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Ligand Dissociation Constants From Competition Binding Assays: Errors Associated with Ligand Depletion

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SUMMARY

The dissociation constant of a ligand at a binding site (e.g., receptor, antibody) is often determined indirectly by competitive displacement of a radioligand. It is well known that such a determination may be seriously in error unless the free concentrations of both the radioligand and the unlabeled ligand can be measured. By means of computer simulations we have explored the conditions under which this error may occur and its magnitude. We offer guidelines for recognizing a probably inaccurate dissociation constant, and we show how, in some cases, a correction can be made. The problem addressed here is not only a theoretical one; it can arise in the ordinary performance of competition binding assays.

Competition binding assays are often used to determine the affinities of various ligands for binding sites (receptors, antibodies). The results of such assays depend on competition equilibria described by the law of mass action. A radioligand interacts reversibly with a binding site, a competing ligand reduces the bound radioactivity in a concentration-dependent manner. *Free* ligand concentrations are required in the mass law equations but, frequently, only *total* concentrations are known. In the Michaelis-Menten approximation for enzyme reactions it is assumed that free and total concentrations of a substrate or inhibitor are virtually the same, i.e., that a negligible fraction is depleted by binding. This same approximation is often employed uncritically by practitioners of competition binding assays.

The errors in estimating ligand affinities that are introduced by ignoring ligand depletion have been considered at length before (1–5), and full theoretical treatment of the problem is to be found in extensive analyses by Rodbard and his co-workers (6–9). All of those analyses, like the present one, are implicit in the mass law equations for two-ligand, one-site competition (10). The popular computer program LIGAND (8) provides a useful prepackaged method of analyzing experimental data according to this model. Nonetheless, experimenters could also benefit from practical guidelines for recognizing when approximations are likely to be valid and when they may produce significant error. The novel approach described here attempts to meet this need.

Our central concern is with the experimental procedures as they are actually carried out in the laboratory. We analyze the magnitude of the sometimes unavoidable systematic error in determining the dissociation constant of an unlabeled ligand by competition with a labeled one, whether in RRAs or RIAs.[1] In this connection, we introduce the concept of "acceptable error," an error of predefined magnitude relative to an empirically measured random experimental error.

Methods

All symbols used in this paper are defined in Table 1. In an experiment one first uses a radioligand by itself and measures the binding (often called B_0). Then one finds the concentration of an unlabeled ligand that at equilibrium reduces the site-bound radioactivity by half. The equilibrium dissociation constant K_X of the competing ligand is computed from this concentration (X_{50}, often called IC_{50}), the radioligand concentration, and the radioligand dissociation constant K_L. Typically, the total added concentrations of radioligand and competing ligand are used in this computation. If there is significant depletion of either, the computed value of K_X may be incorrect. We shall find the magnitude of this error under various relevant conditions.

In our analysis the key parameter is one that is easy to measure—the degree of radioligand depletion, D, in the absence of a competing ligand. D is defined as LS/L_T, where LS is the concentration of site-bound radioligand and L_T is total radioligand (site-bound plus free). We ignore nonspecific binding in this theoretical analysis but, in actual experiments, both free and site-bound radioactivity must be measured to find D. As pointed out by Siiteri (11), many high affinity ligands bind nonspecifically to membranes, proteins, tube walls, and filters, so it is not permissible to equate *free* to *total added minus site-bound*, as

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[1] This error does not arise in the typical use of RIA to compare inhibitory potency with that of a standard, provided that the radioligand, the standard, and the compound being assayed have the same affinity for the antibody-binding sites, as, for example, when they are various concentrations of the same compound.

ABBREVIATIONS: RRA, radioreceptor assay; RIA, radioimmunoassay.

TABLE 1
Definitions of symbols used

K_L	Radioligand dissociation constant, used as the unit in which all concentrations and other dissociation constants are expressed.
K_X	Dissociation constant of unlabeled ligand.
S_T	Total concentration of binding sites.
S	Concentration of unoccupied binding sites.
L_T	Total concentration of radioligand.
LS_0	Concentration of site-bound radioligand in the absence of unlabeled ligand (commonly called B_0).
LS	Concentration of site-bound radioligand (commonly called B).
L_0	Free concentration of radioligand in the absence of competing ligand.
L	Free concentration of radioligand, especially at $B/B_0 = 0.5$ in equilibrium with X_{50}.
D	An index of the depletion of radioligand in the absence of a competing ligand by binding at the binding sites [$=LS/(L + LS)$, or LS/L_T if nonspecific binding can be ignored].
X_T	Total concentration of unlabeled ligand.
XS	Concentration of site-bound unlabeled ligand.
X	Free concentration of unlabeled ligand.
X_{50}	Free concentration of unlabeled ligand when $B/B_0 = 0.5$ (commonly called IC_{50}).

in some computer programs for analyzing binding data. Free radioligand concentrations may be measured in supernatants after centrifugation of membranes or precipitation of antibodies.

We programmed the mass law competition equation (without approximations) on a computer and we solved for the reduction of bound radioactivity by competing ligands with widely differing affinities. We simulated complete competition curves for all plausible values of D, L_T, X (free concentration of competing ligand), K_X, and S_T (total concentration of binding sites). We assumed a single homogeneous noninteracting population of binding sites, and simple competitive equilibrium. Computations were simplified by expressing concentrations and dissociation constants in units of K_L. Logarithms are to the base 10. Derivations of the equations as well as the computer programs themselves (in IBM BASICA) are available on request in exchange for a blank diskette.

The mass law expression $(L_0)(S)/(LS)_0 = K_L$, where subscript 0 denotes initial conditions in the absence of competing ligand, S is the concentration of unoccupied sites, and $(LS)_0 = D(L_T)$ leads to the following expression for the total concentration of binding sites:

$$S_T = [K_L(D)/(1 - D)] + D(L_T) \quad (1)$$

Operationally, L_T will be known, D will be measured, and K_L will have been determined from the binding isotherm or from self-competition (as noted in the Discussion). It should be noted that, in simulations where L_T is fixed, if D is varied, S_T will also vary. Thus, effects and errors ascribed here to D (which is readily measurable) are really due to S_T (which is not).

For each value of K_X (we vary this parameter over many orders of magnitude) we generate a complete competition curve. The free concentration of competing ligand (X) is incremented in steps of 0.2 log units, and for each X we obtain the corresponding total concentration (X_T) by solving for site-bound unlabeled ligand (XS) in $(XS) = S_T - (LS) - S$. S_T was obtained in Eq. 1. LS is derived from the mass law equation for competition equilibrium

$$(LS) = K_X(L)(XS)/K_L(X)$$

by finding the appropriate root of this quadratic equation:

$$(LS)^2 - [S_T + L_T + (K_L/M)](LS) + S_T(L_T) = 0 \quad (2)$$

where $M = K_X/(K_X + X)$.

The solution of Eq. 2 gives $(LS)/(LS)_0$, which is B/B_0, and also the radioligand site occupancy $(LS)/S_T$. Finally, from $S = K_X[S_T - LS - S]/(X)$ we obtain $S = M[S_T - (LS)]$. $(XS)/S_T$ is the site occupancy by unlabeled ligand, and X_T is found as the sum $X + (XS)$. Using X as the independent variable rather than X_T and then solving for X_T (as above) makes these complex equations manageable. X_{50} values are found by iterating with ever smaller increments and decrements of X until B/B_0 is between 49.9% and 50.1%. Slopes and correlation coefficients are computed by least squares linear regression on log-logit transformed data, excluding values of B/B_0 outside the range of 0.05–0.95 (considered to be the meaningful range of experimental observations).

Results

Depletion of radioligand. The slope of the log-logit plot of competition data is sensitive to radioligand depletion. That the slope of such a plot is often close to 1 has led to a widespread belief that it is equivalent to a Hill plot. It is not. The Hill equation is a transformation of the mass law equation for the saturation of sites by a ligand; in the corresponding graphical representation abscissae are logs of the free ligand concentrations and ordinates are logits of the fractional saturation of the sites. In a log-logit plot of competition data, on the other hand, there is typically a low fractional saturation of sites to begin with, and this is reduced toward 0 by competition. Here we have a purely empirical device to obtain a nearly linear transformation of the data. It is not a simple transformation of the mass law competition equations. A slope of 1 is merely an asymptote, and slopes of less than 1 are not unusual.

Fig. 1 illustrates this point. It was constructed by fixing a value L_T and increasing D. At each value of D a complete log-logit competition curve was constructed, the slope of which was

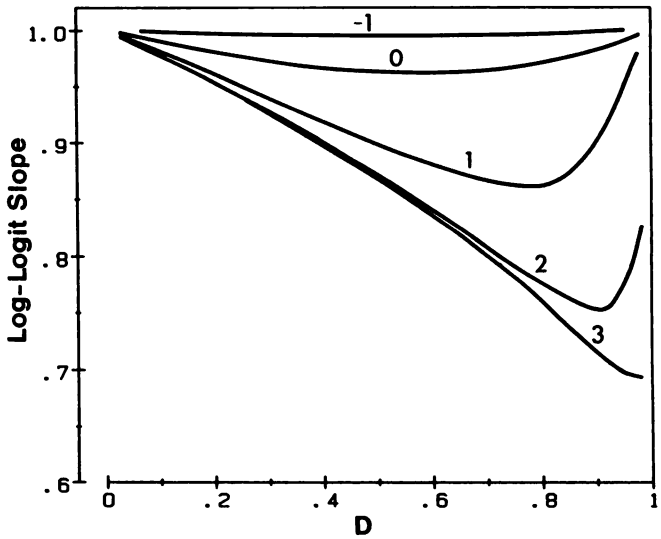

Fig. 1. Theoretical log-logit slopes. For each L_T and D we obtained a complete log-logit competition curve, plotting $\log\{[B/B_0)]/[1 - (B/B_0)]\}$ as ordinates against $\log(X)$ as abscissae. Each slope was then found by linear regression between the limits $B/B_0 = 0.95$–0.05, and plotted against D. Each curve is labeled with the value of $\log(L_T)$. Concentrations are in units of K_L. This figure applies to all values of K_X.

found by linear regression analysis. In that procedure the x axis of the competition curve was $\log(X)$, the *free* concentration of competing ligand, assumed to have been measured or to be virtually the same as the total. Thus, the data for Fig. 1 include no approximations whatsoever. We see that, for the values of L_T displayed here, the slope approaches 1 at low values of D, becomes less than 1 as D increases, and then returns toward 1 at very high D. The slope deviates noticeably from 1 at values of $\log(L_T)$ greater than about -1.0 (i.e., $L_T > 0.1\ K_L$), becoming progressively lower as L_T increases. These same curves apply to all values of K_X, i.e, to all competing ligands. It follows from these results that a log-logit competition slope of less than 1 cannot be considered a reliable indicator of site heterogeneity or negative cooperativity. However, if the degree of depletion of radioligand and the free concentration of unlabeled ligand are both known throughout the span of a competition curve, the information displayed in Fig. 1 may be used to test whether an observed competition slope is compatible with a simple single-site model.

In what follows in this section we assume, as above, that unlabeled ligand is depleted to a negligible extent, or that its free concentration is known. We now further assume that the radioligand may be depleted significantly and that its free concentration is unknown. In a later section we deal with the reverse case, in which radioligand depletion is negligible, or its free concentration is known, and the free concentration of unlabeled ligand is unknown.

The most obvious consequence of ignoring significant radioligand depletion is that in constructing a saturation binding isotherm K_L will seem to be larger than it really is because the concentrations from which it is computed are spuriously high. A more subtle error, which affects the determination of K_X, has often escaped attention. Consider first the rigorous Eq. 3 (from the mass law competition equation) for computing K_X from X_{50}:

$$K_X = \{1/[(2L/L_0) + (L/K_L) = 1]\}X_{50} \quad (3)$$

Here L is free radioligand in equilibrium with X_{50}, when $B/B_0 = 0.5$. In the approximations offered by Cheng and Prusoff (12) and by Chou (13), which are widely applied to competition binding data, the important distinction between L_0 and L is ignored, and the nominal (total) radioligand concentration (L_T) is substituted in Eq. 3, yielding the following simplification:

$$K_X = [K_L/(K_L + L_T)]X_{50} \quad (4)$$

Only if radioligand depletion is negligible ($L \approx L_0$) will Eq. 3 reduce to Eq. 4. For self-competition, where the competing ligand is the same compound as the radioligand ($K_X = K_L$), Eq. 3 becomes:

$$K_X = [X_{50} - L][L_0/(2L - L_0)] \quad (5)$$

If $L \approx L_0$, this reduces to the simpler approximation for self-competition:

$$K_X = X_{50} - L_T \quad (6)$$

In our experience with RRA, using high affinity radioligands at concentrations near K_L, it has not been unusual for measured L at $B/B_0 = 0.5$ to be 1.5 times the original measured L_0, so that K_X determined by self-competition from Eq. 6 is nearly twice its true value given by Eq. 5.

The difference between L_0 and L in Eq. 3 also accounts for the flattening of the competition slopes shown in Fig. 1. The greater the depletion of radioligand and the higher the total radioligand concentration, the greater the increase in free radioligand as LS is decreased by competition. The slope becomes flatter because the increasing concentrations of unlabeled ligand have to compete against progressively higher concentrations of free radioligand. Only at very high S_T, associated with high values of D (cf. Eq. 1), does the competition slope return toward 1.

When D is large but unmeasured, the K_X computed from X_{50} will be erroneous, as has been pointed out before (1, 4–6, 11, 14). But what does "large" mean? How small a depletion can be considered negligible? To answer, we must first decide how great an error in a computed K_X is acceptable. We define *error* as the difference between the log of K_X computed from the approximate Eq. 2 or 4 and the log of the true value of K_X given by Eq. 3 or 5. An objective criterion for *acceptable error* is that it should be a small fraction of the random experimental error that is associated with determining an X_{50} value in the laboratory.

In order to estimate this random experimental error, we reviewed our own experience of recent years with RRA at opioid binding sites on brain membranes and RIA for measuring various opioid peptides and morphinans. We suppose that our mastery of the techniques is fairly average and, therefore, that our experience can be generalized. Our RRA data base contained 111 X_{50} determinations, comprising two to eight replications of 40 independent experiments. Within each experiment we computed the deviations from the mean X_{50} estimate; this yielded an overall SD of 0.090 log unit of concentration with 71 *df*. For RIA the experimental error was nearly the same, an SD of 0.101 log unit based on 370 X_{50} determinations in 91 experiments. What, then, is the smallest difference between two X_{50} values that could be detected with 95% confidence in a typical set of triplicate determinations? Using the RRA result and the one-tail value of $t_{0.05}$ with 4 *df* we obtain 0.111 log unit, corresponding to an error of about 29%. We then choose, arbitrarily, one-fifth of this value as our acceptable error—0.022 log unit, corresponding to about 5% of a measured X_{50} value. In the relevant figures we also indicate an error of 29% for comparison.

In Fig. 2 the inner shaded area includes values of D and L_T that permit computation of K_X without exceeding the acceptable error of 5%. The lighter shaded area includes values for a 29% error. In the region above a shaded area the computed values of K_X are too low; below a shaded area they are too high. It is evident from the interesting shape of this figure that, unless D is extremely small, it is virtually impossible to avoid exceeding the 5% acceptable error; and even at $D = 0.05$, a value that has been recommended for RRA, concentrations of L_T only a few times greater than K_L will lead to erroneous values of K_X.

Without measuring the free radioligand concentration, the extent of depletion, D, will be unknown. Moreover, K_L cannot be determined accurately and, consequently, L_T (which is expressed here in units of K_L) will also be unknown. With D and L_T unknown, there is no way to ascertain if a given binding assay system falls into the shaded area of Fig. 2. We conclude, therefore, as a practical matter, that for accurate results the free radioligand concentrations in the absence of unlabeled ligand and in the presence of X_{50} concentration of unlabeled

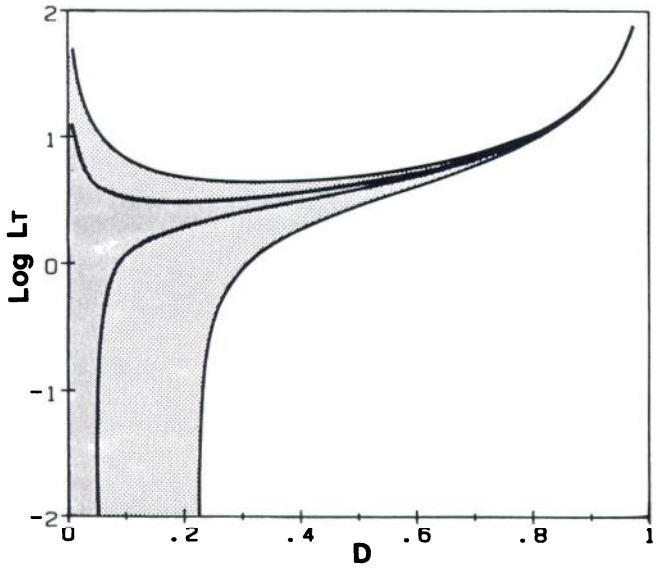

Fig. 2. Critical values of D and of total radioligand concentration for acceptable error of 5% (*inner shaded area*) or 29% (*outer shaded area*) in determining K_X. This shows the combinations of D and L_T that will just result in the specified error in the computed value of K_X when depletion of radioligand is ignored. It is assumed here either that the free concentration of the unlabeled ligand is measured or that it is known to be depleted to only a negligible extent. Erroneous computed values of K_X may be greater than (*below* the shaded area) or less than (*above* the shaded area) the true values. For a given L_T the shaded area gives the highest values of D that can be tolerated without exceeding the acceptable error. Concentrations and K_X are in units of K_L. This figure applies to all values of K_X.

ligand should both be measured. Inasmuch as measurements of free radioligand concentrations present no great technical difficulty, this should be a routine procedure in every competition binding assay, unless depletion is known to be negligible by the criteria illustrated in Fig. 2. The approximate Eqs. 4 and 6, in particular, are not otherwise valid for use in competition binding assays, a limitation acknowledged by Cheng and Prusoff (12) but often ignored.

Depletion of unlabeled ligand. We now address a source of error that is difficult to avoid. As has been noted explicitly or implicitly by others (1–3, 5–8, 15), it is necessary to measure the free concentration of an unlabeled ligand whenever its affinity is greater than that of the radioligand. This is easier said than done. A high affinity competing ligand will necessarily be used at low concentration, favoring depletion, and often leaving a free concentration below sensitivity limits of available assay methods.

To analyze the errors associated with depletion of the competing ligand, we programmed each concentration curve in two ways. With *free* concentration, $\log(X)$, of unlabeled ligand as abscissae and B/B_0 as ordinates, a rigorously correct *theoretical* curve was obtained. With *total* concentrations, $\log(X_T)$, as abscissae, a typical *experimental* curve was obtained. The difference between the two $\log(X_{50})$ values is the *error*, as defined earlier.

Fig. 3 shows a typical result—families of curves for $L_T = 1.0$ (i.e., $L_T = K_L$) and $D = 0.3$, as might be found sometimes in RRAs and often in RIAs. Dissociation constants of the unlabeled ligand, varied by factors of 10, are shown as $\log(K_X)$ on the curves. The *upper panel* of Fig. 3 shows the theoretical curves, all parallel and equally spaced. The *lower panel* shows

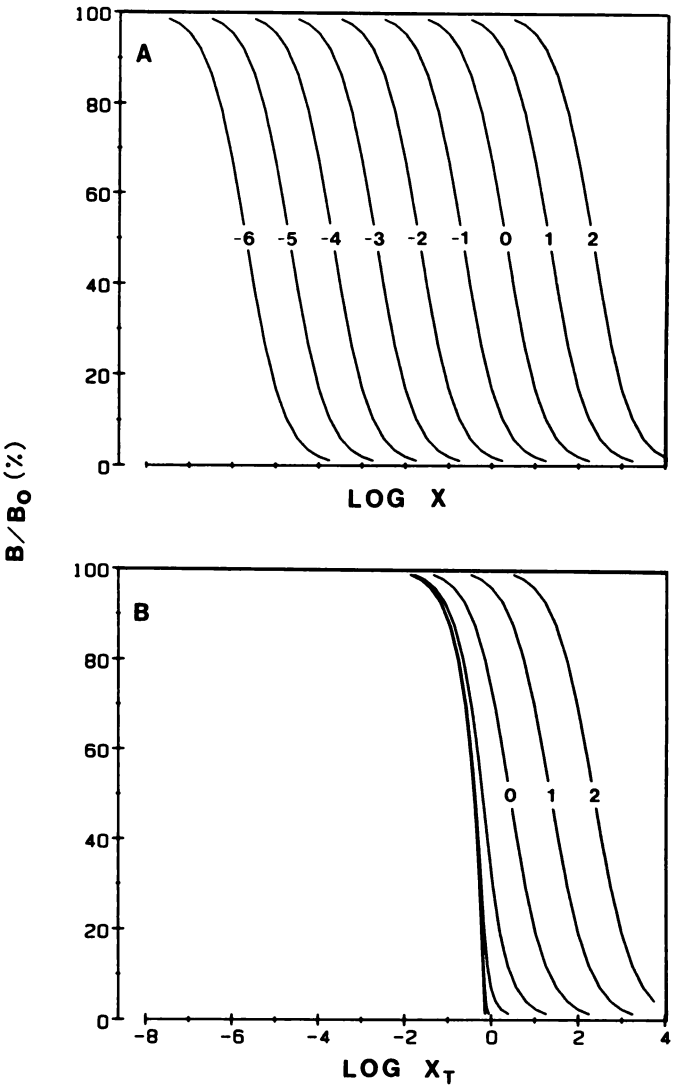

Fig. 3. Effect on competition curves when depletion of competing ligand is ignored. In this example, $D = 0.3$, $L_T = 1.0$, and *numbers curves* denote values of $\log(K_X)$. A. Log of free concentration of competing ligand is plotted on the x axis. B. Log of nominal (total) concentration of competing ligand is plotted on the x axis. The unlabeled curve to *left* of *0* is for $\log(K_X = -1.0$. Concentrations and K_X are in units of K_L.

the corresponding experimental curves. At high values of K_X (to the *right*), virtually all the unlabeled ligand is free and the experimental curves are indistinguishable from the theoretical ones. As K_X becomes smaller than K_L [$\log(K_X) < 0$], the slope increases and a limiting value of X_{50} is approached, so that the error increases rapidly. As an example, for an unlabeled ligand with dissociation constant 1/100 that of the radioligand, the error is 1.27 log units; thus, a computed value of K_X based on an experimentally determined X_{50} will be 19 times greater than the true value. The error becomes more serious the greater the initial radioligand depletion, as shown for several values of D and K_X in Table 2.

Regardless of how small D is, K_X computed from an X_{50} value based on total concentration of unlabeled ligand will be overestimated if K_X is small enough, i.e., if the affinity is high enough. Then what experimentally determined values of K_X can be relied on? When is it safe to ignore the depletion of a competing ligand? We use the acceptable error, as before, to

TABLE 2

Errors in estimating K_x when depletion (D) of radioligand is known but depletion of competing ligand is ignored

Data are antilogs of the error (as defined in text); thus, each number is the ratio of experimentally estimated K_x to true K_x. K_x is expressed in units of K_L. This table applies to all values of L_T.

K_x	D			
	0.01	0.1	0.3	0.5
1.0	1.01	1.05	1.18	1.33
0.10	1.05	1.53	2.76	4.34
0.010	1.50	6.27	18.6	34.4
0.0010	6.03	53.7	177	335

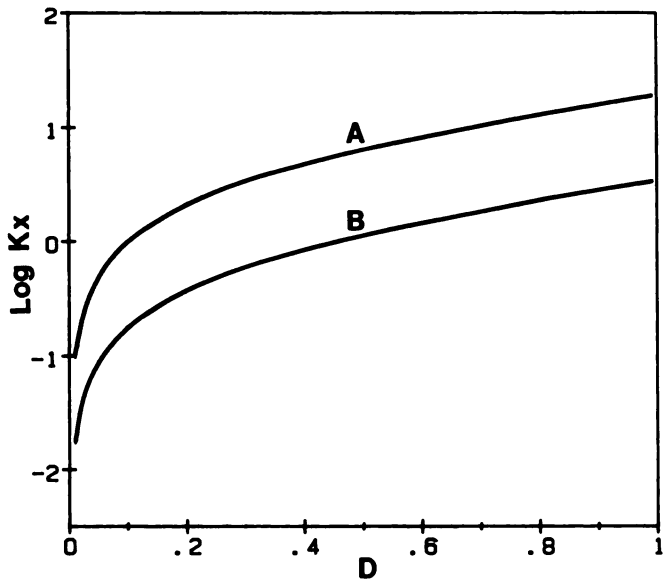

Fig. 4. Critical values of D and of $\log(K_X)$ needed to avoid exceeding an acceptable error in determining $\log(K_X)$ by competition. See the text for definition of "acceptable error," here 5% (curve *A*) or, alternatively, 29% (curve *B*). Here it is assumed that D, K_L, L_0, and L values at 50% reduction of radioligand binding are all known. Then for any true value of $\log(K_X)$ the area *above* the curve includes all the values of D that can be tolerated without exceeding the specified error. Erroneous computed values of $\log(K_X)$ are always greater than the true values. This figure applies to all values of L_T. Concentrations and K_X are in units of K_L.

answer this question. We assume that the free radioligand concentration has been measured, as recommended above, so that D is known. Now we compute for each value of D the smallest K_X that could be determined with a just-acceptable error. The results, in Fig. 4, apply to all radioligand concentrations. The region below each curve includes combinations of $\log(K_X)$ and D that yield an error greater than specified unless the free concentration of the competing ligand can be measured. At $D = 0.1$, for example, any competing ligand with K_X smaller than $K_L[\log(K_X) < 0]$ will give an experimental X_{50} that is more than 5% (the acceptable error)—too high. As D becomes smaller than about 0.1, one's ability to obtain accurate X_{50} values for high affinity competing ligands improves dramatically. Nevertheless, even at $D = 0.05$ (which might be thought low enough), unlabeled ligands with K_X less than about one-half of K_L give unacceptable errors.

A dilution procedure may indicate whether or not an estimate of K_X is valid and may sometimes permit a computed value to be corrected. In this procedure a determination of X_{50} is repeated at a reduced concentration of binding sites (same number of sites in a larger volume) with unchanged total radioligand concentration. In Fig. 5 we illustrate for 10-fold dilution. The panels represent selected values of D, and each panel displays curves for a range of L_T. An observed change in an experimentally determined X_{50} as a result of dilution can be found on the x axis, and the corresponding true value of $\log(K_X)$ can be read from the y axis. Dilution may lead to a lower X_{50} value. The extent of reduction depends primarily on K_X and to some extent on L_T. For competing ligands with K_X greater than about one-tenth $K_L[\log(K_X) > -1]$, the X_{50} does not change markedly on dilution; thus, obtaining such a result would confirm the validity of the K_X estimate already obtained. For a reduction of X_{50} by less than 10-fold, the true $\log(K_X)$ can be read off the appropriate curve. Finally, a reduction in X_{50} comparable to the dilution factor (here 10-fold) indicates that K_X is too low to estimate accurately, but an upper limit can be stated.

We have incorporated the theoretical information about dilution in a computer program that permits the user to define an acceptable error and to enter experimentally measured values of D, K_L, and L_T, and the observed X_{50} for a competing ligand. The outputs of the program are a computed value of $\log(K_X)$ from Eq. 3 and an estimate of the error. If the error is unacceptable, dilution is required, and a new experiment is carried out. The new X_{50} is entered, yielding a new output with a new estimate of $\log(K_X)$ and the error. Now a decision can be made about the acceptability of the new K_X estimate. If the error is still unacceptable, the new estimate of K_X can be taken as an upper limit for the true value.

Discussion

We have addressed three sources of error in competition binding assays. These are implicit in the mass law competition equations and they have, indeed, been pointed out from time

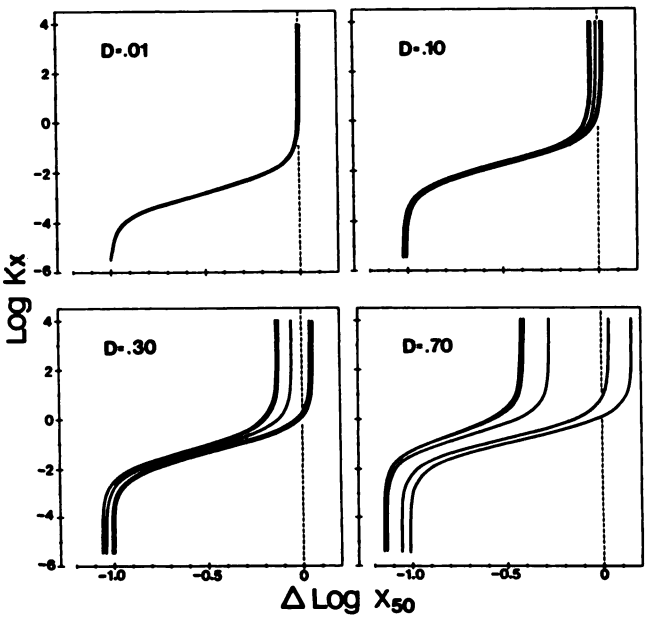

Fig. 5. Effect of the 10-fold dilution of binding sites (S_T) on the experimentally determined X_{50}. Here an X_{50} determination is repeated at a 10-fold lower concentration of S_T without changing L_T. For an observed decrease in $\log(X_{50})$ on the x axis, with a known measured D and a known L_T, the true value of $\log(K_X)$ can be found on the y axis. *Panels* are for four selected D values, and each panel shows curves for a range of $\log(L_T)$, from *left* to *right*: −2.0, −1.0, 0.0, +1.0, +2.0. Concentrations and K_X are in units of K_L.

to time. Our aim was to propose some novel ways of approaching the problem and to offer practical guidance for the experimenter, in view of the widespread use of these assays.

First, we pointed out that when there is radioligand depletion, log-logit competition slopes less than 1 may (contrary to a common misunderstanding) be compatible with homogeneous noninteracting sites.

Second, we showed that unless depletion is known to be negligible under the conditions of a particular assay, it is a virtual necessity to measure the free radioligand concentration. One needs to know the fraction of radioligand that is bound initially in the absence of unlabeled ligand, and then one needs to know the free radioligand concentration when the initial binding has been reduced by 50%. With these values in hand it becomes possible to find K_L by self-competition—sometimes a useful alternative to the saturation binding isotherm. In this case, since the radioligand and the competing ligand are the same, the free fraction of radioligand $(1 - D)$ at 50% displacement can be applied as a correction to the nominal (total) concentration of unlabeled ligand to obtain its free concentration.

Third, we analyzed the unavoidable inaccuracy in deriving K_X from X_{50} in a competition binding assay when $K_X < K_L$ and the free concentration of the unlabeled ligand cannot be measured.

How can competition binding assays be designed to minimize the potential for error, and how can error be detected when it occurs? The key requirement is to have a radioligand with as high a specific radioactivity as possible so that L_T, D, and S_T can be kept as low as possible. The common practice, in RIAs, of adjusting S_T to make D about 0.33 was developed (10, 16) to optimize responsiveness to a change in concentration of a competing ligand in a self-competition paradigm not intended for determining K_X. That recommended value of D has no relevance if RIA is used to determine the affinities of ligands, some of which may have $K_X < K_L$. From the standpoint of minimizing the error in determining K_X, whether in RRA or RIA, the lower the depletion, the better.

A radioligand with the lowest possible K_L is always to be preferred, inasmuch as error in the estimate of K_X is most likely when K_X is less than K_L.

A low concentration of L_T relative to K_L is desirable to improve the assay sensitivity (i.e., to ensure lower X_{50} values for competing ligands); but concentrations lower than about 0.1 K_L offer little incremental improvement, as is evident in the equations of Ekins *et al.* (10). If low L_T results in insufficient bound radioactivity for accurate measurement, a remedy is to increase S_T, but only at the cost of increasing D and thus possibly promoting greater error. Furthermore, it may be impractical to increase S_T for other reasons; binding sites might be in short supply, or the efficiency of separating bound from free might be compromised by filter clogging or incomplete precipitation.

Let us suppose that the best feasible competition binding assay has been developed, accepting the unavoidable trade-offs enumerated above. Is there, then, a way to gauge whether or not an X_{50} value based on total (rather than free) concentration of unlabeled ligand in Eq. 3 will give a valid estimate of K_X? Fig. 4 is not of practical value in answering this question because, although D will be known from measurement, K_X will not—indeed, K_X is what we are trying to estimate. We offer three guidelines:

1) A value of D greater than about 0.1 (cf. Fig. 4) should raise a suspicion of error. However, regardless of how low the value of D is, if the true value of K_X is low enough, the computations will result in an erroneous estimate.
2) A log-logit competition slope greater than 1 is an indication that one is near the limiting competition curve (cf. Fig. 3B). The slope only changes perceptibly after the error has become quite large, so that any slope clearly greater than 1 signifies serious error. However, a slope of exactly 1 is no assurance of the absence of error or of site homogeneity inasmuch as it could result from a combination of slope flattening due to radioligand depletion and slope steepening due to depletion of the competing ligand. Knowing the value of D allows one to estimate the expected degree of slope flattening (cf. Fig. 1) and thus to judge how much slope steepening may be obscured.
3) If an experimentally determined value of X_{50} based on total concentration of unlabeled ligand is not at least 10 times greater than the X_{50} obtained for self-competition (unlabeled form of radioligand as competing ligand), the corresponding value of K_X computed from Eq. 3 is likely to be seriously in error unless D is extremely low.

In summary, a potential limitation in the use of competition binding assays such as RRAs and RIAs is that the true equilibrium dissociation constant of a high affinity competing ligand may not be measured accurately if a significant degree of depletion of radioligand or unlabeled ligand is ignored. An erroneous estimate is obtained—sometimes too low and sometimes too high—if depletion of radioligand is ignored, always too high if depletion of unlabeled ligand is ignored. Although this has been pointed out before, both explicitly and implicitly, it is still not generally recognized.

We have explored systematically the sources and magnitudes of the errors and we find that these may occur under conditions found in ordinary laboratory practice. The errors with which we are concerned here can be avoided by measuring the free concentrations of all ligands. Measurement of free radioligand is a relatively simple procedure; thus, it should be routine in all assays until it is established that depletion is negligible by the criteria developed here. However, because of limited sensitivity of analytical methods, measurement of free concentrations of unlabeled ligands may not be feasible when affinities are high and concentrations low. In that case, if an unlabeled ligand has a higher affinity than the radioligand, the error may be unavoidable. We have offered some practical guidelines for recognizing error in the estimation of K_X, and a practical dilution test and computer program that may sometimes permit error correction.

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