《原 著》

Graphical Determination of Affinity Constant for Labeled and Unlabeled Ligand from Angiotensin I Radioimmunoassay

Isao IKEDA*, Kazushige IINUMA*, Masaru TAKAI* and Kunio KURATA*

**Dainabot Radioisotope Laboratories, Chiba*

Abstract A theoretical procedure for calculating the affinity constant of both labeled and unlabeled ligand has been developed. A linear Scatchard plot is obtained from the dose-response curve with only labeled ligand and the calculated affinity constant for labeled ligand is used to determine the affinity of unlabeled ligand. From the bound/free of dose-response curve with various amount of unlabeled ligand and a fixed amount of labeled ligand, true bound concentrations are corrected against the linear Scatchard plot with only labeled ligand, and then the affinity for unlabeled ligand can be calculated. We applied the method for the determination of affinity constants of angiotensin I and ^{125}I-labeled angiotensin I with antisera.

Introduction

The mathematical theories of radioligand assay have evolved over the last decade since the introduction of Scatchard plot into the field of radioimmunoassay (RIA)[1-3]. The value of K, the affinity constant, is of great importance in the elucidation of the nature of the reaction between a ligand and a binding molecule. In most of the methods, only one ligand binding to one class of site has been considered for the determination of the affinity constant, and also it is assumed that labeled and unlabeled ligand have the same affinity for all sites. However, with the development of RIA for small molecules, various substances having no functional group for iodination such as cyclic nucleotides[4], digoxin[5], estrogens[6] and bile acid[7] have been derivatized as a histamine or succinyltyrosyl methyl ester. In this case, it cannot be assumed that the modified labeled ligand and unmodified ligand are identical with respect to their affinity. In fact, greater affinity of testosterone-3-histamine-^{125}I than testosterone-[^{3}H] in testosterone RIA has been observed[8]. This indicates the affinity of testosterone-3-histamine-^{125}I is different from that of unlabeled testosterone. Cailla et al also pointed out that the linkage by hemisuccinate group for a cAMP RIA increased the immunoreactivity 100 times[9]. Hollemans et al have discussed the heterogeneity in binding affinity of labeled and unlabeled ligand from the Scatchard plot[10]. They evaluated the inequality of the affinity constant even for the labeled ligand itself, depending on its specific activity. On the other hand, it has been reported that immunoreactivity of the labeled ligand is decreased by the chemical effect during the iodination processes rather than the incorporation of iodine atoms into the ligand[11,12]. This indicates that the change of the binding affinity by chemical reaction in iodinated molecules should be discussed more than the incorporation ratios of iodine atoms.

In this paper, we describe a theoretical procedure for calculating the affinity constant of both labeled and unlabeled materials, and the experimental data from angiotensin I RIA was applied in this analysis.

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Materials and Methods

Radioiodination of angiotensin I

Two μg of angiotensin I (Protein Research Foundation, Osaka, Japan) in 0.1 m*l* of 0.5 M phosphate

buffer pH 7.4 was iodinated with 3 mCi of $Na^{125}I$ (Radiochemical Centre, Amersham, England) by the chloramine T method. Immediately after the reaction was complete, ^{125}I-labeled angiotensin I was purified on a Sephadex G-25 column (1×50 cm) with 0.2 M phosphate buffer pH 6.0. The ^{125}I-labeled angiotensin I eluate was diluted with 0.01 M formic acid and stored at −20°C until use.

Standard angiotensin I

The angiotensin I was obtained from Protein Research Foundation and tested against Research Standard A for (asp^{1}ileu^5) angiotensin I (National Institute for Biological Standards and Control, Hally Hill, London).

Immunization

The antibodies to angiotensin I were raised in female New Zealand White Rabbits as described previously[13]. Antisera with final titers ranging from 5000 to 210000 were used in the experiment.

Assay procedure

The assay was performed in 12×75 mm polypropylene tubes in quadruplicate as follows. For dose-response curve with only labeled ligand, 0.2 m*l* of assay buffer (0.01N formic acid) containing 5.0% bovine serum albumin and 0.1 m*l* of ^{125}I-labeled angiotensin I (0.1 M Tris buffer, pH 7.4) at 6 different concentrations, ranging from 20,000 to 170,000 cpm, were incubated with 0.1 m*l* of rabbit anti-angiotensin I anti-serum. On the other hand, for dose-response curve with labeled and unlabeled ligand, 0.2 m*l* of angiotensin I standard (0, 0.1, 0.2, 0.8, 2 and 4 ng/m*l*) in 0.01N formic acid containing 5.0% bovine serum albumin and 0.1 m*l* of ^{125}I labeled angiotensin I (approximately 30000 cpm, 0.1 M Tris buffer, pH 7.4) were incubated with 0.1 m*l* of rabbit anti-angiotensin I anti-serum. In both assays, the tubes were vortex-mixed for 2–3 seconds and allowed to equilibrate for 72 hr at 4°C. In addition, extra assays were performed to define the upper limit (A) with a 50-fold increase in antibody concentration and lower limit (B) with a standard of 100 ng/m*l*, and bound/total (b) is calculated

$$b=\frac{b'-B}{A-B}$$

where b′= apparent bound/total

as introduced by Waler[14]. After incubation, antibody bound ^{125}I labeled angiotensin I was separated from free ^{125}I labeled angiotensin I by adding 0.1 m*l* of rabbit γ-globulin (10 mg/m*l*) and 1 m*l* of 25% polyethylene glycol followed by centrifugation at 2,200×g for 20 min. The radioactivities of the precipitates were counted with a gamma counter.

Theoretical Analysis

In the theoretical model, only a single class of binding sites is considered to be associated with the binder. The labeled and unlabeled ligand are assumed to be different with respect to their affinity for the binder. However, the affinity constant for the labeled material is assumed to be homogeneous regardless of its specific activity. According to the law of mass action, the following equations in the equilibrium state can be defined.

$$K=\frac{[AgAb]}{[Ag][Ab]} \quad \cdots\cdots 1$$

$$K^*=\frac{[Ag^*Ab]}{[Ag^*][Ab]} \quad \cdots\cdots 2$$

$$Ta=[Ag]+[AgAb] \quad \cdots\cdots 3$$

$$Ta^*=[Ag^*]+[Ag^*Ab] \quad \cdots\cdots 4$$

$$Tb=[Ab]+[Ag^*Ab]+[AgAb] \quad \cdots\cdots 5$$

$$b=\frac{[Ag^*Ab]}{Ta^*} \quad \cdots\cdots 6$$

where, K = Affinity constant for the reaction between unlabeled ligand and antibody.

K* = Affinity constant for the reaction between labeled ligand and antibody.

Ta = Total concentration of unlabeled ligand.

Ta* = Total concentration of labeled ligand.

Tb = Total antibody site concentration.

b = Bound/Total of labeled ligand.

[Ag] = Free unlabeled ligand concentration.

[Ag*] = Free labeled ligand concentration.

[Ab] = Free antibody site concentration.

[AgAb] = Unlabeled bound concentration.

[Ag*Ab] = Labeled bound concentration.

Then, these equations can be solved for b,

$$K^*Ta^*(K-K^*)b^3+\{2K^{*2}Ta^*+K^{*2}Tb+K^*-K-K^*KTb-K^*KTa^*+K^*KTa\}b^2-\{K^*+K^{*2}Ta^*+2K^{*2}Tb+K^*KTa-K^*KTb\}b+K^{*2}Tb=0 \quad \cdots\cdots 7$$

As demonstrated in Fig. 1, the dose-response curves (b vs Ta) in $K\neq K^*$ system are significantly deviated from that of $K=K^*$, depending on their differences

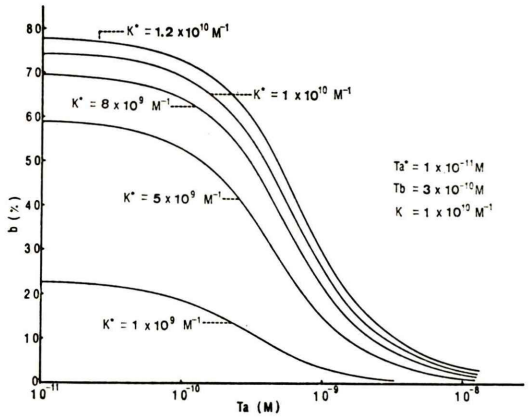

Fig. 1 Theoretical dose response curves for different values of affinity constant of labeled ligand (K*). Affinity constant for unlabeled ligand (K), concentrations of labeled ligand (Ta*) and antibody (Tb) are constant.

of magnitude of affinity.

Substitution of equation 1, 2, 3, 4 and 6 in 5 results in

$$\frac{b}{1-b}=K^*\{Tb-b(Ta+Ta^*)\}+\frac{TaK^*b(1-b)(K^*-K)}{Kb+K^*(1-b)} \quad \cdots\cdots 8$$

when $K=K^*$, equation 8 gives

$$\frac{b}{1-b}=K^*\{Tb-b(Ta+Ta^*)\} \quad \cdots\cdots 9$$

Equation 9 defines the classic linear Scatchard plot of $b/1-b$ vs. $b(Ta+Ta^*)$ with slope equal to $-K^*$. When the labeled and unlabeled ligand are different with respect to their affinity ($K\neq K^*$), marked deviation from linearity will be obtained from equation 8 and 9, which corresponds to

$TaK^*b(1-b)(K^*-K)/\{Kb+K^*(1-b)\}$

Equation 8 can be rearranged by substituting $b/(1-b)=Y$ and $b(Ta+Ta^*)=X$,

$$Y=K^*(Tb-X)+\frac{TaK^*(K^*-K)X}{(Ta+Ta^*)(KY+K^*)} \quad \cdots\cdots 10$$

Then the Y ordinate intercept is

$$\lim_{X\to o} Y=K^*Tb \quad \cdots\cdots 11$$

i.e., this is equal to the y ordinate intercept of Scatchard plot with only the labeled ligand. When $Y=0$, equation 10 can be written as,

$$X=\frac{K^*Tb(Ta+Ta^*)}{TaK+Ta^*K^*} \quad \cdots\cdots 12$$

Generally, bound to free ratios of labeled ligand (Y) can be decreased by increasing the unlabeled ligand (Ta). Thus, the X abscissa intersection can be defined from the equation 12.

$$\lim_{Ta\to\infty} X=\frac{K^*}{K}Tb \quad \cdots\cdots 13$$

It is apparent that the $K=K^*$ gives the Tb as the X intercept shown in Fig. 2.

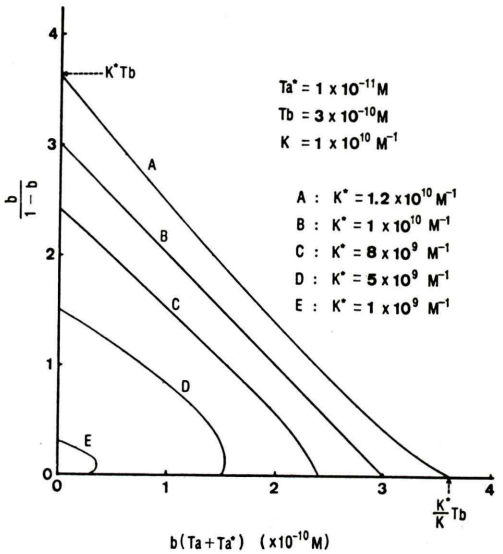

Fig. 2 Scatchard plots calculated at various values of K* in Fig. 1.

Rearranging equation 8 gives,

$$\frac{b}{1-b}=K^*Tb-K^*bTa^*\left\{1+\frac{Tak/Ta^*}{Kb+K^*(1-b)}\right\} \quad \cdots\cdots 14$$

This gives a linear relationship between the $b/(1-b)$ and $bTa^*+bTaK/\{Kb+K^*(1-b)\}$ with slope equal to $-K^*$, and ordinate and abscissa intercept of K^*Tb and Tb, respectively. In fact it can be seen that if one can plot of $b/(1-b)$ on the ordinate vs. $bTa^*+bTaK/\{Kb+K^*(1-b)\}$ on the abscissa, the curve is essentially the same as the Scatchard plot performed with only labeled ligand as indicated in equation 9 (cf Fig. 3). First, measure the bound to free ratios $(b/1-b)$ from the dose-response curve by varying the amount of unlabeled ligand, and then determine the concentration of bound defined as ϕ, by reading the $b/(1-b)$ on the Y axis and the corresponding ϕ on the X axis of the Scatchard plot with only labeled ligand.

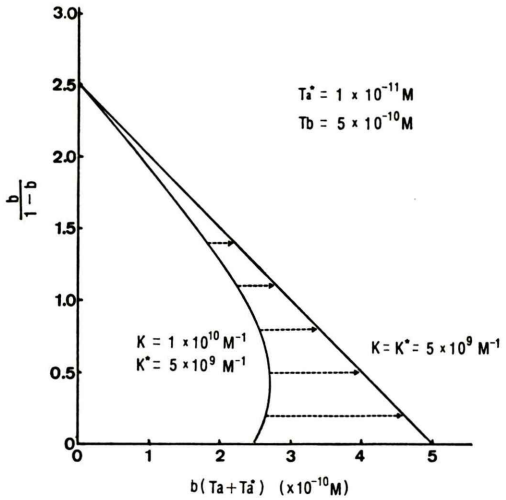

Fig. 3 Scatchard plots for K=K* and K≠K* systems.
In the case of K≠K* system, if b/(1−b) on the ordinate vs.
bTa*+bTak/{Kb+K*(1−b)} instead of b(Ta+Ta*) on the abscissa is plotted, linear straight line which is exactly the same line of K=K* as indicated by arrows can be obtained.

ϕ is expressed as,

$$\phi = bTa^* + \frac{bTaK}{Kb + K^*(1-b)} \quad \cdots\cdots 15$$

The concentration of labeled ligand can be replaced with its specific activity equivalent as follows.

$$ta^* = \beta\, Ta^* \quad \cdots\cdots 16$$

where ta^*=Total concentration of labeled ligand counts (cpm/l)
β=Specific activity (cpm/mole)

Substitution of equation 16 in 15 result in

$$\frac{Ta}{\beta\phi - bta^*} = \frac{1}{\beta} + \frac{1}{K}\left(\frac{K^*}{\beta}\right)\cdot\frac{1-b}{b} \quad \cdots\cdots 17$$

When $Ta/\{\beta\phi - bta^*\}$ is plotted on the Y axis and $(K^*/\beta)\cdot(1-b)/b$ on the X axis, a plot of experimental values should form a straight line with slope 1/K and y intercept of $1/\beta$. For the experimental determination of this relationship, it is required to know the values of K^*/β and $\beta\phi$. These values can be obtained from the slope and X-axis values of equation 9 after substitution of equation 16 as

$$\frac{b}{1-b} = \left(\frac{K^*}{\beta}\right)\{\beta Tb - bta^*\} \quad \cdots\cdots 18$$

Results and Discussion

In the first step, the dose-response curve was obtained by incubating various amounts of ^{125}I labeled angiotensin I with appropriately diluted rabbit anti-angiotensin I anti-serum. Figure 4-(A) shows the Scatchard plot in equation 18. The experimental data, plot of b/1−b *vs.* bta^* showed a good linear relationship. The K^*/β was calculated from the slope, and used for further calculation step. In the second step, another dose-response curve was obtained from various amount of angiotensin I standard (Ta) and a fixed amount of ^{125}I labeled angiotensin I (ta^*) with the antiserum. Then, the $\beta\phi$ was read as the abscissa of Fig. 4-(A) from b/(1−b) of the second step dose-response

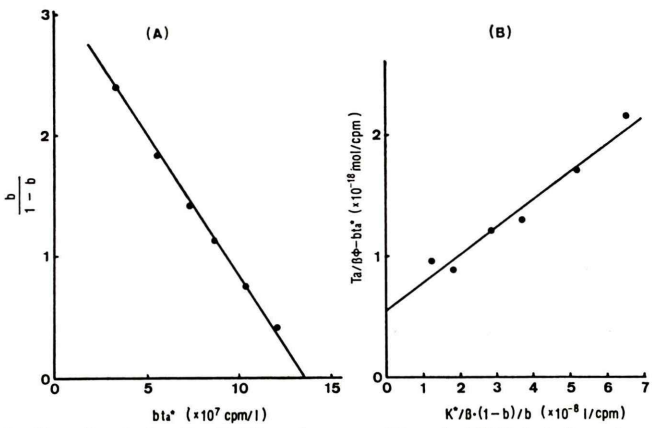

Fig. 4 (a) Scatchard plot for R-75 antiserum with only ^{125}I labeled angiotensin I.
(b) Plot of $Ta/\{\beta\phi - bta^*\}$ vs. $K^*/\beta\cdot(1-b)/b$ according to equation 17 showing a straight line with slope 1/k.

Table 1 Affinity constant and titer of anti-angiotesin I anti-serum with angiotensin I (K) and ^{125}I labeled angiotensin I (K*)

Antiserum	K	K*	Final dilution[a)]
R-45	1.7×10^{11}	1.3×10^{11}	1:210000
R-66	6.3×10^{10}	6.4×10^{10}	1:5000
R-67	6.5×10^{10}	6.3×10^{10}	1:21000
R-68	6.9×10^{10}	6.5×10^{10}	1:28000
R-71	5.7×10^{10}	5.7×10^{10}	1:105000
R-75	4.4×10^{10}	3.2×10^{10}	1:35000

[a)] Dilution of antiserum capable of binding 50% of ^{125}I-labeled angiotensin I is difind.

curve. The plot of the data between $Ta/\{\beta\phi-bta^*\}$ and $K^*/\beta\,(1-b)/b$, according to equation 17, gave good linearity with the slope as 1/K as shown in Fig. 4-(B). The affinity constant of unlabeled angiotensin I (K) and ^{125}I labeled angiotensin I (K*) for the antiserum R-75 were 4.4×10^{10} 1/mol and 3.2×10^{10} 1/mol, respectively.

The affinity constants and titers of various antisera against angiotensin I raised in rabbits are listed in Table 1. Approximately 25% of the affinity of R-45 and R-75 was found to be decreased by labeling with ^{125}I, and 9% for R-68. On the other hand, the antisera R-66, R-67 and R-71 showed no significant difference between K and K*, which indicated that the binding characteristics to angiotensin I by these antisera were not affected by labeling with ^{125}I.

Hollemans et al[10] pointed out that only a small amount of unlabeled ligand in the tracer might interfere with the linearity of the plot, and might result in the underestimation of Tb and consequently overestimation of K. It is true when the unlabeled ligand in the tracer and standard unlabeled ligand are identical, and only the labeled ligand in the tracer is different with respect to their affinity. As a matter of course, the amount of the unlabeled ligand depends on the specific activity of the tracer. However, the immunoreactivity of tracer greatly depends on not only specific activity but also its labeling method. In fact, it is well known that the labeled ligand prepared by chloramine T method has generally lower immunoreactivity and greater susceptibility to damage than by enzymatic radioiodination such as lactoperoxidase[11,12]. This indicates that the immunoreactivity is decreased during the iodination processes rather than the incorporation of iodine atoms into the ligand. Therefore, we assumed that the affinity of the tracer is homogeneous regardless its specific activity in order to evaluate the deviation of affinity between the tracer and the standard more practically.

The specific activity can be calculated from the y intercept of Fig. 4-(B). The specific activity of ^{125}I-labeled angiotensin I calculated was 1.8×10^{18} cpm/mol, which corresponded to 895 μCi/μg of angiotensin I. This indicates that approximately an average of 0.5 iodine atoms are incorporated into one mol of angiotensin I.

The heterogeneity of affinity between testosterone-3-histamine-^{125}I and testosterone has been estimated in testosterone RIA[8]. They observed greater affinity of the derivatized tracer than original ligand. However, they calculated the affinity based on the assumption that the unlabeled ligand and labeled ligand were the same molecular species and K and K* were equal. The method described here can distinguish the K and K* by using the plot of equation 17. Further, if the dose-response is obtained with testosterone-3-histamine-^{125}I as tracer and testosterone as standard, the affinity of tracer (K*) and standard (K) can be directly calculated from the plot. Various haptens having no site for iodination, have been chemically modified by introduction of tyrosine, tyramine, histamine or tyrosine methyl ester, which can be iodinated. These haptens have also been modified as immunogen. Weeman & Schuuers obtained a considerably increased sensitivity for estrogen enzyme-immunoassay by heterologous combination of antibody and estrogen-enzyme conjugate[15]. These results have been explained on the basis that the link between hapten and carrier in the immunogen contributed to the specificity and the affinity of the antibody. This indicates heterogeneity of the affinity can be expected between ligand and the derivatized ligand. The method described here provides an efficient procedure to determine the affinity of unlabeled and labeled ligand regardless of their conformational differences if only complete inhibition by labeled ligand can be achieved.

Berzofsky & Schechter have presented the mathematical assessment of crossreactivity for homogeneous and heterogeneous antibody[16]. For homogeneous antibody, they indicated that the affinity

of cross-reactive ligand can be obtained from the reciprocal concentration of free crossreactive ligand at $b=1/2\ b_0$ only if the concentration of labeled homogeneous ligand is sufficiently infinitestimal. In the case of a single homogeneous antibody having true crossreactivity by complete inhibition of tracer ligand binding, the affinity of the crossreactive ligand with antibody can be readily determined from equation 17 after substitution of $Ta=Tc$ (Tc: Total concentration of crossreactive ligand).

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要 旨

アンジオテンシン I RIA を利用した標識および非標識リガンドの結合部に対する親和定数のグラフ的手法による求め方

池田 勲夫* 飯沼 一茂* 高井 優* 倉田 邦夫*

*ダイナボットラジオアイソトープ研究所

標識および非標識リガンドの結合部に対するおのおのの親和定数の理論的な求め方を開発した．まず最初に，標識リガンドのみを用いてその濃度を変化させ，dose-response 曲線を描き，そのScatchard プロットから標識リガンドの結合部に対する親和定数を求めた．つぎに一定量の標識リガンドに対し，非標識リガンド濃度を変化させて得た Scatchard プロットにおいて，先に求めた標識リガンドのみのプロットを利用し，真の結合濃度を補正することによって非標識リガンドの親和定数を求めることができた．本法を用い，アンジオテンシン I の RIA において，抗血清に対する ^{125}I 標識アンジオテンシン I と非標識アンジオテンシン I のおのおのの親和定数を求めることができた．

Key words: Affinity Constant, Scatchard plot Radioimmunoassay, Angiotensin I.