What Does the Accuracy of the Equilibrium Dissociation Constant of **Affinity Complexes Depend on Fundamentally?**

Tong Ye Wang, Hongchen Ji, Daniel Everton, An T. H. Le, Svetlana M. Krylova, René Fournier, and Sergey N. Krylov*

Department of Chemistry and Centre for Research on Biomolecular Interactions, York University

4700 Keele Street, Toronto, Ontario M3J 1P3, Canada

*Corresponding author's email address: skrylov@yorku.ca

mination involves finding the dependence of a fraction of unbound ligand on the total concentration of target (T_0) when the total concentration of ligand (L_0) remains constant. It is known that K_d determination for highly stable complexes is notoriously inaccurate; however, what the accuracy of K_d depends on fundamentally, i.e., method-independently, is largely unknown. Here we present an error-propagation analysis that answers this question in detail. This analysis explains the critical



importance of the L_0/K_d value for the accuracy of K_d and allows one to define the range of L_0/K_d values required for accurate K_d determination. Our analysis creates a theoretical foundation for improving the accuracy of K_d determination.

Drugs and diagnostic probes (ligands) are developed to form highly stable complexes with their molecular targets.^{1–9} The stability of a target-ligand complex is characterized by the equilibrium dissociation constant (K_d) of the binding reaction between a target and a ligand:

Target + Ligand
$$\underset{K_d}{\underbrace{K_d}}$$
 Complex (1)

$$K_{\rm d}$$
 is defined as:
 $K_{\rm d} = TL/C$ (2)

where T, L, and C are equilibrium concentrations of the target, ligand, and complex, respectively, in the binding reaction (Eq 1). Lower K_d values correspond to greater complex stability and are typically desired.^{10–12} The values of K_d define therapeutic concentrations of drugs and analytical parameters of diagnostic methods.^{12–15} Therefore, they are used not only for relative ranking of the ligands but also for assessment of their suitability for intended applications.^{13,14,16}

The equilibrium (non-kinetic) approach to K_d determination involves preparing a series of equilibrium mixtures of target and ligand in which the total concentration of ligand (L_0) is the same while the total concentration of target (T_0) varies. These mixtures are used to build a binding isotherm (dots in Figure 1), i.e., the experimental dependence of a fraction R of unbound ligand ($R = L/L_0$) on T_0 for constant L_0 . The value of K_d is then typically found by fitting the binding isotherm with the theoretical dependence of R on T_0 :¹

$$R = -\frac{K_{\rm d} + T_0 - L_0}{2L_0} + \sqrt{\left(\frac{K_{\rm d} + T_0 - L_0}{2L_0}\right)^2 + \frac{K_{\rm d}}{L_0}}$$
(3)

while varying K_d until the best fit (red line in Figure 1) is obtained. It is important to emphasize that Eq 3 is obtained by solving a quadratic obtained from an expression for K_d :



Figure 1. An example of determining K_d with a binding isotherm. A binding isotherm is the experimental dependance of the fraction of unbound ligand (R) on total target concentration (T_0), which is represented by black dots. The value of K_d is determined the best fit (red line) of the isotherm with Eq 3.

$$K_{\rm d} = \frac{T_0 - L_0(1 - R)}{(1/R - 1)} \tag{4}$$

which, in turn, is obtained from first principles: definitions of K_d (Eq 2) and R and mass balance for the target and ligand $(T_0 = T + C \text{ and } L_0 = L + C)$. In other words, Eqs 3 and 4 are fundamental, i.e., method-independent and obtained without assumptions which would restrict the generality.

For K_d to be a reliable measure of target–ligand affinity, it must be determined accurately. K_d determination for highly stable complexes is particularly inaccurate.^{19–21} Discrepancies between K_d values reported by different laboratories for the same complex may reach orders of magnitude leading to misconceptions and wrong conclusions about ligand potency.^{22,23} Great variations are often observed even for K_d values determined with the same method, suggesting that fundamental sources of inaccuracy play a critical role in inconsistencies of K_d for highly-stable complexes.^{20,24}

There are two fundamental requirements for K_d accuracy: (i) the binding reaction (Eq 1) must approach the equilibrium and (ii) the study must be done in a so-called binding regime which is assured by satisfying a condition of $L_0/K_d \ll 1.^{20,25-28}$ According to a recent review by Jarmoskaite et al.²⁰ of 100 publications dealing with K_d determination, fewer than 10% and 5% of publications reported satisfying these two requirements, respectively. Failure to satisfy fundamental requirements may deem the vast majority of the published K_d values greatly inaccurate. The alarming level of researchers' ignoring the fundamental requirement is due to the lack of basic knowledge on how this can affect the accuracy of K_d . There are a few important contributions to the field, which assess K_d accuracy specific methods,^{29–34} e.g., isothermal titration for calorimetry^{27,29} and capillary electrophoresis.^{30–32} However, there are no comprehensive studies that would explain what errors of K_d depend on fundamentally in the way that would further guide theorists and instruct experimenters. Accordingly, the field of experimental determination of K_d is largely an art in which researchers rely mostly on mnemonic rules and intuition in designing experiments and interpreting their results. The goal of our work was to initiate a graduate maturation of this field into a solid quantitative science through the understanding of what fundamental errors of K_d depend on and what it means for experimentalists.

 $K_{\rm d}$ values are not measured, they are determined using Eqs 3 or 4 from known values of T_0 , L_0 , and R (in the case of Eq 3 we also need to use non-linear regression). Accordingly, a deviation of the determined K_d value ($K_{d,det}$) from the true K_d value (K_d) is a result of errors of T_0 , L_0 , and R. So, the answer to the question of what the accuracy of K_d depends on is simple: it depends on the accuracy of T_0 , L_0 , and R. However, there is a second important question: how does the error of K_d depend on the errors of T_0 , L_0 , and R? If the errors of T_0 , L_0 , and R are not zero, which is always the case, then the error of K_d is the result of propagation of errors of T_0 , L_0 , and R. Therefore, the answer to the question about how the accuracy of K_d depends on the accuracy of T_0 , L_0 , and R is also conceptually simple: the dependence is defined by the rules of error propagation. Accordingly, we will apply error-propagation rules to Eq 4 to find how the deviation of $K_{d,det}$ from K_d ($\Delta K_d = K_{d,det} - K_d$) depends on deviations of T_0 , L_0 , and R (ΔT_0 , ΔL_0 , and ΔR , respectively, defined similar to ΔK_d) from their true values.

Note that deviations ΔT_0 , ΔL_0 , and ΔR are not random errors with a given error distribution; they can be both positive and negative. ΔK_d is a systematic error, which defines the accuracy of $K_{d,det}$. ΔK_d may be much greater than the random error of $K_{d,det}$. Furthermore, ΔK_d is virtually impossible to determine since the true K_d value is unknown by definition due to the absence of standard reference instruments for determination of K_d or standard reference K_d values.

For a known theoretical dependence of K_d on T_0 , L_0 , and R, we can write the general dependence of ΔK_d on ΔT_0 , ΔL_0 , and

 ΔR :

$$\Delta K_{\rm d} = \left(\frac{\partial K_{\rm d}}{\partial T_0}\right) \Delta T_0 + \left(\frac{\partial K_{\rm d}}{\partial L_0}\right) \Delta L_0 + \left(\frac{\partial K_{\rm d}}{\partial R}\right) \Delta R \tag{5}$$

The three partial differentials in the brackets are found by differentiating Eq 4 allowing us to transform Eq 5 to:

$$\Delta K_{\rm d} = \frac{R}{1-R} \Delta T_0 - R \Delta L_0 + \left(-L_0 + \frac{T_0}{\left(1-R\right)^2} \right) \Delta R \tag{6}$$

Convenient error analysis requires that this dependence of absolute errors be transformed to that of relative errors defined as ΔK_d , ΔT_0 , ΔL_0 , and ΔR divided by their respective true values. To facilitate this analysis, we divide Eq 6 by a true K_d value:

$$\frac{\Delta K_{\rm d}}{K_{\rm d}} = \frac{R}{1-R} \left(\frac{\Delta T_0}{T_0} \right) \left(\frac{T_0}{K_{\rm d}} \right) - R \left(\frac{\Delta L_0}{L_0} \right) \left(\frac{L_0}{K_{\rm d}} \right) + \left[-\frac{L_0}{K_{\rm d}} + \frac{1}{(1-R)^2} \left(\frac{T_0}{K_{\rm d}} \right) \right] \Delta R$$
(7)

To allow the non-numerical analysis of Eq 7, we make a single simplifying assumption that the determined (from measured signals) value of *R* is equal to $\frac{1}{2}$. Note that $R = \frac{1}{2}$ leads to the least erroneous K_d ,¹⁹ thus, we are considering the best-case scenario and finding the lower limit for the error of K_d . For convenience of writing, we will replace $\frac{1}{2}$ with 0.5 assuming its infinite precision (0.500...). Then the true value of *R* is:

$$R = 0.5 - \Delta R \tag{8}$$

By inserting Eq 8 into Eq 4 and re-arranging the latter to express T_0 , we obtain:

$$F_0 = \frac{K_d(0.5 + \Delta R)}{0.5 - \Delta R} + L_0(0.5 + \Delta R)$$
(9)

Finally, by inserting Eq 9 into Eq 7 and naming relative errors of T_0 , L_0 , and R as $t = \Delta T_0/T_0$, $l = \Delta L_0/L_0$, and $r = \Delta R/R$, we obtain:

$$\frac{\Delta K_{\rm d}}{K_{\rm d}} = \left[t + \frac{r(1+r)}{0.5+r}\right] + \left[\frac{0.5(t-l-r)}{1+r} + \frac{0.5r}{0.5+r}\right] \frac{L_{\rm 0}}{K_{\rm d}}$$
(10)

where one should note that, as expected, the absolute value of relative error of *R* (which ranges between 0 and 1) must be less than unity: |r| < 1.

Eq 10 reveals that the relative error of K_d is a linear function of L_0/K_d with an intercept dependent only on relative errors of T_0 and R and the slope dependent on relative errors of all three variables: T_0 , L_0 , and R. For further analysis, we rewrite Eq 10 in a simplified form of a linear function:

$$\frac{\Delta K_{\rm d}}{K_{\rm d}} = a + b \frac{L_0}{K_{\rm d}}$$

$$a = t + \frac{r(1+r)}{0.5+r}$$

$$b = \frac{0.5(t-l-r)}{1+r} + \frac{0.5r}{0.5+r}$$
(11)

where *a* and *b* are the terms in the square brackets of Eq 10. Detailed calculation process for Eqs 6–11 is shown in the Supporting Information (Note S2). Eq 11 explicitly shows the role of L_0/K_d in the relative error of K_d . The value of L_0/K_d is only important when the second term is greater than the first, i.e., when $L_0/K_d > a/b$. In other words, in contrast to a

commonly used requirement of $L_0/K_d \ll 1$, it is unnecessary to decrease L_0/K_d much below a/b. Rather, using $L_0/K_d \ll a/b$ will likely cause the opposite effect; namely, it will lead to an increase in $\Delta K_d/K_d$ due to the increase of r when L_0 is too low, causing an unacceptable signal to noise ratio (S/N), which will be briefly discussed later. On the other hand, when $L_0/K_d > a/b$, the increase in L_0/K_d plays a crucial role in increasing the relative error of K_d ; an order of magnitude increase in L_0 will lead to an order of magnitude increase in $\Delta K_d/K_d$. One should appreciate that if $L_0/K_d > a/b$, then $K_{d,det}$ may differ from true K_d by orders of magnitude.

For a large range of L_0 values, which is typically the case, it is more convenient to present the dependence of $\Delta K_d/K_d$ on L_0/K_d in a double-log scale. A graph of $\log(\Delta K_d/K_d)$ versus $\log(L_0/K_d)$ is triphasic: two asymptotically-linear ranges flank a non-linear transition range (Figure 2a). For small values of L_0/K_d , i.e., for $L_0/K_d \ll a/b$, the dependence is a linear function with no dependence on L_0/K_d : $\log(\Delta K_d/K_d) = \log(a) = \text{const. For}$ a large value of L_0/K_d , i.e., for $L_0/K_d \gg a/b$, the dependence is a linear function $\log(\Delta K_d/K_d) = \log(b) + \log(L_0/K_d)$ with an intercept with the ordinate equal to $\log(b)$ and a slope equal to unity. The abscissa of the intersection of the two asymptotic lines is defined by $\log(L_0/K_d) = \log(a/b)$.

Using Eq 11 and $\Delta K_d = K_{d,det} - K_d$, we can show that not only $\Delta K_d/K_d$ depends linearly on L_0/K_d , but also $K_{d,det}$ is a linear function of L_0 (Note S3):

$$K_{\rm d,det} = c + bL_0, \qquad c = (a+1)K_{\rm d}$$
 (12)

If presented in a double-log scale this dependence is expectedly triphasic (Figure 2b) and has features similar to the dependence of $\Delta K_d/K_d$ on L_0/K_d (Figure 2a).

The dependence in Eq 12 has two known variables $-L_0$ and $K_{d,det}$ – and, therefore, it can be tested experimentally, which we have undertaken in this work. Experimentally, we used a wellestablished solution-based method of nonequilibrium capillary electrophoresis of equilibrium mixtures (NECEEM) as the K_{d} determination method and MutS-aptamer as the binding pair.^{19,35–38} In NECEEM, unbound ligand and complex (bound ligand) are separated by an electric field in a capillary due to their different charge-to-size ratios.35-37 In six NECEEM experiments, the aptamer concentration (L_0) was kept constant at 0.02, 0.05, 0.1, 0.5, 2, and 10 nM, respectively; MutS concentration (T_0) varied from 0 to 312.5 nM (all concentrations are given as nominal values with no significance to the number of digits). The lowest value of L_0 was chosen to be equal to the limit of quantitation (LOQ) linked to S/N as discussed below. The determination of LOQ for NECEEM is described in the Supporting Information (Figure S1). The influence of L_0 on



Figure 2. General trends in dependencies of $\log(\Delta K_d/K_d)$ on $\log(L_0/K_d)$ (**a**) and $\log(K_{d,det})$ on $\log(L_0)$ (**b**). In both (**a**) and (**b**), reasonable relative errors of L_0 and T_0 (5%) and R (2%) were used: $\Delta L_0/L_0 = \Delta T_0/T_0 = 0.05$ and $\Delta R/R = 0.02$. In panel (**b**) K_d is set to be unity (unitless). With these conditions, the parameters *a*, *b*, and *c* are calculated to be 0.089, 0.0094 and 1.1, respectively.



Figure 3. The influence of L_0 on binding isotherms (**a**) and the dependence of $K_{d,det}$ on L_0 (**b**). In panel (**a**), all nonlinear regressions were conducted using OriginPro software with the iteration algorithm of Levenberg Marquardt. See text and Supporting Information for other details.

binding isotherms and the dependence of $K_{d,det}$ on L_0 are shown in Figure 3. Figure 3b shows that (in a double-log scale) $K_{d,det}$ is insensitive to the change of L_0 when L_0 is at low concentrations (e.g., << ~0.1 nM), and $K_{d,det}$ is linearly dependent on L_0 when L_0 is at high concentrations. The results in Figure 3b are consistent with the theoretical predictions shown in Figure 2b, which confirms the validity of our theoretical analysis.

Although Figure 2 suggests that we can use an as-low-aspossible L_0 to avoid the error of $K_{d,det}$ being greatly magnified, the choice of the lowest experimentally-suitable L_0 is dictated by LOQ of an instrument used to measure the signal. The value of LOQ is, by definition, the analyte concentration (ligand concentration L_0 in our case) for which S/N is equal to a certain value X >> 1 which guarantees that the noise does not affect quantitation significantly. If the noise is independent on the signal and the value of LOQ is known, S/N can be calculated for any given concentration of the ligand as (Note S4):

$$\frac{S}{N} = X \frac{L_0}{\text{LOQ}}$$
(13)

The minimum acceptable ligand concentration which satisfies Eq 13 is:

$$L_{0,\min} \approx \text{LOQ}$$
 (14)

Since *R* values are determined based on the measured signals *S*, using $L_0 < \text{LOQ}$ is counterproductive as it will cause a significant increase in the relative error of *R* (*r*), and, accordingly, in the relative error of $K_{d,det}$.

Based on above analysis, to achieve good accuracy of $K_{d,det}$ in an experiment, L_0/K_d should satisfy:

$$\frac{\text{LOQ}}{K_{\rm d}} < \frac{L_0}{K_{\rm d}} < \frac{a}{b} \tag{15}$$

where *a* and *b* are defined by the relative errors of T_0 , L_0 , and *R* (i.e., *t*, *l*, and *r*) as shown in Eq 11. Because *t*, *l*, and *r* are relatively difficult to determine, LOQ can be determined and used as a sole determinant of ligand concentration:

 $L_0 = \text{LOQ} \tag{16}$

to minimize the error of $K_{d,det}$ in a K_d -determination experiment. To conclude, in this study, we investigated the theoretical dependence of the relative deviation of $K_{d,det}$ from K_d ($\Delta K_d/K_d$) on the ratio of L_0/K_d with error propagation. Our analysis shows that, with fixed errors of T_0 , L_0 , and R, $\Delta K_d/K_d$ depends linearly on L_0/K_d , and presents a triphasic dependence in a double-log scale: When L_0/K_d is small, $\Delta K_d/K_d$ is insensitive to the change of L_0/K_d and approaches a constant that is defined by the errors of T_0 and R. When L_0/K_d is large, $\Delta K_d/K_d$ is sensitive to the change of L_0/K_d , i.e., an order of magnitude increase in L_0 will lead to an order of magnitude increase in $\Delta K_d/K_d$. There is a non-linear transition range between the two linear phases. We also theoretically demonstrated that $K_{d,det}$ is a linear function of L_0 and should show the triphasic features in a double-log scale. The theoretical relation between $K_{d,det}$ and L_0 was confirmed with NECEEM experiments using MutS-aptamer as the binding pair. The results of this work suggest that, without knowing the errors of T_0 , L_0 , and R, we should use the smallest L_0 that does not sacrifice the accuracy of R, which is dictated by LOQ of an instrument used. It is important to emphasize that using $L_0 = \text{LOQ}$ does not imply that $L_0/K_d < a/b$ and thus does not guarantee the accuracy of K_d . With the current state of affairs, the only way to confirm (indirectly) that $L_0/K_d < a/b$ is to conduct experiments not only at $L_0 = LOQ$ but also at $L_0 > LOQ$. If K_d is not affected by the value of L_0 then one can assume that $L_0/K_d < a/b$ and deem K_d accurate. However, we think that our findings (e.g., the triphasic dependence of $\Delta K_d/K_d$ on L_0/K_d in Figure 2a) can help create an approach of K_d accuracy assessment from a single binding isotherm if the relative errors in T_0 , L_0 , and R can be estimated.

CONTENT OF SUPPORTING INFORMATION

Derivation of the theoretical dependence of fraction of unbound ligand (*R*) on total target concentration (T_0) (Note S1); Derivation of the dependence of relative systematic error of K_d ($\Delta K_d/K_d$) on L_0/K_d ratio (Note S2); Derivation of the dependence of $K_{d,det}$ on L_0 (Note S3); Derivation of the signal to noise ratio for any ligand concentration L_0 (Note S4); Materials and solutions (Note S5); Details of NECEEM experiments (Note S6, Figure S1, Figure S2, and Table S1).

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