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SHORT COMMUNICATION

FITTING PROGRESS CURVES IN ASSAYS OF SLOW-BINDING ENZYME INHIBITORS

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Practical aspects of fitting progress curves deriving from assays of slow-binding enzyme inhibitors in order to extract characteristic parameters is briefly discussed. An approach little-used, but which may provide more accurate estimates, is the simultaneous analysis of two assays differing from one another in the order of addition of reactants.

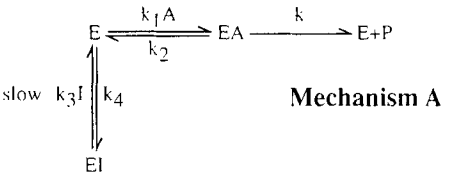
KEY WORDS: Progress curve analysis, slow-binding enzyme inhibitors.

INTRODUCTION

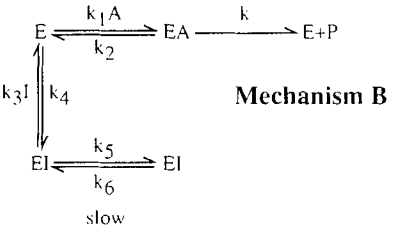
Examples of slow-binding and tight-binding enzyme inhibition have been encountered more frequently as more potent inhibitors have been discovered. When meaningful inhibition of the target enzyme is caused by nearly stoichiometric concentrations of inhibitor, tight-binding inhibition is the characterization. Although slow-binding inhibition might be any process occurring at less than a diffusion-controlled rate, in practice it is an operational appellation which depends on the enzyme assay. What might be considered slow on one time scale might be fast on another. In the typical spectrophotometric enzyme assay applied to characterization of slow-binding inhibitors, generation of product often is monitored continuously over a period of several minutes; an inhibitor causing a steady decline in enzyme activity over the same period would be called slow-binding. Assays that do not permit continuous monitoring of the enzyme-catalyzed reaction are, in general, not useful for such studies. In what follows, a few practical considerations for kinetic characterization of slow-binding inhibitors are presented. Three points in particular are discussed: Data should be collected over an appropriate number of half-lives of the slow process; model parameters may be more accurately defined if calculated from two data sets, identical in concentrations of all reactants but differing in the order of their addition to the assay mixture; and, of the parameters to be determined by data analysis, only one is non-linear, thus simplifying minimization of the sum of squared errors.

RESULTS AND DISCUSSION

Models formulating the kinetic description of slow-binding and tight-binding inhibition require that the interaction of enzyme and inhibitor be reversible. Tight-binding



Mechanism A



Mechanism B

$$P = v_S t + (v_O - v_S)(1 - e^{-kt})/k$$

mechanisms must account for distribution of inhibitor among free and enzyme-complexed species, whereas the most-commonly applied slow-binding models assume a large excess of inhibitor so that the fraction of total inhibitor binding to enzyme is not significant. Often such inhibitors resemble a substrate of the target enzyme, or a transition state intermediate along the reaction pathway and inhibition and binding of substrate are mutually exclusive. Mechanisms for slow-binding inhibition have been concisely described by Morrison,[1] in whose terminology the equations and mechanisms here have been presented, and in greater detail by Williams and Morrison[2] and Morrison and Stone.[3] Mechanisms **A** and **B** differ in the occurrence of one or two enzyme-inhibitor species. In **B**, the first complex is formed rapidly and there is a slow generation of the second; in **A**, there is no rapidly-formed complex. Full characterization of the two-complex model cannot be carried out if inhibitor concentrations necessary to generate appreciable amounts of the first complex are not tested. For both models, the progress curve is expressed by the same integrated rate equation. For the two models, the velocity terms v_0 and v_s have the same meanings, that is, initial and steady state velocities. The k parameter differs for the two mechanisms, as does the formulation of the overall inhibition constant K_i^*. P represents product, t represents time in this equation.

The two velocities that characterize the progress curve of an enzyme assay carried out in the presence of a slow-binding inhibitor are illustrated in Figure 1. Two sorts of assays may be carried out. In one, the enzyme is added last to a solution of inhibitor and substrate and the initial velocity is greater than the steady state velocity. In the second, enzyme and inhibitor are incubated together and substrate is added to complete the assay mixture. In this situation, the duration of the interval during which enzyme and inhibitor were permitted to interact before the addition of substrate will determine whether the initial velocity is greater than the steady-state velocity; if the incubation is carried out until equilibrium between enzyme and inhibitor is achieved, the initial velocity will be less than the steady-state velocity, since the extent of inhibition will be lessened in the presence of substrate. For the two assays then, if identical in all respects save for the order of addition of components to the assay

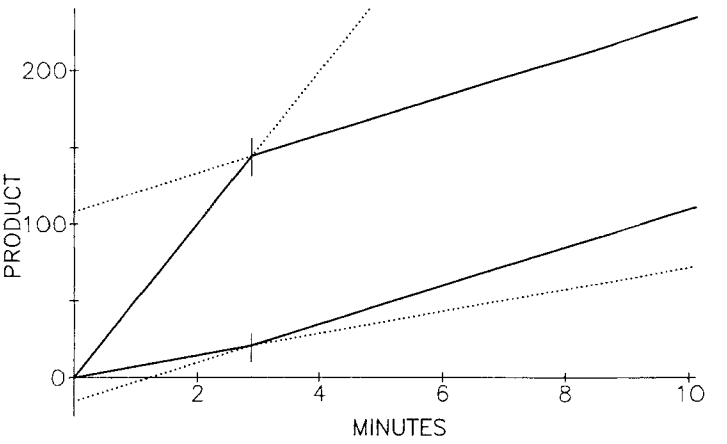


FIGURE 1 Initial and steady-state velocities for an assay in which enzyme was added to a mixture of substrate and slow-binding inhibitor (upper pair of lines) and for another assay in which substrate was added to a mixture of enzyme and slow-binding inhibitor which had been incubated to equilibrium (lower pair). The rate constant for the slow-binding process may be obtained from the intersection of the lines. These lines are those of the model for creation of artificial data sets over five half-lives, as discussed in the text.

mixture, four parameters are needed to describe the two progress curves. There is the initial velocity parameter for the enzyme-last assay and another for the substrate-last assay. For both assays the steady-state velocity must be the same and the rate constant describing the transition between the initial and steady-state velocities is the same. The velocity parameters depend on enzyme, substrate and inhibitor concentrations in the trial; the apparent first-order rate constant for that trial is independent of enzyme concentration. Accordingly, concentrations of enzyme and inhibitor may be chosen to increase or decrease the amount of product formed over the course of the assay. This is most significant in a trial where initial velocity exceeds the steady-state velocity and a reasonable rule of thumb is that substrate depletion of 10% or so does not seriously compromise the assumption of constant substrate concentration.

As inhibitor trials are carried out, it is worthwhile to sketch in, even roughly, tangents to the progress curve at the beginning and the end of the assay, as in Figure 1. Morrison[1] has pointed out that the intersection of the two lines occurs at the point $t = 1/k$. From the intersection then, a quick estimate of the half-life of the slow process may be made. As discussed below, an assay must be carried out for several half-lives to yield believable parameter estimates; the intersection is optimally located 15–25% along the time axis of the progress curve.

It is not necessary to understake mathematical analysis to understand that if an initial velocity is to be estimated with some confidence, assay data must be collected immediately after all components have been added to an assay mixture. Likewise, estimation of a terminal velocity requires data near equilibrium among enzyme, substrate and inhibitor. Obvious also is the need to achieve a measurable degree of inhibition. Practically, 40–90% inhibition, relative to the inhibitor-free control, provides useful data. If the degree of inhibition is too slight, a difference from the control rate may not stand out from experimental uncertainty. If the model with two

enzyme-inhibitor complexes is to be tested, two different ranges of inhibitor concentration may be necessary to establish the effect of each complex.

As an illustration of optimization of an assay, artificial data sets were created for inhibition according to mechanism **A** above. V_{max} was given the value 100 (in appropriate units), the ratio $[I]/K_i^*$ was given the value 6 and the ratio $[A]/K_m$ was given the value 1. A half-life of 2 minutes was chosen; thus the apparent first-order rate constant k had the value $(\ln 2)/2$. With these values, the steady-state velocity was 12.5 (in appropriate units) and the initial velocity, when the assay was started by addition of enzyme, was 50; when substrate was added to equilibrated enzyme-inhibitor complex, the initial velocity was 1/7th as great. Twenty time points were evenly spaced over a two-minute assay interval, corresponding to one half-life. Twenty time points were evenly spaced over a four-minute interval, corresponding to two half-lives. Other sets of twenty time points for three, four and five half-lives were prepared. Morrison and Stone[3] point out that all fitting procedures assume the time variable is known with certainty, so it makes sense to space observations unevenly over time to get approximately evenly spaced increments in the product variable, which is assumed to contain all experimental error. Such spacing would differ for enzyme-last and substrate-last assays, since the rate of product formation is greatest at the outset of the former assay and least at the outset of the latter assay. For this illustration, however, even spacing of time points seemed adequate. Random error was added to the calculated value of product formed at each time point: a relative error of up to plus or minus 4% of the exact value added to an absolute error of up to plus or minus 2 'units'. Two 'units' was about 1% of the total product formed over 10 minutes. The goal was to push to failure fitting of sets because of data variability. Nine replicate sets of enzyme-last assays at each half-life were created and analyzed, calculating initial and steady-state velocity and rate constant k parameters that best fit the data by least-squares techniques. Knowledge of the error structure was not incorporated into the minimization process; the default choice of unweighted data analysis is most often made, in practice. In all cases, initial velocities were adequately determined, since there were always data obtained at the outset of the assay. Estimation of the terminal velocity occasionally was poor when duration of the assay was less than four half-lifes, as illustrated in Figure 2. For assays over 1, 2 or 3 half-lives, fits to two or more of the nine sets generated estimates of poor accuracy and precision. For the same collection of data sets, rate constant estimates were liable to gross imprecision and inaccuracy when the assay covered three or fewer half-lives. Estimates of k are illustrated in Fig. 3.

Analysis of artificial data sets deriving from trials in which substrate was added to equilibrated enzyme-inhibitor solutions led to similar conclusions: the possibility of very misleading estimates when the progress curve was followed for too short an interval. Combining two trials in the analysis process, in contrast, yielded greatly improved results. This was tested with the nine data sets collected for three half-lives. There were 81 pairs of combinations of nine enzyme-last trials and nine substrate-last trials. Each of the 81 analyses yielded two initial velocity estimates, a steady-state velocity estimate and an estimate of the rate constant. These two latter parameters were always well-determined using paired data sets. In Figures 2 and 3 the range of 81 estimates is illustrated near the equivalent unpaired estimates. It can be seen that tight and accurate parameter estimates were derived from paired assay analyses when single assay analysis was prone to unreliable results.

It is not intended that conclusions drawn from examination of such limited artificial data should be considered definitive. The purpose is to support the intuitive

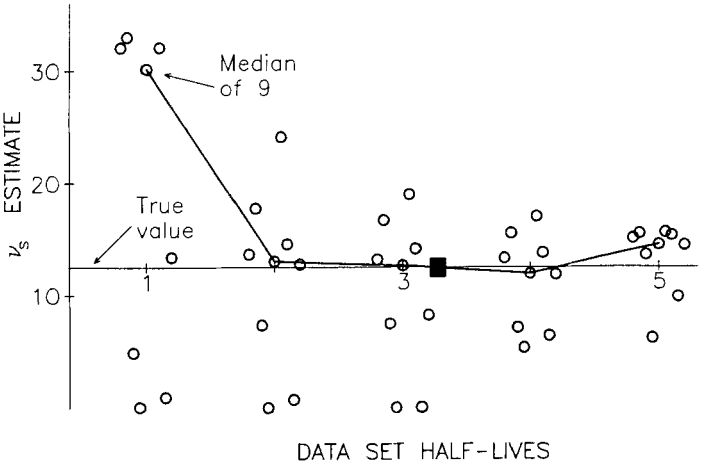


FIGURE 2 Estimates of steady-state velocity for data sets deriving from assays over one, two, three, four and five half-lives of the onset of inhibition of a slow-binding enzyme inhibitor. For each assay interval, nine sets of twenty artificial data values were created. Exact product values calculated according to the model expression were modified by random error as described in the text. Parameter estimation was by non-linear least-squares regression. Median estimates of the nine sets for each assay interval are connected by a line. The horizontal line denotes the exact model value of 12.5 'units'. The solid block represents the range of estimates of steady-state velocity for 81 pairs of all combinations of nine data sets each of artificial data for enzyme-last and substrate-last assays over 3 half-lives as discussed in the text.

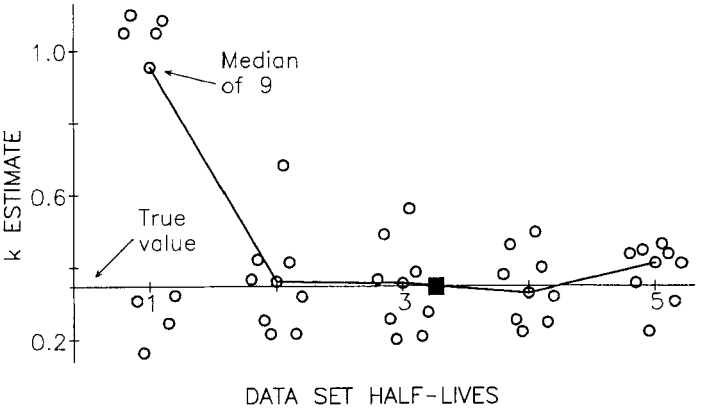


FIGURE 3 Estimates of rate constant k for data sets deriving from assays over one, two, three, four and five half-lives of the onset of inhibition of a slow-binding enzyme inhibitor. Data sets were the same as in Figure 2. Median estimates of the nine sets for each assay interval are connected by a line. The horizontal line denotes the exact model value of (ln 2)/2. The solid block represents the range of estimates of parameter k for 81 pairs of all combinations of nine data sets each of artificial data for enzyme-last and substrate-last assays as discussed in the text.

appreciation that data for several half-lives may be necessary for acceptable parameter estimation and that analysis of two data sets, obtained under identical conditions save for the order of addition of reactants, may provide much more reliable characterization of the slow-binding inhibitor.

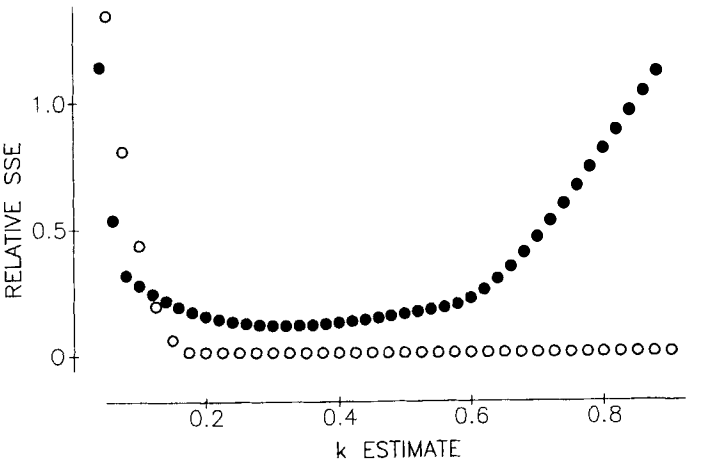


FIGURE 4 Sums of squared errors (SSE) are plotted as a function of rate constant parameter k. For each k estimate, optimum velocity parameters were determined by linear least squares regression. The open circles illustrate fitting results for a data set of twenty values which are so poor they do not enable the fitting process to converge to a satisfactory minimum. The filled circles illustrate simultaneous fitting of that data set (an enzyme-last assay) with another set of twenty values (a substrate-last assay); convergence to best-fitting parameters was obtained. Data sets were derived from artificial data in which $k = 0.347$. Ordinate values are scaled for ease of display.

In the integrated rate equation, the velocity parameters are linear, the rate constant is non-linear. Least-squares fitting of the parameters can be computationally simplified if the linear and non-linear parameters are fitted separately, as described by Lawton and Sylvestre.[4] For each trial estimate of k, the velocity parameters that generate the minimum sum of squared errors are uniquely defined. Iterative fitting is then equivalent to finding a minimum along a single dimension. In the fitting of the data described here, the sum of squared errors was treated as a parabolic function of parameter k and the minimum of the parabola was found within a few iterations – unless the data were too poor to yield a function minimum. Error surfaces are illustrated in Figure 4 for paired data sets which yield a best-fit rate constant and for a single data set which does not. For the single set, low rate constant estimates led to high errors, but there were no meaningful increase in error sum as the estimate was increased beyond reason. That is, only a very slow process was inconsistent with the data set. In contrast, with two sets, only a process neither very slow nor very fast was acceptable. The computer program used for such fitting is available on request: it is written for an MS-DOS personal computer having a graphics display adapter. Sculley and Morrison[5] have described a more sophisticated program which analyzes both slow- and tight-binding inhibition data. General programs such as SAS and Enzfitter are widely available which fit all parameters by non-linear regression.

As an example of analysis of slow-binding inhibition, the effect of MDL 27,013, a derivative of methoxysuccinyl-alanyl-alanyl-prolyl-valine in which the terminal carboxylate has been replaced by a trifluoromethylketone[6], on the enzymatic activity of human leukocyte elastase is illustrated in Figure 5. Parameters for paired assays were obtained using data over about five half-lives. Multiple assays at each of two substrate concentrations were combined in Dixon plots. One plot was made for the effect of inhibitor on initial velocity in enzyme-last assays; from such a plot the

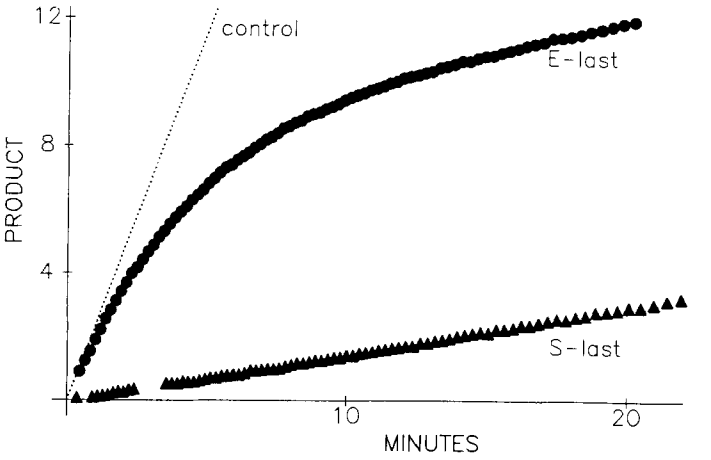


FIGURE 5 Inhibition of human leukocyte elastase by MDL 27,013. Two assays were carried out. In one, enzyme was added to a mixture of 0.2 μM inhibitor and 0.1875 mM succinyl-alanyl-alanine-p-nitroanilide substrate (E-last). In the other, enzyme and inhibitor were incubated together before addition of substrate (S-last). Absorbance at 405 nm was converted to nmols p-nitroaniline product formed. The dashed line indicates the progress curve obained in the absence of inhibitor.

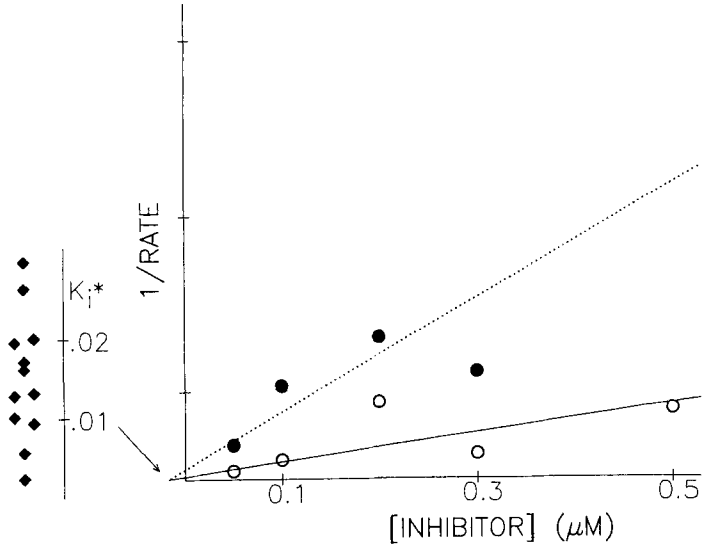


FIGURE 6 Dixon plot of inhibition of elastase by MDL 27,013. Each of the ten steady-state velocities illustrated in reciprocal form in the plot, given inhibitor and substrate concentrations and assuming values of Michaelis-Menten parameters, was put into the equation for simple competitive inhibition and generated a single estimate of the overall inhibition constant K_i*. These estimates (and a few others, from data not shown) are indicated to the left of the figure. The median of the estimates, and the intersection of the two lines, was determined to be 0.015 μM. Open circles, [substrate] = 0.75 mM. Filled circles, [substrate] = 0.1875 mM.

inhibition contant for the quickly-formed enzyme-inhibitor complex could be obtained. One Dixon plot was made using steady-state velocities. This latter plot is illustrated in Figure 6. The abscissa value at the intersection of the two lines is the overall inhibition constant K_i^*. In point of fact, the plots themselves were not used to obtain the desired parameters. Since the rate expression for competitive inhibition involves Michaelis–Menten constants K_m and V_{max}, and since these two had been well-established over many assays in the course of analysis of several inhibitors of this series and could be assumed known with certainty, relative to the value of the inhibition constant, the latter parameter was obtained as the median value by ranking estimates from each (paired) assay. A median-based approach lessens the influence of outliers, compared to least-squares methods, and seemed justified in this situation, where considerable information was available to define Michaelis–Menten parameters. A plot of rate constants k for the same assays is illustrated in Figure 7. The curves in the Figure were drawn in conformity with mechanism **B** and are not linear. Although a median-oriented approach is less justified here, values for inhibition constants for the rapidly-formed complex and for the overall inhibition process were assumed known and the rate constant for dissociation of the slowly-formed complex was obtained as the median of estimates from the assembly of (paired) assay results. This constant corresponds to the intersection of the two curves on the ordinate axis.

In summary, slow-binding enzyme inhibition can be readily characterized when continuously-monitored enzyme assays are carried out over a time interval appropriate to the half-life of the process. For very slow-binding inhibitors, where substrate would be consumed over a long assay, inhibition constants and rate constants are more suitably determined in separate equilibrium and pre-equilibrium trials. When the mode of inhibition is not of the tight-binding type, and inhibitor can be used

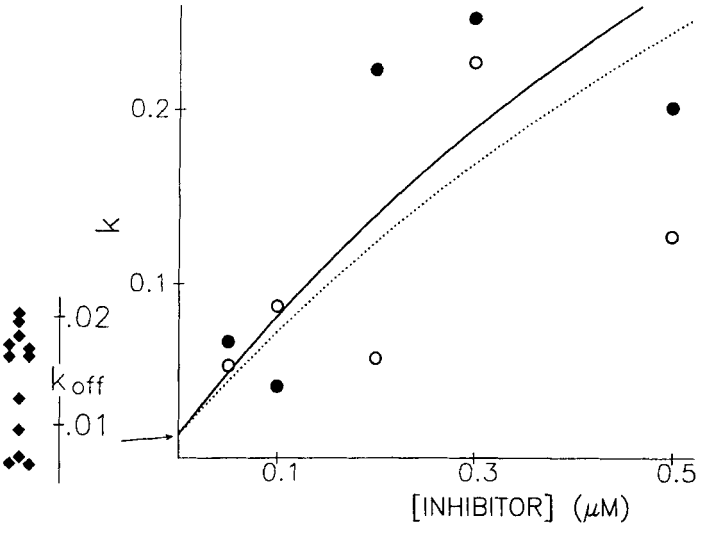


FIGURE 7 Observed first-order rate constants for inhibition of elastase by MDL 27,013 at substrate concentreations of 0.75 mM (open circles) and 0.1875 mM (filled circles). The median of the estimates of the dissociation constant of the slowly-formed enzyme-inhibitor complex (k_{off} or k_s in Mechanism **B**), which is also the intersection of the two curves on the ordinate, was determined to be 0.014 min^{-1}. The curves are drawn according to inhibition parameters for mechanism **B**.

in considerable excess over enzyme, one non-linear parameter is involved in the mathematical descriptions, permitting straight-forward least-squares fits.

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