A NEW APPROACH TO THE CURVE FITTING OF IN VITRO RADIOASSAYS BASED ON THE MASS ACTION LOW

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ABSTRACT

A new curve fitting method with its mathematical models derived from the mass action law is presented which is applicable to several in vitro radioassays including RIA and RBA. Experiments revealed that the robustness of this method is better than the conventional methods like Woolf plot in RBA and 4- parameter logistic plot in RIA. However, the robustness of this method is only relative: in some cases of RIA and RBA, the bias of the results may still be too large to be acceptable. Further improvement is expected to be studied.

Key words: Vitro radioassays Curve fitting Mathematical models Mass action law

I. INTRODUCTION

Many important in vitro radioassays like radioimmunoassay (RIA), immuno-radiometric assay (IRMA) and radioligand binding assay of receptors (RBA) have their fundamental working principle based on the mass action law. However, most of the mathematical models commonly used in RIA and IRMA are not strictly derived from this law and the calibration curves are usually fitted with least square regression or its modifications. The combined use of these models and fitting methods may result in significant errors of unknown samples when the assay procedure is not typical (for example, nonequilibrium RIA) or when there is (are) outlier(s) in the calibration curve. In RBA, the curve fitting is accomplished either by linearization (e.g., Scatchard or Woolf plot) or by direct least square regression. With these methods, outlier(s) may also lead to marked errors in assay results. It is therefore claimed that new mathematical models based on the mass action law and more robust curve fitting methods should be developed[1-3]. At least one new model for RIA and one new fitting method is already in use[4], though the method of curve fitting has not yet been reported. In this paper, a method applicable to several kinds of in vitro radioassays will be described. The mathematical models are derived from the mass action law and the curve fitting method is entirely different from least square regression. The advantages and disadvantages of the method studied with the aid of Lotus- 123 software will also be discussed.

II. PRINCIPLE AND FUNDAMENTAL METHOD

1. Mathematical models deduced from the mass action law

In most radioassays, the specific binding agent (antibody in RIA and IRMA, receptors in RBA) has only one high affinity binding site toward the corresponding ligand (antigen in RIA and IRMA, agonist or antagonist in RBA). The binding is usually 1:1 in molecular ratio. In such systems, when equilibrium is achieved, the following relation can be derived from the mass action law:

$$KD = \frac{[\text{Free ligand}]\,[\text{Free binding agent}]}{[\text{Specific complex}]} \qquad (1)$$

where KD is the equilibrium dissociation constant, or the reciprocal of the equilibrium association constant KA.

In RBA, $[RT]$ and $[LT]$ are usually used to represent the total concentrations of receptor binding sites and ligand respectively. When equilibrium is achieved, the radioactivities of total and nonspecific bindings are measured and the concentration of the receptor– ligand complex $[RL]$ is calculated. Thus, equation (1) may be rewritten as:

$$KD = [RT - RL]\,[LT - RL]/[RL]$$

which may be further developed into:

$$[RL]^2 - [RL]\,[LT + RT + KD] + [LT]\,[RT] = 0 \qquad (2)$$

For a specified system, $[RT]$ and KD are two parameters with fixed values while $[LT]$ and $[RL]$ are the independent and dependent variables. The plot should be an ascending curve which approaches saturation with increasing $[LT]$.

In RIA, the total concentrations of labelled antigen, unlabelled antigen and antibody are usually expressed by $[p]$, $[D]$ and $[q]$, while the concentrations of specific and non– specific binding are expressed by $[SB]$ and $[NSB]$. In many cases, the radioactivities of total binding and non– specific binding are measured from which the ratio (R) of free to specifically bound antigen may be calculated. With these factors introduced into equation (1), equation (3) can be deduced:

$$R^2 [q] + R [q + NSB - p - D - KD] - KD = 0 \qquad (3)$$

where $[q]$, $[p]$, $[NSB]$ and KD are four parameters with fixed values while $[D]$ and R are independent and dependent variables.

According to R.Ekins et al[3], $[NSB]$ is not a fixed value in RIA systems. Instead, it should be a certain percentage (b) of the concentration of free antigen. Thus, they calculated the "apparent R" from the free and total bound fractions and expressed $[NSB]$ as $b[p]R/(1+R)$. When these factors are introduced into equation (3), a slightly different expression (equation (4)) may be obtained.

$$R^2 [b(KD + p + D) + q] + R [KD(b - 1) + q - p - D] - KD = 0 \qquad (4)$$

The use of R instead of B (the bound % of antigen) as the dependent variable

simplifies the computing procedure. When B values are finally obtained from the R values, the plot of B against D should be a descending curve.

The mathematical model of IRMA may be deduced in a similar manner. However, since the measured non-specific binding comes from labelled antibody q and there is no labelled antigen p, the equation obtained is slightly different (e.g., equation (5)).

$$R^2 [\, b\,(q + KD) + D] + R [\, KD\,(b \quad 1) - q + D] - KD = 0 \qquad (5)$$

2. Curve fitting

The basic principle of the curve fitting procedure used in this method may be described briefly as follows:

(1) If the number of parameters in the mathematical model is m, a set of simultaneous equations can be established by substituting the independent and dependent variables with m sets of experimental data. Solving the simultaneous equations will yield a set of definite values of the parameters. When the number of experimental points is n ($n \geqslant m$), there will be a total of $n!/m!(n-m)!$ sets of solutions for the parameters. Any single set of the solutions will suffice the needs of the mass action law.

(2) Since the sum of squared residual errors is sensitive to outlier(s), the alternative criterion suggested by R.Ekins et al.[3] is adopted. This is to minimize the sum of absolute residual errors (Sum RE) and is less strongly influenced by outliers. If there is certain reliable method to estimate the weight of response errors at different doses of ligands, it is usually better to use weighted instead of unweighted absolute residual errors.

(3) The mathematical models derived from the mass action law are hyperbolic functions. Hence the fitting procedure involves a selection of one curve out of the two which should conform to the reality of the corresponding radioassay. For example, the estimated R's of RIA should be positive and rise with the increase of dose and the estimated $[RL]$'s in RBA should approach saturation with the increase of ligand concentration.

(4) In order to solve the parameters in a more rapid and simple way, the original function is first rearranged in accordance with different combinations of the two variables and the various combinations of parameters are substituted by X, Y, Z etc to give a more simple equation.

For RBA, equation (2) is rearranged to give equation (6) and then transformed to equation (7).

$$[\, RL]\,[\, RT + KD] \quad [\, LT]\,[\, RT] = [\, RT]^2 - [\, LT]\,[\, RL] \qquad (6)$$

$$a_i X - b_i Y = c_i \qquad (7)$$

where $X = [\, RT + KD]$, $Y = [\, RT]$, $a_i = [\, RL]$, $b_i = [\, LT]$, $c_i = [\, RL]^2 - [\, RL]\,[\, LT]$, and i is the serial number of experimental points.

For RIA, equation (4) is rearranged and transformed to equations (8) and (9).

$$R^2 [\, b(KD + p) + q] + R^2 D [\, b] + R[\, KD(b - 1) + q - p] - KD = RD \quad (8)$$

$$a_i X + b_i Y + c_i Z + W = e_i \quad (9)$$

where $X = b(KD + p) + q$, $Y = b$, $Z = KD(b - 1) + q - p$, $W = -KD$, $a_i = R^2$. $b_i = R^2 D$, $c_i = R$, and $e_i = RD$.

The program solves X, Y, Z etc first, and then the parameters of the original equation.

III. EXAMPLES OF EXPERIMENTAL RESULTS

1. Radioligand binding assay of receptors

Table 1

RBA of M- cholinergic receptors of mouse brain

LT (nmol/l)	*RL* (cpm) (observed)	[*RL*] (nmol/l) (observed)	[*RL*](nmol/l) (estimated)	Abs.RE (nmol/l) (absolute)
0.0656	651	0.0551	0.0545	0.0006
0.0984	801	0.678	0.0805	0.0127
0.1639	1389	0.1176	0.1295	0.0119
0.2459	2279	0.1930	0.1834	0.0096
0.3278	2683	0.2272	0.2272	0.0000
0.4918	3307	0.2800	0.2844	0.0044
0.6557	3827	0.3240	0.3138	0.0102
0.8196	3893	0.3296	0.3296	0.0000
Sum of absolute residual error	=			0.0494

Sp. Act. of ^{3}H- QNB: 1.4TBq /mmol Counting efficiency:35%
Assay volume: 0.4ml Protein content: 0.152 mg

Table 2

Curve fitting result of the raw data listed in Table 1

Set of exp.points	[*RT*] (nmol/l)	*RT* (fmol/mg prot)	*KD* (nmol/l)	Sum of Abs. RE (nmol/l)
5 - 8	0.3731	981.9	0.065	0.049
1 - 8	0.3697	973.0	0.060	0.053
6 - 8	0.3810	1002.7	0.076	0.054
5 - 7	0.3980	1047.4	0.076	0.058
1 - 7	0.3852	1013.6	0.063	0.063
...	...	...	...	...
Final result: *RT* = 981.9 fmol/mg prot,			*KD* = 0.065 nmol/l.	

Only the first 5 sets with least sums of absolute residual errors were listed

M- cholinergic receptors of mouse brain were assayed with tritiated QNB at 8 different concentrations of *LT*. After incubation, the bound radioactivities were measured and, with the corresponding radioactivities of NSB deducted, turned to *RL* concentrations. The raw data are listed in Table 1.

From the raw data of the 8 experimental points, 28 sets of [*RT*] and *KD* values were

obtained by the fitting method. The first 5 sets with least sums of absolute residual errors are listed in Table 2. The final result is : RT = 981.9 fmol/mg prot and KD = 0.065 nmol/l. The estimated RL values and absolute residual errors calculated with these parameters are listed in the right half of Tab.1.

The robustness of this method was examined by the effect of outliers on the curve fitting result. 16 sets of "raw data", each with one artificial outlier, were produced by adding each time a +25% or − 25% error to one of the observed [RL] values of the 8 experimental points. As shown in Fig.1, among the 16 final results obtained from these "raw data", 9 sets of [RT] and KD values were exactly the same as without outlier. The bias of the other 7 final results, though present, were smaller than those obtained with other curve fitting methods (e.g., Woolf plot). However, it can also be seen in Fig.1 that one bias of the 16 results obtained with this method is not small enough. Further improvement of the method is therefore expected to be studied in the future.

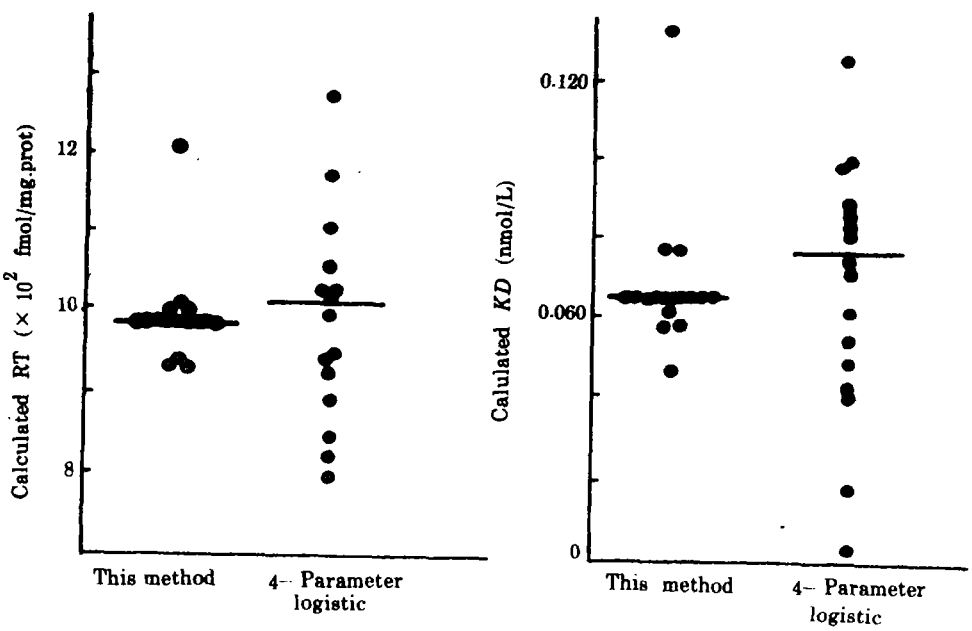

Fig. 1 The effect of outliers on the results of RBA obtained with differentcurve fitting methods

Each point represents the result with one "outlier"

The horizontal lines indicate the "unbiased" level

2. Fitting of the calibration curve of RIA

Radioimmunoassay of Adenosine– 3′, 5′– cyclic nucleotide (cAMP) was carried out with ^{3}H– cAMP. The raw data of the calibration curve are listed in Table 3.

From the 7 experimental points, 35 (7!/4!3!) sets of X, Y, Z, W values and 35 sets of

b, KD, p, q values were obtained. The first 5 sets with least sums of weighted absolute residual errors are listed in Table 4 and the first set was chosen as the final result. The individual estimated *B*'s and their errors obtained with this set of *b, KD, p* and *q* values are listed in the right half of Table 3.

Table 3

A set of raw data of the calibration curve of cAMP RIA

Dose (pmol)	Observed B (bound %)	Observed R (F/B)	Estimated B (bound %)	Weight RE	Wt Abs
0	46.476	1.152	46.476	1.52	0.000
0.4	23.494	3.256	23.494	1.56	0.000
0.8	16.112	5.207	16.203	1.63	0.148
1.6	10.371	8.643	10.371	1.70	0.000
2.4	7.971	11.546	7.863	1.83	0.197
4.0	5.576	16.932	5.576	1.96	0.000
8.0	4.000	24.000	4.323	2.22	0.717

^{3}H– cAMP in each tube: 17000cpm

The weight is calculated from the average SEM of the B's of each point obtained in several previous batches

Table 4

Result of curve fitting of the raw data listed in Table 3

Set of exp points	Parameter values		fromcurve fitting		Sum of wt. Abs. RE.
	b	*KD*	*p*	*q*	
1-2-4-6	0.0158	-0.024	0.4419	0.1803	1.062
1-2-4-7	0.0100	-0.067	0.5552	0.1970	1.146
1-2-3-6	0.0171	-0.002	0.3856	0.1736	1.215
1-3-4-6	0.0151	-0.059	0.5152	0.1847	1.317
1-2-5-6	0.0128	-0.076	0.5726	0.1964	1.324
...	...	...	...	...	...

Only the first 5 sets with least sums of weighted absolute residual errors are listed

Artificial outliers were also used to examine the robustness of the fitting. 14 modified calibration curves of the original curves were established by introducing each time a +30% or – 30% error to one of the observed *B* of the 7 standards. The observed *B*'s of a set of unknown samples were then substituted into each curve to yield a set of corresponding dose values. As can be seen in Fig.2, the bias of the doses of unknown samples obtained with this method were significantly smaller than those obtained with the conventional 4- parameter Logistic method[6]. Such an improvement is apparently due to the small effect of outliers on the shape and corrdinates of the calibration curve (Fig.3).

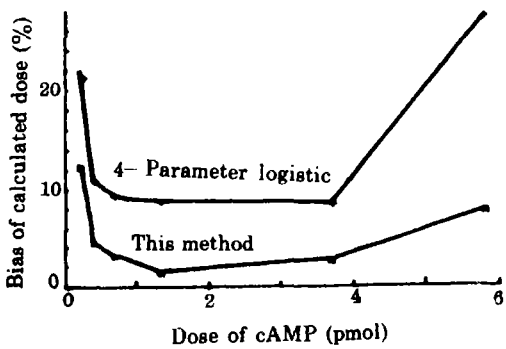

Fig. 2 The bias of doses of unknown samples obtained from calibration curves fitted with different methods

Each point is the mean of 14 values from the 14 curves with outliers as described in the text

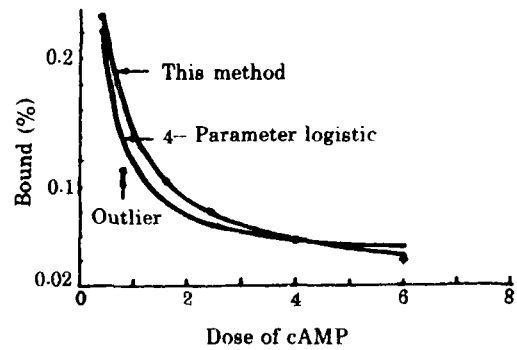

Fig. 3 The difference of the shape of curves obtained with different fitting method from the same set of standards with one outlier

The O– dose point is not shown in the figure

IV. DISCUSSION AND CONCLUSION

1. A new curve-fitting method with its mathematical models derived from the mass action law is presented in this paper. The method is applicable to several in vitro radioassays.

2. The curve fitting method is entirely different from the conventional least square regression. The sum of absolute instead of squared residual errors is used as the fitting criterion and the selection of a best solution from several possible solutions is used instead of iteration. Experiments revealed that the robustness of this method is better than the conventional methods like Woolf plot in RBA and 4– parameter Logistic plot in RIA.

3. The 4– parameter mathematical model of RIA of this method is derived according to the principle described by R.Ekins et al. However, the middle term of the original model of Ekins et al is $R[\ KD\ (b\ +\ 1)+\ q\ -\ p-\ D]$, and in this paper it is $R\ \{\ KD\ (\ b\ -\ 1)\ +\ q\ -\ p-\ D]$.

4. In non–equilibrium RIA, the forward velocity v_1 is not equal to the backward velocity v_2. When this factor is introduced into the 4–parameter model, KD may be written as $(v_1/v_2)\ KD$. Therefore, this model may also be used in non– equilibrium RIA if we define *"KD"* as the apparent KD and keep in mind that when $v_1 \neq v_2$, it will be greater than the real KD.

5. It should be emphasized that the robustness of this method is only relative. It depends on the number of experimental points and the number, location and magnitude of outlier(s). In some cases with outliers, the bias of the results may be too

large to be acceptable. In addition, the robustness is, to some degree, also determined by the number of parameters in the model. Thus, with the 4– parameter model of RIA, although the robustness of the solution of unknown doses is usually satisfactory, the values of the parameters themselves may fluctuate markedly with outliers.

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