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Hook effect in radioligand assay for Anti Glutamic Acid Decarboxylase (Anti-GAD65). Influence of temperature and physicochemical interpretation

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RESUMEN

Objetivo: El análisis por radioligando es uno de los métodos principales utilizados en la determinación analítica del Anti-GAD_{65}. Se ha estudiado la influencia de la temperatura sobre las gráficas de calibración obtenidas por dicha técnica.
Material y Métodos: Usamos un kit comercial para Anti-GAD_{65} y un contador gamma. Los resultados son analizados mediante el programa Statistica.
Resultados y Discusión: Las actividades ligadas al anticuerpo aumentan con la temperatura. Se observa una disminución de la actividad para altas concentraciones atribuible al llamado "efecto anzuelo". Se propone un sencillo modelo fisicoquímico que justifica satisfactoriamente los resultados

PALABRAS CLAVE: Curvas de calibración. Anti-GAD_{65}. Efecto anzuelo

ABSTRACT

Background: Radioligand assay is one of the principal methods used for analytical determination of the Anti-GAD_{65} concentration. We studied the influence of temperature on the calibration curves obtained by such a method
Matherial and Methods: We used a commercially available RIA kit for Anti-GAD_{65} and a gamma counter. Data was analyzed using Statistica software.
Results and Discusion: Activities bound to the antibody increase with temperature. There was a decrease in activity for high concentrations attributable to the "hook effect". We propose a simple physicochemical model that justifies satisfactorily the results

KEYWORDS: Calibration curves. Anti-GAD65. Hook effect

INTRODUCTION

The glutamic acid decarboxylase (GAD) is the enzyme that catalyzes the conversion of

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glutamate to gamma amino butyric acid (GABA) and has been identified in two isoforms of molecular masses 65,000 (GAD65) and 67,000 (GAD67). Autoantibodies against GAD 65 are present in 70-80% of diagnosed patients with type 1 mellitus diabetes (insulin-dependent diabetes) and those with diabetes type 2 (non insulin dependent diabetes) that evolve into type 1 diabetes or latent autoimmune diabetes in adults (LADA). The presence of auto antibodies against GAD65 in serum of such patients is a sensitive and specific marker for future insulin dependency.

Immunoradiometric analysis (IRMA) is a modification of the radioimmunoassay (RIA)1 due to Miles and Hales2, who submitted some criticisms of the famous regression of calibration curves in RIA. In the IRMA antigen present in the sample (analyte) reacts with a generally immobilized antibody and with a second radiolabelled antibody (tracer). Then measure the radioactivity of the immobilized antibody-antigen complex antibody-marked ("sandwich"), which naturally increases with the concentration of antigen. As shown, the fundamental difference between RIA and IRMA is that the radioactive tracer is antigen in the first and antibody in the second, with the already pointed result that the calibration graphs are decreasing in the first case and increasing in the second. It has been described in some cases a decrease of the curves at high concentrations called "hook effect", which is interpreted as a blockade affecting the labelled antibody binding caused by excessive analyte.

For the determination of serum, anti-GAD65 it is used a modification of IRMA, which uses a suspension of Protein A (ligand-specific anti-GAD65) in place of the immobilized antibody.

OBJECTIVE

In precedent papers4-9 we have studied the influence of some physicochemical factors on the kinetics of the reactions involved in immunoanalytical techniques, as well as a theoretical justification of the equation of four parameters from the model of ligand-receptor10. In this case we intend to analyze the influence of temperature on the calibration graphs obtained in the case of the Anti GAD65, trying to justify and give meaning to the physiochemical found variations.

MATERIAL AND METHODS

Reagents:

- 125I-GAD65 Solution (<0.05 MBq)
- Protein A-suspension
- Anti-GAD65 standard solutions. Concentrations: 0.1- 1.0- 3.0- 30- 300 U/mL

All reagents used were included in the kit CentAK ® anti-GAD65, supplied by MEDIPAN GMBH, Berlin

Instruments:

Gammamaster LKB Automatic Gamma Counter

Experimental Procedure:

20 µL of 125I-GAD65 and 50 µL of Anti-GAD65standard solution were added to each tube and incubate for 1 hour. Then added 50 µL of Protein A-suspension and incubate for 2 hours

for 1 hour, after that added 1 mL of buffer and the tubes were centrifuged for 20 minutes at 1500 g. Finally, supernatant was removed by decantation and counted radioactivity in the gamma counter. The experiments were performed in duplicate at temperatures of 4, 25, 30 and 37 ºC (277, 298, 303 and 310 K)

Analysis of results:

We used the program Statistica (Copyright© StatSoft, Inc:, 1993) with specific equations for nonlinear regression. To choose between the various equations is used the GraphPad Software Quick Calcs11, that used Akaike's Information Corrected Criterion (AICC), expressed as AICC = N ·ln (SS / N) +2 P +2· P (P +1) / (N - P-1) where N is the number of points, SS the sum of squares of residuals and P the number of parameters in the equation, being selected the model giving a lower AICC. It is also used ANOVA (F test).

GENERAL MODEL

In the first stage of the studied process the Anti-GAD65 (A, Analyte) reacts with 125I-GAD65 (G, Tracer) to give a Complex (AG):

$$\mathbf{A + G \leftrightarrow AG} \qquad \mathbf{K = (AG)/(A)(G)} \qquad \mathbf{(1)}$$

The complex can accept a second molecule A:

$$\mathbf{AG + A \leftrightarrow A_2G} \qquad \mathbf{K' = (A_2G)/(AG)(A)} \qquad \mathbf{(2)}$$

In the second stage, the AG complex binds to the protein P:

$$\mathbf{P + AG \leftrightarrow PAG\ (sandwich\ radiactivo)} \qquad \mathbf{K'' = (PAG)/(P)(AG)} \qquad \mathbf{(3)}$$

The conservation of G requires:

$$\mathbf{G_0 = (G) + (AG) + (A_2G) + (PAG)} \qquad \mathbf{(4)}$$

If one assumes a large excess of P, the formed AG complex becomes fully in the PAG radioactive sandwich. A2G cannot link to P. Therefore, if you despise (AG) and introduce the values of (G) (A2G) and (PAG) given by (1), (2) and (3), equation (4) becomes:

$$\mathbf{G_0 = (AG)/K(A) + K'(AG)(A) + K''(AG)(P) = (AG)[1/K(A) + K'(A) + K''(P)]} \qquad \mathbf{(5)}$$

From (3) and (5) is obtained:

$$\mathbf{(PAG) = KK''(P)G_0(A)/[1+ KK''(P)(A)(1+ KK'(A)/K''(P))]} \qquad \mathbf{(6)}$$

The radioactive sandwich is separated by centrifugation and its activity is measured in cpm. Activity "y" (cpm) measured should be a linear function of the concentration of PAG:

$$\mathbf{y = a + m(PAG)} \qquad \mathbf{(7)}$$

Substituting in (6) the value of (CPA) given by (5), becomes:

$$\mathbf{y = a+ mKK''(P)G_0(A)/[1+ KK''(P)(A)(1+ K'(A)/K''(P)]} \qquad \mathbf{(8)}$$

The equilibrium constant is related to temperature through the equation of van t'Hoff13:

$$\mathbf{K = C\cdot exp(-\Delta H^0/RT)} \qquad \mathbf{(9)}$$

Introducing the value of K given by (9) in (8) grouping the constants and representing the concentration of analyte (A) for x, is:

$$y = a+b\cdot x\cdot \exp(-c/T)/(1+d\cdot x\cdot \exp(-c/T)\cdot(1+e\cdot x)) \qquad (10)$$

RESULTS AND DISCUSSION

Table 1 shows the results for the calibration curve at different temperatures.

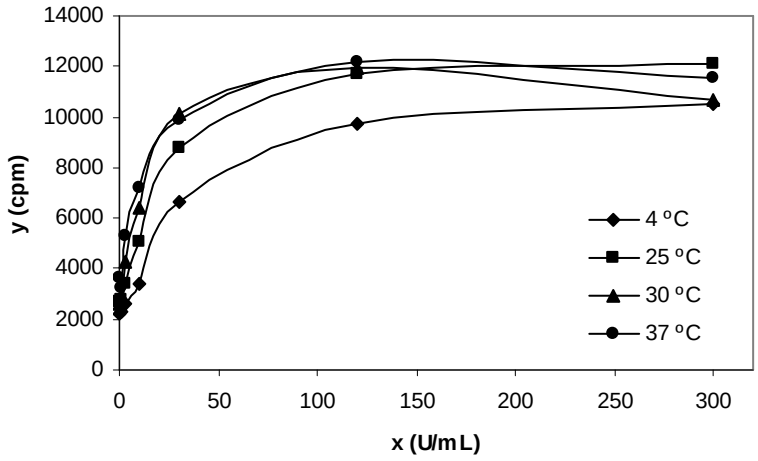
x = concentration patterns in U / mL
y = activity in counts per minute (cpm) obtained for each pattern at the temperatures indicated.

Table 1. y values (cpm) obtained for different concentrations (x) and temperatures

	T = 277 K		T = 298 K		T = 303 K		T = 310 K	
x = 0 U/mL	2224,5	2227,5	2599,5	2815,5	2680,5	2608,5	3568,5	3706,5
x = 1 U/mL	2245,5	2401,5	2866,5	2737,5	2974,5	2911,5	3229,5	3217,5
x = 3 U/mL	2582,8	2639,2	3346,0	3462,1	4251,9	4242,0	5264,0	5313,8
x = 10 U/mL	3445,8	3415,7	5054,9	5031,7	6233,0	6584,7	7288,2	7119,0
x = 30 U/mL	6530,9	6687,3	8963,7	8614,2	10168,8	10125,2	9695,9	10008,7
x = 120 U/mL	9566,1	9902,2	11769,3	11639,5	12108,8	11795,9	12554,7	11875,8
x = 300 U/mL	10643,1	10351,8	12137,5	11997,0	10766,5	10523,1	11479,4	11654,2

The graphical representation of the values contained in Table 1 is shown in Figure 1. The values represented correspond to the average results for each temperature.

Figure 1. Calibration curves obtained for different temperatures



In Figure 1 shows a tendency to decrease and for high values of x, particularly noticeable at

temperatures of 30 and 37 ºC. This is defined as the "hook effect" and is attributed to the inhibition of the binding protein-analyte-tracer caused by an excess of analyte molecules. For this reason, the general pattern includes the formation of the species A2G, which would not join P, because the binding site is occupied by the second molecule A. Another aspect to consider is the possibility of cooperation phenomena taking place in the analyte-tracer and analyte-protein joints. This would result in the appearance of an exponent affecting x in the equations. Accordingly, the equations in Table 2 are proposed for the adjustment of the results set out. All are variants of equation (10), whose meaning includes the cooperative (12) or non cooperative nature of the analyte-tracer binding, the influence of temperature and hook effect.

Table 2. Equations used to adjust the results

Ecuación	Significado
$y = a+b\cdot x\cdot \exp(c/T)/(1+d\cdot x\cdot \exp(c/T))$ **(01)**	Unión no cooperativa dependiente de la temperatura
$y = a+b\cdot x\cdot \exp(c/T)/(1+d\cdot x\cdot \exp(c/T)\cdot(1+e\cdot x))$ **(02)**	Unión no cooperativa dependiente de la temperatura con efecto anzuelo
$y = a+b\cdot x\cdot \exp(c/T)/(1+d\cdot x\cdot \exp(c/T)\cdot(1+e\cdot x\cdot \exp(f/T)))$ **(03)**	Unión no cooperativa con efecto anzuelo. Unión y efecto dependientes de la temperatura
$y = a+b\cdot(x^c)\exp(d/T)/(1+e\cdot(x^c)\cdot \exp(d/T)\cdot(1+f\cdot x^c))$ **(04)**	Unión cooperativa dependiente de la temperatura con efecto anzuelo
$y = a+b\cdot(x^c)\cdot \exp(d/T)/(1+e\cdot(x^c)\cdot \exp(d/T)\cdot(1+f\cdot(x^c)\cdot \exp(g/T)))$ **(05)**	Unión cooperativa con efecto anzuelo. Unión y efecto dependientes de la temperatura

More satisfactory adjustment taking into account the criteria AICC and F test was obtained for the equation (02):

$$y = a+b\cdot x\cdot \exp(-c/T)/(1+d\cdot x\cdot \exp(-c/T)\cdot(1+e\cdot x))$$

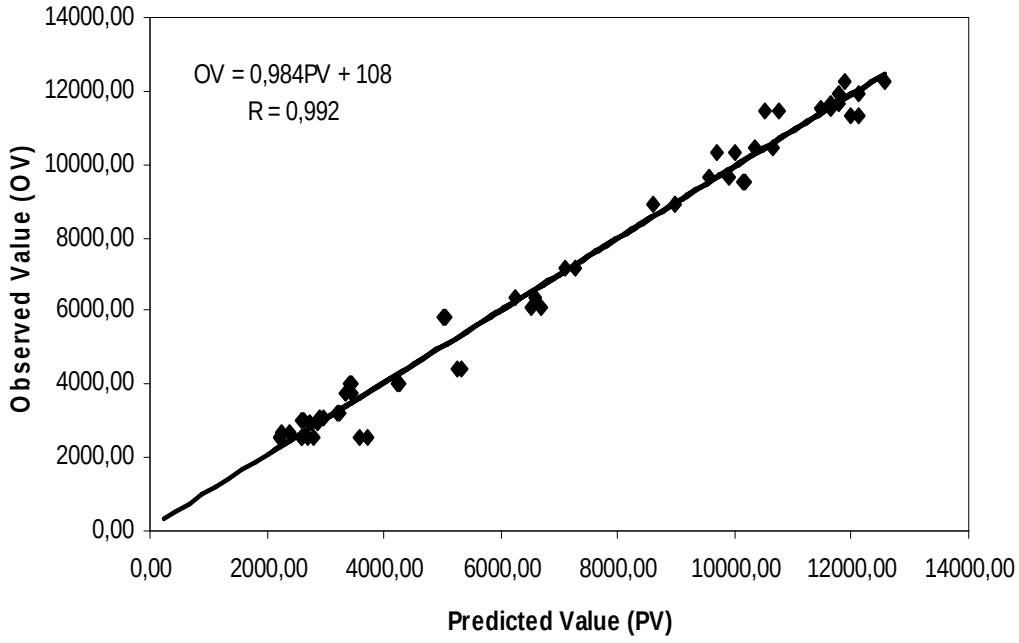
Whose parameters, correlation coefficient (r) and residual sum of squares (ss) are:

$$a= 2526 \quad b=1.977\cdot 10^{8} \quad c=3866 \quad d=16153 \quad e=0.001006 \quad r=0.992 \quad ss=1.208\cdot 10^{7}$$

The equation (02) is identical to equation (10), and contains the effect of temperature, including the exponential terms, and the hook effect in (1 + e·x). It is noteworthy that the latter term is relevant only to high concentrations.

Figure 2 shows the correlation between the observed values and the predicted ones by equation 02.

Figure 2. y values observed in experiments (Table 1) vs. values predicted for equation 02



CONCLUSIÓN

The general concepts related to chemical equilibrium and the influence of temperature on it has been applied to obtain five equations potentially applicable to the experimental calibration graphs obtained for the Anti-GAD65 presenting hook effect. By applying statistical criteria an equation was chosen that fits well with the results and allows to:

- Justify the influence of temperature on the binding of anti-GAD65 and GAD65-125I to calculate the $\Delta H0$ value as: $\Delta H0 = 8.31 \cdot 3866 = 32126$ J mol-1

- Justify the hook effect by the formation of a complex containing two molecules of anti-GAD65 -Establish the absence of cooperation phenomena.

- From the practical point of view, it is inadvisable to perform the technique at temperatures above the atmosphere, as this increases the hook effect.

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