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Calculation Of Results In Immunoanalysis: Modification Of The Four Parameters Equation Showing The Tracer Concentration And The Temperature Influence

R.Díez Montoro[1], M.T.Salabert Salvador[1], J.L.Moreno Frigols*[1,2]
[1]Department of Physical Chemistry, Faculty of Pharmacy, Valencia, (SPAIN)
[2]Radiopharmacy Unit, Radioisotope Service, Valencia Hospital Clinico, (SPAIN)
Tel.: +34963543289 ; Fax : +34963544892
E-mail: jose.l.moreno@uv.es
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ABSTRACT

The activity in the equilibrium after the displacement of ^{125}I-T4 and ^{125}I-digoxin by the T4 and digoxin is measured. The results adjust to a variant of the four parameters equation that includes the influence of the concentration of tracer and the temperature. Such variant is used to predict the calibration curves used in the determinations of these substances by radioimmunoassay.

KEYWORDS

Four parameters model;
Tracer concentration;
Temperature.

INTRODUCTION

Radioimmunoassay(RIA) is a competitive technique in which the antigen molecule to be determined(Ag) competes with a radioactive tracer(labelled antigen: Ag*) in order to bind to a specific antibody(Ab) that reacts to both antigens until equilibrium is reached for both immunocomplexes-the radioactive one and the non-radioactive or 'cold' one- to coexist[1]:

Ag+Ab+Ag*⇔(Ag—Ab)+(Ag—Ab)*

By keeping tracer(Ag*) and antibody(Ab) quantities constant, the higher or lower proportion in the immunocomplexes formed will solely depend on the amount of cold antigen(Ag) in the sample to be analysed. If the tracer behaves similarly when bound and not bound, then the separation of the bound and free fractions is essential. In the technique observed in this research, separation is accomplished by fixation on a second antibody coated on the tube wall

The determination of the samples concentration is made by means of its reading in the corresponding curve of calibration, which is always decreasing, since a great amount of substance in the sample will tend to saturate the antibody, reason why the its activity will be low. If the amount is small, will happen the opposite.

Almost all the analyses with great amounts of samples in clinical chemistry use the calculations of automated results, what increase the yield by eliminating the manual process, lowering the error of the observer and diminishing the presence of the trascription errors. The calibration curve, based in the points obtained by measurement of standard samples, needs an adjustment whose quality conditions the exactitude of the obtained result.

The nonlinear model most used in RIA is the logistic model of the four parameters[2], that calculates the results by application of eq 1:

$$y=D+(A-D)/(1+(x/C)^B) \quad (1)$$

y=Activity(cpm) bound to antibody, x=Concentration of the sample, D=non specific binding (NSB). It agrees with the activity corresponding to a sample with infinite concentration, A=maximum activity, corresponding to a sample with zero concentration. C=concentration of a sample to which it corresponds y=(A+D)/2, B=parameter that depends on the process characteristics

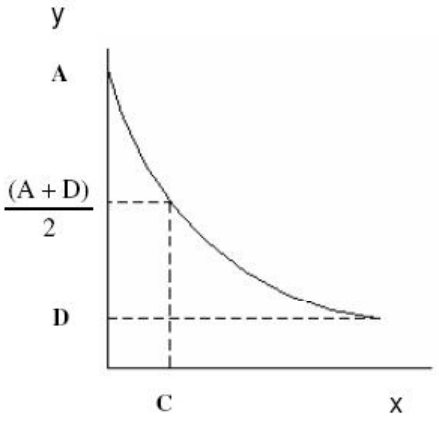

Figure 1 :Plot of four parameters model

This model has been successful because it simulates the physical characteristics of radioimmunoassay systems. However, until the widespread use of computers, eq.1 was not used directly. Its use results in a linearization by means of the input of a new variable Y defined as:

$$Y=(y-D)/(A-D)$$

From which it is obtained:

$$\ln (Y/(1-Y))=\text{logit}(Y)=\ln (c^b) - b\cdot\ln x \quad (2)$$

According to eq 2 a plot of logit (Y) versus ln x must be linear, which facilitates the graphical calculation.

In our previous research[3-5] different features relative to the kinetics of antigen-antibody reactions used by immunoanalytical techniques were analysed. Theoretical models were prepared for an application to the immunocomplex formation processes produced in RIA (Radioimmunoassay) and IRMA(Immunoradiometric assay). We also studied the fitting of equilibrium results to several pre-set equations, and a mathematical deduction that justifies them theoretically was obtained.

We seek to develop a general model applicable to competitive immunoassays including the influence of several variables. Its validation comes from the fitting of the results to the equations obtained. The models of Stenberg, Rabany, and those of Zuber refer to the formation of the radioactive immunocomplex but not to the competition between labelled and unlabelled antigen, which is the basis of competitive immunoassays. Such models do not determine the influence of the variables studied here.

In line with the above research, this paper aims to:

- Obtain equations showing the influence of the tracer concentration(m) and the temperature(T) on the equation parameters.
- Predict the calibration curves based on such variables
- Make the results potentially applicable to the design of immunoanalytical techniques

Theoretical model

The four parameters equation is:

$$y=D+(A-D)/(1+(x/C)^B) \quad (1)$$

Term 'D' represents the activity corresponding to a sample of infinite concentration. Supposing that this term is directly proportional to the activity of tracer (m), it is had:

$$D=d\cdot m \quad (3)$$

The term 'A' corresponds to the maximum activity bound to the antibody. Therefore, also it can hope that it is directly proportional a m. In addition, it must contain the equilibrium constant corresponding to the formation of inmunocomplex. Expressing this constant in function of the temperature by means of the van't Hoff equation[6], it results:

$$A = a\cdot m\cdot\exp(e/T) \quad (4)$$

It seems reasonable to suppose that the quotient x/C includes the relation between the concentrations of analyte(x) and tracer(m). The exponent 'B' is related to the degree of cooperativity of the process. An exponent different to B can be assigned to m, with which C is in the form:

$$C=c\cdot m^f \quad (5)$$

Introducing (3), (4) and (5) in (1) it is:

$$y = d\cdot m+(a\cdot m\cdot\exp(e/T)-d\cdot m)/(1+(x/(c\cdot m^f))^b) \quad (6)$$

Full Paper

Assuming d≈0, f=0, eq 6 becomes:

$$y = (a \cdot m \cdot \exp(e/T))/(1+(x/c)^b) \quad (7)$$

EXPERIMENTAL

Instruments

ILKB gammamaster automatic gamma counter.

Reagents

PT=Plastic tubes with rabbit anti-digoxin immuno globulin or rabbit anti-T4(Anti-Thyroxine) immuno globulin immobilized to the inside wall, DM=solution of ^{125}I-labelled substance(Digoxin or T4) in a protein-based buffer, DQ=digoxin or T4 standard solution

These reagents were included in the cot-a-count digoxin kit or cot-a-count T4 provided by DPC

Experimental procedure

Several tube series were prepared as per the TABLE 1

TABLE 1: Preparation of tubes

PT	1	2	3	4-10
DM(mL)	0.25	0.5	0.75	1
H_2O(mL)	0.75	0.5	0.25	0

They were left to react overnight. The next day, they were decanted and washed with 2ml distilled water. Activity was measured using a gamma counter.

Solutions were as per the TABLE 2

TABLE 2 : Composition of solutions

Solution	1	2	3	4
DQ(μL)	25	50	75	100
H_2O(mL)	7.975	7.950	7.925	7.900

Reaction kinetics were studied by placing 1mL of the previously mentioned solutions in the plastic coated tubes and letting them react at 24hours, this being considered infinite time. Each tube was washed to remove any unbound labelled antibody. Any radioactivity present in the remaining bound labelled antibody was then measured using a gamma counter.

For T4, 10 experiments were performed, arranged as follows:

- **Experiments 1T4, 2T4, 3T4, 4T4:** Study of the influence of T4 concentration(x) upon the global reaction using tubes 4,5,6,7 and solutions 1,2,3,4
- **Experiments 5T4, 6T4, 7T4:** Study of the influence of ^{125}I- T4 concentration(m) upon the global reaction using tubes 1,2,3 and solution 4.
- **Experiments 8T4, 9T4, 10T4:** Study of the influence of temperature(T) using tubes 8,9,10 and solution 4.

For digoxin a parallel series was performed, following the same procedure experimental. The experiments for this series are represented as 1Dig-10Dig

Data analysis

The Statistica programme(Copyright © StatSoft, Inc:, 1993) was used with specific non-linear regression equations. As the statistical criterion that allows a choice from different equations, SS and corrected Akaike's information criterion(AIC_c) was used, expressed as

$$AIC_C = N \cdot In\left(\frac{SS}{N}\right) + 2P + \frac{2p(p+1)}{N-p-1}$$

where N is the number of points, SS the addition of residual squares, and p the number of parameters in the equation. The fitting with the lowest AIC_c must be chosen. In order to distinguish equations from monoexponential and biexponential models, AICc and ANOVA(F test) were used[7,8].

RESULTS AND DISCUSSION

T4 results

The results of experiments 1T4-10T4 are shown in TABLE 3

TABLE 3 : y values for experiments 1T4-10T4

Experiment	y	x	m	T
1T4	13578.0	25	100	318
2T4	126901.5	50	100	318
3T4	7667.8	75	100	318
4T4	5208.4	100	100	318
5T4	1745.4	100	25	318
6T4	4196.0	100	50	318
7T4	5826.1	100	75	318
8T4	7393.3	100	100	310
9T4	11542.4	100	100	305
10T4	16672.5	100	100	299

The results of TABLE 3 are fitted to the equation

$$y = d \cdot m + (a \cdot m \cdot \exp(e/T) - d \cdot m)/(1+(x/(c \cdot m^f))^b) \quad (6a)$$

Its parameters, coefficient of correlation (r) and sum of squares of residuals (ss) are: d=34.6 $a=6.41 \cdot 10^{-10}$, e=8299 c=391, f=-0.365, b=4.47 r=0.991, ss= $3.62 \cdot 10^6$

The eq 6a is identical to eq 6. The adjustment of the data to eq 6a can be seen in figure 2

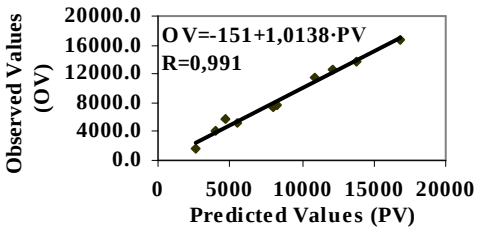

Figure 2 : y values observed in experiments 1T4–10T4(TABLE 4) vs. values predicted for equation (6a)

Conclusion 1-The equation (6a) predicts the values of y with a mean deviation that can be estimated as: $(3.62\cdot10^6/10)^{1/2}$= 602cpm. This represents a relative deviation of about 9%

Digoxin results

The results of experiments 1Dig-10Dig are shown in TABLE 4:

TABLE 4 : y values for experiments 1Dig-10Dig

Experiment	y	x	m	T
1Dig	9168,3	25	100	318
2Dig	8888,3	50	100	318
3Dig	7740,6	75	100	318
4Dig	6058,6	100	100	318
5Dig	1650,6	100	25	318
6Dig	3087,5	100	50	318
7Dig	4564,8	100	75	318
8Dig	6961,9	100	100	310
9Dig	8829,4	100	100	305
10Dig	10350,6	100	100	299

The results of TABLE 4 are fitted to the equation

$$y = (a\cdot m\cdot\exp(e/T))/(1+(x/c)^b) \qquad (7a)$$

Its parameters, coefficient of correlation (r) and sum of squares of residuals (ss) are:a=0.01609, e=2752, c=117.6, b=3.72, r=0.998, $ss=3.43\cdot10^5$

The eq(7a) is identical to eq(7). The adjustment of the data to eq(7a)can be seen in figure 3.

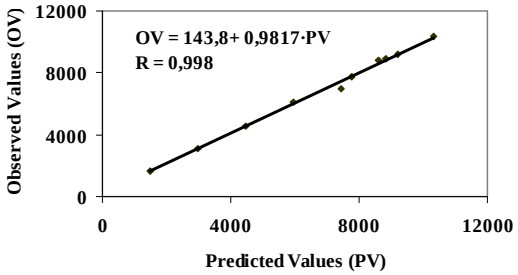

Figure 3 : y values observed in experiments 1Dig-10Dig (TABLE 4) vs. values predicted for equation (7[a])

Conclusion 2-The equation 7a predicts the values of y with a mean deviation that can be estimated as: $(3.43\cdot10^5/10)^{1/2}$= 185cpm. This represents a relative deviation of about 3%

Prediction of calibration curves

y values are calculated by aplication of the eq (6a) and(7a) for different x values as is plotted in the calibration curves of RIA. Figures 4,5,6,7 represent such curves and they show the influence of the studied variables

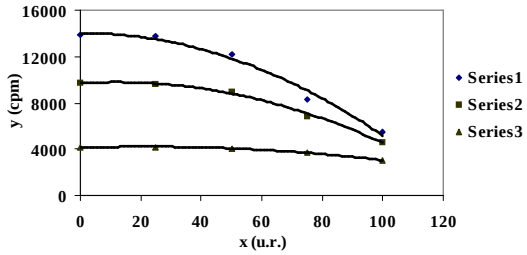

Figure 4 : Calibration curve calculated with the equation 6a for different ^{125}I-T4 concentrations (m). Series 1: m=100 u.r., Series 2: m=70u.r., Series 3: m=30u.r

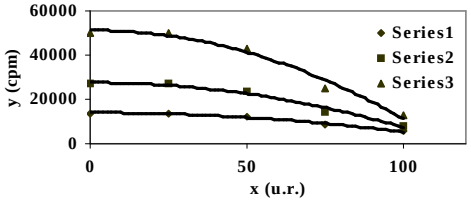

Figure 5 : Calibration curve calculated with the equation (6a) for T4 at different temperatures. Series 1: T=45^{0}C, Series 2: T=37^{0}C, Series 3: T 30^{0}C

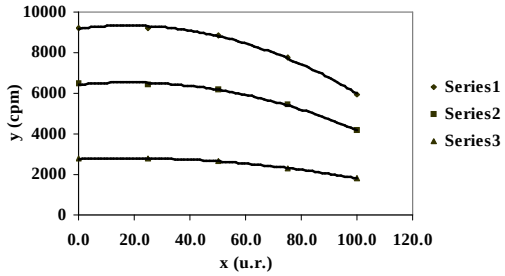

Figure 6 : Calibration curve calculated with the equation (7a) for different ^{125}I-digoxin concentrations (m). Series 1: m=100u.r, Series 2:m=70u.r, Series 3:m=30u.r.

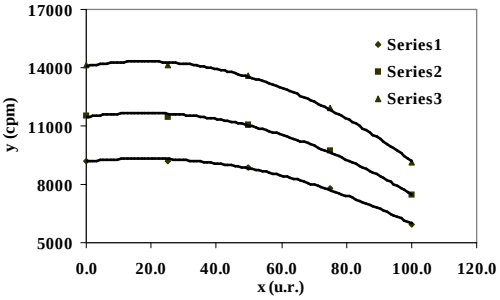

Figure 7 : Calibration curve calculated with the equation (7a) for digoxin at different temperatures. Series 1: T=45^{0}C., Series 2: T=37^{0}C., Series 3: T 30^{0}C

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- **Conclusion 3-The curves predicted by the equations (6a) and (7a) show greater slope for high values of M. Hence, the sensitivity of the method is greater if high concentrations of tracer are used**
- **Conclusion 4-The curves predicted by equations (6a) and (7a) exhibit larger slope for lower temperatures. Therefore, the sensitivity of the method is greater if the incubation is carried out at room temperature. Moreover, it will be necessary to increase the incubation time, because the process becomes slower**
- **Conclusion 5-The activity corresponding to concentration zero increases at low temperatures. It suggests that the displacement of tracer by the analyte is an endothermic process in both cases studied**

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