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Statistical error in isothermal titration calorimetry: Variance function estimation from generalized least squares

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Abstract

The method of generalized least squares (GLS) is used to assess the variance function for isothermal titration calorimetry (ITC) data collected for the 1:1 complexation of Ba^{2+} with 18-crown-6 ether. In the GLS method, the least squares (LS) residuals from the data fit are themselves fitted to a variance function, with iterative adjustment of the weighting function in the data analysis to produce consistency. The data are treated in a pooled fashion, providing 321 fitted residuals from 35 data sets in the final analysis. Heteroscedasticity (nonconstant variance) is clearly indicated. Data error terms proportional to q_i and q_i/v are well defined statistically, where q_i is the heat from the ith injection of titrant and v is the injected volume. The statistical significance of the variance function parameters is confirmed through Monte Carlo calculations that mimic the actual data set. For the data in question, which fall mostly in the range of $q_i = 100$–2000 μcal, the contributions to the data variance from the terms in q_i^2 typically exceed the background constant term for $q_i > 300$ μcal and $v < 10$ μl. Conversely, this means that in reactions with q_i much less than this, heteroscedasticity is not a significant problem. Accordingly, in such cases the standard unweighted fitting procedures provide reliable results for the key parameters, K and ΔH° and their statistical errors. These results also support an important earlier finding: in most ITC work on 1:1 binding processes, the optimal number of injections is 7–10, which is a factor of 3 smaller than the current norm. For high-q reactions, where weighting is needed for optimal LS analysis, tips are given for using the weighting option in the commercial software commonly employed to process ITC data.
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In the method of isothermal titration calorimetry (ITC)[1], reaction enthalpy q is estimated for sequential addition of one reactant (titrant) to the other (titrate), producing a titration curve of q versus extent of reaction. Analysis of such data provides estimates of the enthalpy change ΔH° and the equilibrium constant K for the reaction. Although titration calorimetry dates back more than 40 years [1], the current burst of interest in this method can be traced to the more recent develop-

ment of instruments that permit relatively rapid generation of titration curves for small samples (∼1 ml volume, ∼1 mM concentration of titrate) [2–7]. Such instruments are now widely used to study biochemical binding processes. As an indication of the breadth of interest in this method, a recent check of the Science Citation Index for articles citing "isothermal titration calorimetry" in the titles, abstracts, or keywords turned up works in more than 60 different journals in the year 2004 alone.

ITC data are analyzed by least squares (LS) fitting, with most workers using nonlinear algorithms provided by the manufacturers of the instruments. By default, these packages use unweighted fitting, which tacitly assumes that the statistical error in the data is constant.

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[1] *Abbreviations used:* ITC, isothermal titration calorimetry; LS, least squares; GLS, generalized least squares; MC, Monte Carlo.

In recent works, I have noted that uncertainty in the delivered volume of titrant from the syringe can be a significant source of random error, leading to noise proportional to q in the data [8,9]. When this type of error predominates, the fitting should employ weights, $w_i \propto q_i^{-2}$, or in one possible model of the volume error, correlated fitting. In a subsequent extensive study of the Ba^{2+} complexation with 18-crown-6 ether [10], the data were fitted in turn to the three limiting models: constant error, proportional error, and correlated error. The results showed a preference for the proportional error model, but it was acknowledged that this model could not be completely valid because every experimental technique is limited by constant error in the small-signal limit.

In the current work, I return to the data from [10] for a more extensive examination of the statistical error. To this end, I use the method of generalized least squares (GLS), in which the variance function itself is estimated as a part of the analysis [11–13]. Usually the goal of a GLS analysis is optimal estimation of the adjustable parameters in the LS fit model, but here the goal is the variance function itself. Confident knowledge of the nature of the statistical error in ITC data can be used to optimize the design of experiments, which was the underlying purpose of my original theoretical investigation [8]. For example, one important result of that study was the realization that as few as five to seven injections may be optimal for 1:1 complexation ($X + M \rightleftarrows MX$) when constant error dominates. Because ITC experiments typically take 5–10 min per injection, this result can mean a tremendous boost to throughput in production run work, where 20–30 injections seems to be the current norm.

In the GLS method, the variance function is estimated in a sort of back-and-forth iterative analysis of the data. The LS residuals from one analysis are themselves subjected to an LS fit to the variance model, the results of which are then used to redefine the weights in the data fit for the next cycle. Convergence typically occurs in four to eight cycles. The capabilities of this method have been fairly well characterized for linear LS fit models, but less well for nonlinear ones, so the GLS method itself is examined in the current work through Monte Carlo (MC) calculations on an ITC model representative of the actual experimental data. The method is applied to the data from [10] for several different proposed variance functions that include constant and proportional error. The results support the expectation of both constant and proportional contributions to the data error. However, for the data used in this study (which involved q values mostly in the range of 100–2000 µcal and injection volumes of 4–40 µl), a simple scale error turns out to be a more important source of proportional error than the volume error identified in [8].

In following sections, I first review the essential aspects of nonlinear LS needed here and then do the same for the GLS method. The latter is tested for a representative ITC data set through MC methods typically involving 30 nine-point data sets at a time. The 37 actual Ba^{2+}/crown ether data sets are then analyzed in a pooled fashion, under the assumption they all are characterized by the same variance function. This analysis is repeated for a half-dozen different proposed variance functions, of which the preferred one to emerge consists of a sum of three variances: one constant and two proportional to q^2. The implications of these results for experiment design are then considered briefly, with an updating of some key results found earlier [8]. Particularly important is the result already noted, namely that heat-starved reactions are better done with few injections.

Materials and methods

Nonlinear least squares and Monte Carlo

The nonlinear LS calculations are done using standard methods, including some that are readily available [14,15]. The specific codes are written in FORTRAN and are similar to those that have been described in studies of bias and non-Gaussian parameter distributions in linear and nonlinear LS [16,17]. At the heart of the calculations is the evaluation of the matrix $\mathbf{A}$, given by

$$\mathbf{A} = \mathbf{X^T W X}, \tag{1}$$

where the matrix $\mathbf{X}$ contains elements $X_{ij} = (\partial F_i / \partial \beta_j)$, in which F expresses the fit function in terms of the fit variables (x and y) and the adjustable parameters $\boldsymbol{\beta}$. In the current work, no correlated models are considered, so the weight matrix $\mathbf{W}$ is diagonal, with elements $W_{ii} = w_i = \sigma_i^{-2}$.

If we take the view that the data error σ_i is known, we can define an a priori variance–covariance matrix $\mathbf{V}_{\text{prior}}$:

$$\mathbf{V}_{\text{prior}} = \mathbf{A}^{-1}. \tag{2}$$

$\mathbf{V}_{\text{prior}}$ is exact for linear fit models conducted under the usual assumptions of normal unbiased data with an error-free independent variable. For example, it can be used to check a MC code, the output of which should agree (statistically) with $\mathbf{V}_{\text{prior}}$ for a linear model. We can similarly define an "exact" $\mathbf{V}_{\text{prior}}$ for exactly fitting data in a nonlinear model. This nonlinear $\mathbf{V}_{\text{prior}}$ is not truly exact, because nonlinear LS parameters are not normally distributed and might not even have finite variance. However, examination of a number of representative nonlinear problems has led to a 10% rule of thumb that seems to be generally reliable: if the relative error in a parameter is less than 10%, the confidence limits can