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Immunocomplex formation and radioactive antigen displacement in C-peptide RIA

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ABSTRACT

The double antibody radioimmunoassay (RIA) is one of the main methods used in the analytical determination of C-Peptide. The influence of initial concentrations of labelled (M), the binding of the immunocomplex (PM) to the second antibody (J) immobilised on a bead, and the replacement of M by Q in the PMJ immunocomplex have been studied. In order to study the effect of such variables, 30 experiments were conducted and an explanation based on a physical-chemical model is proposed. The model used successfully reproduces the results. © 2010 Trade Science Inc. - INDIA

KEYWORDS

Kinetics;
RIA;
Double antibody;
C Peptide.

NOMENCLATURE

P = antibody in solution, Q = unlabelled antigen, M = ^{125}Iodine-labelled antigen, J = second antibody coated on plastic beads, P_0, M_0, Q_0 = initial concentrations in arbitrary units, PQ, PQJ = non-radioactive immunocomplexes, PM, PMJ = radioactive immunocomplexes, [P], [Q], [M], [PQ], [PM],[PQJ], [PMJ] = concentrations in mol / L, [J] = concentration of vacant biding sites in antibody J, Z = cpm activity in each tube after reaction ($Z = Z_{sp} + Z_0$). The tables include a sub-index indicating the experiment number. Z_{sp} = cpm activity from the radioactive immunocomplex corresponds to specific binding. Z_0 = value of Z at t = 0, corresponds to non-specific binding. $Z\infty$ = value of Z obtained at t infinity. Z_e = value of Z at equilibrium ($Z_e = Z\infty - Z_0$), t = time in minutes, k = rate constant, K = equilibrium constant, r = correlation coefficient.

INTRODUCTION

C-peptide is a polypeptide (31 amino acid residues) with a relative molecular mass (RMM) of 3018 Dalton. It is part of the proinsulin molecule and has the following structure: B chain – Arg – Arg -C-peptide – Lys – Arg – A chain.

In the pancreatic β-cells, proinsulin is enzymatically cleaved into insulin (A chain and B chain) and the C-peptide molecule. Both are simultaneously secreted in equimolar concentrations into blood. Insulin has a rather short half-life -5 minutes- while the half-life of C-peptide is 30 minutes.

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Therefore, the molar ratio between C-peptide and insulin in peripheral blood ranges between 3:1 and 5:1. The main degradation site for C-peptide is the kidney. Consequently, patients with renal dysfunction have a longer half-life and higher basal values. Among other reasons, its determination is indicated in the study of pancreatic reserves in individuals with diabetes and pancreatectomy patients, and in insulinoma diagnosis.

Radioimmunoassay (RIA) is used in C Peptide assessment. It 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 binds to both antigens until equilibrium is reached, in which circumstance both immunocomplexes -the radioactive one and the non-radioactive or "cold" one- can coexist[1]:

$$Ag + Ab + Ag^* \Leftrightarrow (Ag—Ab) + (Ag—Ab)^*$$

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 or in solution, then the separation of the bound and free fractions is essential. In our case, separation is accomplished by fixation on a second antibody coated on a plastic bead.

Kinetics and equilibrium in antigen-antibody reactions are determining factors of the rapidity, analytical range, and reliability[2-15] of immuno-analytical techniques. Likewise, the search for more reliable faster immunoassays is one of the main development areas in this field. This has caused the overall process to be progressively automated, from sample handling to statistical assessment of results. Yet, despite the large number of immunoanalytical systems developed in recent years, very few of them include kinetic analysis.

In our previous research[16-21], different characteristics related to the kinetics of antigen-antibody reactions used in analytical techniques were studied, incorporating radioactivity as a measurable magnitude. Theoretical models were prepared applicable 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.

In line with our previous research, this paper focuses on the kinetics of the reactions between C-Peptide and its specific antibody. The aim is to characterise radioimmunoanalytical reactions and in particular those used in the RIA determination of C-Peptide. This determination uses the binding of C Peptide present in the sample (Q) to an antibody in solution (P) and another antibody immobilised on a bead (J) in the presence of ^{125}I (M)-labelled C Peptide.

OBJECTIVES

To that end, we intend to:

1 Obtain kinetic and equilibrium experimental data to illustrate the formation of the PMJ immunocomplex for several concentrations of P, M and Q.

2 Obtain kinetic and equilibrium experimental data to illustrate the displacement of M by Q in the pre-formed PMJ immunocomplex.

3 Produce a general model to justify the results obtained in 1 and 2. The model is presented in the next section, but it was actually produced after obtaining the results.

GENERAL MODEL

Influence of initial concentrations of solution antibody and labelled antigen on reaction kinetics

For the process: P + M ⇔ PM this rate equation is obtained[22]:

$$Z = Z_{e1} \cdot \left[1 - \exp\left[-t \cdot k_{D1} \cdot \left(\frac{P_{01} \cdot M_0}{Z_{e1}}\right)\right]\right] + Z_{e2} \cdot \left[1 - \exp\left[-t \cdot k_{D2} \cdot \left(\frac{P_{02} \cdot M_0}{Z_{e2}}\right)\right]\right] + pM_0 \quad (1)$$

The equilibrium constant for this process is:

$$K = \frac{[PM]}{[P] \cdot ([M]_0 - [PM])}$$

$$K \cdot [P] \cdot [M]_0 - K \cdot [P] \cdot [PM] = [PM]$$

$$[PM] \cdot (1 + K \cdot [P]) = K \cdot [P] \cdot [M]_0$$

$$[PM] = \frac{K \cdot [P] \cdot [M]_0}{1 + K \cdot [P]} = \frac{[P] \cdot [M]_0}{\frac{1}{K} + [P]}$$

By assuming that $[P] = \alpha \cdot [P]_0$ and if $\frac{1}{K} = \beta$, then the following is obtained:

$$[PM] = \frac{\alpha \cdot [P]_0 \cdot [M]_0}{\beta + \alpha \cdot [P]_0} \quad (2)$$

The equilibrium constant for the last stage is:

$$K_J = \frac{[PMJ]}{[PM] \cdot [J]} \qquad [PM] = \frac{[PMJ]}{K_J \cdot [J]}$$

By replacing this [PM] value in Eq.2, and bearing in mind that Ze is directly proportional to [PMJ], after simplification we have:

$$Z_e = \frac{a \cdot P_0 \cdot M_0}{P_0 + b} \quad (3)$$

By substituting the value of Z_e drawn from Eq.3 in Eq.1 and by simplifying, we have

$$Z = \frac{a \cdot P_0 \cdot M_0}{P_0 + b} \cdot [1 - \exp[-t \cdot k_{D1} \cdot (P_0 + b)]] + \frac{c \cdot P_0 \cdot M_0}{P_0 + d} \cdot [1 - \exp[-t \cdot k_{D2} \cdot (P_0 + d)]] + pM_0 \quad (4)$$

Binding of the immunocomplex to the antibody immobilised on a bead

This is the last stage in the mechanism:

$$PM + J \underset{k_{-3}}{\overset{k'_3}{\rightleftharpoons}} PMJ$$

Its rate is expressed as follows:

$$\frac{d[PMJ]}{dt} = k'_3 \cdot [PM] \cdot [J] - k_{-3} \cdot [PMJ]$$
$$= k_3 \cdot [PM] - k_{-3} \cdot [PMJ]$$
$$= k_3 \cdot ([PM]_0 - [PMJ]) - k_{-3} \cdot [PMJ]$$
$$= k_3 \cdot [PM]_0 - (k_3 + k_{-3}) \cdot [PMJ]$$

By integration, it leads to:

$$[PMJ] = \frac{k_3 \cdot [PM]_0}{k_3 + k_{-3}} \cdot [1 - \exp(-(k_3 + k_{-3}) \cdot t)]$$

By transforming concentrations into cpm and including the unspecific calculations, then:

$$Z = a \cdot [1 - \exp(-b \cdot t)] + pM_0 \quad (5)$$

Displacement of labelled antigen by unlabelled antigen in the pre-formed immunocomplex

The process could be expressed as follows:

$$PMJ + Q \underset{k'_{-1}}{\overset{k'_1}{\rightleftharpoons}} PQJ + M$$

Its equilibrium constant being $K_{MQ} = \frac{[PQJ] \cdot [M]}{[PMJ] \cdot [Q]}$

The rate equation for this mechanism is:

$$\frac{d[PMJ]}{dt} = -k'_1 \cdot [PMJ] \cdot [Q] + k'_{-1} \cdot [PQJ] \cdot [M]$$

Matter conservation requires the following:

$[PMJ]_0 = [PMJ] + [PQJ]$

$[Q]_0 = [Q] + [PQJ]$

Calling: $[PMJ]_0 = f$

$[Q]_0 = g$ $[PQJ] = [M] = x$ $[PMJ] = f - x$

$[Q] = g - x$

the rate expression is

$$-\frac{dx}{dt} = -k'_1 \cdot (f - x) \cdot (g - x) + k'_{-1} \cdot x^2 = -k'_1 \cdot f \cdot g + k'_1 \cdot (f + g) \cdot x - k_1' \cdot x^2 + k'_{-1} \cdot x^2 \quad (6)$$

By integration of Eq. 6, if the labelling is assumed not to significantly alter the properties of the antigen, then the following can be accepted: $k'_1 \approx k'_{-1}$, it results:

$$x = x_e \cdot \left(1 - \exp\left(-k'_1 \cdot f \cdot t\right)\right) \quad f - x = f - x_e \cdot \left(1 - \exp\left(-k'_1 \cdot f \cdot t\right)\right)$$

Coming back to the starting notation, we have

$$[PMJ] = [PMJ]_0 - ([PMJ]_0 - [PMJ]_e) \cdot (1 - \exp(-k'_1 \cdot [PMJ]_0 \cdot t))$$

By transforming concentrations into cpm and simplifying, we have:

$$Z = Z_e + (Z_0 - Z_e) \cdot \exp(-k_D \cdot t) \quad (7)$$

Since α is the factor for cpm conversion of the concentrations, the value of Z_e can be calculated as follows:

$$K_{MQ} = \frac{[PQJ] \cdot [M]}{[PMJ] \cdot [Q]} = \frac{\alpha \cdot [PQJ] \cdot \alpha \cdot [M]}{\alpha \cdot [PMJ] \cdot \alpha \cdot [Q]} = \frac{(Z_0 - Z_e)^2}{Z_e \cdot \alpha \cdot [Q]}$$
$$= \frac{(Z_0 - Z_e)^2}{Z_e \cdot [\alpha \cdot [Q]_0 - (Z_0 - Z_e)]} = \frac{Z_0^2 - 2 \cdot Z_0 \cdot Z_e + Z_e^2}{(\alpha \cdot [Q]_0 - Z_0) \cdot Z_e - Z_e^2}$$
$$= \frac{1 - 2 \cdot \frac{Z_e}{Z_0} + \left(\frac{Z_e}{Z_0}\right)^2}{\left(\frac{\alpha \cdot [Q]_0}{Z_0} - 1\right) \cdot \frac{Z_e}{Z_0} - \left(\frac{Z_e}{Z_0}\right)^2}$$

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By assuming $\frac{Z_e}{Z_0} < 1$, the quotient $\left(\frac{Z_e}{Z_0}\right)^2 <<< 1$, finding the value of Z_e, substituting the value of Z_e in Eq. 7 and simplifying, we have:

$$Z = \frac{a}{[Q]_0 + b} + \left(Z_0 - \frac{a}{[Q]_0 + b}\right) \cdot \exp(-k_D \cdot t) \quad (8)$$

For two binding sites, Eq. 8 takes the expression

$$Z = \frac{a}{[Q]_0 + b} + \left(Z_{01} - \frac{a}{[Q]_0 + b}\right) \cdot \exp(-k_{D1} \cdot t) + \frac{c}{[Q]_0 + d} + \left(Z_{02} - \frac{c}{[Q]_0 + d}\right) \cdot \exp(-k_{D2} \cdot t) \quad (9)$$

Equilibrium equations

These are obtained from rate equations by making time tend to infinity. By doing this, exponential terms containing such a variable disappear., Addiotionally the unspecific activity is subtracted

EXPERIMENTAL

Reagents

The reagents used belong to the RIA-coat® C-Peptid kit, manufactured by Byk-Sangtec Diagnostica GMBH & Co.KG. The kit includes:

-A polyclonal antiserum obtained by immunising goats with synthetic human C-Peptide

-A second monoclonal antibody (mouse anti goat) coated on a plastic bead

-^{125}I-C-Peptide: a vial with lyophilised labelled C-Peptide

-Unlabelled C-Peptide vials in different concentrations, with which different solutions were prepared, 0, 0.533 and 5.917μg/mL being the final concentrations for the determination of the influence of initial labelled and unlabelled antigen concentrations, and 1, 3, 10 and 30μg/mL for the study of the labelled antigen displacement resulting from the addition of the unlabelled antigen.

Instrumentation

LKB Gammamaster Automatic Gamma Counter, fitted with a computer with a Riacalc programme.

Computer programme

Statistica (Copyright© StatSoft, Inc.1993). It allows the fitting of experimental data using specific non-linear regression equations, and the production of the corresponding tables. As a statistical criterion for equation selection in the different models, AIC (Akaike's Information Criterion)[23] was observed; it can be expressed as follows: AIC = N·lnS + 2·P, where N is the number of points, S the addition of the squares of the residuals, and P the number of parameters in the equation. The equation with the lowest AIC in the fitting must be chosen.

Experimental procedure

Experiments 1-9

Study of the influence of the concentration of the antibody in solution and the labelled antigen on reaction rate and equilibrium. Labelled C Peptide and antibody concentrations varied in the first five series while conditions were the opposite in the last four.

Experiment 10

Study of the binding of the dissolved immunocomplex to the bead-coating antibody. 100μL of labelled C Peptide solution was left to react with 100μL anti-C peptide antibody solution at their maximum concentrations for 2 hours, after which a bead was added in each tube, and then everything was kept in agitation for different time periods. Next, the tubes were washed and the radioactivity bound to the beads measured.

Experiments 11-14

To study the substitution of M by Q in the pre-formed PMJ immunocomplex, 100μL of labelled C Peptide solution, 100μL anti-C peptide antibody solution at their maximum concentrations and one bead was left to react in agitation for 24 hours. Once this period elapsed, 100μL unlabelled C peptide solution in different concentrations was added at different times, after which tubes were washed and the radioactivity on the bead measured.

In all cases, the total added radioactivity was measured as an indirect measurement of the initial labelled antigen concentration.

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RESULTS AND DISCUSSION

Influence of initial concentrations of antibody in solution (P_0) and labelled antigen (M_0) on reaction kinetics and equilibrium

This was studied in Experiments 1-9, their results being shown in TABLE 1.

TABLE 1 shows that, for a given P value, if M concentration increases, the amount of radioactive immunocomplex also increases for all times. Keeping the concentration of M constant, the influence of solution P antibody is seen in the fact that, when its concentration is increased, the amount of radioactive immunocomplex increases for all times.

TABLE 1 : Influence of P_0 and M_0

t (min)	0	10	30	60	120	180	∞	M_0(cpm)	P_0(u.r.)
Z_1	195.0	3074.0	8600.7	12083.5	16703.5	17335.0	22916.7	26719.0	100
Z_2	93.1	2471.2	5886.9	10108.0	12963.0	13489.2	18802.5	21438.0	100
Z_3	54.9	1628.0	4659.4	74947.7	9472.4	11073.0	14375.0	16159.6	100
Z_4	27.0	1287.3	3030.9	4960.7	6650.5	7219.0	9273.7	10291.5	100
Z_5	2.1	663.4	1641.0	2515.0	3325.9	3670.5	4726.2	5270.8	100
Z_6	95.0	2168.4	6339.5	9910.8	14466.9	15223.0	20153.0	25984.3	80
Z_7	135.9	1569.1	4730.8	8349.5	11692.8	12960.6	16602.8	25984.3	60
Z_8	113.5	1278.0	3322.0	5780.6	7744.3	9597.7	13008.1	25984.3	40
Z_9	50.8	537.4	1670.4	2831.5	4248.0	4658.0	6552.2	25984.3	20

The data in Table 1 have been fitted in with Eq. 4, save for the Z_0 unspecific activity term, which has been taken as equal to $p \cdot M_0$. These are its parameters and coefficients:

a	b	$k_{D1} \cdot 10^2$	c	d	$k_{D2} \cdot 10^2$	p	r	AIC
1.220	92.0	0.01058	0.590	73.9	0.000457	0.000983	0.997	997

From this equation, we note that when P_0 is increased, the apparent kinetic parameters and the dissociation equilibrium parameters rise. The consistency between the values observed (TABLE 2) and those calculated by Eq. 4 is shown in figure 1.

The results at infinite time, corresponding to equilibrium, fit in with the following equation

$$Z_e = \frac{1.22 \cdot P_0 \cdot M_0}{P_0 + 92} + \frac{0.590 \cdot P_0 \cdot M_0}{P_0 + 73.9} \qquad r = 0.999$$

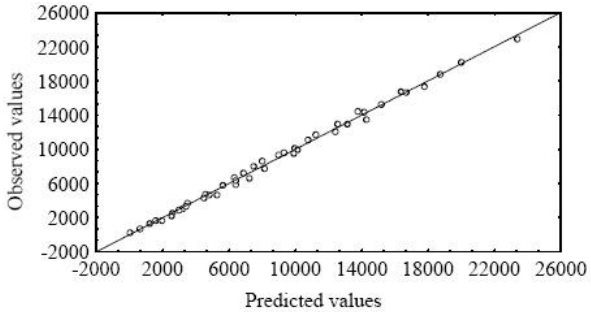

Figure 1 : Observed values (TABLE 1) vs. Predicted values (Eq.4)
Observed values = -13.32 + 1.0019 · Predicted values, r = 0.999

Binding of the immunocomplex to the antibody immobilised on a bead

This was studied in experiment 10, and the following values were obtained:

TABLE 2 : Binding of the immunocomplex PM to the antibody J

t (min)	0	10	20	30	40	50	$_0$(cpm)	P_0(u.r.)
Z_{88}	776.0	8700.0	11366.9	13379.1	14875.3	14863.8	25984.3	100

They are in line with Eq.5, save for the Z_0 unspecific activity term, which has been taken as equal to p·M0. These are its parameters and correlation coefficients:

The graphic representation can be seen in figure 2.

a	b	p	r
14430	0.0713	0.0339	0.998

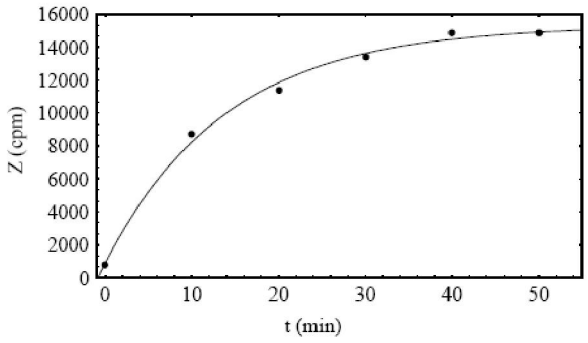

Figure 2 : Z values vs. t. TABLE 2, Eq. 5

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The binding of the immunocomplex dissolved antibody - C Peptide to the second antibody immobilised on a bead is a quick process that does not constrain the global reaction rate.

Displacement of M by Q in the pre-formed PMJ immunocomplex

This was studied in Experiments 11 – 14, their results being shown in TABLE 3.

TABLE 3 : Displacement of M by Q

t (min)	0	10	30	60	120	180	∞	Q_0 (nmol/L)
Z_{11}	18541.1	18198.2	17336.9	17087.0	16001.0	15676.4	14378.6	0.331
Z_{12}	18658.7	18003.2	17475.5	17154.6	15451.0	14301.3	12290.0	0.994
Z_{13}	18420.0	17945.0	17402.3	16260.8	14392.5	13721.2	10584.9	3.313
Z_{14}	18784.5	17620.2	16617.0	15806.0	14789.2	13752.3	10049.6	9.94

TABLE 3 shows that, when Q concentration is increased, the amount of radioactive immunocomplex also decreases for all times. The data in the table are in line with Eq.9, whose parameters and correlation coefficients are in bellow TABLE.

The consistency between the values observed (TABLE 3) and those calculated by Eq. 9 is shown in figure 3.

The results at infinite time, corresponding to equilibrium, are in line with the following equation

$$Ze = \frac{3732}{Q_0 + 0.462} + 9650 \qquad r = 1.000$$

a	b	Z_{01}	k_{D1}	c	d	Z_{02}	k_{D2}	r	AIC
2931	0.336	6536	0.00353	$3.20 \cdot 10^6$	320	11962	0.0143	0.995	416

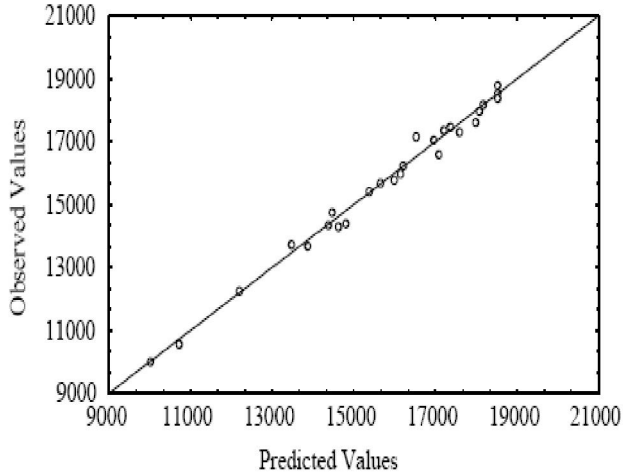

Figure 3 : Observed values (TABLE 3) vs. Predicted values (Eq.9) Observed values = -0.536 + 1.000 · Predicted values, r = 0.995

CONCLUSIONS

1 A theoretical model was prepared to study the kinetics of the formation reaction of immunocomplex antibody-labelled C-Peptide (PMJ) an the substitution reaction in the immunocomplex antibody-labelled C-Peptide (PMJ) by unlabelled C-Peptide (Q).

2 When its concentration is increased, the influence of the dissolved antibody shows in an increased amount of radioactive immunocomplex for all times and the apparent kinetic and dissociation equilibrium parameters.

3 In the formation of the PMJ immunocomplexes, an apparently irreversible biexponential behaviour is found, corresponding to two binding site types.

4 Equilibrium data do not allow us to distinguish single site from double site binding models. However, a distinction was possible between both models when kinetic data were used.

5 The displacement of M by Q in the preformed PMJ immunocomplex follows a reversible second order kinetics in both directions.

6 Experimental results were satisfactorily fitted to the theoretical model.

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