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Deterministic error propagation in ITC: Revealing multi-fold errors in K_d values under standard conditions

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ABSTRACT

Accurate determination of the equilibrium dissociation constant (K_d) is essential in fields such as drug discovery and molecular diagnostics, where a rigorous understanding of molecular interactions drives critical decisions. Among established techniques, isothermal titration calorimetry (ITC) is highly valued for its ability to directly capture binding thermodynamics without the need for labeling or immobilization. However, while ITC is often praised for its precision, potential inaccuracies due to the systematic errors in experimental variables (analyte concentrations and measured heat) are frequently overlooked. One key reason for this oversight is the lack of a deterministic framework that explicitly demonstrates how ITC-derived K_d values can be affected by these errors. To address this gap, we derived a closed-form mathematical model for error propagation in ITC-based K_d determination, quantifying the impact of inaccuracies in analyte concentrations and measured heat on the resulting K_d. This framework provides a robust foundation for understanding and predicting the influence of these systematic errors on K_d accuracy. Using this solution, we demonstrate that even within the conventionally recommended *c*-value range of 10–100, expected systematic errors in concentrations and heat can potentially lead to significant multi-fold deviations in K_d. These findings underscore the need for quantitative accuracy assessments in ITC experiments and highlight the importance of developing practical tools to support such evaluations.

1. Introduction

Non-covalent molecular binding, which drives the formation of affinity complexes, is fundamental to both biological processes and technological applications [1–3]. Understanding the strength of these complexes, whether in cellular signaling pathways or ligand–target interactions in pharmaceutical development, is essential for advancing biological research and therapeutic innovation [4,5]. The equilibrium dissociation constant (K_d) is a critical parameter that characterizes the affinity between two binding partners, and its accurate determination is critical for fields such as drug discovery, molecular diagnostics, and biomolecular engineering [6,7].

It is important to distinguish between accuracy and precision in K_d determination. Accuracy of K_d refers to how close the experimentally determined K_d ($K_{d,det}$) is to its (unknown) true value, reflecting the presence of systematic errors in $K_{d,det}$. Precision of K_d, on the other hand, relates to the reproducibility of K_d determinations, often assessed through nonlinear regression and is expressed in the standard deviation (σ) of experimentally determined K_d values [8–10]. Accuracy and precision are key characteristics of exactness and both are essential for reliable use of determined K_d values. Importantly, even highly precise measurements can be significantly inaccurate if the systematic errors are not addressed [11,12]. Unfortunately, many researchers report determined K_d only as $K_{d,det} \pm \sigma$, focusing primarily on precision while completely overlooking the critical factor of accuracy of $K_{d,det}$. Inaccuracies in $K_{d,det}$ have far-reaching consequences, potentially leading to misguided conclusions in molecular interaction studies and misinformed decisions in developing drugs and diagnostics [9,13,14]. To improve and evaluate the accuracy of $K_{d,det}$, it is essential to first maximize the accuracy of the input variables involved in K_d determination, such as analyte concentrations and experimental signals [9]. This requires minimizing systematic errors and estimating the confidence intervals of any remaining systematic errors in these variables [9,11]. These intervals should then be propagated into the final $K_{d,det}$ values to account for their cumulative effect. Moreover, given that the probability distribution of $K_{d,det}$ is often non-Gaussian [10,11], it is scientifically more

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rigorous to report $K_{d,det}$ as a confidence range accompanied by a specified confidence level (e.g., 95 %).

The most common methods for K_d determination, for which specialized commercial equipment is available, include isothermal titration calorimetry (ITC), surface plasmon resonance (SPR), bio-layer interferometry (BLI), fluorescence anisotropy (FA), and microscale thermophoresis (MST) [9,15]. Each method offers unique advantages, e.g., SPR and BLI can also provide kinetic data, while MST requires smaller sample volumes [16–18]. However, many of these methods, such as SPR, BLI, FA, and MST often require labeling or immobilization, which can potentially alter the natural interaction between binding partners [17–21]. ITC, on the other hand, has gained widespread use in the past few decades due to its ability to directly measure the heat changes associated with binding without the need for labels or immobilization [18,22,23]. Additionally, ITC provides a comprehensive thermodynamic profile, including enthalpy (ΔH°), Gibbs free energy (ΔG°), and entropy (ΔS°), making it a preferred method for thoroughly studying molecular interactions in their native, unmodified states [24,25]. Major limitations of ITC include low throughput and the need for large amounts of materials [18,23]. This balance of advantages and limitations makes ITC well-suited for confirmatory studies aiming at obtaining accurate and comprehensive binding data for binders identified in high-throughput screening by other methods [23,26].

In a classic (non-titration) equilibrium approach to determining K_d, e.g., FA and MST, multiple equilibrium mixtures are prepared, where the concentration of one component (the ligand) is kept constant, while the concentration of the other component (the target) is varied over a wide range [17,27]. For each mixture, the fraction of the free (unbound to the target) ligand is measured. Finally, the resulting binding isotherm, depicting the dependence of this fraction on the target concentration, is analyzed using nonlinear regression to obtain $K_{d,det}$ [17,27–30].

We have recently published an error-propagation analysis for this classic approach [31]. This analysis has shown that the relative systematic error in $K_{d,det}$ becomes increasingly sensitive to systematic errors in the variables — both concentrations and the measured unbound fraction — as the ratio between the true (and, thus, unknown) values of ligand concentration and K_d grows [31]. This ratio is called a c-value in ITC; we will use this notation for the non-titration approach discussed here as well. Even small inaccuracies in the variables can be greatly amplified when propagated into error in K_d [31]. This amplification leads to significant systematic errors in $K_{d,det}$ when the c-value is high.

It is generally recommended that the c-value be kept below unity for the non-titration approach [28,29,32]. However, this recommendation is an oversimplification and may not always be applicable. The error-propagation analysis revealed that the c-value should instead be compared to a threshold determined by the magnitude of errors in the variables — concentrations and the unbound fraction [31]. This threshold can be significantly smaller than unity if these errors are substantial [31]. In other words, when the errors in the variables are high, the relative systematic errors in $K_{d,det}$ can be significant, even when $c < 1$. Experimental observations have shown that differences in $K_{d,det}$ for varying c-values can span several orders of magnitude despite the relative standard deviation remaining below 50 % [29,31].

Unlike the classic approach discussed above, ITC utilizes a titration method enabling dynamic variation in target concentration over successive injections. The concentrated solution of the target (titrant) is titrated into the ligand (titrand) solution so that the concentration of the ligand gradually and slightly decreases while the concentration of the target increases over a wide range. The successive injections of the target generate a binding isotherm from the heat release or consumption measured after each injection.

For ITC, it is recommended that the c-values for a 1:1 binding process fall within the range of 10 to 100 [33]. This range ensures that the ITC binding curve contains sufficient information to reliably determine thermodynamic parameters, such as the enthalpy change (ΔH°), while avoiding overly steep curves that could compromise the accurate determination of K_d at higher c-values [24,33]. Interestingly, this recommendation to maintain $c > 10$ stands in stark contrast to the consensus recommendation of satisfying $c < 1$ in non-titration methods, suggesting that standard conditions for ITC may potentially compromise the accuracy of $K_{d,det}$.

The nonlinear regression model in ITC differs from the one used in the non-titration approach. Instead of the target concentration, it utilizes the molar (target-to-ligand) ratio and usually includes three unknowns: K_d, ΔH°, and the binding stoichiometry (n) [24,33]. Therefore, the result obtained for error propagation in the non-titration K_d-determination approach (connecting the relative systematic error in $K_{d,det}$ to systematic errors in concentration and fraction unbound) is not directly applicable to ITC. This represents a significant methodological gap.

To address this limitation, we derived a closed-form expression to model error propagation in ITC-based K_d determination, as presented here. This expression specifically focuses on how systematic errors in key variables, such as the concentrations of the ligand and target, and the measured heat changes, propagate through the nonlinear regression model to affect $K_{d,det}$. Our approach involves deriving equations that explicitly link these systematic errors to the resulting relative systematic error in $K_{d,det}$, taking into account the unique aspects of the ITC model, including its dependence on the molar ratio and additional unknown parameters, such as ΔH°.

We incorporated experimentally realistic systematic errors in reactant concentrations and ITC-measured heat into the derived closed-form expression to evaluate the magnitude of relative errors in $K_{d,det}$ across a wide range of c-values. For the recommended range of c-values (10 to 100), we found that the accuracy of $K_{d,det}$ can be poor, with deviations from the true K_d values reaching multi-fold levels, consistent with certain experimental findings reported in the ITC field [25,34]. This finding underscores that ITC can be prone to gross inaccuracies in $K_{d,det}$ within its recommended operating conditions. It also highlights the necessity for a quantitative evaluation of $K_{d,det}$ accuracy in ITC experiments. While this evaluation has been addressed in a number of publications [35–38], it remains underutilized by practitioners, largely due to a lack of awareness of the potential magnitude of errors in $K_{d,det}$ [39].

2. Results and discussion

2.1. Mathematics of classic equilibrium K_d determination

To understand the foundational mathematics of K_d determination, we consider the reversible binding of a target (T) to a ligand (L), forming a 1:1 complex (C):

$$\mathrm{T} + \mathrm{L} \underset{K_d}{\rightleftharpoons} \mathrm{C} \tag{1}$$

The equilibrium dissociation constant, K_d, is expressed as:

$$K_d = TL/C \tag{2}$$

where T, L, and C are the equilibrium concentrations of the target, ligand, and complex, respectively. Using total concentrations T_0 and L_0 of the target and ligand:

$$T_0 = T + C, \quad L_0 = L + C \tag{3}$$

and the fraction of unbound ligand, R:

$$R = L/L_0 \tag{4}$$

we derive the dependence of K_d on T_0, L_0, and R: [40]

$$K_d = \frac{T_0 - L_0(1 - R)}{(1/R - 1)} \tag{5}$$

By solving eq. 5 for R, we obtain the dependence of R on K_d, T_0, and L_0: [30,31,41,42]

$$R = -\frac{K_d + T_0 - L_0}{2L_0} + \sqrt{\left(\frac{K_d + T_0 - L_0}{2L_0}\right)^2 + \frac{K_d}{L_0}} \tag{6}$$

This equation represents a model for nonlinear regression of an experimental binding isotherm "R vs T_0" (with constant L_0) obtained by the classic equilibrium K_d-determination approach.

The nonlinear regression procedure uses K_d as a sought parameter, R as a dependent variable, and T_0 and L_0 as independent variables to compute $K_{d,det}$ and its standard deviation σ (Fig. 1a) [9,11,31]. This completes the consideration of fundamental math of K_d determination by nonlinear regression of a binding isotherm in the non-titration approach.

2.2. Mathematics of K_d determination with ITC

In ITC, the binding process is monitored by measuring the heat change during each injection of the target into the ligand [22–24]. For clarity, in this work, we refer to the injected reactant (titrant) — whose concentration varies throughout the experiment — as the target, while the reactant (titrand) in the ITC sample cell, which remains at a near-constant concentration, is referred to as the ligand, aligning with the terminology used in our previous studies [9,11,31].

In ITC experiments, the total heat absorbed/released after i-th injection (Q_i) is directly proportional to the total amount of complex formed: [43,44]

$$Q_i = nV_0\Delta H^{\circ} C \tag{7}$$

Here, n represents the number of binding sites per ligand molecule (analyte in the ITC sample cell), V_0 is the sample cell volume, ΔH° denotes the enthalpy change of complex formation (heat absorbed/released per mole of the complex formed), and C is the equilibrium concentration of the complex in the sample cell after the i-th injection. In this study, we focus on the one-site binding model (eq. 1) with n theoretically equal to unity, as this is the most common interaction type in both biological and technological contexts [45,46].

By combining eqs. 3 and 4, we obtain:

$$C = (1 - R)L_0 \tag{8}$$

Then, for $n = 1$, eq. 7 can be rewritten as:

$$Q_i = V_0\Delta H^{\circ} L_0(1 - R) \tag{9}$$

By substituting the dependence of R on K_d, L_0, and T_0 (eq. 6) into eq. 9, the fundamental equation used to derive the practical nonlinear regression models for ITC data is:

$$Q_i = V_0\Delta H^{\circ}\left[\frac{(K_d + L_0\Gamma + L_0)/2-}{\sqrt{(K_d + L_0\Gamma - L_0)^2/4 + K_dL_0}}\right] \tag{10}$$

with K_d and ΔH° as sought parameters and the molar ratio $\Gamma = T_0/L_0$ used instead of T_0 as the varied independent variable [44]. Note that, according to eq. 10, if the ITC measurements reach saturation (no unbound ligand remains) at the highest target concentration used in the experiment ($T_0 >> L_0, K_d$), the R values can be back-calculated from Q_i, and the total heat changes measured at the initial (zero) and saturation target concentrations (Note S1).

In most ITC data-fitting processes, the first derivative of eq. 10 with respect to T_0 serves as the fitting model [24,44]. Furthermore, due to slight dilution of reactants during the titration process and the overflow design of modern ITC instruments, small corrections to L_0 and T_0 after each injection are introduced when applying nonlinear regression to process the experimental data [24,44]. Nonetheless, these adjustments do not alter the fact that the practical fitting models used for ITC data analysis fundamentally originate from eq. 10 and align with the principles of the classic non-titration equilibrium K_d-determination approach outlined in eqs. 1–6. This equivalence underscores that the insights derived from the classic method are applicable to ITC. To streamline this study, here we conduct a semi-quantitative analysis leveraging the close relationship between eq. 10 and eq. 6, which is central to interpreting experimental data from the classic K_d-determination approach.

2.3. Propagation of systematic errors for K_d determination in ITC

Error propagation in K_d determination using the non-titration equilibrium approach has been explored in our recent studies [9,11,31]. In these studies, error propagation rules for strongly and weakly correlated error sources were applied to the dependence of K_d on L_0, T_0, and R (eq. 5) to establish the relationships between the accuracy of $K_{d,det}$ ($|\Delta K_d/K_d|$, where $\Delta K_d = K_{d,det} - K_d$) and the c-value, defined as the ratio between the true values of L_0 and K_d (L_0/K_d) [31]. As a result, we found that regardless of the relationships between error sources, the dependence of $|\Delta K_d/K_d|$ on the c-value (L_0/K_d) exhibits a triphasic shape, best seen in double-log coordinates (Fig. 1b). In the first phase, at low c-values, $|\Delta K_d/K_d|$ demonstrates a linear relationship with a negligible dependence of $|\Delta K_d/K_d|$ on c. In the third phase, at high c-values, another linear relationship emerges, showing a high sensitivity of $|\Delta K_d/K_d|$ to the c-value. Between these two linear phases lies a nonlinear transition phase. These dependencies were derived for a typical case where the experimentally determined R is 0.5 assuming the relative systematic errors in L_0, T_0, and R (denoted as $\Delta L_0/L_0$, $\Delta T_0/T_0$, and $\Delta R/$

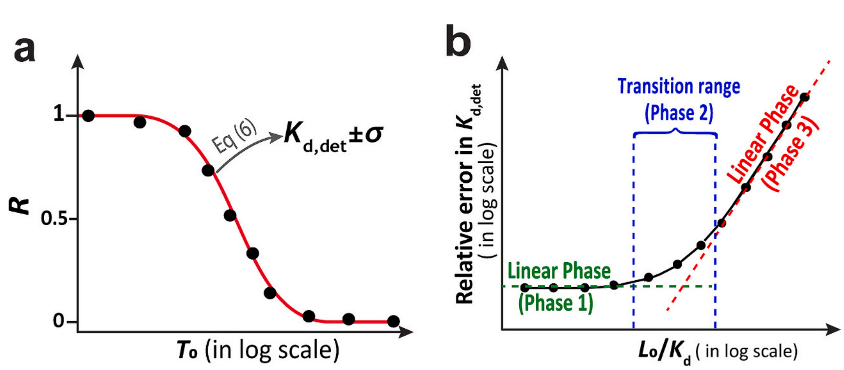

Fig. 1. A schematic illustration of K_d determination. **Panel a** shows a binding isotherm (dots), i.e., the dependence of R on T_0, and the result of fitting the isotherm with eq. 6 (red line); **Panel b** illustrates the triphasic dependence of relative error in $K_{d,det}$ on L_0/K_d. This relationship was derived by applying error propagation rules to the theoretical dependence of K_d on L_0, T_0, and R (eq. 5), under the assumption that the errors in T_0, L_0, and R remain constant across the entire studied L_0/K_d range. Here, L_0 and K_d represent the true values of the total ligand concentration and the equilibrium dissociation constant, respectively. (This figure is modified with permission from **ref** 31. Copyright 2023 American Chemical Society).

R) remain constant across the entire range of studied c-values [31].

This triphasic dependence (Fig. 1b) suggests that minimizing $|\Delta K_d/K_d|$ requires lowering the c-value through reducing L_0 and/or minimizing the relative systematic errors in L_0, T_0, and R [31]. Since decreasing L_0 is limited by the instrument's limit of quantitation (LOQ) for detecting ligand signals, in our previous work, we have systematically explored strategies to reduce the relative systematic errors in L_0, T_0, and R, and to determine their confidence intervals [9].

As demonstrated earlier (eqs. 1–10), the core principles of data analysis remain consistent between ITC and the classic non-titration approach. Therefore, the results of error propagation for ITC data can be derived based on the findings from error-propagation studies of non-titration equilibrium methods.

In this study, which focuses exclusively on analyzing the accuracy of K_d, we assume that the parameter ΔH° is directly measurable from the data points of the initial injections and set $n = 1$ without systematic error. Here, n is treated as the number of binding sites on the ligand, rather than a compensatory parameter used to address inaccuracies in ligand concentration, as is commonly practiced by many ITC practitioners [37,47,48]. Pre-setting n to its theoretical value of 1 (for the studied 1:1 binding process) does not imply that the ligand concentration is accurate. Instead, systematic errors in ligand concentration are explicitly included in the error propagation analysis. Under these assumptions, K_d becomes the sole parameter being determined. The systematic error in the $K_{d,det}$ (ΔK_d) arises exclusively from the systematic errors in the measured heat (Q_i), measured ΔH°, nominal total ligand concentration (L_0), and the calculated molar ratio (Γ), denoted as ΔQ_i, $\Delta(\Delta H^\circ)$, ΔL_0, and $\Delta \Gamma$, respectively. While $K_{d,det}$ can also be affected by inaccuracies in injection volumes and sample cell volume (V_0), these sources of error are tied to fixed instrumental limitations and cannot be directly quantified or corrected by experimentalists. Therefore, this study focuses on evaluating the impact of systematic errors in analyte concentrations and measured heat, and how these systematic errors propagate into ΔK_d.

Through comprehensive mathematical analysis (Notes S2 and S3), we found that the relative systematic errors in T_0, L_0, and R (which are the error sources for non-titration equilibrium methods) are interconvertible with the relative systematic errors in Q_i, ΔH°, L_0, and Γ. As a result, if ΔQ_i, $\Delta(\Delta H^\circ)$, ΔL_0, and $\Delta \Gamma$ are strongly correlated (which occurs when similar procedures are used for ligand and target solution preparation, and ΔQ_i and $\Delta(\Delta H^\circ)$ are mainly the consequence of ΔL_0 and $\Delta \Gamma$), the relationship between the accuracy of the determined K_d ($|\Delta K_d/K_d|$) and the c-value is derived as a linear equation (Notes S2):

$$|\Delta K_d/K_d| = A + Bc \tag{11}$$

where both constants A and B depend on the relative systematic errors in all four variables: $|\Delta Q_i/Q_i|$, $|\Delta(\Delta H^\circ)/\Delta H^\circ|$, $|\Delta L_0/L_0|$, and $|\Delta \Gamma/\Gamma|$. Note that c is the true c-value, defined as the ratio between the true values of L_0 and K_d (L_0/K_d), which are unknown due to the unavoidable systematic errors in them.

In contrast, if ΔQ_i, $\Delta(\Delta H^\circ)$, ΔL_0, and $\Delta \Gamma$ are weakly correlated (occurring when different procedures are used for preparing ligand and target solutions, and ΔQ_i and $\Delta(\Delta H^\circ)$ are independent of ΔL_0 and $\Delta \Gamma$), the dependence of the accuracy of $K_{d,det}$ on the true c-value (for weakly correlated error sources) is derived to be a square root of a quadratic function for the c-value (Notes S3):

$$|\Delta K_d/K_d| = \sqrt{\Omega^2 c^2 + \Lambda c + \Theta^2} \tag{12}$$

with constants Ω, Λ, and Θ being defined by different combinations of $|\Delta Q_i/Q_i|$, $|\Delta(\Delta H^\circ)/\Delta H^\circ|$, $|\Delta L_0/L_0|$, and $|\Delta \Gamma/\Gamma|$, respectively.

To illustrate these relationships, we plotted the dependencies described by eqs. 11 and 12 for constant A and B (strong correlation) and for constant Ω, Λ, and Θ (weak correlation) in Fig. 2 (and Fig. S1). As expected, the resulting curves resemble the triphasic behavior shown in Fig. 1b, characterized by two linear phases at low and high c-values, separated by a nonlinear transition phase. The threshold, defined as the intercept of the two linear regions, depends on the relative errors in the variables and is given by A/B for eq. 11 and Θ/Ω for eq. 12.

2.4. Changing relationship between error sources with changing c-value

In the earlier analysis, we assumed that error sources were either fully correlated or completely uncorrelated across the entire range of c-values, leading to the derivation of eq. 11 and eq. 12, respectively. However, as we explain below, in practical ITC experiments, the relationship between error sources evolves with changing c-values. Specifically, eq. 11 applies to high c-values, where the systematic errors in all variables are predominantly influenced by inaccuracies in analyte concentrations. In contrast, eq. 12 is appropriate for low c-values, where weaker heat signals and incomplete binding isotherms become the dominant sources of errors in the measured heat, independent of the error sources for concentrations.

While modern ITC instruments are capable of accurately measuring the experimental heat change for low c-values (e.g., $0.1 < c < 1$) [49], it is widely accepted that the lowest c-value suitable for producing a relatively complete binding curve to measure ΔH° reliably is around 10 [49–52]. The accuracy and precision of measured ΔH° decrease rapidly when $c < 10$ [49]. Therefore, for $c \geq 10$, it is reasonable to assume that the systematic errors in measured heats (ΔQ_i and $\Delta(\Delta H^\circ)$) are primarily

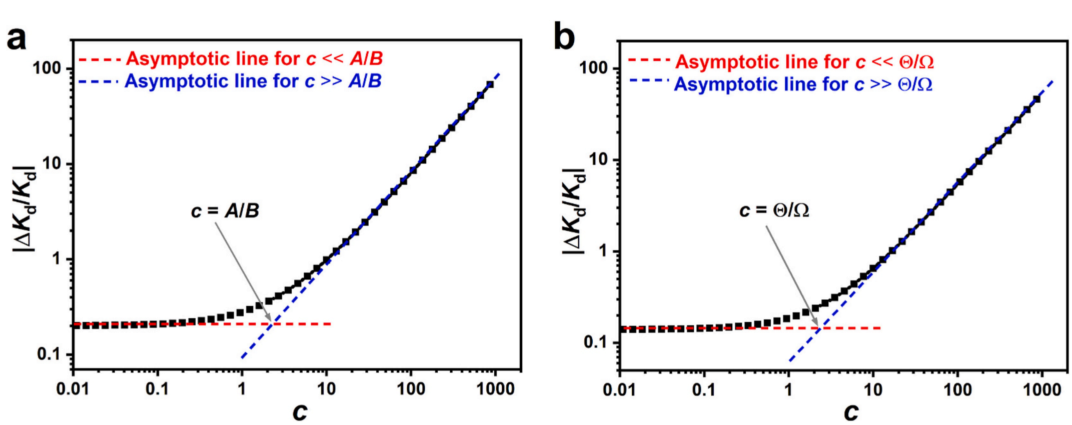

Fig. 2. General trends in the dependence of $|\Delta K_d/K_d|$ on c-value for constant relative systematic errors in variables. Reasonable values were used for A, B, Θ, Λ, and Ω, calculated from typical values of $|\Delta Q_i/Q_i|$, $|\Delta(\Delta H^\circ)/\Delta H^\circ|$, $|\Delta L_0/L_0|$, and $|\Delta \Gamma/\Gamma|$ as explained in Sections 2.5 and 2.6. These values are: $|\Delta Q_i/Q_i| = |\Delta(\Delta H^\circ)/\Delta H^\circ| = 0.01$, $|\Delta L_0/L_0| = 0.05$, and $|\Delta \Gamma/\Gamma| = 0.1$. The dependence of $|\Delta K_d/K_d|$ on c is shown for panel **(a)** using eq. 11 and for panel **(b)** using eq. 12. Data are presented on a log-log scale to enhance the clarity of details for low c-values.

driven by ΔL_0 and $\Delta \Gamma$, and error propagation can be derived using eq. 11. However, when $c < 10$, the primary source of systematic errors in the measured heat shifts toward weaker heat signals and incomplete binding isotherms. This results in a gradual increase in the magnitude of ΔQ_i and a rapid increase in the magnitude of $\Delta(\Delta H^{\circ})$, both of which become independent of ΔL_0 and $\Delta \Gamma$. In this case, eq. 12 should be used to produce error propagation.

Notably, for a molecular binding pair, since the true K_d value is a constant, a decrease in the true c-value (L_0/K_d) corresponds to a decrease in ligand concentration L_0. Consequently, to maintain the same molar ratio range, the target concentration in the syringe must also decrease if the injection volume and number of injections remain unchanged. This reduction in concentration poses greater challenges in accurately preparing low-concentration samples and amplifies the impact of unavoidable reagent loss during sample preparation. As a result, lower c-values are often associated with increased systematic errors in concentrations and, consequently, in molar ratios [53]. Nevertheless, for $0.1 < c < 10$, compared to the rapid increase of $\Delta(\Delta H^{\circ})$, the increase of systematic errors in concentrations and Q_i is much less significant [37]. For $c \geq 10$, relative systematic errors in concentrations and heat measurements are primarily constrained by the maximum accuracy limits of the experimental equipment, which remain approximately constant.

In this study, we focus on the broader c-value range of $0.1 < c < 1000$, which extends significantly beyond the commonly recommended range of $10 \leq c \leq 100$. Within this range, we assume that $|\Delta L_0/L_0|$, $|\Delta \Gamma/\Gamma|$, and $|\Delta Q_i/Q_i|$ remain constant. However, $|\Delta(\Delta H^{\circ})/\Delta H^{\circ}|$ exhibits a rapid increase as the c-value decreases within $0.1 < c < 10$. For $10 \leq c < 1000$, we approximate $|\Delta(\Delta H^{\circ})/\Delta H^{\circ}| \approx |\Delta Q_i/Q_i|$.

2.5. Realistic relative systematic errors in variables for ITC experiments

To analyze how the relative systematic error in K_d ($|\Delta K_d/K_d|$) determined by ITC depends on the true c-value (L_0/K_d), we first need to examine the experimentally realistic relative systematic errors in the variables: $|\Delta Q_i/Q_i|$, $|\Delta(\Delta H^{\circ})/\Delta H^{\circ}|$, $|\Delta L_0/L_0|$, and $|\Delta \Gamma/\Gamma|$.

Modern ITC instruments offer high precision in injection volumes, leading to highly precise calculated values of T_0 and L_0, as well as their molar ratios, with relative random errors typically within 1–2 % [12]. However, after eliminating common sources of systematic errors in analyte concentrations (e.g., reagent impurity and adsorption), the accuracies of T_0 and L_0 are constrained by sample preparation procedures. Given the accuracy limitations of preparation instruments (e.g., analytical balances and pipettes) and the unavoidable impurities in reagents, the relative systematic error in these concentrations ($|\Delta L_0/L_0|$ and $|\Delta T_0/T_0|$) is unlikely to fall below 5 % [12,54]. When considering error propagation in calculating molar ratios from the initial ligand concentration (in the sample cell before titration starts) and the target concentration (in the syringe), along with injection and sample cell volumes, achieving a relative systematic error below 10 % in the computed molar ratio ($|\Delta \Gamma/\Gamma|$) becomes a considerable challenge, given the additive nature of error propagation ($|\Delta \Gamma/\Gamma| > |\Delta L_0/L_0| + |\Delta T_0/T_0|$). However, with a well-calibrated ITC instrument and careful baseline adjustment, the relative systematic error in measured heat change can be effectively controlled within 1–2 % [55].

Regarding the relative systematic error of ΔH° ($|\Delta(\Delta H^{\circ})/\Delta H^{\circ}|$), we assume the following: (*i*) when $0.1 < c < 10$, the relative systematic error for measured ΔH° ($|\Delta(\Delta H^{\circ})/\Delta H^{\circ}|$) increases exponentially as the c-value decreases, such as $|\Delta(\Delta H^{\circ})/\Delta H^{\circ}| = \exp(-4.6c)$ and (*ii*) when $c \geq 10$, $|\Delta(\Delta H^{\circ})/\Delta H^{\circ}|$ remains constant and equal to a typical relative systematic error of Q_i, i.e., 1 %. The constant 4.6 in assumption (*i*) defines the rate at which $|\Delta(\Delta H^{\circ})/\Delta H^{\circ}|$ increases as the c-value decreases (for $0.1 < c < 10$) and, importantly, ensures that $|\Delta(\Delta H^{\circ})/\Delta H^{\circ}|$ approaches 0.01 (1 %) when $c = 10$ for a smooth transition between the two parts of the dependence.

2.6. Dependence of relative systematic errors in K_d on c-value in a wide range of c-values

Based on the discussions in the previous section, we assign reasonable relative systematic errors to ligand concentration, molar ratio, and heat change as $|\Delta L_0/L_0| = 0.05$, $|\Delta \Gamma/\Gamma| = 0.1$, and $|\Delta Q_i/Q_i| = 0.01$, respectively, and establish the dependence of $|\Delta K_d/K_d|$ determined by ITC on the c-value, as illustrated in Fig. 3 (and Fig. S2). In this analysis, we assume that all common and controllable sources of systematic error, such as those arising from reagent impurities, dilution effects, and instrument mis-calibration, have been eliminated. The remaining systematic errors are assumed to originate from unavoidable limitations in sample preparation instruments and baseline selection. To streamline the semi-quantitative analysis, we introduce realistic systematic errors only into L_0, Γ, Q_i, and ΔH°, while omitting potential errors from injection and cell volumes. This simplified approach allows us to illustrate the general dependence of $|\Delta K_d/K_d|$ on the true c-value and to estimate the potential magnitude that $|\Delta K_d/K_d|$ can reach.

The results in Fig. 3 demonstrate that the dependence of $|\Delta K_d/K_d|$ on the true c-value in ITC follows a U-shaped trend. At very low c-values (e.g., $0.1 < c < 1$), $|\Delta K_d/K_d|$ increases as c decreases, primarily because the exponentially increasing relative systematic error of ΔH° becomes the dominant factor in error propagation. As c increases to a certain range (e.g., around $c \approx 1$), $|\Delta K_d/K_d|$ reaches its minimum, suggesting that this range offers an optimal balance for accurate K_d determination, providing sufficient accuracy in the measured ΔH° while the amplification of systematic errors in variables (by c-value) remains insignificant. However, as c-values exceed this optimal point, $|\Delta K_d/K_d|$ starts to increase once again. This is due to the significant magnification of systematic errors at high c-values, as suggested by eq. 11.

In this demonstration shown in Fig. 3, we did not examine the dependence of $|\Delta K_d/K_d|$ on c for $c < 0.1$, as this range is well below the typical lower limit ($c = 10$) for standard ITC operating conditions. At such extremely low c-values ($c < 0.1$), $|\Delta(\Delta H^{\circ})/\Delta H^{\circ}|$ increases rapidly, and the relative systematic errors in Q_i and concentrations also rise significantly, albeit at unknown rates.

The general trend in the dependence of $|\Delta K_d/K_d|$ on the c-value, as illustrated in Fig. 3, qualitatively agrees with the results of previous numerical studies [56,57]. To our best knowledge, however, this is the first time this trend has been revealed through an error propagation. It is

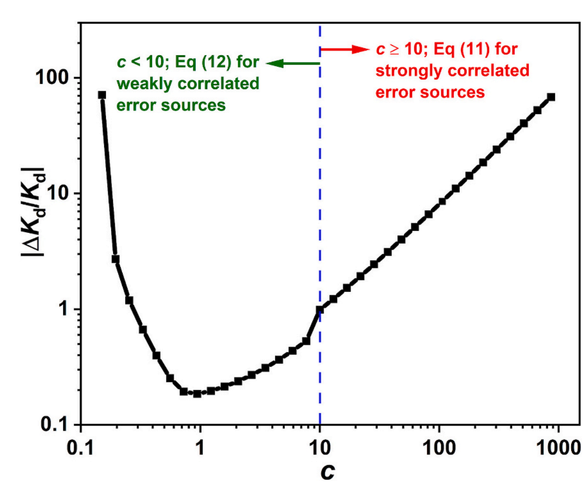

Fig. 3. General trends in the dependence of $|\Delta K_d/K_d|$ on c-value under realistic experimental conditions. The parameters A, B, Θ, Λ, and Ω were calculated using typical values for $|\Delta Q_i/Q_i|$, $|\Delta(\Delta H^{\circ})/\Delta H^{\circ}|$, $|\Delta L_0/L_0|$, and $|\Delta \Gamma/\Gamma|$, consistent with those in Fig. 2, and held constant across the entire c-value range. For $|\Delta(\Delta H^{\circ})/\Delta H^{\circ}|$, we assume $|\Delta(\Delta H^{\circ})/\Delta H^{\circ}| = \exp(-4.6c)$ for $c < 10$ and $|\Delta(\Delta H^{\circ})/\Delta H^{\circ}| = 0.01$ for $c \geq 10$. The dependence of $|\Delta K_d/K_d|$ on c was calculated using eq. 12 for $0.1 < c < 10$ and eq. 11 for $10 \leq c < 1000$. Data are presented on a log-log scale to enhance the clarity of details for low c-values.

important to emphasize that the results presented in Fig. 3 may differ quantitatively from actual experimental outcomes of K_d accuracy assessments, which are typically presented as the dependence of $K_{d,det}$ on the determined c-value. Several factors can contribute to such quantitative differences, and we list a few of them below.

First, the relative systematic errors assigned to the variables used to generate Fig. 3 may differ from the actual errors in experiments. Second, the error propagation in this study is applied only to the specific case where the fraction of unbound ligand R is close to 0.5, rather than across the full range of R in nonlinear regression. Third, we assume that ΔH° is directly measurable, whereas, in real ITC experiments, ΔH° is a parameter to be determined. Finally, ITC data analysis often includes stoichiometry n as an additional parameter to estimate, alongside K_d and ΔH°, even though n is theoretically equal to unity in a 1:1 binding model [37,47,48].

These four factors prevent a direct comparison of the theoretical results in Fig. 3 with the experimental dependencies of ITC-determined K_d values on experimentally determined c-values. However, none of these factors significantly alters the overall trend depicted in Fig. 3, as they cannot eliminate the systematic errors in the experimental variables embedded within the constants of eqs. 11 and 12. For instance, while the mutual interactions among the uncertainties of the three common ITC-derived parameters — K_d, ΔH°, and n — are difficult to capture through analytical error propagation, there is robust evidence that these interactions do not negate the trends shown in Fig. 3. Specifically, in most cases, allowing n to float as a fitting parameter tends to further increase the uncertainty in the determined K_d [37]. Additionally, the uncertainty in K_d has been shown to correlate positively with the uncertainty in ΔH° [58], indicating that the uncertainties of these two parameters propagate to one another rather than compensating each other.

Furthermore, in ITC data fitting, some researchers incorporate additional parameters, such as a constant or polynomial representing the heat of dilution or baseline offset, into the regression model [53]. While increasing the number of fitting parameters may improve the apparent fitting quality, there is no mathematical evidence that this approach enhances the accuracy of critical fitted parameters, such as K_d and ΔH°. In fact, unknown interactions between the systematic errors of various parameters may degrade the reliability of all fitted values [37,53]. To ensure robust and meaningful results, the number of parameters included in the ITC data fitting model should be minimized [59]. Parameters that can be determined experimentally or independently should be measured separately rather than estimated during the fitting process. For example, the heat change resulting from dilution is best addressed by conducting a control titration to directly measure the dilution heat and then subtracting it from the ITC data [60,61]. Similarly, baseline determination is an essential step in ITC data analysis but should be performed independently of the binding isotherm fitting process. This can be achieved through manual baseline selection or rigorous baseline-determination procedures [62], rather than incorporating baseline as an additional parameter in the regression model. Thus, separating baseline determination and other extraneous factors from the fitting process helps avoid overfitting and ensures that the determined binding parameters, such as K_d and ΔH°, are more accurate and reliable.

Therefore, the purpose of Fig. 3 here is not to provide quantitative guidance on an optimal c-value for practitioners because the actual experimental systematic errors for each variable and the true c-values (without systematic errors) are unknown in real experiments. Instead, Fig. 3 demonstrates that within the traditionally recommended optimal c-value range of 10 to 100, the K_d values determined by ITC may still deviate significantly from the true K_d.

2.7. Improving the reliability of experimentally determined K_d values in ITC

The findings in Fig. 3 underscore that even for the widely regarded K_d-determination method, ITC, significant systematic errors can persist in the determined K_d values, even when experiments are conducted under recommended conditions. This highlights the critical need to rigorously assess the accuracy of ITC-derived parameters and also to develop other reliable techniques for K_d-determination.

While several Monte Carlo-based tools, such as SEDPHAT and *pytc*, have been developed for assessing the accuracy or precision of ITC-derived parameters [35–38], they are underutilized by ITC practitioners. This lack of adoption can be attributed to two primary factors: limited awareness of the potential magnitude of inaccuracies in $K_{d,det}$ and the relatively steep learning curve required to effectively use these programs. Moreover, many existing statistical methods fail to clearly define the necessary input information for accurately assessing ITC-derived parameters. Consequently, there is a strong demand for a comprehensive study on the necessary inputs for accuracy assessment and the development of a more user-friendly, accessible statistical tool tailored to the needs of the field.

It is important to emphasize that while ITC is arguably the primary confirmatory method, there are other techniques that offer comparable or even superior accuracy. Although a comprehensive analysis of these methods is beyond the scope of this work — a topic deserving of a dedicated review — we highlight a few notable alternatives, including thermal shift analysis [63,64], kinetic capillary electrophoresis (KCE) [65,66], and accurate constant via transient incomplete separation (ACTIS) [41,67]. Each of these methods has distinct advantages and limitations. Achieving the highest accuracy in K_d determinations often requires employing multiple complementary methods with meticulous attention to experimental design, implementation, and data analysis [15].

3. Conclusions

Building on our previous work [31], we investigated the theoretical relationship between the accuracy of ITC-derived K_d ($|\Delta K_d/K_d|$) and the true c-value using deterministic error propagation. Our analysis reveals a U-shaped dependence of $|\Delta K_d/K_d|$ on the c-value (Fig. 3). At very low c-values, $|\Delta K_d/K_d|$ increases significantly as c decreases. This trend is primarily due to the dominance of the rapidly increasing relative systematic error in ΔH°, which drives the overall propagation of errors. As c increases, $|\Delta K_d/K_d|$ reaches a minimum in a certain range, indicating an optimal balance where the amplification of systematic errors in variables by the c-value (according to eq. 11) is insignificant, and ΔH° measurements remain sufficiently accurate. However, as c-values exceed this optimal range, $|\Delta K_d/K_d|$ increases once more due to the amplification of systematic errors at high c-values. These findings confirm conclusions from the previous numerical studies but, to our knowledge, represent the first demonstration of the described trends using deterministic error propagation.

While our theoretical results may differ quantitatively from experimental observations due to variations in experimental and data processing methods, the overall trends shown in Fig. 3 remain robust. These trends underscore that even within the traditionally recommended c-value range of 10 to 100, ITC-derived K_d values can deviate significantly — by several folds — from the true K_d values. These findings highlight the critical need for assessing the accuracy of K_d values derived from ITC experiments.

Although Monte Carlo-based tools for accuracy assessment are available, they are largely underutilized by ITC practitioners. This is partly due to a tendency to overestimate the accuracy of ITC measurements, and the challenging learning curve associated with mastering these software programs. Additionally, many statistical methods lack clear input requirements for accuracy evaluation. While a recently introduced tool for computing the accuracy confidence interval (ACI) for K_d using the classic non-titration approach represents a significant advancement [11], it is not directly applicable to ITC. This highlights the urgent need for a user-friendly, accessible tool and a clearer understanding of necessary inputs for assessing the accuracy of ITC-derived parameters.

CRediT authorship contribution statement

Tong Ye Wang: Writing – original draft, Validation, Formal analysis. **Sergey N. Krylov:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of generative AI and AI-assisted technologies in the writing process

The authors utilized ChatGPT during manuscript preparation to refine sentence structure and enhance overall readability through removing redundancies. All content influenced by the tool was thoroughly reviewed to ensure that no changes to the original meaning were introduced. The authors take full responsibility for the content of the submitted manuscript.

Declaration of competing interest

The authors declare no competing financial interest.

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Appendix A. Supplementary data

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