

# Chapter 2

## Fundamentals of Enzyme Kinetics

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### Abstract

This chapter provides a general introduction to the kinetics of enzyme-catalyzed reactions, with a focus on drug-metabolizing enzymes. A prerequisite to understanding enzyme kinetics is having a clear grasp of the meanings of “enzyme” and “catalysis.” Catalysts are reagents that can increase the rate of a chemical reaction without being consumed in the reaction. Enzymes are proteins that form a subset of catalysts. These concepts are further explored below.

**Key words** Enzyme, Catalysis, Reaction rate, Rate constant, Binding constant, Michaelis–Menten equation, Half-life, Lineweaver–Burk plot, Eadie–Hofstee plot, Nonlinear regression, Substrate

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### 1 General Introduction: Activation, Energy, Catalysts, and Enzymes

In any chemical reaction, the reactants and products are separated by an energy barrier, known as the free energy of activation. This barrier represents the energy difference between the reactants and the transition state, which is a high-energy intermediate that occurs during the formation of the product [1]. Catalysts function by lowering the activation energy of a reaction [2]. Catalysts, however, do not alter the reaction equilibria [3] and can only facilitate reactions that are already chemically feasible. Chemical catalysts are in general not very specific to the reactions that they catalyze.

Enzymes are specialized protein catalysts. In contrast to chemical catalysts, enzymes are often highly specific, and many enzymes catalyze a single reaction [4]. For example, carbonic anhydrase catalyzes the conversion of carbon dioxide and water to carbonate and protons. This highly specific enzyme increases the rate of the reaction by  $10^8$ -fold over the uncatalyzed reaction [5]. Drug-metabolizing enzymes are also protein catalysts, but in contrast to very substrate-specific enzymes like carbonic anhydrase, they generally metabolize a wide variety of substrates. This ability to metabolize more than one specific substrate, sometimes referred to as

“promiscuity,” is beneficial in breaking down the myriad of exogenous compounds (including drugs) to which the body is exposed daily. However, in exchange for this ability to metabolize a broader range of substrates, drug-metabolizing enzymes are much less catalytically efficient than single-substrate enzymes due to their generally larger and less specific active sites.

Drug-metabolizing enzymes generally fall into two categories: oxidative, reductive, and hydrolytic (often referred to as phase I reactions) or conjugative enzymes (referred to as phase II reactions). As the name implies, oxidative enzymes, like the cytochromes P450 (*see* Chapter 8), through their catalytic activity cause oxidation of the substrate, generally to a more water-soluble form that is more easily excreted. In the case of conjugative enzymes, such as the glucuronosyl transferases (*see* Chapter 11), these enzymes catalyze the conjugation of a more water-soluble molecule (e.g., glucuronic acid) with the substrate, again producing a more water-soluble product.

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## 2 Introduction to Kinetics

### 2.1 Reaction Rates, Rate Constants, and Binding Constants

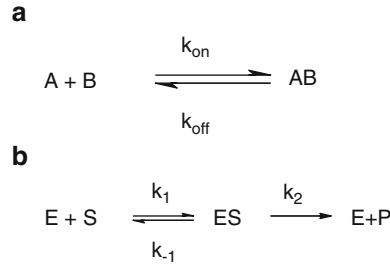
A rate reflects the speed or the velocity of a reaction. Rates have units of concentration of product formed or reactant converted per unit time. The rate constant defines the relationship between the substrate concentration and the rate. A rate constant is typically expressed as a lower case “ $k$ .” Rate constants for association and dissociation of two molecules, such as an enzyme and a substrate, can be defined as  $k_{\text{on}}$  and  $k_{\text{off}}$ . For many reactions, “on” rates are diffusion limited as the ability of two molecules to find one another is limited by their diffusion through the solvent [5]. The dissociation constant,  $K_d$ , can be derived from the “on” and “off” rate constants as shown in Eq. 1. Lower values of  $K_d$  reflect a tighter binding affinity:

$$K_d = \frac{[A][B]}{[AB]} = \frac{k_{\text{off}}}{k_{\text{on}}} \quad (1)$$

In Fig. 1a, it is assumed that a single molecule of A binds to a single molecule of B. In cases where one or both of the interacting partners have multiple binding sites, cooperative or allosteric binding is possible. Cooperative interactions as they relate to enzyme kinetics will be discussed in Chapter 3.

### 2.2 Reaction Order

The reaction order defines the relationship between the rate of product formation and the rate constant,  $k$ . In a zero-order reaction, the rate of product formation (and the rate of substrate depletion) does not depend on the concentration of substrate. Thus, the reaction rate is constant regardless of increases in



**Fig. 1** Panel **a** depicts the scheme for a bimolecular interaction. Panel **b** depicts the scheme for a single-enzyme, single-substrate reaction

**Table 1**  
**Reaction orders**

Reaction order	Zero order	First order	Second order
Differential equation	$\frac{d[S]}{dt} = -k$	$\frac{d[S]}{dt} = -k \cdot [S]$	$\frac{d[S]}{dt} = -k \cdot [S]^2$
Integrated rate expression	$[S] = [S]_0 - kt$	$[S] = [S]_0 \cdot e^{-kt}$	$\frac{1}{[S]} = \frac{1}{[S]_0} + kt$
Half-life	$t_{1/2} = \frac{[S]_0}{2k}$	$t_{1/2} = \frac{\ln(2)}{k}$	$t_{1/2} = \frac{1}{k \cdot [S]_0}$
Units for $k$	$M \cdot \text{min}^{-1}$	$\text{min}^{-1}$	$M^{-1} \cdot \text{min}^{-1}$

substrate concentration. For a first-order reaction, there is a linear relationship between the rate of product formation and the substrate concentration. An increase in substrate concentration will result in a corresponding, proportional increase in the reaction rate. Understanding the reaction order is also important in assigning the units to the rate constant. Since the units of rate (molar/time) and substrate concentration (molar) are known, the units of the rate constant can be readily determined from the rate expression. The differential equations and half-life calculations for various reaction orders are shown in Table 1 [2].

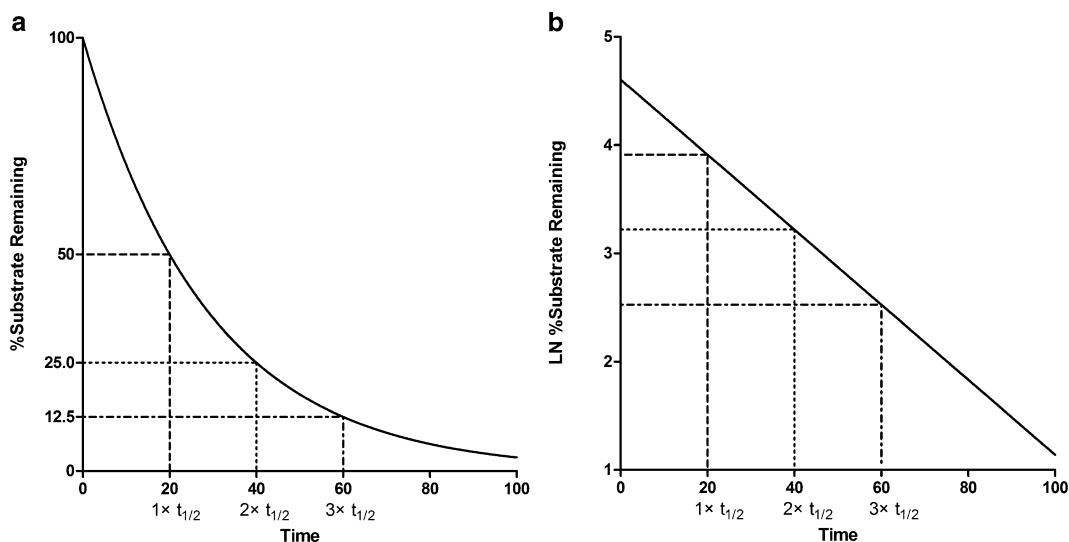
### 2.3 Half-Life

Half-life ( $t_{1/2}$ ) is the amount of time required for the substrate concentration to decrease to 50 % of its original concentration (Fig. 2). For a first-order reaction, the half-life is readily derived from the rate equation (Eq. 2)

$$[S] = [S]_0 \cdot e^{-kt} \quad (2)$$

At the half-life, the concentration of substrate ( $[S]$ ) is equal to exactly half of its concentration at time zero ( $[S]_0$ ):

$$0.5 \cdot [S]_0 = [S]_0 \cdot e^{-kt_{1/2}} \quad (3)$$



**Fig. 2** Determination of half-life for a first-order reaction. Panels **a** and **b** depict the  $y$ -axes on linear and logarithmic scales, respectively

Solving Eq. 3 for  $t_{1/2}$  yields Eq. 4, in which  $k$  is the first-order rate constant for substrate depletion:

$$t_{1/2} = \frac{\ln(2)}{k} \quad (4)$$

During the drug development process, it is frequently useful to estimate the in vitro half-life of the new chemical entity for the purposes of predicting (through in vitro–in vivo correlations; *see* Chapter 13) whether it might possess satisfactory in vivo pharmacokinetic properties. In vitro half-life values can be determined for new chemical entities using a variety of drug-metabolizing enzyme sources, such as recombinant human enzymes, human liver fractions (microsomes, cytosol, S9), or human hepatocytes (*see* Case Studies 1 and 3). The  $t_{1/2}$  value can be determined by calculating the fraction of substrate remaining at successive time points and fitting the data to Eq. 2. An example plot is shown for a first-order reaction in Fig. 2. On a linear scale, the curve follows an exponential decay. When the natural logarithm of the  $y$ -values is plotted, the curve behaves linearly. The data derived from these experiments can be scaled up to predict in vivo hepatic clearance (*see* Chapter 13) [6, 7].

## 2.4 Michaelis–Menten Kinetics

Michaelis–Menten kinetics describe enzyme processes at steady state. The term steady state refers to a process in which formation and consumption rates of a reactive intermediate are identical. Thus, its concentration never builds up to an appreciable level [8]. Typically, the formation of product is measured in these experiments, and enzyme kinetic parameters are determined. A single-enzyme, single-substrate reaction is shown in Fig. 1b, in which E, S,

and P represent the enzyme, substrate, and product, respectively. The product may also be referred to as the metabolite, particularly in the context of drug-metabolizing enzymes and their reaction products.

It is assumed that the enzyme, substrate, and enzyme–substrate complex are in rapid equilibrium. Thus, the dissociation rate of the enzyme–substrate (ES) complex back to enzyme and substrate is much faster than the formation of product [8].

At steady state, there is no change in the concentration of the ES complex, which can be considered to be a reactive intermediate, and any change in the substrate concentration is negligible. This assumption is the reason why one attempts to assure minimal turnover of the substrate (<10 %) in an experiment to determine enzyme kinetic parameters. This assumption also implies that the substrate concentration must be much higher than the enzyme concentration. Consequently, changes in the substrate concentration, as a result of the formation of the ES complex, are considered negligible [8]. To satisfy this requirement, the enzyme concentration should be kept as low as possible. The equations describing the steady-state conditions are shown below:

$$\frac{d[ES]}{dt} = 0 \quad (5)$$

$$\frac{d[S]}{dt} \approx 0 \quad (6)$$

## 2.5 Derivation of the Michaelis–Menten Equation

The Michaelis–Menten equation relates substrate concentration to reaction rate via two kinetic parameters,  $K_m$  and  $V_{max}$  (see Eq. 16 and Fig. 3). The differential equation describing time-dependent changes in the ES complex can be written based on Fig. 1b and takes into account both the formation and consumption of this complex (Eq. 7):

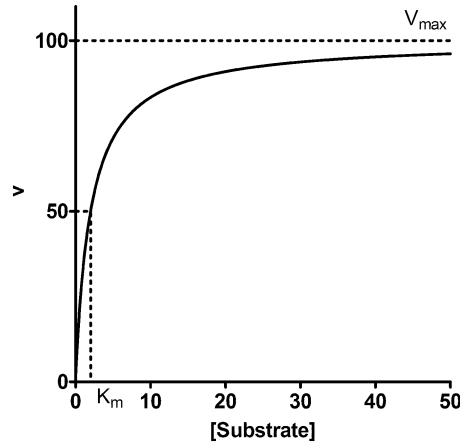
$$\frac{d[ES]}{dt} = k_1[E]_f[S] - k_{-1}[ES] - k_2[ES] \quad (7)$$

At steady state, the rate of [ES] consumption is equal to the rate of [ES] formation. Thus, the net change in [ES] over time is zero (Eq. 5 and Eq. 7) and can be rewritten as Eq. 8:

$$k_1[E]_f[S] = (k_{-1} + k_2)[ES] \quad (8)$$

The conservation equation for the enzyme is given by Eq. 9 and defines the total enzyme concentration as the sum of the free (unbound) enzyme concentration  $[E]_f$  and the concentration of ES complex:

$$[E] = [E]_f + [ES] \quad (9)$$



**Fig. 3** Example of Michaelis–Menten kinetics. In this figure, the  $K_m$  and  $V_{\max}$  values are 2 and 100, respectively

Substituting Eq. 9 into Eq. 8 and dividing through by  $k_1$  yields Eq. 10:

$$([E] - [ES])[S] = \left(\frac{k_{-1} + k_2}{k_1}\right)[ES] \quad (10)$$

A new constant,  $K_m$ , can be defined as in Eq. 11 and substituted into Eq. 10 to yield Eq. 12:

$$K_m = \left(\frac{k_{-1} + k_2}{k_1}\right) \quad (11)$$

$$([E] - [ES])[S] = K_m[ES] \quad (12)$$

The rate of product formation,  $v$ , can be defined as in Eq. 13:

$$v = k_2[ES] \quad (13)$$

The expression for  $[ES]$  from Eq. 13 can be substituted into Eq. 12 to yield Eq. 14:

$$[E][S] - \frac{v}{k_2}[S] = K_m \frac{v}{k_2} \quad (14)$$

The maximum velocity for an enzyme-catalyzed reaction occurs when all of the enzyme is complexed with substrate. Thus,  $V_{\max}$  is theoretically achieved when  $[ES] = [E]$ . Equation 13 is written for this condition in Eq. 15:

$$V_{\max} = k_2[E] \quad (15)$$

Multiplying both sides of Eq. 14 by  $k_2$  and substituting the expression for  $k_2$  from Eq. 15 yields the Michaelis–Menten equation (Eq. 16)

$$v = \frac{V_{\max}[S]}{K_m + [S]} \quad (16)$$

## 2.6 Interpretation of $K_m$ and $V_{max}$ Parameters

The Michaelis–Menten constant,  $K_m$ , represents the substrate concentration at which the reaction velocity is half-maximal ( $V_{max}/2$ ). Since  $K_m$  represents a concentration, it has units of concentration. As defined in Eq. 11,  $K_m$  is a collection of rate constants. It is not the binding constant for the interaction between enzyme and substrate. This binding constant,  $K_s$ , is defined in Eq. 17. The  $K_m$  value will always be greater than  $K_s$  by the factor  $(k_2/k_1)$ . When  $k_2$  is sufficiently small relative to  $k_{-1}$ , the  $K_m$  and  $K_s$  values will be approximately equal. In some instances,  $K_m$  is erroneously described as the affinity of the enzyme for the substrate:

$$K_s = \frac{k_{-1}}{k_1} \quad (17)$$

$V_{max}$  is a rate, and therefore, it has units of concentration per unit time. In the field of drug metabolism, the  $V_{max}$  value is often divided by the concentration of enzyme used in the experiment as a form of normalization. Thus, it is common to see  $V_{max}$  with units of pmol/min/mg (of tissue or enzyme protein). This normalized form of  $V_{max}$  is not to be confused with  $k_{cat}$  (or  $k_2$ ; see Fig. 1b), which represents the turnover number of the reaction and has units of inverse time.

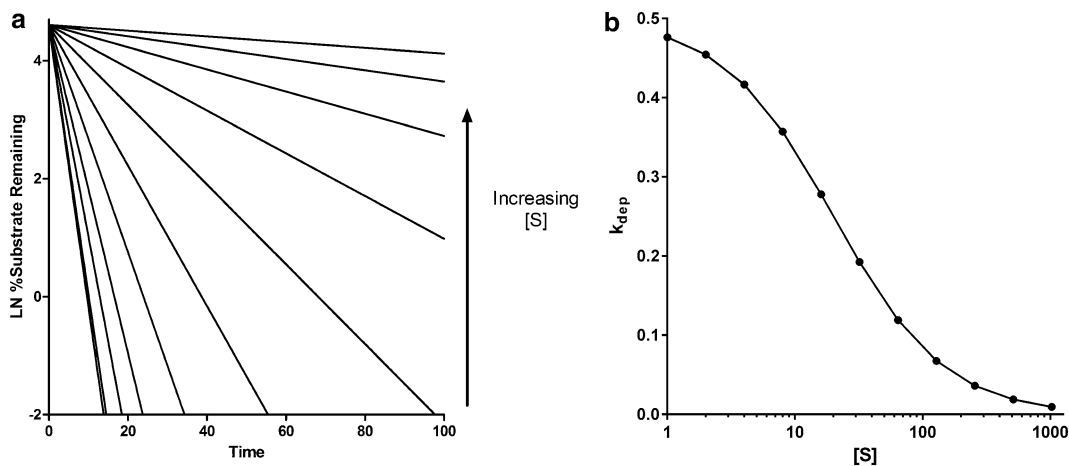
In Fig. 3, reaction velocity ( $v$ ) is plotted versus substrate concentration. At low concentrations, the increase in velocity is roughly linearly proportional to the change in substrate concentration. Thus, in this region of the curve, first-order kinetics are approximated. At substrate concentrations much greater than the  $K_m$  value, there is very little further increase in velocity, and in this region of the curve, the kinetics can be considered zero order [9].

The  $K_m$  and  $V_{max}$  values can be used to determine intrinsic clearance ( $CL_{int}$ ), which is the capacity of an enzyme or an organ to clear the substrate in the absence of blood flow or protein binding limitations [10]. Intrinsic clearance is expressed as the ratio of the product formation rate to the substrate concentration (Eq. 18):

$$CL_{int} = \frac{v}{[S]} = \frac{V_{max}}{K_m + [S]} \quad (18)$$

When the substrate concentration is much lower than the  $K_m$  value, the  $[S]$  in the denominator is negligible, and Eq. 18 simplifies to Eq. 19. Intrinsic clearance can be scaled up to total clearance:

$$CL_{int} \approx \frac{V_{max}}{K_m} \quad (19)$$



**Fig. 4** Determination of  $K_m$  values by the substrate depletion method. Panel **a** depicts the individual depletion curves at varying substrate concentrations. Panel **b** depicts a plot of the  $k_{dep}$  values versus substrate concentration

### 2.7 Alternative Approach to Determining $K_m$ and $V_{max}$ Values

The kinetic parameters,  $K_m$  and  $V_{max}$ , can also be determined by monitoring depletion of substrate [11, 12]. This method is useful when authentic metabolite standards are not available and for the determination of an “overall” apparent  $K_m$  value. To determine these parameters, the substrate concentration is varied and the first-order rate constant for depletion is calculated at each concentration. These rate constants ( $k_{dep}$ ) are then plotted versus substrate concentration (Fig. 4b). The data can be fit to Eq. 20 to determine the  $K_m$  value and the  $k_{dep}([S] \rightarrow 0)$  value, which represents the rate constant for depletion at an infinitely low substrate concentration.  $V_{max}$  can be calculated from these two constants, as shown in Eq. 21:

$$k_{dep} = k_{dep}([S] \rightarrow 0) \left( 1 - \frac{[S]}{[S] + K_m} \right) \quad (20)$$

$$V_{max} = K_m \cdot k_{dep}([S] \rightarrow 0) \quad (21)$$

As illustrated in Fig. 4a, the half-life values for substrate depletion are larger at higher substrate concentrations.

### 2.8 Practical Considerations in the Determination of Kinetic Parameters

The  $K_m$  and  $V_{max}$  values for a given enzyme–substrate system can be estimated in an experiment using an in vitro system. As the  $V_{max}$  value is a rate, and represents turnover per unit time, linearity with respect to incubation time should be determined as a prerequisite to determining  $K_m$  and  $V_{max}$  values (*see* Case Studies 1 and 3). Since  $V_{max}$  will most likely be normalized to protein concentration, linearity with respect to enzyme concentration should also be established.



In order to satisfy the steady-state assumption, the concentration of substrate should not change significantly over the course of the incubation. Up to 10 % of substrate consumption during the experiment is generally considered acceptable and will have minimal impact on the estimated  $K_m$  and  $V_{max}$  values. Greater fractions of substrate consumption will result in larger errors in the estimated kinetic parameters. In cases where more substantial substrate consumption is unavoidable, an integrated form of the Michaelis–Menten equation may be used to estimate  $K_m$  and  $V_{max}$  values [9].

Additionally, as described above, the concentration of enzyme should be much lower than that of the substrate in order to satisfy the steady-state approximation. When performing drug metabolism studies, it is also advantageous to keep the protein concentration as low as possible to avoid nonspecific binding of the substrate as this can affect the free concentration of substrate accessible to the enzyme and lead to falsely elevated  $K_m$  estimates (*see* Chapter 7).

## 2.9 Data Analysis

Before the advent of modern computing technology, linear transformations of the Michaelis–Menten equation were used to estimate the parameters,  $K_m$  and  $V_{max}$ . The Lineweaver–Burk plot is a double-reciprocal plot, in which  $1/v$  is plotted versus  $1/[S]$  to yield a linear transformation (Eq. 22) [13]. In this transformed equation, the slope and y-intercept describe  $(K_m/V_{max})$  and  $V_{max}$ , respectively (Fig. 5). As the Lineweaver–Burk transformation relies on the use of a reciprocal plot, it is very sensitive to change at low concentrations, where errors in rate determinations are likely to be higher due to assay sensitivity limits:

$$\frac{1}{v} = \frac{K_m}{V_{max}} \frac{1}{[S]} + \frac{1}{V_{max}} \quad (22)$$

For the Eadie–Hofstee transformation, the Michaelis–Menten equation is rearranged to yield Eq. 23 [14]. In the Eadie–Hofstee plot, as shown in Fig. 6, the velocity ( $v$ ) is plotted versus the ratio of the velocity to the substrate concentration ( $v/[S]$ ). In this transformation, the slope and y-intercept describe  $-K_m$  and  $V_{max}$ , respectively. A disadvantage to using the Eadie–Hofstee transformation to determine enzyme kinetic parameters is that velocity is included in both the dependent and independent variables [15]. Thus, the errors in the  $x$ - and  $y$ -variables are not independent, as is required for linear regression analyses:

$$v = -K_m \frac{v}{[S]} + V_{max} \quad (23)$$

Linear transformations are useful tools for detecting trends in the data and for evaluating different kinetic models. Atypical kinetics can be diagnosed with linear transformation plots as they demonstrate very systematic deviations from the standard profile,

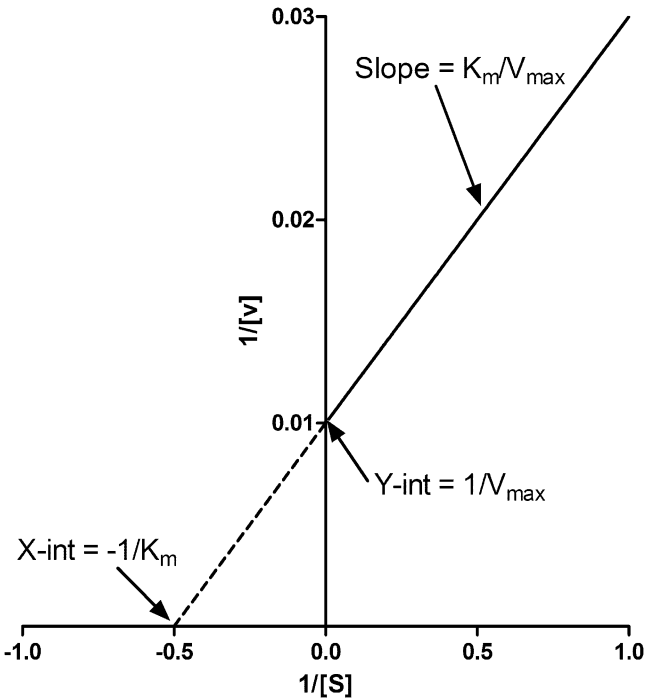


Fig. 5 Lineweaver–Burk transformation

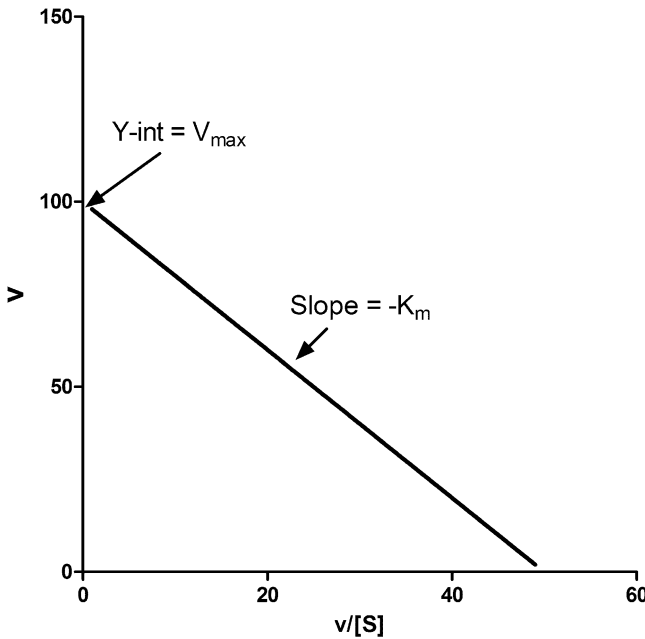


Fig. 6 Eadie–Hofstee transformation

especially in the Eadie–Hofstee plots [16]. Atypical kinetics will be further described in Chapter 3. As described above, each of these transformations suffers from shortcomings, and thus, it is not recommended to use these transformations to determine the Michaelis–Menten constants.

Nonlinear regression analysis is the preferred approach to estimating  $K_m$  and  $V_{max}$  values. A number of commercially available software packages can be used for nonlinear regression analysis of enzyme kinetics data such as GraphPad Prism<sup>®</sup>, GraFit<sup>®</sup>, SigmaPlot<sup>®</sup> (with Enzyme Kinetics module), WinNonlin<sup>®</sup>, and XLFit<sup>®</sup>. Ideally, the selected software should utilize the Marquardt–Levenberg algorithm for least-square minimization. This algorithm combines the best features of the gradient and grid search methods and provides robust parameter estimates [17]. For more details on the various nonlinear regression algorithms, the reader is referred to [17].

When performing nonlinear regression analysis, it is important to assess the goodness of fit. A visual inspection of the data points with the regression line should be performed, and any systematic deviations or trends should be noted as they may suggest that the best model has not been utilized. The residuals, which are the differences between the data points and the fitted values, should be randomly distributed, and no trends should be apparent. In nonlinear regression analysis,  $R^2$  is the coefficient of determination, which provides a comparison of the regression line to an arbitrary horizontal line drawn at the mean of the  $y$ -values [18]. For a perfect fit to the data, the  $R^2$  value is unity. Significantly lower  $R^2$  values imply a poorer fit to the data.

In some cases, it is helpful to compare two models in order to determine which one provides a better fit. If the models contain the same number of fitted parameters, the  $R^2$  value can be used to select the better fit. In cases where there are different numbers of parameters in the two models, statistical tests are needed. For a comparison of two nested models, an  $F$ -test may be used. In an  $F$ -test, the null hypothesis, which states that the simpler model fits the data better, is tested. If the null hypothesis is rejected, the more complex model is considered to provide a better fit to the data. The  $F$ -ratio is calculated as described in Eq. 24 [18], where  $SS$  and  $DF$  represent residual sum of squares and degrees of freedom, respectively:

$$F = \frac{(SS_{null} - SS_{alt})/SS_{alt}}{(DF_{null} - DF_{alt})/DF_{alt}} \quad (24)$$

The  $p$ -value, which enables a decision to be made as to whether to accept or reject the null hypothesis at a given confidence level, can be determined by using an  $F$ -table.

The Akaike information criterion (AIC) [19] can be used to compare nested or non-nested models [18]. The AIC takes into account the number of data points ( $N$ ), residual sum of squares (SS), and number of fitted parameters. It has been shown that for small samples, it is preferable to use a second-order corrected AIC ( $AIC_c$ ). In Eq. 25,  $K$  represents the number of fitted parameters plus one:

$$AIC_c = N \cdot \ln\left(\frac{SS}{N}\right) + 2K + \frac{2K(K+1)}{N-K-1} \quad (25)$$

The model that produces the lower  $AIC_c$  value is considered to provide a better fit to the data.

### 2.10 Example Experimental Protocol

An example experimental protocol for the determination of  $K_m$  and  $V_{max}$  values for a CYP-catalyzed reaction using human liver microsomes is provided below. Additional considerations for these reactions are provided in Chapters 8–11. Prior to conducting the experiment to determine  $K_m$  and  $V_{max}$  values, it is recommended that time and protein linearity are assessed. The protocol below can be modified based on the needs of a specific project and knowledge about the compound's interactions with the enzyme.

[Microsomes (HLM)]	0.025, 0.050, 0.100 mg/mL (stock suspensions prepared at $1.02 \times$ final concentrations).
[S]	1, 10, 100, 1,000 $\mu$ M (stock solutions prepared at $100 \times$ final concentrations), such that concentration of organic solvents is minimal (refer to Chapter 16 and Case Study 1 for details).
[NADPH]	2 mM (stock solution is prepared at $100 \times$ final concentration, i.e., 200 mM in the reaction buffer).
Buffer	50 mM potassium phosphate, pH 7.4.
Time points	0, 3, 6, 10, 15, 20, 40, 60 min.
Quench solution	40 % (v/v) ACN, 0.05 % (v/v) acetic acid, with internal standard

- Thaw HLM immediately before dilution, and keep diluted HLM suspensions on ice until use.
- Aliquot 490  $\mu$ L HLM suspension to appropriate wells of a 96-well plate (incubation plate).
- Add 5  $\mu$ L substrate to appropriate wells of the incubation plate.
- Pre-warm the incubation plate in a 37 °C water bath for 5 min.
- Initiate the reactions with the addition of 5  $\mu$ L NADPH, and thoroughly but gently mix the samples.

- At indicated time points, remove 50  $\mu\text{L}$  aliquots from the incubation plate and transfer to a recipient plate containing a glass fiber filter with 100  $\mu\text{L}$  of quench solution.
- Centrifuge the recipient plate.
- Prepare a standard curve by diluting metabolite standard into HLM suspension.
- Submit samples for LC/MS/MS analysis of metabolite.

Following analysis of the time and protein linearity samples, a single protein concentration and one to three time points may be selected for use in the determination of  $K_m$  and  $V_{\max}$  values. The data generated in the preliminary experiment may also provide a rough estimate of the  $K_m$  value. Substrate concentrations ranging from 0.1- to 20-fold of this value are then used in the determination of  $K_m$  and  $V_{\max}$  values.

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### 3 Questions

1. In a preliminary experiment, you determine the rate of formation of a metabolite at different incubation time points (table below). Based on these data, which time point would you recommend for conducting the definitive  $K_m$  and  $V_{\max}$  determination and why?

Time (min)	Normalized rate (pmol/min/mg)
2	0.521
4	0.526
8	0.439
20	0.304

2. Referring back to the table above, which additional pieces of information would be required before finalizing the experimental design for the  $K_m$  and  $V_{\max}$  determination?

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<http://www.springer.com/978-1-62703-757-0>

Enzyme Kinetics in Drug Metabolism

Fundamentals and Applications

Nagar, S.; Argikar, U.; Tweedie, D. (Eds.)

2014, XVIII, 538 p. 134 illus., 36 illus. in color.,

Hardcover

ISBN: 978-1-62703-757-0

A product of Humana Press