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A Useful Microsoft Excel Add-in Program for Modeling Steady-state Enzyme Kinetics

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Abstract

In vitro metabolism and inhibition studies, which serve as the basis of predicting pharmacokinetic events *in vivo*, are an essential part of pharmaceutical development and research. With the increasing occurrences of a typical kinetic profiles, modeling of enzyme kinetics is no longer a one-step operation of fitting classical Michaelis-Menten equation to the data. It involves considerable computational works regarding model selection and discrimination. This study presented XL Kinetics, a free Microsoft Excel add-in program written in Visual Basic for Application (VBA), for enzyme kinetic analysis. The program provides 11 most frequently used enzyme (stead-state) kinetic models including the models describing atypical kinetics (i.e., substrate inhibition, sigmoidal and biphasic models), a bisubstrate compulsory ordered model, and four reversible inhibition models. To evaluate the program, modeling results from XL_Kinetics and the commercial software packages (i.e., GraphPad Prism and Sigma Plot) were systematically compared. The results show that the kinetic parameters and their respective standard errors derived using XL_Kinetics are essentially the same as those obtained with the commercial software's. In conclusion, XL_Kinetics automates enzyme kinetic analysis in MS Excel, and may provide drug researchers and students with a fast, reliable and easy-to-use tool for routine analysis of enzyme kinetic data.

Introduction

Kinetic modeling is an indispensible part of *in vitro* drug metabolism and/or inhibition studies. The determined parameters provide substantial insights into the underlying mechanisms of an enzyme, and may allow prediction of *in vivo* drug disposition [1,2]. Modeling of a steady-state kinetic profile essentially refers to the use of curve fitting (or nonlinear regression) to describe the experimental data. The "curve" is defined by a mathematical equation in the form of y = f(x), where x is the independent variables, such as the substrate and/or inhibitor concentrations. Y is the initial reaction rate measured by the formation of the metabolite(s) or the disappearance of the substrate. And f is the function which includes the parameters (e.g., K_m, K_i and V_{max}) used to depict the data.

Fitting data with non-linear functions can be handled by a variety of outstanding programs such as GraphPad Prism, Sigma Plot, Origin, and Graf it. Their applications to enzyme kinetic modeling were widely performed [3-5]. However, as mentioned by Brown [6] access to these software's requires extra expenses in the range of $500. For those who just want to carry out a non-linear regression, they have to pay for a vast excess of redundant features. While for novice or occasional users, learning/re-learning tends to be difficult and time-consuming.

Microsoft (MS) Excel' spreadsheet has become the standard platform for data collection, graphing and analysis, and majority of original data are kept as MS Excel files in pharmaceutical field. The advantages of Excel include (1) the user-friendly interface and ease of use; (2) it offers many built-in mathematical and graphical routines which can be called in user-defined functions; and (3) it provides tremendous customization through macros and Visual Basic add-ins, for those users with specific needs and programming experience. By far, numerous Excel-based spreadsheet templates and add-ins have been programmed for data analyses of *in vitro-in vivo* correlations [7-

11] pharmacokinetics and/or pharmacodynamics [12-17]. Hernández and Ruiz formatted a simple Excel spreadsheet template for modeling kinetics data using Michaelis-menten equation [18]. However, this template appears to be limited for real-word use in drug metabolism studies as it only considers one model equation.

The objective of this study is to provide a tool for the statistical analysis of enzyme kinetics that is accurate and free, and, at the same time, easy to use and manage. To this end, we developed a Visual Basic for Application (VBA) program, XL_Kinetics, for kinetic modeling of drug metabolism. The encoded 11 models include (1) Michaelis-Menten equation; (2) the atypical kinetics (i.e., substrate inhibition, sigmoidal and biphasic models); (3) a bisubstrate compulsory ordered model; and (4) four reversible inhibition models (i.e., competitive, noncompetitive, uncompetitive and mixed-type inhibition). It is noteworthy that the models (relating reaction rate V to the concentration of the substrate [S]) here are used to describe steady-state enzyme kinetic data. Thus, they appear in a regression form but not in a differential form. The program featured in automated generation of the diagnostic diagrams for model selection, and approximation of the standard errors for the modeled parameters. Validation of XL_Kinetics was performed by comparing its modeling results to those from the commercial software packages (i.e., GraphPad Prism 5.0 and Sigma Plot 11.0).

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Materials and Methods

Automatic nonlinear regression

Enzyme kinetic modeling is implemented using Excel SOLVER, which is based on the robust and reliable generalized reduced gradient method (GRG) [19]. This optimization algorithm requires the preset initial parameter values to start the iterative process. The iterative calculation stops and provides the solutions, when the target (i.e., sum of residuals or weighted sum of residuals) converges at a predefined value. A detailed illustration of this nonlinear regression method in Excel was reported previously [6]. For the ease of use, kinetic modeling is performed via an interactive Excel UserForm, where data input and modeling settings are specified. Each integrated module is executed to tabulate data, call and run SOLVER, and generate graphs, as described earlier [17]. Initial parameters can be specified by the users; otherwise the program uses their estimates according to the schemes depicted in Table 1.

Sigmoidal kinetics modeling

Sigmoidal kinetics, also known as auto activation or homotropic cooperativity, has been seen more frequently in phase I and phase II drug metabolism [20]. The underlying mechanisms for this kinetic phenomenon were discussed in literature [21-23]. The Hill equation (Equation (1)) or two sites model (Equation (2)) including different parameters with distinct implication each can be applied to describe the relationship between the initial reaction rate (V) and the concentration of a substrate ([S]). The fitting equations are shown as follows,

$$V = \frac{V_{max} \times [S]^n}{K' + [S]^n} \tag{1}$$

where V_{max} is the maximum enzyme velocity; K' (also depicted as S_{50}^n) is related to the K_m, but is not an equal of the substrate concentration needed to achieve a half-maximum enzyme velocity (unless n=1); n is the Hill coefficient, indicative of the degree of curve sigmoidicity and/or cooperativity.

$$V = \frac{\dfrac{V_{max1} \bullet [S]}{K_{m1}} + \dfrac{V_{max2} \bullet [S]^2}{K_{m1} \bullet K_{m2}}}{1 + \dfrac{[S]}{K_{m1}} + \dfrac{[S]^2}{K_{m1} \bullet K_{m2}}} \tag{2}$$

where K_{m1} and K_{m2} are the Michaelis-Menten constants for the binding of the first and second substrate molecules, V_{max1} and V_{max2} are their respective maximal velocities.

Biphasic kinetics modeling

Biphasic kinetics is characterized by a typical biphasic curve: a hyperbolic-like pattern at initial stage, followed by a near linear portion at later stage. The hypothesis for occurrence of this atypical kinetics was discussed elsewhere [20,24]. The kinetic equations widely applied to this type of data include the two sites model (equation (3)) and the linear portion model (equation (4)). Although mathematically appear to be different, both models are essentially developed based on an identical putative assumption. That is the enzyme possesses two distinct binding sites, which are responsible for the "early phase" and "later phase" metabolism, respectively. These two models are shown as follows:

$$V = \frac{V_{max1} \bullet [S]}{[S] + K_{m1}} + \frac{V_{max2} \bullet [S]}{[S] + K_{m2}} \tag{3}$$

where K_{m1} and K_{m2} are the Michaelis-Menten constants for the binding of the first and second substrate molecules, V_{max1} and V_{max2} are their respective maximal velocities.

$$V = \frac{V_{max1} \bullet [S] + CL_{int} \bullet [S]^2}{[S] + K_{m1}} \tag{4}$$

where, K_{m1} and V_{max1} are defined the same as above, and represent the pseudo-hyperbolic part of the profile; CL_{int} represents the slope of the near linear portion.

Bisubstrate compulsory ordered model

Phase II drug metabolism such as glucuronidation (mediated by UDP-glucuronosyltransferases (UGTs)) occurs by transferring a hydrophilic entity (e.g. glucuronic acid) from the cofactor to the substrate. As two substrates and two products are involved, this enzymatic conjugation is also described as a bi bi reaction [25]. The catalytic mechanisms for bi bi reactions such as ping pong (or Theorell-Chance), random ordered and compulsory ordered had been extensively proposed [25,26]. However, UGT isoforms catalyzed conjugation tends to follow a compulsory ordered mechanism [2].

Kinetics Models	Initial parameter values			
Michaelis-Menten	V_{max} = intercept of linear plot*	K_m = slope of linear plot*		
Substrate inhibition	V_{max} = max (v)	K_m = 0.2 × max ([S])	K_s = 3 × K_m	
Sigmoidal Hill equation	V_{max} = max (v)	K_m = 0.2 × max ([S])	n = 1	
Sigmoidal two sites model	V_{max1} = 0.1 × V_{max2}	V_{max2} = max (v)	K_{m1} = 0.2 ×max ([S])	K_{m2} = 10 × K_{m1}
Biphasic two sites model	V_{max1} = 0.1 × V_{max2}	V_{max2} = max (v)	K_{m1} = 0.2 ×max ([S])	K_{m2} = 10 × K_{m1}
Biphasic linear portion model	V_{max1} = max (v)	K_{m1} = 0.2 × max ([S])	CL_{int} = V_{max1}/K_{m1}	
Bisubstrate compulsory ordered	V_{max} = max (v)	K_{mB} = 0.2 max ([B])	K_{AX} = 0.2 ×max ([AX])	K_{mAX} = K_{AX}
Competitive inhibition	V_{max} = max (v)	K_m = 0.2 × max ([S])	K_i = 3 × K_m	
Noncompetitive inhibition	V_{max} = max (v)	K_m = 0.2 × max ([S])	K_i = 3 × K_m	
Uncompetitive inhibition	V_{max} = max (v)	K_m = 0.2 × max ([S])	K_i = 3 × K_m	
Mixed-type inhibition	V_{max} = max (v)	K_m = 0.2 × max ([S])	K_i = 3 × K_m	Alpha (α) = 1

* Eadie-Hofstee plot; v, observed initial rates; [S], substrate concentrations; [B], aglycone substrate concentrations; [AX], cofactor concentrations.

Table 1: Schemes for auto-estimating initial parameter values in XL_Kinetics.

The relationship of the independent variables (i.e., the concentrations of the aglycone substrate [B] and the cofactor [AX]) to the dependent variable (i.e., initial reaction rate (v)) can be depicted by equation 5 using a steady state assumption [25].

$$v = \frac{V_{max}[AX][B]}{(K_{AX}K_{mB} + K_{mAX}[B] + K_{mB}[AX] + [AX][B])} \quad (5)$$

where, V_{max} is the maximal enzyme velocity; K_{AX} is the dissociation constant for the enzyme-AX complex; and K_{mAX} and K_{mB} are the Michaelis-Menten constants for AX and B, respectively.

Model discrimination

As Tracy and co-worker stated [26,27], selecting equations that adequately describe a kinetics profile was necessary in order to facilitate estimation of the relevant kinetic parameters. Mis-identification of kinetic profiles can lead to inaccurate predictions of intrinsic clearance. To select an appropriate model, XL_Kinetics provides the statistics for goodness-of-fit assessment such as determination of coefficient (R^2), sum of squares of residuals (SS), standard error of weighted residuals (SE), Akaike's information criterion (AIC) and Schwarz criterion (SC). Among those statistics, AIC and SC include a penalty term for the number of modeled parameters [28], and are computed the same way as described earlier [17]. In addition, XL_Kinetics generates Eadie-Hofstee (V is plotted against V/[S]) or the double-reciprocal plots (for inhibition module) to assist in model selection. Specific description of the corresponding diagnostic diagrams for different kinetic profiles is beyond the scope of this article. One can refer to the literature for details [20].

Standard error of the estimated parameters

Although being capable of estimating the parameters, SOLVER does not approximate the standard errors of estimated parameters. A special macro therefore is encoded in XL_Kinetics to provide the standard errors with respective to the parameters resulted from SOLVER.

The standard error of the parameter a_i is given by [29].

$$\sigma_i = \sqrt{p_{ii}^{-1}} \times SE$$

Where P_{ii}^{-1} is the ith diagonal element of the inverse of the P_{ij} matrix, SE is the standard error of weighted residuals.

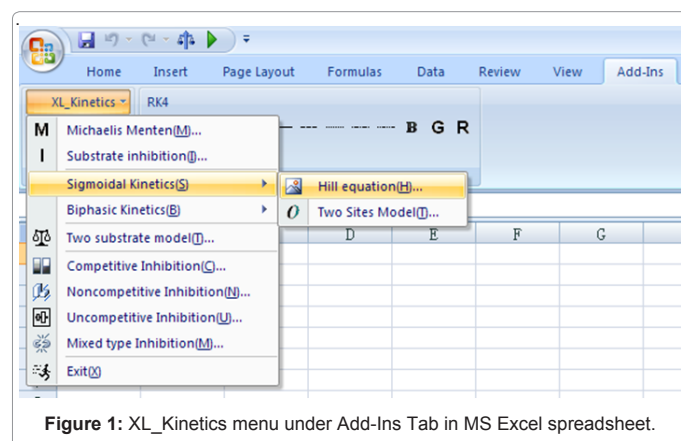$$P_{ij} = \sum_{n=1}^{N} \frac{\delta F_n}{\delta a_i} \frac{\delta F_n}{\delta a_j}$$

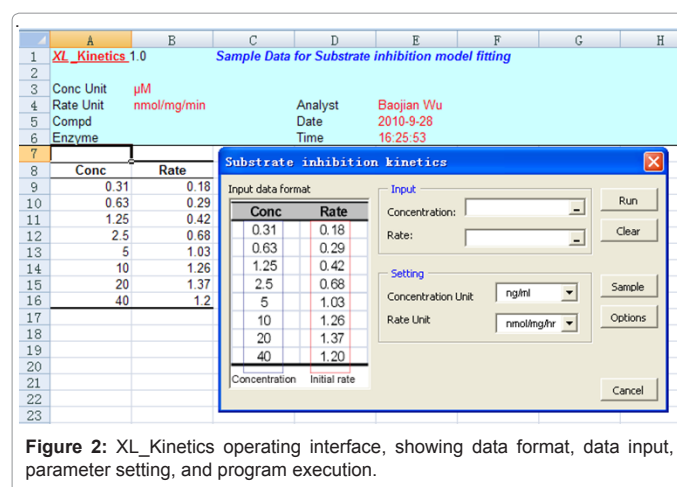**Figure 1:** XL_Kinetics menu under Add-Ins Tab in MS Excel spreadsheet.

Figure 2: XL_Kinetics operating interface, showing data format, data input, parameter setting, and program execution.

N is the number of observations. $\delta F_n/\delta a_i$ is the partial derivative of the function with respective to a_i evaluated at x_n (x, independent variables).

## Results and Discussion

Operating interface in excel

A pull-down XL_Kinetics menu is embedded under the Add-Ins tab, after installation of the program in MS Excel, as illustrated in Figure 1. By clicking a model of interest, users will be prompted to specify the data range, as well as other parameter settings (Figure 2). Users are also allowed to use different weighting schemes (1, 1/y and $1/y^2$; y is the measured rates to be fitted by the model or the predicted ones from the model) and adjust the SOLVER features such as the minimization method, precision, convergence and iterations (by activating "Options" button) (Figure 3). The modeling computation is started instantaneously by clicking the "run" button. Extensive calculations will proceed in the newly created sheet "Kinetics_Result" (by default), where the results are presented (Figure 4). In order to facilitate a quick experience and learning, sample data are included in each module, which can be invoked simply by clicking the "Sample" button (Figure 2).

Encoded models

As seen in Figure 1, the program encompasses most of the frequently used models in drug metabolism and/or inhibition studies. Specifically, they are the classic Michaelis-Menten equation, the substrate inhibition model, the sigmoidal kinetics (including Hill equation and two sites model), the biphasic kinetics (including two sites and linear portion models), a bisubstrate model (compulsory ordered mechanism) and four reversible inhibition models (i.e., competitive, noncompetitive, uncompetitive and mixed-type inhibition). The models in the XL_Kinetics memu are fully named to avoid any confusion. In addition, a key model description is included for each model, which can be accessed by clicking "options" button (Figure 3).

Specific features

As scientists are more educated in the application of MS Excel, the add-in program is much user-friendly. Kinetic modeling implemented in Excel avoids generating extra files with different extension, and might ease organization of scientific data. One unique feature of XL_Kinetics is its ability to provide accurate standard errors for estimated
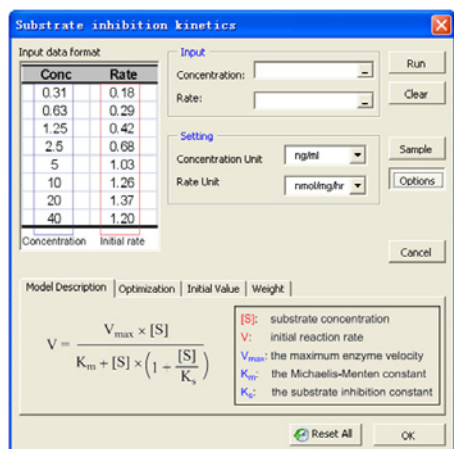
Figure 3: In the "Options" panel of XL_Kinetics, model description is provided; modeling settings (SOLVER configuration, initial parameter values, and weighting) can be adjusted.
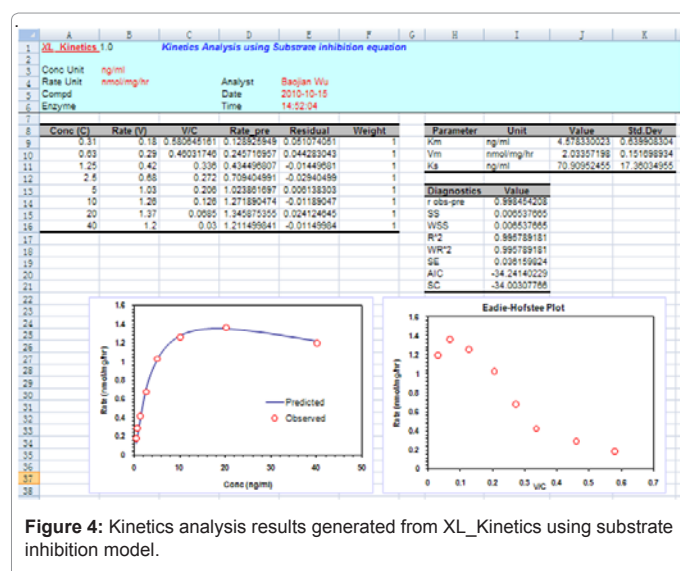
Figure 4: Kinetics analysis results generated from XL_Kinetics using substrate inhibition model.

parameters, without which, the parameter estimates are less valuable. This is especially the case when a comparison of a single parameter from the different datasets is needed.

In addition, XL_Kinetics provides some important models such as the two sites model (sigmoidal kinetics), the linear portion model (biphasic kinetics) and the bisubstrate compulsory ordered models, which somehow are not yet included in the commercial software packages (e.g., GraphPad Prism and Sigma Plot). Although writing new model equations is allowed in those software's, it usually takes long learning cycle for non-professionals. Therefore, frequent use of this feature does not occur, as pointed out by Meineke and Brockmöller [16]. Also, XL_Kinetics offers the important diagnostic values, AIC and SC, which however are not provided in these commercial software packages.

Lastly, XL_Kinetics package offers a mini toolkit "Trimmer" for kinetic graph editing. With the toolkit, one can easily add the error bars to a particular data series, and change the marker or line types, etc.

"Trimmer" appears as a few buttons in the region [Custom Toolbars] under Add-Ins tab, after a quick installation. Instruction of "Trimmer" is included in the supplementary files.

Example program runs

Validation of the Excel Add-in program was performed by directly comparing the results of XL_Kinetics with those from the commercial software packages (i.e., Graphpad Prism and/or SigmaPlot). Although being rather simple as designed, the standard operating procedures of kinetic modeling are outlined as follows,

1. Prepare data in a proper format, which is instructed on the model operating interface (Figure 2);

2. Pull down the XL_Kinetics menu and select the module of interest;

3. Input data range, and other settings if required;

4. Execute the modeling process by clicking the "run" button.

The datasets (i.e., the sample data) used for illustration were adapted from UGT metabolism studies in this laboratory (both published and unpublished). As mentioned earlier, the dataset can be recalled by clicking the "sample" button.

Michaelis-Menten and atypical kinetics analyses

The first example run was the classic Michaelis-Menten model (or a hyperbolic curve), one of the most widely used model for describing enzyme kinetic data. Two key parameters (K_m and V_{max}) were estimated. The results (counting four decimals) were numerically identical among XL_Kinetics, Graphpad Prism and SigmaPlot (Table 2).

Next, the atypical kinetics (or non-hyperbolic curves) including substrate inhibition, Hill equation, and biphasic two sites model were tested. The results are summarized in Table 2-4. For the substrate inhibition model (Table 2), XL_Kinetics, Graphpad Prism and SigmaPlot gave essentially identical parameter estimates and the fitting statistics. Slight differences were also noted: K_m of Graphpad Prism was 0.0001 (or 0.02 ‰) higher than those of XL_Kinetics and SigmaPlot, whereas K_s of Graphpad Prism was 0.0002 (or 0.003 ‰) lower than those of XL_Kinetics and SigmaPlot.

For Hill equation fitting (Table 3), the results were numerically identical among XL_Kinetics, Graphpad Prism and SigmaPlot. As for biphasic two sites model, the results were essentially identical (Table 4). However, K_{m1} of XL_Kinetics was 0.0008 (or 0.5 ‰) lower than that of SigmaPlot. Standard error of V_{m2} was 0.0001 (or 0.6 ‰) higher than that of SigmaPlot.

Reversible inhibition analyses

The mechanisms regarding inhibition of an enzyme by reversible inhibitors usually are classified into competitive, noncompetitive, uncompetitive and mixed-type inhibition. A single dataset (i.e., the sample data in the inhibition module) was fitted to all four models using both XL_Kinetics and Graphpad Prism. The results are summarized in Table 5. For competitive, noncompetitive and uncompetitive inhibition model, identical results were observed between XL_Kinetics and Graphpad Prism. For mixed-type inhibition model, most of results were the same, but slight differences (< 0.7 ‰) were also noted for K_m, Alpha (α), K_i and its standard error.

Parameters	Michaelis-Menten model			Substrate inhibition model		
	XL_Kinetics	Graphpad Prism	SigmaPlot	XL_Kinetics	Graphpad Prism	SigmaPlot
K_m	0.5268 ± 0.0433	0.5268 ± 0.0433	0.5268 ± 0.0433	4.5783 ± 0.6399	4.5784 ± 0.6399	4.5783 ± 0.6399
V_{max}	4.4013 ± 0.1113	4.4013 ± 0.1113	4.4013 ± 0.1113	2.0336 ± 0.1517	2.0336 ± 0.1517	2.0336 ± 0.1517
K_s				70.9095 ± 17.3603	70.9093 ± 17.3602	70.9095 ± 17.3603
Diagnostics						
R^2	0.9955	0.9955	0.9955	0.9958	0.9958	0.9958
SS	0.0632	0.0632	0.0632	0.0065	0.0065	0.0065
SE	0.1026	0.1026	0.1026	0.0362	0.0362	0.0362
AIC*	-18.0978			-34.2414		
SC*	-17.9389			-34.0031		

*AIC and SC are not provided in Graphpad Prism and SigmaPlot.

Table 2: Detailed comparison of XL_Kinetics results with those of Graphpad Prism 5.0 and SigmaPlot 11.0 (Michaelis-Menten and substrate inhibition models)

Parameters	Hill equation		
	XL_Kinetics	Graphpad Prism	SigmaPlot
K'	0.5730 ± 0.0334	0.5730 ± 0.0334	0.5730 ± 0.0334
V_{max}	4.8048 ± 0.0610	4.8048 ± 0.0610	4.8048 ± 0.0610
n	1.6801 ± 0.0566	1.6801 ± 0.0566	1.6801 ± 0.0566
Diagnostics			
R^2	0.9995	0.9995	0.9995
SS	0.0116	0.0116	0.0116
SE	0.0482	0.0482	0.0482
AIC*	-29.6357		
SC*	-29.3974		

*AIC and SC are not provided in Graphpad Prism and SigmaPlot.

Table 3: Detailed comparison of XL_Kinetics results with those of Graphpad Prism 5.0 and SigmaPlot 11.0 (Sigmoidal-Hill equation).

Parameters	Biphasic two-binding site model	
	XL_Kinetics	SigmaPlot
K_{m1}	1.4812 ± 0.8036	1.4820 ± 0.8039
V_{m1}	1.1559 ± 0.1241	1.1559 ± 0.1241
K_{m2}	0.0316 ± 0.0060	0.0316 ± 0.0060
V_{m2}	2.2529 ± 0.1618	2.2529 ± 0.1617
Diagnostics		
R^2	0.9966	0.9966
SS	0.0097	0.0097
SE	0.0494	0.0494
AIC	-29.0455	
SC	-28.7278	

*AIC and SC are not provided in SigmaPlot.

Table 4: Detailed comparison of XL_Kinetics results with those of SigmaPlot 11.0 (Biphasic two sites model).

The XL_Kinetics package (zipped file) is available in supplementary materials. The package contains the XL_Kinetics, Trimmer, and instructions after unzipping the file. Interested users can download and install it for free. The package was developed and tested in Excel 2007 under Microsoft Windows XP service pack 2. The models were also successfully run in Excel 2010 beta version. Other Excel or Windows versions were not tested. It is noteworthy that this add-in program does not work with MS Office 2008 for Mac, since Microsoft prevents the use of visual basic add-in in this version. We are looking forward to receiving any suggestions from our program users. Since XL_Kinetics essentially is an MS Excel SOLVER implementation, all limitations of SOLVER is also applied to this add-in program. For example, (1) the greater the number of parameters in the model, the longer the program will take; (2) the number of observations cannot exceed the

Parameters	Competitive inhibition model		Noncompetitive inhibition model		Uncompetitive inhibition model		Mixed-type inhibition model	
	XL_Kinetics	Graphpad Prism	XL_Kinetics	Graphpad Prism	XL_Kinetics	Graphpad Prism	XL_Kinetics	Graphpad Prism
K_m	3.4968 ± 0.8099	3.4968 ± 0.8099	5.5511 ± 0.5512	5.5511 ± 0.5512	7.2424 ± 1.1466	7.2424 ± 1.1466	4.8663 ± 0.6438	4.8664 ± 0.6438
V_{max}	1.6238 ± 0.0978	1.6238 ± 0.0978	1.8835 ± 0.0671	1.8835 ± 0.0671	2.0026 ± 0.1251	2.0026 ± 0.1251	1.8194 ± 0.0739	1.8194 ± 0.0739
K_i	0.1657 ± 0.0394	0.1657 ± 0.0394	0.8417 ± 0.0701	0.8417 ± 0.0701	0.5942 ± 0.0817	0.5942 ± 0.0817	0.5268 ± 0.1466	0.5269 ± 0.1467
Alpha (α)							1.9835 ± 0.8329	1.9834 ± 0.8329
Diagnostics								
R^2	0.9302	0.9302	0.9302	0.9767	0.9492	0.9492	0.9792	0.9792
SS	0.2825	0.2825	0.0944	0.0944	0.2053	0.2053	0.0843	0.0843
SE	0.1160	0.1160	0.0670	0.0670	0.0989	0.0989	0.0649	0.0649
AIC*	-24.3368		-50.6453		-31.9966		-51.3623	
SC*	-20.8026		-47.1111		-28.4624		-46.6501	

*AIC and SC are not provided in Graphpad Prism.

Table 5: Detailed comparison of XL_Kinetics results with those of Graphpad Prism 5.0 (inhibition modules)

limit of 200. It is also noted that the program is sensitive to given initial parameter value. If the initial parameters were inappropriate, SOLVER either proceeds in the wrong direction and a solution cannot found, or converges at a local minimum and provides a biased solution.

Conclusion

This study presents XL_Kinetics, a free MS Excel add-in program, for modeling enzyme kinetics. The program provides 11 most frequently used enzyme kinetic models including atypical kinetics, bisubstrate, and reversible inhibition models. Although the program is satisfactory regarding the estimated parameters and their respective standard errors for a limited number of example runs, further testing is required for additional models and datasets. We anticipate that XL_Kinetics is good choice for drug metabolism researchers in routine data analyses.

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