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ARTÍCULO

Mono and biexponential models in radioimmunoassay of insulin

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ABSTRACT

Radioimmunoassay (RIA) is a principal method for quantifying serum Insulin concentration. We studied the influence of initial concentration of ^{125}I-labeled antigen (M) and unlabeled Insulin (Q), viscosity and temperature on the substitution reaction between Q and the immunocomplex (PM) formed by M and the anti-Insulin antibody (P). The accuracy of this method is critically dependent on such factors. In addition, we propose a kinetic model for this reaction. We used a commercially available RIA kit for Insulin, a gamma counter, and a viscosimeter to study the effect of initial concentration of M, ionic strength, viscosity, and temperature on the substitution reaction between M and Q. Data were analyzed using Statistica software. The apparent rate constant for the reaction between PM and Q is dependent on the initial concentrations of M and Q, and the viscosity of the reaction medium, and temperature, and independent of the ionic strength.

A kinetic model for the displacement of the ^{125}I-Insulin by the Insulin in its union to a specific antibody is proposed. Such model adjusts satisfactorily to the results and shows the influence of the variables studied on the sensitivity of the method of RIA on which the analytical determination of the Insulin is based.

Key Words: Concentration; Immunocomplex; Ionic Strength; Kinetics; Substitution Reaction; Temperature; Viscosity.

RESUMEN

Modelos mono y biexponencial en Radioinmunoanálisis de Insulina

El Radioinmunoanálisis (RIA) es uno de los principales métodos para la cuantificación de la concentración sérica de Insulina. Se ha estudiado la influencia de la concentración inicial de ^{125}I- Insulina (M) e Insulina no marcada (Q), viscosidad y temperatura en la reacción de sustitución entre Q y el inmunocomplejo (PM) formado por M y el anticuerpo anti Insulina (P). La exactitud de este método es críticamente dependiente de dichos factores. Además se propone un modelo cinético para esta reacción. Se utilizó un kit comercial de RIA para Insulina, un contador gamma y un viscosímetro para estudiar el efecto de las variables indicadas anteriormente sobre la reacción de sustitución entre M y Q. Los datos se analizaron usando el programa Statistica. La constante aparente de velocidad para la reacción entre PM y Q depende de las concentraciones iniciales de M y Q, de la viscosidad del medio y de la temperatura. Es independiente de la fuerza iónica.

Se propone un modelo cinético para el desplazamiento de la ^{125}I-Insulina por la Insulina en su unión a un anticuerpo específico. Dicho modelo se ajusta satisfactoriamente a los resultados y muestra la influencia de las variables estudiadas sobre la sensibilidad del método de RIA en que se basa la determinación analítica de la Insulina.

Palabras Clave: Concentración; Inmunocomplejo; Fuerza Iónica; Cinética; Reacción de Sustitución; Temperatura; Viscosidad.

1. INTRODUCTION

Radioimmunoassay (RIA) is used in Insulin assessment. The procedure is a solid-phase radioimmunoassay (1), wherein ^{125}I-labeled insulin competes for a fixed time with insulin in the patient sample for

antibody sites. Because the antibody is immobilized to the wall of a polypropylene tube, simply decanting the supernatant suffices to terminate the competition and to isolate the antibody-bound fraction of the radiolabeled insulin. Counting the tube in a gamma counter then yields a number, which converts by way of a calibration curve to a measure of the insulin present in the patient sample. Rodbard, Munson, Ekins (2-4) and others suggested some models for the adjustment of calibration curves, the most widespread and used in current software is that of the four parameters.

Kinetics and equilibrium in antigen-antibody reactions are determining factors in the sensitiveness and accuracy of immunoanalytical techniques (5-7). A diffusion-controlled process must meet some typical requirements such as a considerable reaction rate decrease when medium viscosity is greater, and scarce temperature influence with a reduced energy demand with regards activation, this causing activation enthalpy values to be the same order as the solvent's viscous flow energy (19000 J mol^{-1} for water) (8). Diffusion control for this type of processes has been theoretically studied by Nigren, Stenberg et al (9-13). They proposed an application model for reactions produced in the solid-liquid interphase which provided an equation containing four diffusion influence parameters. Raman (14) also observed diffusion control for monoclonal antibody binding to cytochrome C. Xavier and Wilson (15) studied the association and dissociation reactions of Anti-Hen Egg Lysozyme (HEL) with two of its specific antibodies (HyHEL-5 and HyHEL-10) under pseudo first order conditions for the association, and found diffusion control. The decrease in the reaction rate constants as a result of viscosity turned out to be more drastic than theoretically expected, this aspect being put down to potential osmotic effects. In addition, rate constants were found to approximately double when ionic strength goes down from 500 mM to 27 mM, which indicates that the process occurs between species with opposite charges that affect the orientational requirements of association.

Equilibrium data analysis is largely used in determining the capacity of a substance to bind to one or several receptor populations. Nonetheless, as pointed out by Weber (16), detecting two binding sites through such an assay requires the ligand to have very different affinity for the two binding sites.

In our previous research (17-27) 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 (9-13), Rabany (7), and those of Zuber (6) 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:

— Produce a kinetic model applicable to the substitution of the labelled antigen bound to the antibody by the unlabelled one, this process being at the foundations of RIA.

— Distinguish between single-site and two-site binding models by analysing kinetic data.

— Determine potential diffusion control.

This must be done in different stages:

— Obtaining integrated rate equations for the overall processes.

— Studying the medium's viscosity influence on reaction kinetics.

— Complementary analysis of ionic strength influence in order to include or rule out the effect of electrical charges.

— To predict the calibration curves showing the influence of the mentioned variables.

— The results must be potentially applicable to the design of immunoanalytical techniques.

2. THEORETICAL MODEL

This is the reaction studied:

$PM + Q \leftrightarrow PQ + M$

P = Anti-Insulin antibody immobilised on the tube wall.

M = ^{125}I-Insulin.

PM = Radioactive immunocomplex.

Q = Insulin.

PQ = Non-radioactive immunocomplex.

The treatment of the reaction kinetic model was exposed in a paper published earlier (24). As it appeared there, the activity of PM in cpm (z) varies over time according to the equation:

$$z = z_e + (z_0-z_e)(\exp(-t\ (z_0 q \varepsilon k'_1/(z_0-z_e)+(z_0-z_e)(k'_{-1}-k'_1)))) \quad \text{Eq. 1}$$

z = Activity of PM in cpm, z_0 = initial value of z, z_e = z value in the equilibrium, q = Q concentration (pmol/L), ε= Conversion factor concentration-activity, k'_1 = rate constant for the forward reaction, k'_{-1} = rate constant for the reverse reaction, $z_0 q \varepsilon k'_1/(z_0-z_e)+(z_0-z_e)(k'_{-1}-k'_1)$ = Apparent rate constant.

Or, for two processes:

$$z = z_{e1} + (z_{01}-z_{e1})(\exp(-t\ (z_{01}k'_1+q\varepsilon\ (k'_{-1}-k'_1)))) + z_{e2} + (z_{02}-z_{e2})(\exp(-t\ (z_{02}k'_2+q\varepsilon\ (k'_{-2}-k'_2)))) \quad \text{Eq. 2}$$

3. MATERIALS AND METHODS

3.1. Instruments

ILKB Gammamaster Automatic Gamma Counter.

Brookfield DV–II digital viscosimeter. Viscosity measurements were performed at 60 rpm with a UL ADAPTER at room temperature.

3.2. Reagents

DM = Solution of ^{125}I-labelled Insulin in a protein-based buffer. Estimated concentration ≈ 50 pmol/L.

PT = Plastic tubes with rabbit anti-Insulin immunoglobulin immobilized to the inside wall.

DQ = Insulin standard solutions.

These reagents were included in the Insulin RIA DPC kit.

GL = Glycerol Merck pro analysis.

DS = Solution of NaCl 2.05 M.

Several tube series were prepared as per the Table I.

Table I. **Preparation of Coated Tubes Containing ^{125}I-Insulin**

PT	1-6	7-12	13-18	19-100
DM (mL)	0.25	0.50	0.75	1
H20 (mL)	0.75	0.50	0.25	0

They were left to react overnight. The next day, they were decanted and washed with 2 mL distilled water.

Solutions were prepared as per Table II.

Table II. **Preparation of Solutions Containing Insulin, Glycerol and NaCl**

Solution	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
DQ (µL)	25	50	75	100	100	100	100	100	100	800
GL (mL)	0	0	0	1	2	3	0	0	0	0
DS (µL)	100	100	100	100	100	100	200	300	400	800
H_2O (mL)	7.875	7.850	7.825	6.8	5.8	4.8	7.7	7.6	7.5	62.4
q (pmol/L)	7.84	15.7	23.5	31.4	31.4	31.4	31.4	31.4	31.4	31.4

q = Concentration of Insulin

3.3. Experimental Procedure

Activity was measured on tubes 1, 7, 13 and 19 at 0 minutes using a gamma counter. Reaction kinetics were studied by placing 1 mL of the previously mentioned solutions in the plastic coated tubes and letting them react at different times and at 48 hours, this being considered infinite time. Each tube was washed to remove any unbound labeled antibody. Any radioactivity present in the remaining bound labeled antibody was then measured using a gamma counter.

16 experiments were performed, arranged as follows:

— Experiments 1, 2, 3, 4

Study of the influence of ^{125}I- Insulin concentration (m) upon the global reaction using tubes 1-28 and solution 10.

— Experiments 4, 5, 6, 7

Study of the influence of Insulin concentration (q) upon the global reaction using tubes 22-46 and solutions 10,1, 2, 3.

— Experiments 4, 8, 9, 10

Study of the influence of ionic strength (I), using tubes 22-28, 47-64 and solutions 10, 7, 8, 9.

— Experiments 4, 11, 12, 13

Study of the influence of viscosity (η) using tubes 22-28, 65-82 and solutions 10, 4, 5, 6. The final viscosity of the solutions obtained in this manner was determined by comparison with a calibration curve drawn from standard glycerol-water mixes.

— Experiments 4, 14, 15, 16

Study of the influence of temperature (T) using tubes 22-28, 83-100 and solution 10.

3.4. Data Analysis

The Statistica programme (Copyright © StatSoft,Inc:, 1993) was used with specific non-linear regression equations. As the statistical criterion (28,29) that allows a choice from different equations, SS and Corrected Akaike´s Information Criterion (AIC_c) was used, expressed as $AIC_C = N \cdot \ln\left(\frac{SS}{N}\right) + 2P + \frac{2P(P+1)}{N-P-1}$ 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.

4. RESULTS

Results of z values for experiments 1-16 are shown in Table III.

5. DISCUSSION

5.1. Influence of m (Experiments 1, 2, 3, 4)

The results of experiments 1, 2, 3, 4 are fitted to the equation:

$z=a\cdot m/(m+b)+(c\cdot m/(m+d)-a\cdot m/(m+b))\cdot(\exp(-t\cdot e))$ Eq. 3

Its parameters, coefficient of correlation (r), sum of squares of residuals (s) and AICc are:

a = 4399, b = 80.4, c = 51993, d = 341, e = 0.0858, r = 0.984, s = $5.59\cdot10^6$, AICc = 357.72.

Or to:

$z=a\cdot m/(m+b)+(c\cdot m/(m+d)-a\cdot m/(m+b))\cdot(\exp(-t\cdot e))+f\cdot m+(g\cdot m/(m+h)-f\cdot m)\cdot(\exp(-t\cdot j))$ Eq. 4

Its parameters, coefficient of correlation, sum of squares of residuals and AICc are:

a = 5577, b = 92.0, c = 52007, d = 340, e = 0.1053, f = 0.462, g = 1.295, h = -69.6, j = -0.00269, r = 0.998, s = $7.785\cdot10^5$, AICc = 319.46

Table III. **z values for experiments 1-16**

T (min)	0	15	30	45	60	75	Infinite	m	Q	I	η	T
z_1	3423	1683	1326	1000	930	925	761	25	31.4	0.0256	1.385	318
z_2	6673	2744	2574	2354	2131	1928	660	50	31.4	0.0256	1.385	318
z_3	9581	3870	3058	2504	2471	2291	1750	75	31.4	0.0256	1.385	318
z_4	11747	4679	3404	2900	2898	2878	913	100	31.4	0.0256	1.385	318
z_5	11747	6379	5317	4647	3005	2966	1836	100	7.84	0.0256	1.385	318
z_6	11747	5223	4852	4285	3775	3614	2662	100	15.7	0.0256	1.385	318
z_7	11747	4824	3977	3500	2917	2746	2379	100	23.5	0.0256	1.385	318
z_8	11747	4606	4128	3451	2501	2363	2066	100	31.4	0.0513	1.385	318
z_9	11747	4626	3115	2996	2758	2595	2147	100	31.4	0.0769	1.385	318
z_{10}	11747	4818	3264	2807	2692	2411	1756	100	31.4	0.1026	1.385	318
z_{11}	11747	4711	4123	3368	3136	2846	1794	100	31.4	0.0256	1.478	318
z_{12}	11747	5048	4074	3357	2806	2353	1740	100	31.4	0.0256	1.677	318
z_{13}	11747	6058	4827	3928	3122	2692	1407	100	31.4	0.0256	1.980	318
z_{14}	11747	8429	6085	5845	5527	5190	2478	100	31.4	0.0256	1.385	310
z_{15}	11747	8891	8645	8224	7993	7370	4132	100	31.4	0.0256	1.385	303
z_{16}	11747	10466	9776	9713	8464	8423	5593	100	31.4	0.0256	1.385	295

z = activity (cpm) of PM immunocomplex. The subscript indicates the experience number.
m = M initial concentration (relative units).
Q = Q initial concentration pmol/L).
I = ionic strength ($mol \cdot L^{-1}$).
η = viscosity (mPa·s).
T = Temperature (K).

The values of AICc indicate that the Eq. 4 is significantly better than Eq. 3. The fact that the ecuation Eq. 4 is biexponential indicates that the process consist of two different chemical reactions. It also happens with the other studied variables, althoug the comparison is included only in this case.

Eq. 4 is obtained from Eq. 2 by substitution:

$z = z_{e1} + (z_{01}-z_{e1})(\exp(-t\ (z_{01}k'_1+q\lambda\ (k'_{-1}-k'_1)))) + z_{e2} + (z_{02}-z_{e2})(\exp(-t\ (z_{02}k'_2+q\varepsilon\ (k'_{-2}-k'_2))))$ Eq. 2

$z_{e1} = a\cdot m/(m+b)$ (Langmuir)

$z_{01} = c\cdot m/(m+d)$ (Langmuir)

$z_{01}k'_1+q\varepsilon\ (k'_{-1}-k'_1) = e$

$z_{e2} = f\cdot m$

$z_{02} = g\cdot m/(m+h)$ (Langmuir)

$z_{02}k'_2+q\varepsilon\ (k'_{-2}-k'_2) = j$

Eq. 4 shows that the initially obtained z values are dependent of m as per Langmuir model (30). The z values at equilibrium are directly proportional to z_0 (empirical observation). The apparent rate constants are independent from $z_{0,}$ and therefore of m. This is explained admitting that the used concentrations of tracer are significantly inferior to those of Insulin.

5.2. Influence of q (Experiments 4, 5, 6, 7)

The results of experiments 4,5,6,7 are fitted to the equation:

$z=(a-b\cdot q)+(c+d\cdot q)\cdot\exp(-t\cdot(e+q\cdot f))+(g+h\cdot q)\cdot\exp(-t\cdot(j+q\cdot k))$ Eq. 5

Its parameters, coefficient of correlation and sum of squares of residuals are:

$a = 2554$, $b = 32.3$, $c = 1976$, $d = 193$, $e = 5.17$, $f = -50.5\cdot10^{-3,}$ $g = 7216$, $h = -160.7$, $j = 0.0297$, $k = -0.669\cdot10^{-3}$, $r = 0.995$, $s = 2.84\cdot10^{6}$

Eq. 5 is obtained from Eq. 2 by substitution:

$z_{e1} + z_{e2} = a-b\cdot q$

$z_{01}-z_{e1} = c+d\cdot q$
$z_{01}k'_1+q\varepsilon\ (k'_{-1}-k'_1) = e+q\cdot f$
$z_{02}-z_{e2} = g+h\cdot q$
$z_{02}k'_2+q\lambda\ (k'_{-2}-k'_2) = j+q\cdot k\cdot 0.001$

The negative values of f and k indicate that k'_1 is greater than k'_{-1}, and k'_2 greater than k'_{-2}. Values of z in the equilibrium depend linearly on q. This suggests that used values of q are in the range of low concentrations. The apparent rate constant for the process (kf) is linearly dependent on the initial concentration of insulin.

5.3. Influence of I (Experiments 4, 8, 9, 10)

The results of experiments 4, 8, 9, 10 are fitted to the equation:

$z = a+b\cdot\exp(-t\cdot c)+d\cdot\exp(-t\cdot e)$ Eq. 6

Its parameters, coefficient of correlation and sum of squares of residuals are:

$a = 1722$, $b = 7549$, $c = 0.1364$, $d = 2476$, $e = 0.01455$, $r = 0.996$, $s = 2.217\cdot 10^6$

The rate constant is independent of the ionic strength. It indicates that the reaccionantes species do not have electrical charge.

5.4. Influence of η (Experiments 4, 11, 12, 13)

The results of experiments 4, 11, 12, 13 are fitted to the equation:

$z = a/(1+b\cdot\eta)+c\cdot\exp(-t\cdot d/\eta)+e\cdot\exp(-t\cdot f/\eta)$ Eq. 7

Its parameters, coefficient of correlation and sum of squares of residuals are:

$a = 1214$, $b = -0.1093$, $c = 6595$, $d = 0.231$, $e = 3673$, $f = 0.0235$, $r = 0.996$, $s = 2.071\cdot 10^6$

For reactions between spherical molecules, nonionic and of similar sizes, (31) one is fulfilled:

$k = 8RT/3\eta$

Eq. 7 is obtained from Eq. 2 by substitution:

$z_{e1} + z_{e2} = a/(1+b\cdot\eta)$

$z_{01}-z_{e1} = c$

$z_{01}k'_1+q(k'_{-1}-k'_1) = d/\eta$

$z_{02}-z_{e2} = e$

$z_{02}k'_2+q(k'_{-2}-k'_2) = f/\eta$

The rate and equilibrium constants are inversely proportional to the viscosity of the medium, as it corresponds to molecules with spherical symmetry, nonionic and of similar sizes. For constant values of m, q and T, the activity in the equilibrium diminishes when it increases viscosity.

5.5. Influence of T (Experiments 4, 14, 15, 16)

The results of experiments 4, 14, 15, 16 are fitted to the equation:

$z = a\cdot\exp(b/T)+c\cdot\exp(d/T)\cdot\exp(-t\cdot e\cdot\exp(-f/T))+g\cdot\exp(h/T)\cdot\exp(-t\cdot j\cdot\exp(-k/T))$ Eq. 8

Its parameters, coefficient of correlation and sum of squares of residuals are:

$a = -0.445\cdot10^{-12}$, $b = 11355$, $c = 1.893\cdot10^{9}$, $d = -3913$, $e = 3.21\cdot10^{7}$, $f = 6215$, $g = 0.1279\cdot10^{-6}$, $h = 7737$, $j = 29.7\cdot10^{7}$, $k = 8601$, $r = 0.994$, $s = 3.208\cdot10^{60}$

The equilibrium constant is related to the temperature according to the equation of van t'Hoff (32):

$K = A\cdot\exp(-\Delta H^0/RT)$

The rate constant is related to the temperature according to the equation of Eyring (33):

$k = B \cdot T \cdot \exp(-\Delta H^{\ddagger}/RT)$

Eq. 8 is obtained from Eq. 5 introducing:

K (van t'Hoff) in b, d, h,

k (Eyring) in e and j, grouping the constants and simplifying.

The activation parameters are:

$\Delta H^{0}_{1} = 3913 \cdot 8.31 = 32517$ J mol^{-1} $\Delta H^{0}_{2} = -7737 \cdot 8.31 = -64294$ J mol^{-1}

$\Delta H^{\ddagger}_{1} = 6215 \cdot 8.31 = 51647$ J mol^{-1} $\Delta H^{\ddagger}_{2} = 8601 \cdot 8.31 = 71474$J mol^{-1}

When temperature increases, the concentration of PQ in equilibrium and the apparent rate constant increase, according to Eyring and van't Hoff equations.

Process 1 is endothermic and 2 exothermic. The diminution of ze when increasing the temperature can be explained admitting that process 2 predominates on the 1. The activation energies are much greater than the energy of viscous flow of the water. It indicates that the process is not controlled by diffusion.

5.6. Influence of m, q, I, η, T (Experiments 1–16)

The results of experiments 1-16 are fitted to the equation:

$$z = (a-b \cdot q) \cdot m \cdot \exp(c/T) + d \cdot \exp(e/T) \cdot (m/(m+f)) \cdot \exp(-t \cdot (g/\eta) \cdot \exp(-h/T)) + j \cdot (m/(m+k)) \cdot \exp(n/T) \cdot \exp(-t \cdot (u/\eta) \cdot \exp(-w/T)) \quad \text{Eq. 9}$$

Its parameters, coefficient of correlation and sum of squares of residuals are:

$a=-0.1529 \cdot 10^{-15}$	$b=0.234 \cdot 10^{-15}$	c=11595	$d=2.87 \cdot 10^{9}$	e=-3492
f=469	$g=47.5 \cdot 10^{7}$	h=6995	$j=0.1892 \cdot 10^{-6}$	k=188.3
n=7919	$u=0.1042 \cdot 10^{7}$	w=6152	r= 0.992	$s= 1.885 \cdot 10^{7}$

Eq. 9 contains the influence of all the studied variables. The adjustment of the data of Table III (n = 112) to the Eq. 9 can be observed in Figure 1.

6. CONCLUSIONS

— **Conclusion 1.** In the reaction two process occur simultaneously, that could correspond to differents epitopes. The initial activity of the radioactive immunocomplex (z_0) is dependent on m, as per Langmuir's model. The apparent rate constants for the process (kf) is independent of m.

— **Conclusion 2.** Activity in equilibrium (z_e) is directly proportional to z_0, and therefore it also depends on m, as per Langmuir's equation. As a consequence, the RIA calibration curves obtained with these reagents must follow the model of the four parameters and provide a good logit-log linear fit.

— **Conclusion 3.** The sensitivity of the method is greater at the most small ist he value of z_e for a given value of q. Since z_e increases if it makes m, it agress to use low values of m. In this way increases sesitivity without the reaction becomes slower.

— **Conclusion 4.** The modification of the ionic strength does not contribute any advantage from the practical point of view.

— **Conclusion 5.** An increase in viscosity provides a greater sensitivity of the technique. Nevertheless, it must be valued that the reaction would become slower, with the consequent increase of the incubation time.

— **Conclusion 6.** The sensitivity of the method is increased making the incubation to temperatures superior to the one of the room, whenever it does not put in danger the thermal stability of the insulin. In addition, the reaction becomes faster.

— **Conclusion 7.** A theoretical model was prepared to study the kinetics of the substitution reaction in the immunocomplex antibody-labelled Insuline (PM) by unlabelled Insuline (Q). Equations linking PM concentration with time, M and Q concentrations, ionic strength, viscosity, and temperature were obtained. Experimental results were satisfactorily fitted to the theoretical model.

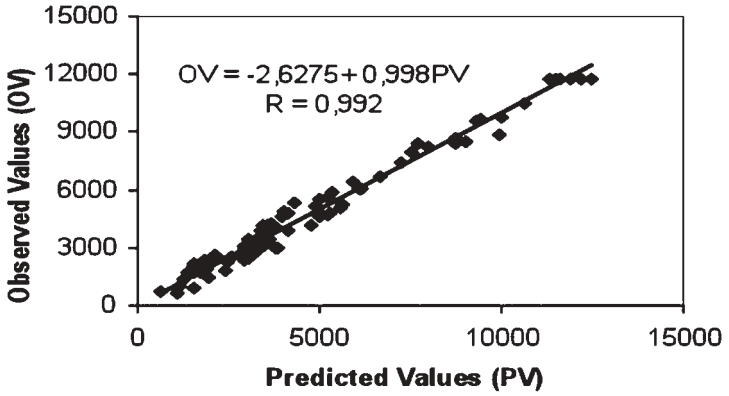

Figure 1. z values observed in experiments 1 – 16 (Table III) vs. values predicted for Eq 9.

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