U}/\text{ml}$	8.0	10.0	10.7	10.5	8.8	11.7	10.7	10.05 \pm 0.15

^a Individual determinations were made with 0.05 to 0.5 ml serum.

Table 3. *Insulin assays on the same serum — Reproducibility at different dilutions*

Serum dilution	1/20	1/10	1/5	1/3.3	1/2.5	1/2
Insulin ($\mu\text{U}/\text{ml}$) $\bar{x} \pm \text{S.E.}$	11.2 \pm 1.58	10.2 \pm 0.95	8.6 \pm 0.68	8.4 \pm 1.36	8.5 \pm 0.5	10 \pm 1.16
<i>n</i>	3	5	4	3	3	3

Table 4. *Recovery of porcine insulin added to human serum*

μU insulin recovered	charcoal-dextran technique	Serum volumes	μU porcine insulin added				
			2.5	5	10	20	40
		0.1 ml	2.5	5.0	8.7	18.4	43.7
			0.3 ml	2.8	5.0	8.7	20.0
	Double-antibody technique	0.1 ml	1.5	3.8	9.0	21.4	40.2
			0.2 ml	2.8	4.6	10.0	21.8

tained in suspension by constant shaking) were added to each tube, which was then agitated with a vortex mixer and centrifuged at room temperature for 20 min at 2000 rpm. The supernatant of each tube was then counted in a scintillation counter. The graph of the standard curve was plotted according to the original Herbert technique (Co/Cx). Under these con-

ditions, the assay of insulin present in one sample of serum shows little dispersion in the course of two months (Table 2). Tables 3 and 4 illustrate reproducibility of results with different volumes of serum, and recovery of porcine insulin added to human serum, respectively; as well as a comparison of values obtained by the charcoal-dextran and double antibody techniques.

Fig. 3 shows the correlation between insulin values assayed by the charcoal-dextran and double antibody methods. A series of sera were assayed by both techniques for that purpose. The correlation found was $r: 0.98$ ($P < 0.001$).

Discussion

Ideal conditions in the charcoal-dextran technique would be complete adsorption of free insulin and no adsorption at all of antibody. We believe our method is a simple way of approaching this ideal.

Under the working conditions described above, free insulin is almost completely adsorbed, the values in the supernatant being only 5 to 10%. After incubation this figure may rise to 15% if determinations are made on large volumes of serum (0.5 ml). Serum exerts no

influence on adsorption of free insulin nor on separation of fractions when volumes below 0.2 ml are used.

Separation of free from bound insulin also depends on the quality of the labeled insulin. Ageing of the insulin (over 20 days after being prepared) alters the results obtained by the charcoal-dextran technique for reasons still unknown.

Under the conditions described above, the good separation of free from antiserum-bound insulin allows us to carry out assays on unknown sera at 1/20 or 1/10 dilutions with a sensitivity of 0.5 μ U.

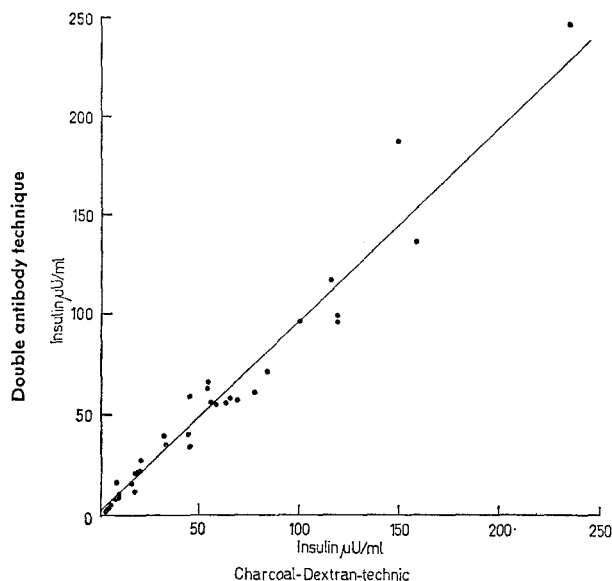


Fig. 3. Linear correlation between the charcoal-dextran technique and the double-antibody technique; $r=0.98$; $y = 0.967x + 0.615$

Appendix

Buffer, albumin buffer, charcoal, dextran, and charcoal-dextran were prepared according to Herbert *et al.* [1] with the following chemicals: Charcoal (activated charcoal, neutral, Merck (R)); Dextran (dextran, MW

80 000, Pharmacia (R)); Human Albumin (Behringwerke (R)).

Porcine 125 I-Insulin (Hoechst glucagon-free porcine insulin, recrystallized seven times) with specific activity 150 to 200 mC/mg was provided by the Comisión Nacional de Energía Atómica de la República Argentina (C.N.E.A.), Labeled Molecules Division.

The standard used was porcine insulin (Hoechst) 27 U/mg.

AI5 was prepared by inoculation of 15 guinea pigs with porcine insulin in Freund's adjuvant. Each animal received 0.5 mg insulin in 0.5 ml saline plus 0.5 ml Freund's adjuvant in monthly applications for five months. One ml epinephrine (1/100 000) was administered prior to each inoculation. One week after the last inoculation the guinea pigs were exsanguinated by cardiac puncture.

The double antibody technique was followed according to Morgan and Lazarow, with slight modifications.

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