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ESTIMATION OF K_I IN A COMPETITIVE ENZYME-INHIBITION MODEL: COMPARISONS AMONG THREE METHODS OF DATA ANALYSIS

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ABSTRACT:

There are a variety of methods available to calculate the inhibition constant (K_i) that characterizes substrate inhibition by a competitive inhibitor. Linearized versions of the Michaelis-Menten equation (e.g., Lineweaver-Burk, Dixon, etc.) are frequently used, but they often produce substantial errors in parameter estimation. This study was conducted to compare three methods of analysis for the estimation of K_i: simultaneous nonlinear regression (SNLR); nonsimultaneous, nonlinear regression, "$K_{M,app}$" method; and the Dixon method. Metabolite formation rates were simulated for a competitive inhibition model with random error (corresponding to 10% coefficient of variation). These rates were generated for a control (i.e., no inhibitor) and five inhibitor concentrations with six substrate concentrations per inhibitor and control. The K_M/K_i ratios ranged from less than 0.1 to greater than 600. A total of 3 data sets for each of three K_M/K_i ratios were generated (i.e., 108 rates/data set per K_M/K_i ratio). The mean inhibition and control data were fit simultaneously (SNLR method) using the full competitive enzyme-inhibition equation. In the $K_{M,app}$ method, the mean inhibition and control data were fit separately to the Michaelis-Menten equation. The SNLR approach was the most robust, fastest, and easiest to implement. The $K_{M,app}$ method gave good estimates of K_i but was more time consuming. Both methods gave good recoveries of K_M and V_{MAX} values. The Dixon method gave widely ranging and inaccurate estimates of K_i. For reliable estimation of K_i values, the SNLR method is preferred.

The complete in vitro characterization of substrate metabolism involves determination of both a qualitative profile and quantitative parameters. The former includes identification of isozymes involved in metabolic reactions and identification of biotransformation products. Quantitative parameters generally include estimation of the maximal rate of metabolism (V_{MAX}),[1] the Michaelis constant (K_M), and intrinsic clearance ($CL_{int} = V_{MAX}/K_M$). The latter is also, or should be, expressed in unbound form (i.e., $CL_{u,int} = CL_{int}/f_u$; where, f_u, is the unbound fraction of substrate in the in vitro incubation fluid milieu) (Obach, 1996, 1997).

An important practical goal of drug metabolism research is to relate in vitro findings to in vivo results, either within or across animal species. The former might include experiments conducted using a rat hepatocyte preparation and relating those results to findings from an in vivo dosing experiment in the rat. The latter would be exemplified by relating parameters obtained from a rat hepatocyte preparation to in vivo values in humans. Such within- and between-species correlations are also of interest in predicting alterations in drug metabolism (e.g., enzyme inhibition and induction).

One of the important outcomes of identifying isozymes responsible for metabolism, is the prediction and assessment of potential drug (or nutrient) interactions. The questions, "will an existing drug alter the metabolism of the new agent?" and "will the new agent alter the metabolism of existing drugs?" are only partially answered, however, by identification of the substrate isozyme responsible for metabolism. The other quantitative part of the question that needs to be answered is, "is the interaction likely to occur?" The latter is generally assessed by a comparison of the observed (or expected) plasma concentrations of the compound relative to its enzyme inhibition constant (K_i). Thus, if plasma concentrations are greater than K_i, an interaction is likely. Conversely, if the plasma concentrations are less than K_i, an interaction is unlikely.

Enzyme inhibition studies are routinely conducted to assess the presence and magnitude of drug-drug interactions. To characterize the inhibition process (i.e., competitive, noncompetitive, or uncompetitive) and to determine the inhibition constant (K_i), data are often analyzed by techniques that linearize inherently nonlinear relationships. These techniques were developed before the general use of computers and programs capable of analyzing nonlinear functions. Surprisingly, the linear forms of these relationships are in common use today, despite the ready availability of suitable computer programs. There has been increasing recent evidence of a greater reliance on nonlinear fitting routines for the analysis of enzyme kinetic data (Daigle et al., 1997; Scholten et al., 1997). Other investigators use another approach that relies upon a nonsimultaneous, nonlinear re-

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[1] Abbreviations used are: SNLR, simultaneous nonlinear regression; $K_{M,app}$, nonsimultaneous nonlinear regression; K_i, inhibition constant; K_M, Michaelis constant; V_{MAX}, maximal rate of metabolism.

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TABLE 1

Three different sets of parameter values examined in study

Set	K_M	V_{MAX}	K_i
	μM	*nmol/min · mg protein*	*μM*
1	184	0.27	0.3
2	0.34	0.14	4
3	10	100	5

gression method in combination with linear regression (Bourrie et al., 1996; Copeland, 1996).

The aim of the present study was to compare three methods of analysis of competitive enzyme inhibition data for the estimation of K_i (as well as K_M and V_{MAX}). Simulated data sets were generated assuming a competitive inhibition model and the resulting findings compared to assess which is the better, most reliable approach to use.

Materials and Methods

Assuming that a single metabolite is produced from the substrate, metabolite formation rates were simulated using a competitive enzyme-inhibition model. These rates were generated using an Excel spreadsheet (Microsoft, Redmond, WA) and the following equation (Segel, 1975),

$$v = \frac{V_{MAX} \cdot [S]}{k_M\left(1 + \frac{[I]}{K_i}\right) + [S]} \quad (1)$$

where: v is the rate of metabolite formation, V_{MAX} is the maximal rate of metabolism, K_M is the Michaelis constant, K_i is the inhibitor constant, $[S]$ is substrate concentration, and $[I]$ is inhibitor concentration.

Normally distributed [N(0,1)] random error was added to the exact (i.e., perfect) metabolite formation rate data (v_{exact}). The normally distributed random errors were obtained from random numbers (rn) created in Excel. The following error was added to each simulated perfect value of rate: 0.1 · rate · random number. This method corresponds to using an additive error of 10% coefficient of variation. The resulting value for rate is represented as: $v_{obs} = v_{exact} + 0.1 \cdot v_{exact} \cdot rn$; where v_{obs} is the value for rate that was used in the subsequent analyses.

Three different sets of parameter values were examined in this study (Table 1). Set 1 corresponds to values obtained from an in vitro study of an interaction between tolbutamide and sulfaphenazole (Bourrie et al., 1996). Set 2 corresponds to an in vitro interaction study of coumarin and pilocarpine (Bourrie et al., 1996). The two previous studies were conducted using human liver microsomes. Set 3 represents an example cited by Copeland (1996). The units have been defined here only to be consistent with the first two experimentally based literature examples. The range of K_M/K_i ratios varies from 0.085 to 613.

For each of the above three sets of parameters, three complete series of experiments were conducted (in triplicate) such that each experiment comprised five inhibitor concentrations and one control (i.e., no inhibitor), with six substrate concentrations per inhibitor and control. That is, a total of 108 average rates of metabolite formation with error (v_{obs}) were generated per set of parameters. The averages of the observed rates for the three experiments at a given substrate concentration were obtained and used in the subsequent analyses.

The above data were analyzed using the following methods.

Simultaneous Nonlinear Regression (SNLR). The average observed rate versus substrate concentration data (in the presence and absence of inhibitors) were fit simultaneously according to the full nonlinear expression for competitive enzyme inhibition as expressed in eq. 1. This is an exercise in fitting only one complete data set. Nonlinear regression fitting was accomplished with use of the WinNonlin program (Scientific Consulting, Inc., Cary, NC) with a weighting function of $1/Y^2$. Two approaches were applied to this analysis. In one instance all of the data were analyzed simultaneously, as noted above, for all parameters (i.e., V_{MAX}, K_M, and K_i). In the other instance, the control data (i.e., no inhibitor) were first analyzed to obtain estimates of V_{MAX} and K_M. Those values were then fixed in fitting the average rate data obtained from the inhibition experiments for estimation of K_i.

Another question that was addressed was the influence of the number of inhibitor concentrations on the estimated value of K_i. This effect was evaluated by deleting results from the inhibition experiments, beginning with six values and decreasing until only one inhibitor concentration was used in the analysis.

"$K_{M,app}$" Method. This method employs nonsimultaneous, nonlinear regression analyses. Each of the six independent experimental data sets, for the control and each of the five inhibitor concentrations, were fit individually by nonlinear regression to the Michaelis-Menten expression,

$$v = \frac{V_{MAX} \cdot [S]}{K_{M,app} + [S]} \quad (2)$$

Nonlinear regression fitting was accomplished with use of the WinNonlin program with a weighting function of $1/Y^2$. The control data results in a value for K_M and V_{MAX} and each of the inhibitor data sets results in a value for V_{MAX} and an apparent "K_M", which we refer to as $K_{M,app}$. As noted above for the SNLR method, two approaches were applied here with respect to V_{MAX}. In one case the V_{MAX} value obtained from the control experiment was fixed and used for all subsequent fitting of the inhibitor concentration data sets. In the other instance, estimates of V_{MAX} were obtained from the individual fitting of the inhibition data. An example of the type of curves obtained is shown in Fig. 1A.

Estimates of K_i were obtained from the linear regression plot of $K_{M,app}/V_{MAX}$ as a function of the inhibitor concentration, [I]. Such a plot is illustrated in Fig. 1B. The equation describing this relationship is (Copeland, 1996),

$$\frac{K_{M,app}}{V_{MAX}} = \frac{K_M \cdot [I]}{K_i \cdot V_{MAX}} + \frac{K_M}{V_{MAX}} \quad (3)$$

The y values are obtained from the fitting procedure described above. Two graphs were obtained for each data set. In one case, the V_{MAX} obtained from the control data was used to divide all derived $K_{M,app}$ values. In the second instance, the V_{MAX} values were those obtained in conjunction with the individual fitted data, which provided estimates of $K_{M,app}$. The y-axis values may also be obtained from the reciprocal of the slope of a Lineweaver-Burke plot (i.e., 1/v versus 1/[S]), but we elected not to employ that method of analysis for estimating $K_{M,app}/V_{MAX}$.

The value for K_i is obtained from the intercept on the x-axis (i.e., when y = 0, [I] = $-K_i$) or it is calculated from the slope. The data were fit by linear regression (with a weighting function of 1/Y) and solution for the intercept provided an estimate of K_i. The STATA program (Stata Corp., College Station, TX) was used for this purpose. As with the assessment of the SNLR approach, the influence of the number of inhibitor concentrations on the resulting estimate of K_i was determined.

Dixon Method. The Dixon (1953) plot is frequently used for both identification of the likely mechanism of enzyme inhibition and for estimation of K_i. Plots were prepared of the reciprocal of rate of metabolite formation ($1/v_{obs}$) versus inhibitor concentration at each substrate concentration. The equation governing this relationship is given below,

$$\frac{1}{v} = \frac{K_M \cdot [I]}{V_{MAX} \cdot [S] \cdot K_i} + \frac{1}{V_{MAX}}\left(1 + \frac{K_M}{[S]}\right) \quad (4)$$

The resulting straight lines were analyzed by linear regression using the STATA program. Estimates of K_i were obtained by simultaneously solving two sets of equations sequentially; one equation for each of the straight lines equated with the line that represented the highest substrate concentration (this line has the smallest slope). The point of intersection of these pairs of lines represents the value of K_i. The latter values are reported as means or as a range of values. A graphical illustration of this plot is presented in Fig. 2.

Results

Table 2 summarizes the findings of this study for one of the three sets of parameter values (set 3; $K_M/K_i = 2.0$) and for the three methods of analyses examined. For the SNLR and $K_{M,app}$ methods there are three values of K_i listed under the column headings, Fixed and Varying and the resulting means and S.D.s of those values. The term "fixed" indicates that the control data were analyzed by nonlinear regression to obtain the best fitting values for V_{MAX} and K_M.

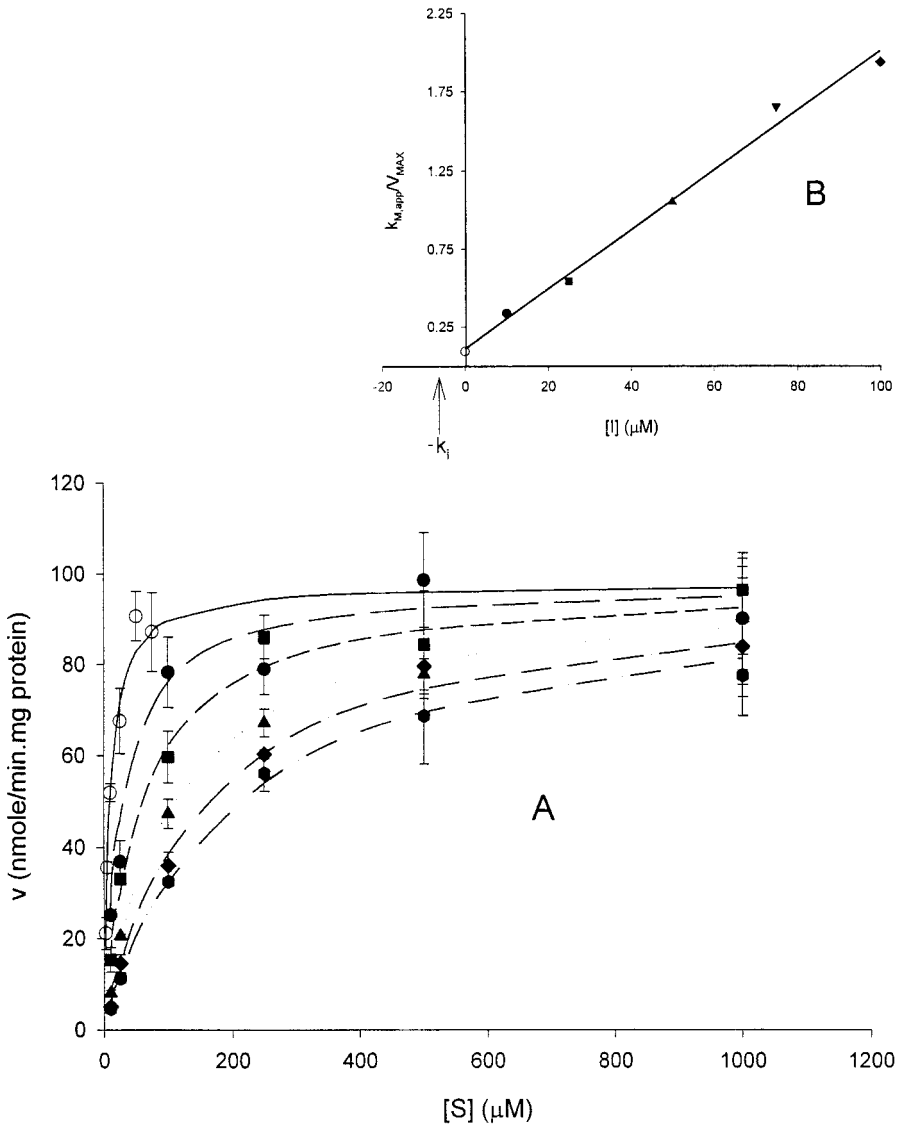

FIG. 1. *Illustration of application of $K_{M,app}$ method for analysis of enzyme inhibition data.*

A, each line represents nonlinear regression analysis of average simulated rates of metabolite formation (v) as a function of substrate concentration [S] for parameter data set 3 (K_M = 10 μM; V_{MAX} = 100 nmol/min · mg protein; K_i = 5 μM). Individual "experimental" data sets were fit to the full Michaelis-Menten equation (see eq. 2 in text) from which estimates of $K_{M,app}$ and V_{MAX} were obtained. Each point represents mean and S.D. (cross-hatched bars) of three experiments. B, plot of $K_{M,app}/V_{MAX}$ versus inhibitor concentration [I] used to estimate inhibition constant, K_i. Estimates of $K_{M,app}/V_{MAX}$ were obtained from analysis of rate data shown in A. Solid line is linear regression analysis of data. Intercept of line on *x*-axis represents $-K_i$ (ca., 5 μM). Inhibitor concentrations: 0 μM (○, control), 10 μM (●), 25 μM (■), 50 μM (▲), 75 μM (▼), and 100 μM (♦).

Those values were then fixed in the subsequent SNLR analysis of all of the data (i.e., control and inhibition data). The term "varying" indicates that values for V_{MAX} and K_M were obtained from the simultaneous fit of all of the data (including the control experiment) and there was no attempt made to fix the values.

Each of the K_i values listed was obtained from the analysis of a complete data set that included triplicate determinations of metabolite formation rates per substrate concentration. Thus, the values 4.71, 4.60, and 4.92 (under SNLR, Fixed column) are K_i estimates obtained from three different data sets, each involving triplicate estimations of rate versus substrate concentrations for a control and five inhibitor concentrations. Furthermore, the table is divided into rows according to the number of inhibitor concentrations used in the analysis. As an example, 4.74 (±0.16) and 4.95 (±0.27) are the means and S.D.s for K_i when the SNLR method is used to analyze the data from five inhibitor concentrations and one control experiment and when V_{MAX} and K_M are either fixed or allowed to vary, respectively.

Considering the SNLR results, the mean estimates of K_i are not very different from the perfect value of 5.0; regardless of whether V_{MAX} and K_M were fixed or allowed to vary. Furthermore, the values for K_i do not vary greatly from the correct number as one moves down the table; that is, as fewer inhibition curves are generated. The S.D.s, however, do increase as the total number of experiments are reduced. Thus, using only the control experiment and one inhibition curve, the mean K_i value is 4.71 (range, 3.91–5.61) with a coefficient of variation of 18%. The latter range and variation are considerably greater than those associated with estimates of K_i that rely upon more inhibition curves.

The column designated $K_{M,app}$ is divided into two components, as with the SNLR method, one in which the value for V_{MAX} was fixed

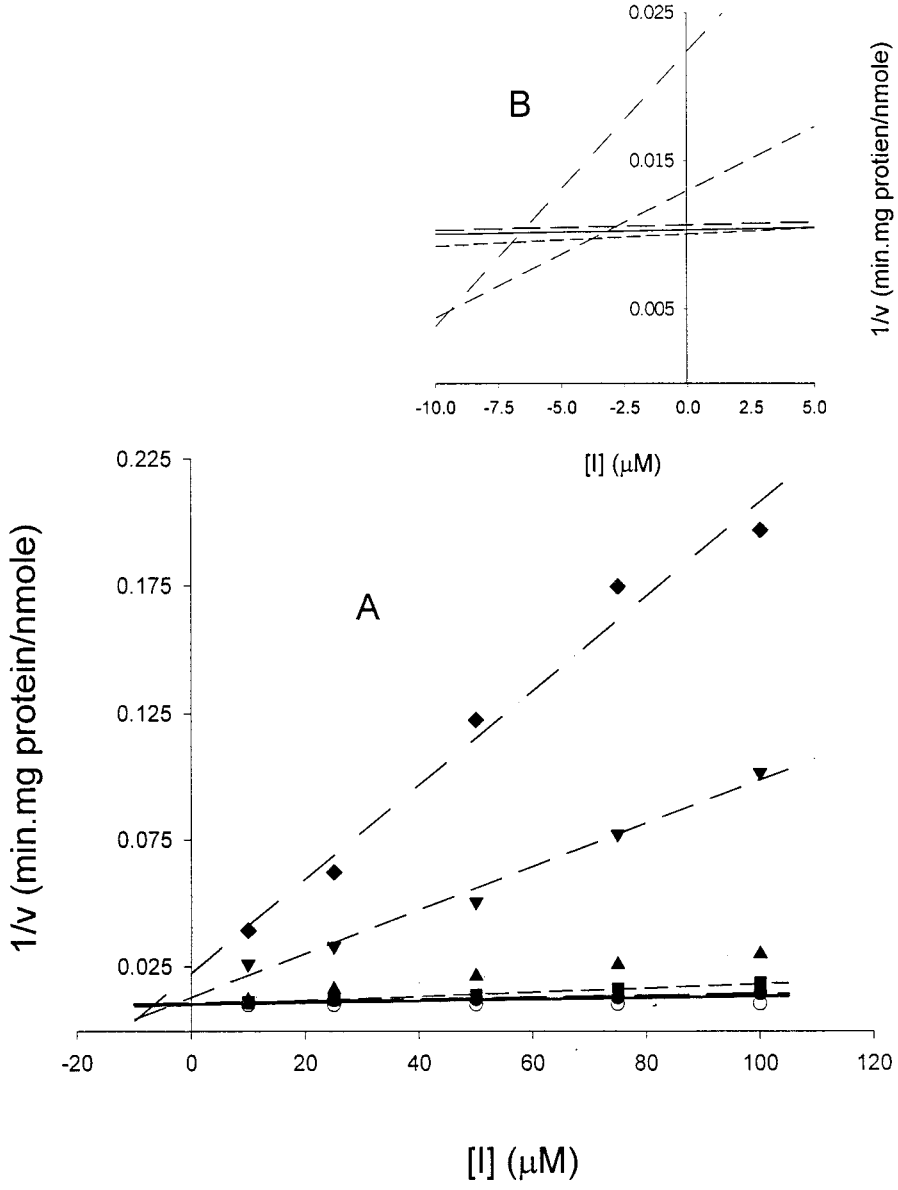

FIG. 2. *A, Dixon plot of reciprocal rates of metabolite formation (1/v) as a function of inhibitor concentration [I].*

Each line represents linear regression analysis of reciprocal of average simulated rates of metabolite formation for different substrate concentrations as a function of inhibitor concentration. These simulated data correspond to parameter data set 3 (K_M = 10 μM; V_{MAX} = 100 nmol/min · mg protein; K_i = 5 μM). Substrate concentrations: 10 μM (♦), 25 μM (▼), 100 μM (▲), 250 μM (■), 500 μM (●), and 1000 μM (○). B, enlarged view of negative *x*-axis from graph A indicating that there are several intersections of lines.

and the other in which it was allowed to vary. The influence of the use of a weighting function (no weight or 1/Y) for the linear regression analysis of the $K_{M,app}/V_{MAX}$ versus [I] data (as shown in Fig. 1B) is presented in the table. The mean values for K_i when a weighting function is applied is shown in parentheses. The mean estimates of K_i are similar to the correct value of 5.0 and weighting generally improves the estimate and reduces its variance. As noted for the SNLR results, the S.D.s increase as one moves down the column; that is, as fewer inhibition curves are used in estimation of K_i.

The results from the Dixon analysis are shown on the right side of Table 2. Each column corresponds to a substrate concentration and the values for K_i are listed for each data set. The values listed for K_i were obtained from the intersection of the line described by that substrate concentration and the line having the least slope (the greatest substrate concentration; [S] = 1000 μM). Thus, the average value for K_i of 4.28 μM (under the column [S] = 10 μM) is the average of the three intersections of the lines described by that substrate concentration and the line of substrate concentration [S] = 1000 μM. The lines corresponding to the lowest substrate concentrations (these are the lines with the steepest slope) provide reasonable but highly variable estimates of K_i. The best estimates of K_i were obtained from the intersection of the lines representing [S] = 25 and [S] = 1000 μM. The corresponding values of K_i ranged from 3.21 to 8.06 with a mean of 5.37 $\pm$ 2.47 μM. The higher the substrate concentration, going from left to right in the table, the less reliable the values for K_i; indeed, in some instances, negative values were obtained, which is clearly meaningless. Applying a weighting function to the regression analysis of the Dixon plot did not substantially improve the estimates of K_i and,

TABLE 2

Estimates of K_i *(μM) obtained from three methods of data analysis applied to one set of parameters*[a]

Data Set[b]	SNLR: V_{MAX} and K_M		$K_{M,app}$: V_{MAX}		Dixon [S]				
	Fixed	Varying	Fixed	Varying	10	25	100	250	500
[I] = 0–100 (6)			unwtd (wt = 1/y)		[S] = 10–1000 (6)[c]				
1	4.71	4.69	5.03	5.06	4.30	8.06	5.31	21.14	−1.94
2	4.60	5.24	4.54	4.31	1.93	4.82	1.05	−9.73	497.00
3	4.92	4.91	4.58	4.96	6.60	3.21	2.29	−5.49	57.93
Mean	4.74	4.95	4.72 (4.74)	4.78 (5.09)	4.28	5.37	2.88	1.97	184.33
S.D.	0.16	0.27	0.27 (0.25)	0.41 (0.23)	2.34	2.47	2.19	16.74	272.43
[I] = 0–75 (5)									
1	4.66	4.67	4.07	3.66					
2	5.43	5.28	4.60	5.18					
3	4.90	4.90	3.87	3.94					
Mean	5.00	4.95	4.18 (4.65)	4.26 (5.05)					
S.D.	0.40	0.31	0.38 (0.16)	0.81 (0.54)					
[I] = 0–50 (4)									
1	4.77	4.82	5.39	5.43					
2	5.55	5.46	5.69	6.38					
3	5.05	5.07	4.98	5.07					
Mean	5.12	5.12	5.35 (4.97)	5.62 (5.49)					
S.D.	0.39	0.32	0.36 (0.29)	0.68 (0.47)					
[I] = 0–25 (3)									
1	4.79	4.86	6.56	6.45					
2	5.27	5.20	3.91	5.53					
3	5.14	5.19	6.05	6.48					
Mean	5.06	5.08	5.50 (4.92)	6.15 (5.56)					
S.D.	0.25	0.19	1.41 (0.80)	0.54 (0.17)					
[I] = 0–10 (2)									
1	3.91	3.90							
2	5.61	5.64							
3	4.60	4.60							
Mean	4.71	4.71							
S.D.	0.85	0.88							

[a] Perfect simulated data were generated with following values: V_{MAX} = 100 nmol/min · mg protein; K_M = 10 μM; K_i = 5.0 μM.
[b] Range and number (in parenthesis) of inhibitor ([I]) concentrations used, including a control.
[c] Range and number (in parenthesis) of substrate ([S]) concentrations used.

therefore, only the estimates obtained without weighting are presented. The graph depicting the Dixon analysis is illustrated in Fig. 2.

Table 3 further summarizes the results of the analyses for all three sets of parameter values. The data are presented as ranges of values and incorporate the identical, extensive analyses as indicated in Table 2. The values for K_i encompass a rather narrow range and approximate the correct value for each data set when the data are analyzed by either the SNLR or $K_{M,app}$ methods. The Dixon method, on the other hand, provides for wide variation in values; often different by an order of magnitude. The Dixon method gave the best estimates of K_i for the strongest inhibitor (K_i = 0.3) but only in two cases; 0.35 ± 0.05 and 0.25 ± 0.16 μM from intersections of lines drawn at [S] = 200 and 500 μM with the line of [S] = 1000 μM, respectively. Furthermore, the Dixon method provides a range of K_i values that encompass zero (i.e., negative values).

The ranges of the recovered values for K_M and V_{MAX} were rather narrow and all ranges encompassed the correct value. Although the ranges were somewhat less variable for the SNLR method, the $K_{M,app}$ method provided essentially identical ranges. No attempt was made to estimate K_M and V_{MAX} from the Dixon analysis.

Discussion

The purpose of this study was to examine and contrast three different methods that are used for the analysis of in vitro enzyme inhibition data to obtain estimates of the inhibition constant, K_i. The primary questions posed were, which procedure: a) provides the most accurate and least variable estimates of K_i (as well as K_M and V_{MAX}); b) is least affected by a wide range of enzyme parameter values; c) requires the least amount of information (and, therefore, a minimal amount of experimentation); and d) is the easiest to implement and use routinely?

There were two issues responsible for prompting these questions. First, although it is generally well known that linear representations of inherently nonlinear relationships (e.g., Lineweaver-Burk plot) will often provide poor estimates of parameter values, there is ample evidence in the recent literature of data analysis using linearized expressions. A specific example is the analysis of enzyme inhibition data with use of, for example, the Dixon plot. Although the latter is often relied upon in making decisions concerning the mechanism of inhibition, it would not be expected to provide robust estimates of the inhibition constant, K_i. The surprising aspect of this situation is that we no longer need to rely upon linearized analyses, as was necessary before the availability of computers (mainframes or personal) and the necessary software packages capable of performing nonlinear regression analyses. Personal computers and software packages are now commonplace and, although there are indications of many groups now using these more sophisticated and appropriate methods of analyses, the current literature is replete with examples where the linearized methods are still being relied upon. Perhaps this is simply an expression of old habits disappearing slowly.

The second issue is best understood with reference to the phenomenal efforts being made in the drug discovery process in the pharma-

TABLE 3

Summary of range of estimates for K_i, V_{MAX}, *and* K_M *obtained from three methods of data analysis applied to three sets of parameters*

Set	Perfect Values[a]			Estimated Values						Dixon K_i
				SNLR			$K_{M,app}$			
	K_i	K_M	V_{MAX}	K_i	K_M	V_{MAX}	K_i	K_M	V_{MAX}	
1	0.3	184	0.27	0.25–0.35	177–222	0.27–0.28	0.25–0.39	184–215	0.24–0.30	−5.04–1.65
2	4.0	0.34	0.14	3.45–5.07	0.34–0.38	0.14–0.15	2.76–6.50	0.34–0.42	0.12–0.16	−38.0–32.5
3	5.0	10.0	100	3.90–5.64	8.96–11.08	97.0–105.8	4.66–6.04	9.36–11.72	92.8–109.3	−9.73–497

[a] K_i = μM; K_M = μM; V_{MAX} = nmol/min · mg protein.

ceutical industry. The creation of large numbers of new chemical entities, especially as a result of efforts from combinatorial chemistry, has created the need for high-throughput screening for numerous drug properties/characteristics such as pathways of metabolism and potential drug-drug interactions. In that regard, the current investigation might prove useful if we were able to identify approaches to data analysis that are not only robust but that are able to reduce the current experimental burden in in vitro screening for interactions.

The results of this investigation provide several answers to the basic questions posed. Regarding the method of analysis of enzyme inhibition data assuming, as we have here, a competitive inhibition model, the classic approach of Dixon is the least acceptable among the three methods examined. The approach used here to estimate K_i from a Dixon analysis is that suggested by Segel (1975): determine the point of intersection of substrate lines either with the highest substrate line ([S] = 1000 μM, in this case) or with a horizontal line drawn through the value of $1/V_{MAX}$ on the *y*-axis (assuming that V_{MAX} is known). Although the Dixon plot may serve to suggest the most likely mechanism of enzyme inhibition and provide for initial estimates of K_i, it does not share the desirable attributes of the alternative methods: accurate and robust estimates of K_i, less demand for experimental data, and simplicity. The latter points are addressed below. Even if one is only interested in a "ballpark" estimate of K_i, the Dixon approach may provide values that are incorrect by an order of magnitude or greater.

The simulations conducted here and analyzed by the Dixon method indicate that the substrate concentration used will have a marked effect on the accuracy of the K_i value. As noted in Table 2, the higher the substrate concentration (moving from left to right in the table), the greater the error and variance in estimation of K_i. This problem can be seen graphically in Fig. 2B. Furthermore, negative values are sometimes encountered: a meaningless result. The most accurate but still variable estimates of K_i appear to occur at low substrate concentrations ([S] = 10 or 25 μM). For the three parameter sets evaluated here and summarized in Table 3, the Dixon method results in ranges of values that encompass zero.

In marked contrast to the Dixon method and, we suspect, other approaches that are a combination of linearized forms of the enzyme inhibition relationship, both the SNLR and the $K_{M,app}$ methods do an excellent job of accurately estimating K_i. The ranges of estimates of K_i encompass the correct value, never include zero, and there is generally less than a 2-fold range in values (Table 3). The accuracy in estimation of K_i appears not to depend upon the enzyme parameter values as a wide range of values were used (i.e., parameter sets 1–3, Table 3). Furthermore, those methods perform very well in accurately recovering values of V_{MAX} and K_M. As noted in Table 3, the ranges for the estimated values of V_{MAX} and K_M are narrow and always encompass the correct value.

Of the two nonlinear approaches examined here, the SNLR method, is recommended because it is easy to implement and it performs the analysis using the full nonlinear expression for enzyme inhibition. In contrast, the other nonlinear approach, referred to here as $K_{M,app}$, although providing equally good estimates of all three parameters, is more demanding of analysis and requires a two-step procedure. That method is more demanding of analysis in that each of the control and inhibitor curves must be individually fit by nonlinear regression to obtain estimates of K_M and V_{MAX} (from the control data) and values for $K_{M,app}$ (with or without estimates of V_{MAX} from the inhibition curves; Fig. 1A). The latter values and K_M must then be plotted on a linear scale for an estimate of K_i to be obtained (Fig. 1B). In the SNLR approach, all parameter values are obtained from a single data fitting session.

The SNLR and the $K_{M,app}$ methods, in addition to providing more accurate and reliable estimates of K_i in comparison with the Dixon method, will permit a reduction in the amount of experimental data needed. This is exemplified in Table 2 where, moving down the table of values, the number of inhibitor concentrations have been reduced from a maximum of five sets of inhibitor curves to only one inhibitor curve for the SNLR method or two inhibitor curves for the $K_{M,app}$ method. In the former case and using our experimental paradigm, this would translate to reducing the experimental burden from 270 values (i.e., 6 substrates × 5 inhibitor concentrations × 3 experiments × 3 determinations) to 54 values (i.e., 6 substrates × 1 inhibitor concentration × 3 experiments × 3 determinations). A control experiment would need to be added to each of the preceding designs. Although the paradigm selected here for simulation purposes is likely to be more extensive and demanding than one chosen to be practical by an experimenter, there will be, nonetheless, a reduction in the total number of experiments and an accompanying savings of time and costs. We are not endorsing the use of only one inhibitor concentration curve at this time, because we have conducted only limited simulations and have not yet experimentally tested this idea. Our limited results (Table 2) suggest that such an approach would provide reasonably accurate but variable estimates of K_i. The $K_{M,app}$ method could in theory also only require one inhibitor curve, because this would provide two points to form a straight line in the linear analysis to calculate K_i. We chose, however, to use two inhibitor curves as the minimum number, which will provide three points (including the control) in the linear analysis.

It is, in fact, possible to use an even less demanding experimental design to obtain reasonable estimates of K_i (as well as K_M and V_{MAX}). For example, rather than conduct an entire inhibition curve (i.e., one inhibitor concentration with a range of substrate concentrations), which is currently our minimal experimental design, would it be feasible to use (in addition to the control experiment) only one substrate concentration with one inhibitor concentration in triplicate (i.e., only three values plus the control experiment)? Preliminary evidence from simulations suggests that the latter is, in fact, plausible and will provide reasonable estimates of the parameter values (Y. Pak, T.K., H.B. and M.M., unpublished observations).

In conclusion, we conducted extensive simulations to compare three methods of analysis that may be used for estimation of the

inhibition constant, K_i, from data obtained in an enzyme inhibition experiment. The two methods that use a nonlinear expression more accurately estimate K_i as well as values for K_M and V_{MAX} in comparison with the linearized approach of Dixon. The latter technique is not recommended for that purpose. The nonlinear approaches also offer, compared with the Dixon method, the potential advantage of substantial reduction in experimental time and costs. The latter is being pursued to determine the minimal experimental design that is compatible with estimation of accurate parameter values. We have not, of course, exhausted the possible combinations of parameter values in our simulations. Nor have we examined the effect of an introduction of larger random errors into the simulated data. However, the final conclusions will most likely remain the same: the nonlinear approach to the estimation of enzyme kinetic parameters is expected to be more accurate and robust.

Note Added in Press: We recently came across a paper by Nimmo and Atkins [*Biochem J* (1976)**157:**489–492] who analyzed data in a manner similar to that reported in this communication. Unfortunately, that paper appears to have been largely ignored by most investigators. Although there are differences to be found between these reports, those investigators deserve credit for the earliest reporting, to our knowledge, of what we have referred to as the SNLR method (their "direct" method).

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