*Short communication*

# Estimation of Kinetic Parameters for Enzyme-Inhibition Reaction Models Using Direct Time-Dependent Equations for Reactant Concentrations

**Marko Goličnik**

*Institute of Biochemistry, Faculty of Medicine, University of Ljubljana, Vrazov trg 2, 1000 Ljubljana, Slovenia*

** Corresponding author: E-mail: marko.golicnik@mf.uni-lj.si*
*Phone: +386-1-5437669, Fax: +386-1-5437641*

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## Abstract

To facilitate the determination of a reaction type and its kinetics constants for reversible inhibitors of Michaelis-Menten-type enzymes using progress-curve analysis, I present here an explicit equation for direct curve fitting to full time-course data of inhibited enzyme-catalyzed reactions. This algebraic expression involves certain elementary functions where their values are readily available using any standard nonlinear regression program. Hence this allows easy analysis of experimentally observed kinetics without any data conversion prior to fitting. Its implementation gives correct parameter estimates that are in very good agreement with results obtained using both the numerically integrated Michaelis-Menten rate equation or its exact closed-form solution which is expressed in terms of the Lambert W function.

**Keywords:** Enzyme kinetics, inhibition, nonlinear regression, Lambert W function, integrated Michaelis-Menten equation, progress curve analysis

## 1. Introduction

Enzymes represent the functional units of cell metabolism. They are remarkable catalysts because under mild operating conditions they show high specificity and activity towards their substrate. A quantitative approach towards the characterization of the activity of enzymes is essential for a detailed understanding of their reaction dynamics, which is itself crucial to several research fields, including biochemistry, biotechnology, pharmacy and medicine. Furhermore, the rates of enzyme-catalyzed reactions can be regulated by different modifiers. Drug discovery particularly focuses on the identification and design of such modifiers, which are generally inhibitors, as a means to perturb enzyme function. Out of the approximate 3,000 "drugable" proteins in humans, enzymes represent a large and diverse class of proteins that are being exploited in drug development, as almost half of all of the marketed small-molecule drugs act on enzymes.[1] Therefore, it is of no surprise that the identification and development of unique small-molecule enzyme inhibitors continue to grow, through systematic medicinal chemistry and pharmacological efforts.[2]

However, in enzyme kinetics studies for drug candidates, there is the need to minimize the use of costly substrates, inhibitors and enzymes, and to minimize the analysis time. Hence, it would be advantageous to be able to determine the correct reaction type and appropriate kinetics constants for enzyme inhibitors with a method that requires the minimal number of experimental assays[3] and that allows direct fitting of the predicted model explicit equations to the raw data using standard software.

Traditionally, the quantitative kinetics of inhibited enzyme-catalyzed reactions have been studied in terms of the correlation between initial rate measurements and substrate concentrations according to the expression given in Eq. (1)[4]:

$$v = -\frac{d[S]}{dt} = \frac{V^* \cdot [S]}{K_m^* + [S]} \tag{1}$$

where the kinetics parameters $V^*$ and $K_m^*$ are apparent (inhibitor-dependent) constants for the limiting rate and the Michaelis constant, respectively (see Table 1). The use of nonlinear regression analysis to Eq. (1) has increased dramatically over the past 10 years[3], as methods that use

simultaneous nonlinear regression provide more accurate estimated values for the kinetics parameters. This analysis procedure is easy to perform and is indeed well established, although initial-rate measurements require a high number of individual experiments, due to the high sensitivity of the reaction velocities to noise. On the other hand, analyses of complete progress curves can provide the same information, although this can be achieved with only a fraction of the number of separate measurements, as any single experimental assay measures the kinetics data at every concentration between the initial value and that at the end of the reaction. Hence, instead of differentiation of the measured reactant concentrations, the kinetics law has to be integrated. This is usually achieved by integrating the rate equation, Eq. (1), numerically, although it was demonstrated recently that this approach has several drawbacks.[5] However, it is also possible to obtain an algebraic solution to Eq. (1) as variables of time t and substrate concentration [S] can be separated, and direct integration of the expression that results gives the integrated Michaelis-Menten equation:[6]

$$V^* \cdot t = [S]_0 - [S]_t + K_M^* \cdot \ln\left(\frac{[S]_0}{[S]}\right) \quad (2)$$

where $[S]_t$ and $[S]_0$ are the substrate concentrations at time t and zero, respectively. The inconvenience of this exact solution is that it is an implicit nonlinear equation; i.e. time-dependent variable $[S]_t$ is not given as a function of independent variable t, and Eq. (2) has to be solved numerically again. Thus, a better alternative might be that the total time-course of the reactants in the enzymatic reaction model is reduced to the explicit solution of Eq. (1), as follows:[7]

$$[P]_t = [S]_0 - [S]_t = [S]_0 - K_m^* \cdot W(x(t)) \quad (3)$$

where $[P]_t$ is product concentration, W is the Lambert W function and time-dependent argument of W (variable x(t)) is given by Eq. (4) as:

$$x(t) = \frac{[S]_0}{K_m^*} \cdot \exp\left(\frac{[S]_0 - V^* \cdot t}{K_m^*}\right) \quad (4)$$

The kinetic parameters in this equation are adequately inhibitor-concentration-dependent for diverse reaction models (see Table 1) that obey the rate equation, Eq. (1). However, nonlinear regression curve-fitting programs that can perform the calculation of W(x) in the exact form of Eq. (3) are not widely available. Therefore, I have introduced a simple, yet accurate, function that can satisfactorily approximate, and thus substitute, W(x) in Eq. (3) with a relative error of < 0.2%:[8]

**Table 1.** The modifications of Eqs. (1) and (3) according to the three standard types of reversible inhibition.

| **Rate equation** | **Solution to rate equation** | | |
|---|---|---|---|
| $v = -\frac{d[S]}{dt} = \frac{V^* \cdot [S]}{K_m^* + [S]}$ | $[P]_t = [S]_0 - K_m^* \cdot W\left\{\frac{[S]_0}{K_m^*} \cdot exp\left(\frac{[S]_0 - V^* \cdot t}{K_m^*}\right)\right\}$ | | |
| **Type of Inhibition** | **Reaction scheme** | $V^*$ | $K_m^*$ |
| Competitive inhibition model | $E + S \underset{}{\overset{K_S}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P$; $E + I \overset{K_i}{\rightleftharpoons} EI$ | $V$ | $K_S \cdot \left(1 + \frac{[I]}{K_i}\right)$ |
| Non-competitive inhibition model | $E + S \overset{K_S}{\rightleftharpoons} ES \xrightarrow{k_{cat}} E + P$; $E + I \overset{K_i}{\rightleftharpoons} EI$; $ES + I \overset{K_i}{\rightleftharpoons} ESI$; $EI + S \overset{K_S}{\rightleftharpoons} ESI$ | $\frac{V}{\left(1 + \frac{[I]}{K_i}\right)}$ | $K_S$ |
| Uncompetitive inhibition model | $E + S \overset{K_S}{\rightleftharpoons} ES \xrightarrow{k_{cat}} E + P$; $ES + I \overset{K_i}{\rightleftharpoons} ESI$ | $\frac{V}{\left(1 + \frac{[I]}{K_i}\right)}$ | $\frac{K_S}{\left(1 + \frac{[I]}{K_i}\right)}$ |

$$W(x) \approx 1.4587 \cdot \ln\left\{\frac{1.2 \cdot x}{\ln\left[2.4 \cdot \left(x/\ln\left(1+2.4 \cdot x\right)\right)\right]}\right\} - 0.4587 \cdot \ln\left\{\frac{2 \cdot x}{\ln\left(1+2 \cdot x\right)}\right\} \quad (5)$$

To verify the accuracy and efficiency of this approximation for estimation of the kinetics parameters for reversible enzyme-inhibition reaction models, I analyzed progress curves using the modified Eq. (3) for direct fitting to reactant concentrations, and compared the estimates obtained for V, $K_m$ and $K_i$ with those determined by applying the numerical integration approach and with the direct integrated rate equation, Eq. (3).

## 2. Methods and Data Analysis

### 2. 1. Data

The data analysis was carried out on time-courses (see Fig. 1) from a reaction catalyzed by the enzyme pepsin. These data are included in the example problems in the DynaFit academic free nonlinear regression software.[9] This case-problem illustrates one of the most common tasks in an enzymology laboratory: the determination of a competitive inhibition constant. However, to test and to justify the use of the described method, I fitted the three classical Michaelis-Menten enzyme-inhibition models shown in Table 1 to these progress curves.

### 2. 2. Nonlinear Regression Fitting Using Numerical Integration

The data analyses were first performed using the available DynaFit computer program,[9] which combines numerical integration with nonlinear regression. The classic Michaelis-Menten enzyme-inhibition mechanisms and the initial estimates of the kinetics constants for the fitting were entered into the program input script files according to the instructions in the DynaFit manual. Afterwards, the iterative fitting was run until the parameter values that generated the best-fit curve to the data were obtained.

### 2. 3. Nonlinear Regression Fitting Using Explicit Equations

The solutions for the product concentration as a function of time were computed using the direct model Eq. (3) in the Wolfram Mathematica 7 software package. Eq. (3) was fitted directly to the time-course data, and the sum of the squares of the differences between the product-concentrations data and the calculated model values was minimized with the Mathematica NonLinearModelFit routine.

The approximation of Eq. (5) to the Lambert W(x) function of modified Eq. (3) for the product accumulation was implemented into the GraphPad Prism 5 software package as a user-defined built-in explicit model equation (see Appendix) for calculating theoretical product concentrations. This standard curve-fitting computer program has an all-user interface that allows users to easily set-up global least-squares nonlinear regression curve fitting.

## 3. Results and Discussion

The analyses of the time-course data shown in Figure 1 were carried out using nonlinear regression, where the theoretical curves for the various reaction models were computed according to different calculation techniques. Table 2 summarizes the values of the fitted estimates of the kinetics parameters. The best parameter values shown in Table 2 yielded almost identical good fits to the experimental data for all of the computing methods. Discrimina-

**Table 2**. Parameters aquired by global (simultaneous) multiple progress-curve fitting. Comparison of fitted values obtained using the numerical integration approach (*DynaFit 3*), the exact model of Eq. (3) with the Lambert W(x) function (*Mathematica 7*), and the approximation of W(x) (Eq. (5)) of the modified Eq. (3) (*Prism 5*), with the absolute sum of squares (SSQ) of all of the fits. The substrate and inhibitor concentrations were set as constants (for values, see Fig. 1). Data are means ± SD.

| | | Numerical integration (DynaFit) | Lambert W function (Mathematica) | Lambert W approx. (Prism) |
|---|---|---|---|---|
| **Competitive inhibition model** | $K_m$ **(μM)** | 65.0 ± 2.6 | 67.5 ± 2.2 | 67.6 ± 2.4 |
| | $V$ **(μM/s)** | 0.570 ± 0.011 | 0.585 ± 0.009 | 0.586 ± 0.010 |
| | $K_i$ **(μM)** | 0.155 ± 0.003 | 0.164 ± 0.002 | 0.165 ± 0.003 |
| | **SSQ** | 48.2 | 29.5 | 31.9 |
| **Non-competitive inhibition model** | $K_m$ **(μM)** | 98.4 ± 6.4 | 99.3 ± 5.4 | 93.0 ± 5.3 |
| | $V$ **(μM/s)** | 0.706 ± 0.028 | 0.714 ± 0.023 | 0.688 ± 0.023 |
| | $K_i$ **(μM)** | 0.349 ± 0.004 | 0.361 ± 0.003 | 0.363 ± 0.003 |
| | **SSQ** | 133.6 | 92.9 | 92.4 |
| **Uncompetitive inhibition model** | $K_m$ **(μM)** | 89.6 ± 9.4 | 95.7 ± 9.4 | 96.3 ± 9.6 |
| | $V$ **(μM/s)** | 0.659 ± 0.039 | 0.689 ± 0.039 | 0.693 ± 0.041 |
| | $K_i$ **(μM)** | 0.174 ± 0.011 | 0.173 ± 0.011 | 0.170 ± 0.011 |
| | **SSQ** | 299.4 | 246.4 | 224.5 |

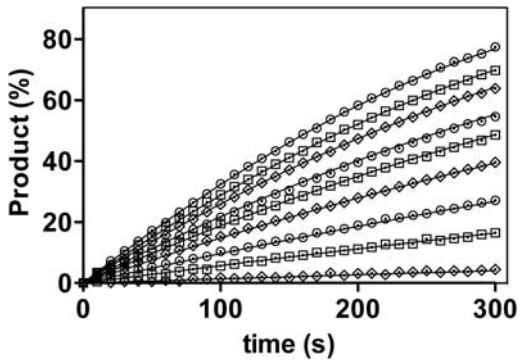


**Figure 1.** Time-courses of product concentrations. The symbols represent the data for the percentage substrate reduction, and the lines represent the theoretical concentrations obtained from the approximation of W(x) (see Eq. (5) in the text and Appendix) of modified Eq. (3) with the parameter values shown in Table 2. Although the parameters and the sum of the squares are given in Table 2 for three tested inhibition models, only the calculated lines for the competitive type of reaction are shown here. The progress curves refer to the following conditions: constant initial substrate concentration $[S]_0$ = 100 μM, with the inhibitor concentrations varying as [I] = 0, 0.05, 0.10, 0.20, 0.30, 0.50, 1.0, 2.0, 10.0 μM. Only 10% of the data points are shown, for clarity.

tion among the inhibition mechanisms (Table 2, SSQ) was achieveable in any case, and it can be seen that the competitive-inhibition reaction model best delineates the experimental progress curves.

These results suggest that applying the approximation of Eq. (5) provides an excellent approach for progress-curve analysis of classical enzyme-inhibited reaction systems that obey the rate equation, Eq. (1). Hence, this Eq. (5) can now be used as an equivalent alternative approach to fit such experimental data, without the need to rely on highly specialized numerical algorithms and powerful mathematical software packages (e.g. Mathematica, Matlab, Maple). This can thus be achieved simply by encoding it into any standard spreadsheet data-fitting computer program that is user friendly (e.g. Prism, SigmaPlot, KaleidaGraph). This approach is a particularly important improvement as most of the available curve-fitting programs are not set-up to handle equations that involve the W(x) function, as Eq. (3). At the same time, although the integrated Michaelis-Menten rate equation is usually known only in the implicit form, which is not suitable for direct fitting, the use of the Lambert W(x) function to provide the explicit solution to Eq. (1) has been reported increasingly in recent years.[7,10,11] This means that the formalism of Eq. (5) described here can be extended to deal with several Michaelis-Menten kinetics problems where the analysis is amenable to combinations of multiple substrate concentrations[12] or even dose bolus regimes.[13]

It should also be emphasized that real-world enzymes generally do not obey the irreversible substrate-conversion mechanism of $E + S \rightleftarrows ES \rightarrow E + P$, as proposed in the reaction models of this report, although experimental conditions can sometimes be manipulated so that this is a very good approximation. Instead, forward velocities of many enzyme-catalyzed reactions are affected by product inhibition if the enzyme and product form an unproductive EP complex, although more realistic reactions are further reversible. However, also the generalized integrated Michaelis-Menten equation that describes time-courses of such mechanisms, together with simple, partial or mixed-type reversible inhibitor effects on them,[4] can be transformed into closed-form W(x)-type solutions.[12] Consequently, the use of the approximation to the W(x) of Eq. (5) would also allow progress-curves analysis of all these reaction models to be performed by applying standard nonlinear regression software. This approach could become an easy, but universal, short-cut for determining kinetics parameters that would also facilitate the characterization of various drugs that perturb enyzme kinetics. Howe-

**Table Appendix 1.** Software user-defined built-in approximations of W(x) of modified Eq. (3) for product accumulation, in GraphPad Prism 5.

| **Reaction type** | |
|---|---|
| **Competitive** | Kapp=Km*(1+I/Ki)<br>Vapp=V<br>x=S0/Kapp*exp((S0-Vapp*t)/Kapp)<br>y=S0-Kapp*(1.4586887*ln(1.2*x/ln(2.4*x/ln(1+2.4*x)))–0.4586887*ln(2*x/ln(1+2*x))) |
| **Non-competitive** | Kapp=Km<br>Vapp=V/(1+I/Ki)<br>x=S0/Kapp*exp((S0-Vapp*t)/Kapp)<br>y=S0-Kapp*(1.4586887*ln(1.2*x/ln(2.4*x/ln(1+2.4*x)))–0.4586887*ln(2*x/ln(1+2*x))) |
| **Uncompetitive** | Kapp=Km/(1+I/Ki)<br>Vapp=V/(1+I/Ki)<br>x=S0/Kapp*exp((S0-Vapp*t)/Kapp)<br>y=S0-Kapp*(1.4586887*ln(1.2*x/ln(2.4*x/ln(1+2.4*x)))–0.4586887*ln(2*x/ln(1+2*x))) |

* – t, x and y represent time, variable x(t) given by Eq. (4) and explicit Eq. (3) for product concentration, respectively, where W(x) in Eq. (3) is substituted by approximation Eq. (5).

ver, there are many other advantages of using this method that are discussed more in details in literature,[12] although there are experimental conditions that need to be avoided; e.g. substrate inhibition deviates initial rate versus substrate concentration profile from standard hyperbolic pattern based on Eq. (1), and enzyme instability leading to non-substrate depletion based rate changes would invalidate the results.

## 4. Conclusions

In conclusion, the present note describes an accurate and efficient progress-curve analysis of inhibited enzyme reactions within the Michaelis-Menten framework for the determination of the type of inhibition and the extraction of the kinetic parameters. Therefore, the presentation of the implementation of Eq. (5) with an instructive enzyme-inhibition example, and the providing of the approach presented here as readily accessible to the readership of this journal can most appropriately be taken as the main aim and result of this report.

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## Povzetek

Najpreprostejši način za določitev tipa in konstante reverzibilne inhibicije ter kinetičnih parametrov encimske reakcije, ki sledi Michaelis-Mentenovi kinetiki, je neposredna analiza progresivnih krivulj. V prispevku je prikazana eksplicitna enačba, ki se lahko neposredno prilega na časovne podatke inhibiranih encimsko kataliziranih reakcij. Prednost enačbe je v tem, da je izražena z elementarnimi matematičnimi funkcijami. Zaradi tega je njeno prileganje na podatke mogoče v vseh računalniških programih z algoritmi nelinearne regresije. Hkrati se analiza poenostavi, ker ni potrebna predhodna transformacija podatkov. Opisan pristop analize kinetičnih podatkov daje rezultate, ki so v skladu s tistimi, ki so določeni s pristopi numerične ali algebrske integracije Michaelis-Mentenove hitrostne enačbe. Pri tem je slednja izražena z Lambertovo W funkcijo, katere uporaba je nemogoča s standardnim računalniškim programjem.