3D Kinetic Analysis: A Rapid, Model Independent, Method for Enzyme Kinetics

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Abstract

In this work, we present the 3D Kinetics approach as a step forward in the field of enzyme kinetics. Normally in enzyme kinetics, it is first assumed that the kinetics will conform to the assumption of Michaelis and Menten and an experiment is conducted at various concentrations around the concentration that gives half-maximal velocity. Often, these experiments could be compromised by having too much substrate, nonlinear reaction over time, time-dependent or substrate inhibition, or several other kinetic models. Herein, we present a general strategy that will decrease the number of experiments required to develop an accurate representation of the kinetics of an enzymatic reaction. We show that with a single experimental protocol, we can fit a number of the most common kinetic models associated with enzyme-catalyzed reactions. Through this experiment, we introduce the effect of time on saturation curves by modeling the reaction velocity over time and across a set of substrate concentrations. Michaelis-Menten (MM) kinetics and other analytical solutions used to solve more complex kinetic models were introduced to the field of enzymology over a hundred years ago and have only marginally changed over the years with each analytical model requiring a different set of experiments and concentration ranges. Although this approach was necessary at the time, the computational power today makes any such simplifying and limiting efforts unnecessary and avoidable. In this study, we use a single experimental protocol and fit a number of different models to the resulting data. We present four different case studies to compare and contrast the outcomes of 3D Kinetics with MM analysis for different kinetic scenarios such as enzymatic reactions with linear kinetics, biphasic kinetics, substrate inhibition, and time-dependent inhibition (TDI) to confirm the advantage of the 3D Kinetics method to the long-established MM analysis.

Introduction

Victor Henri first came up with the equation now known as the Michaelis-Menten (MM) equation while working with the enzyme invertase which catalyzes the reaction that inverts sucrose to fructose and glucose ¹. The reason Michaelis and Menten gained credit for the equation was due to a few critical mistakes that Henri made. His first mistake was not accounting for instability and mutarotation of α -D-glucose to β -D-glucose despite using a polarimetric method. He also did not control the pH of his experiments disallowing him from fitting the data to his proposed model ². After considering these points and coming up with the initial rate measurements to avoid complexities of enzymatic reaction (including the enzyme inhibition by the products of the reaction), Michaelis and Menten set the basis for parameterizing kinetic measurements for the next hundred-plus years ³.

The MM equation was originally solved by assuming that the ES complex is at equilibrium with the enzyme and the substrate, but other scientists believed that the first step of the mechanism should be irreversible ⁴. Around a decade later, Briggs and Haldane introduced the steady-state approximation in which the concentration of any intermediate complex made during the enzymatic reaction is assumed to be constant over time ⁵. Therefore, for any enzyme complex, one can solve a steady state equation ⁶. The

steady state assumption made it possible for enzymologists to come up with more complex kinetic schemes that resultantly required even more complex analytical solutions.

Solving algebraic equations might have been feasible for simple systems, though more complex kinetic mechanisms required better solutions. One of these solutions was the introduction of schematic methods by King and Altman, which were refined by Cleland a few years later^{7,8}. Further work by Cleland came by way of net rate constants, which take advantage of enzyme form partitioning, and describes a net flux through each enzyme population. This technique was especially useful for solving V or V/K as it did not involve a derivation of the complete rate equation⁹. The rapid equilibrium method was later developed by Cha to further simplify some portions of King-Altman and Cleland's methods¹⁰. Logarithmic and double-reciprocal plotting techniques have since further reinforced the importance of canonical parameters in the enzyme kinetics field. Some of these linearization methods were developed and are still being used for purposes such as studying enzyme inhibition, studying the mechanism of multi-substrate reactions, or checking whether or not saturation data follows a perfect hyperbola^{11–13}. The efforts of these scientists alongside many others have led to the appearance of many analytical methods and equations which are commonplace in deriving kinetics parameters. The outcome of this narrative has manifested in the gradual decrease in attentiveness to the validity of the foundational assumptions supporting each of these techniques.

The motivation for the historical preference of parameterizing reactions by way of initial rate determination is clear; it is the approach that simplifies data treatment to such a degree that analytical mathematics can be done without the need for an abundance of computing power. Here, substrate concentrations remain insignificantly different from their initial values, complications due to potentially inhibitory products are minimized, and there is no great opportunity for the protein to undergo partial inactivation¹⁴. One confounding incompatibility for initial-rate techniques is that these analyses attempt to remove the variable time from data treatment, opting instead for key assumptions that allow abstraction of a reaction rate from the remaining substrate or product concentration (by way of differentiation with respect to a constant incubation time). While canonical approaches such as those proposed by Michaelis and Menten are adequate within their respective approximations, such assumptions are commonly unrealistic. An especially scrutinized limitation of classical models is the steady state approximation, stating that model validity is constrained to periods in which the enzyme-substrate complex concentration remains stable¹⁵. An additional challenge when applying classical models is the prevalence of atypical kinetic behaviors, which are commonplace in drug metabolizing systems^{16–19}. Furthermore, for high-affinity substrates, where the K_m is in the range of nanomolar, the pseudo-first-order is compromised since substrate concentration in the reaction is no longer in excess and cannot be maintained at a constant concentration compared with the enzyme concentration²⁰. While adaptations to the original modeling protocols have become popular (e.g. inhibition and activation equations), modest increases in model detail require increasingly complex analytical solutions.

Alternatively, it has been proposed that the integration of time-dependent models would allow for a much greater amount of information to be obtained from simple progress curve experiments. In 1995, Duggelby offered that this strategy would give scientists the ability to contemplate the dependence of both substrate and product concentrations on the enzymatic activity as they evolve over time, but that the complexities in data analysis were prohibitive to such a procedure^{6,14}. Recently, new tools have become available that have allowed the field of enzyme kinetics to evolve toward computational competence. By deprioritizing the classical analytical problem-solving methodologies in favor of numerical modeling, scientists are now able to parameterize realistically complex kinetic reaction mechanisms without a need for the assumptions implicit in the classical approach. Such procedures have been described in theory and applied toward *in vivo-in vitro* extrapolation for time-dependent-inhibition models, but a more widely applicable approach is yet undefined^{21,22}. With experiments that monitor the evolution of product as a function of both substrate concentration and incubation time, numerical modeling has enabled the development of a new protocol named 3D Kinetics. In the current publication, we seek to present a strategy that simplifies the experimental aspects of enzyme kinetics while allowing a parameterization of unconstrained kinetic mechanisms.

Herein, we describe a universal approach unchallenged by substrate depletion and nonlinear rates over time that allows for the simple analysis of even complex kinetic models.

Materials And Methods

Chemicals and Reagents

O⁶-benzylguanine (O6BG), its metabolite, 8-oxo-benzylguanine, and zaleplon were purchased from Toronto Research Chemicals (North York, ON). N-(2-dimethylamino)ethyl)acridine-4-carboxamide (DACA) and its metabolite (DACA-acridone) were synthesized as described previously²³. Phenacetin as the internal standard, catalase, phthalazine, 1-phthalazinone, chlorzoxazone (CZN), and 3,4-Methylenedioxymethamphetamine (MDMA) were purchased from Sigma-Aldrich (St. Louis, MO). 5-oxo-zaleplon was biosynthesized and purified as previously described²³. Dihydroxymethamphetamine (DHMA) was purchased from Cayman Chemical (Ann Arbor, MI). CYP2D6 BACULOSOMES® Plus Reagents and tris(2-carboxyethyl) phosphine were purchased from Thermo Fisher (Waltham, MA). Formic acid was purchased from Fischer Scientific (Pittsburgh, PA). Acetic acid, acetonitrile, superoxide dismutase, and NADPH were purchased from EMD Millipore Corp. (Billerica, MA) and EMD Chemicals (San Diego, CA), respectively. The monobasic potassium phosphate, dibasic potassium phosphate, and EDTA used for the potassium phosphate buffer were purchased from JT Baker (Center Valley, PA). Human CYP 2E1 SupersomesTM was purchased from BD Gentest (Franklin Lakes, NJ). Expression and purification of recombinant human aldehyde oxidase (hAO) was performed following the method previously described^{23,24}.

3D Kinetics Analysis

A schematic representation of the whole assay development and analysis process is presented in Figure 1. Below, each step is described in more detail.

3D Kinetics Assay

For substrates metabolized by aldehyde oxidase (AO), the serially diluted stock solutions of each substrate were added to separate vials containing 100 mM potassium phosphate buffer (pH= 7.4) and 0.1 mM EDTA to have a final volume of 1.5 mL and the final concentration of each substrate in the range of 0.1-10 times the K_m with K_m of 125 mM for O6BG ²⁵ and 12 mM for DACA ²³. After 5 minutes of preincubation at 37°C, the kinetic reactions were initiated by the addition of hAO (0.0192 mM final concentration) and 100 μ L of the reaction was then quenched in 25 mL of 1 M formic acid containing a known concentration of the phenacetin internal standard at times 10 s, 3, 5, 10, 20, 30, 45, and 60 minutes. The total amount of AOX in hAO was quantified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) ²⁶.

For substrates metabolized by CYP450 enzymes, the serially diluted stock solutions of each substrate were added to separate vials containing 100 mM potassium phosphate buffer (pH= 7.4), 2 mM tris(2-carboxyethyl) phosphine, 125 U/mL catalase, and 0.5 mM EDTA to have a final volume of 1.5 mL and the final concentration of each substrate in the range of 0.1-10 times the K_m with K_m of 2.2 mM for MDMA ²⁷ and 39 mM for CZN ²⁸. After 5 minutes of preincubation at 37°C with 10 nM of baculosomes (2E1 with CZN and 2D6 with MDMA), the kinetic reactions were initiated by the addition of 1mM NADPH. 100 μ L of the reaction was then quenched in 25 mL of 1 M formic acid containing a known concentration of the phenacetin internal standard at times 10 s, 3, 5, 10, 20, 30, 45, and 60 minutes.

Liquid Chromatography-Mass Spectrometry

Samples were analyzed using an LC-20AD series high-performance LC system (Shimadzu, Columbia, MD) fitted with an HTC PAL autosampler (LEAP Technologies, Carrboro, NC). Chromatography was performed using a Kinetex® 2.6 mm C18 100 Å column (100× 2.1 mm; Phenomenex). Mobile phase A consisted of 0.05% (by volume) formic acid and 0.2% acetic acid in water, and mobile phase B comprised 90% acetonitrile, 9.9% water, and 0.1% formic acid. The quantitation was conducted on an API 4000 Q-Trap MS system (Applied Biosystems/MDS Sciex, Foster City, CA) with turbo spray electrospray ionization operating in positive ion mode. The program gradient timing of the mobile phases and the MS tuning parameters were similar to a method previously published ²⁹⁻³¹ only using different Q1 and Q3 for various substrates. The flow rate was 0.25 mL/min and the metabolites and internal standard were detected using multiple reaction monitoring mode following these m/z transitions: DACA-acridone, 310.2 → 265.0 ²⁶; 8-oxo-benzylguanine 258.2 → 91.0 ²⁵; 5-oxo-zaleplon, 322.3 → 280.1 ³²; 1-phthalazinone, 147.1 → 118; DHMA 182.1 → 105.0 ²⁹; hydroxychlorzoxazone, 185.9 → 130.1, and phenacetin (internal standard), 180.2 → 110.1.

Mathematica 11.0.1.0 (Wolfram Research, Champaign, IL) was the software platform of choice for data analysis. NonlinearModelFit function with 1/Y weighting was used to fit sets of $([S]_0, v)$ to the Michaelis-Menten equation for each time point, ending up with a total of 8 saturation curves with each providing k_{cat} and K_m values. Biophysical measurements can be used to directly measure the on and off rates and should be used therein if available, however, in most cases, the on and off rates are far greater in magnitude than the rate-limiting step rate constants and thus have a marginal impact on the overall data fit. This is usually checked by changing the on and off rates by 10% and observing the changes in the results. Therefore, the on rates are usually set to a significantly higher value than the initial guesses for the rate constants that are solved for, and the average K_m of all the progress curves, minus the outliers is used to set the off rates, $k_2 = k_1 \times K_{m,avg}$. In cases where substrate inhibition occurred, the k_6 value was treated as an on rate and was therefore set to a similar value as k_1 . The average of the calculated K_i values through fitting the progress curves to Equation 1 was then used to calculate the off rate, $k_7 = k_6 \times K_{i,avg}$.

$$v = \frac{V_{max} \times S}{K_m + \frac{S \times (1 + S)}{K_i}} \quad (\text{Equation. 1})$$

The rate constants corresponding to kinetic events suspected to participate in rate limitation require only a reasonable initial guess and should be fitted numerically. Next, numerical fitting on the whole dataset was performed using the NDSolve function with MaxSteps $\rightarrow$ 10000 and PrecisionGoal $\rightarrow \infty$ on various models. The decision on what model to choose for each dataset was made by comparing the Akaike Information Criterion values (AIC) obtained through the fits to each model. Models were mostly developed based on previous knowledge of the enzymatic reactions and later modified as needed and the goodness of fits were evaluated by AIC and RSquared commands. Modified activity model (MAM), the linear (MM) model, the dead enzyme model (DEM), the Michaelis-Menten substrate inhibition model (MM-SIM) and the substrate inhibition model 1 and 2 (SIM1, SIM2), and the slowly reversible inhibition model (RevTDI) were among the best performing models used for different case studies (Fig. 2).

For the ES simulations (Fig. 3), the same dataset as previous work by Abbasi et al. (2019) was used for O6BG, zaleplon, phthalazine, and DACA. For further details, please refer to this publication³⁰. Briefly, product formation for these substrates was monitored and fitted to MAM to derive the rate constants associated with these reactions. For the simulation section in the present work, the fitted values were used to simulate the changes in the concentration of the ES complex with time for the same reactions.

Results

Model Assessment

A principle task in using the 3D kinetics approach is selecting an appropriate kinetic model to fit the experimental dataset. Models as simple as the MM scheme are recommended to begin data fitting so that a nested approach can be taken, wherein each addition of model complexity contains the entire subset of parameters from the model preceding it. All the models developed for various enzymatic reactions are presented in Figure 2. While AIC is the primary metric by which model fits were compared in the present research, additional tools were used to ensure the correct model and fit. Correlation matrices were used to establish unnecessary micro-rate constants (Supplemental Table. 1). Overfitting the data with an overly complex kinetic scheme will result in highly correlated rate constants. Correlation matrices allowed these relationships to be monitored while adding complexity to each kinetic model. As always, a visual assessment of the fitting over the dataset must accompany each model output. Plotted residuals can aid visual assessment and allow the researcher to observe systematic discrepancies between the model and data (Supplemental Fig. 1). The process of numerical fitting, then, begins with the selection and mathematical description of a kinetic mechanism, including initial rate constants and guesses. The initial guesses are then optimized to allow the best (lowest) AIC score before the model is changed and reoptimized. Once a model is deemed sufficient, it can then be improved with fit-weighting protocols.

Improving Fits by Weights

Once a kinetic reaction is chosen and initial guesses optimized, a weighting can be implemented to improve the fit of the model to the data. Model weights can operate on each of the variable populations being modeled: substrate concentration, time, and product concentration. Weighting evaluations in the current project were limited to direct and inverse scalar variations of each of the three available populations. Due to some disagreement between weight-dependent AIC and R^2 outputs a single metric was chosen for weight type assessment. While AIC is the preferred measure by which a model can be selected in comparison to other models, it cannot be compared for different weighing schemes and R^2 is the appropriate weight-scheme metric due to its focus on goodness-of-fit. Based on the results of all of the enzymatic reactions in this study, weighting with respect to $[P]_t$ or $[S]_0$ yielded the best R^2 values. A comparison between different weighting schemes of DACA metabolism by aldehyde oxidase is provided in Figure 4 as an example of using different weighting schemes and fit outputs are provided in the Table 1.

Table 1. Investigating the effect of different weighing schemes on the output of the DACA fit to SIM1

Weight	R ²	k3	k4	k5
1/[P] _t	0.983	22.5	0.220	4.90
1/[S] ₀	0.991	19.0	0.167	4.65
1/t	0.863	17542	1180	8.42
N/A	0.994	22.0	0.198	4.67
[P] _t	0.996	21.2	0.173	4.34
[S] ₀	0.996	97.86	0.24	17.49
t	0.993	51.8	0.786	5.57

Case Studies to Compare 3D Kinetics With Traditional MM Kinetics

a.) CZN and CYP2E1: linear kinetics

Chlorzoxazone is a common probe for measuring the CYP2E1 activity³³ due to its high selectivity and low interindividual differences³⁴. Since linear product release can be observed through visual inspection of the dataset, the linear MM model was the model of choice for analysis (Fig. 5). An average K_m of 50 μ M was obtained from the 3D dataset, which stands in agreement with the K_m range published in the literature (40 μ M - 135 μ M)^{35,36}. As expected, the k_{cat} derived from MM analysis of this enzymatic reaction was consistent with the k_3 rate constant derived from numerical analysis (Table 2).

b.) O6BG and AO: nonlinear kinetics

Since the plot of product formation over time for most substrates of AO is nonlinear, a kinetic scheme was developed to include a pathway in which a modification in enzyme activity occurs. Although one can come up with many kinetic pathways that may describe this behavior, two simple models were tested. First, it was hypothesized that the enzyme undergoes irreversible inactivation; this model was named the dead enzyme model (DEM). The second hypothesis was that the enzyme undergoes some undefined modification that leads to product formation at a slower rate, which is referred to as the modulated activity model (MAM). These models were studied previously using the time-course data of six different substrates of AO. In short, the differential equations for each of the kinetic models were solved numerically and the goodness of fits were assessed both visually and with AIC. The results by Abbasi et al. (2019) showed that MAM gives the closest fit to time-course data for five of the presently studied substrates, motivating the decision to continue with MAM for 3D Kinetics experiments. The 3D plots for O6BG is provided as an example of the time-course in AO (Fig. 6). The k_{cat} derived from MM analysis was

consistent with the k_3 rate in MAM, disregarding the slower k_5 rate of product formation for the later time points in the reaction (Table 2).

C.) DACA and AO: substrate inhibition

Another substrate of AO which deserved further investigation was DACA. This enzymatic reaction has been reported to exhibit substrate inhibition²³. Some modifications were needed in the MAM model in order to fit the substrate inhibition data observed in DACA (Fig. 7). Three different kinetic schemes were proposed and tested to fit substrate inhibition data (SIM1, SIM2, MM-SIM). The first two variations gave very close AIC values, -19.1 and -11.7 respectively, while MM-SIM performed poorly (AIC= 155). Also, adding the substrate inhibition pathway requires assigning a reasonable value to the new rate constants corresponding to the second substrate binding events (k_6 and k_7). This goal can be achieved by fitting the velocity vs. substrate concentration plots to a substrate inhibition equation (Equation 1). The resulting average K_m and K_i outputs with values of 12.7 and 184 respectively, can then be used to inform the new binding event rates. Parameter output variance highlights the importance of choosing an appropriate kinetic mechanism to fit the 3D data.

D.) MDMA, and CYP2D6: time dependent inhibition

MDMA was selected as a substrate of CYP2D6 demonstrating slowly-reversible time dependent inactivation, as was reported recently³⁷. Publication of a kinetic mechanism of 2D6 inhibition by MDMA necessitated a relevant kinetic scheme (Fig. 8). Standard MM hyperbola fits were used to obtain a mean K_m of 3.11 μ M. A kinetic scheme was constructed in congruence with that reported by Rodgers et al. (2019), featuring a reversible ES-complex inactivation pathway as well as an irreversible death pathway for unbound protein. Upon fitting this kinetic scheme to the 3D plot, an inhibition rate of $k_4 = 0.44 \pm 0.05 \text{ min}^{-1}$ was obtained which was consistent with earlier reports of $k_{\text{inact}} = 0.309 \pm 0.008 \text{ min}^{-1}$ for this interaction²⁹.

Table 2. A comparison between numerical analysis and steady-state Michaelis-Menten analysis

Substrate	Model (AIC)	Numerical Analysis Rates (min^{-1})				Michaelis-Menten Analysis		
		k_3	k_4	k_5	k_6	K_m (μM)	K_i (μM)	k_{cat} (min^{-1})
CZN*	MM (-419)	0.221 $\pm$ 0.005	---	---	---	71 $\pm$ 18	---	0.25 $\pm$ 0.03
	MAM (14.6)	13.5 $\pm$ 0.8	0.13 $\pm$ 0.02	3.1 $\pm$ 0.4	---	6.5 $\pm$ 2.6	---	13.5 $\pm$ 1.6
DACA	MM-SIM (155)	200.004 $\pm$ 0.008	---	---	---	---	---	---
	SIM1 (-19.1)	22.4 $\pm$ 1.2	0.22 $\pm$ 0.03	4.9 $\pm$ 0.4	---	21.2 $\pm$ 7.7	92 $\pm$ 46	28.9 $\pm$ 7.2
	SIM2 (-11.7)	20.6 $\pm$ 1.4	0.25 $\pm$ 0.04	5.4 $\pm$ 0.4	---	---	---	---
O6BG	MAM (176)	119 $\pm$ 12	0.24 $\pm$ 0.07	40.7 $\pm$ 3.4	---	---	---	---
	DEM (184)	86.8 $\pm$ 5.1	0.035 $\pm$ 0.006	---	---	78.7 $\pm$ 14.4	---	131 $\pm$ 11
	MM (217)	57.1 $\pm$ 2.3	---	---	---	---	---	---
MDMA	RevTDI (-283)	155 $\pm$ 8	0.44 $\pm$ 0.05	---	0.031 $\pm$ 0.003	3.5 $\pm$ 0.6	---	10.1 $\pm$ 0.5

*The rate constants are not corrected by a metabolite standard curve for this substrate due to the unavailability of the metabolite.

Discussion

Steady state kinetics experiments have not changed notably from the early years of Michaelis and Menten. Traditionally, a time-course is first performed with a saturating concentration (to minimize the probability of significant depletion) of the substrate to allow determination of the steady state time frame, wherein product formation is linear. This process requires an approximate knowledge of the substrate's K_m so that a saturating concentration may be determined. In the case of substrates with no reported K_m , saturation experiments are first necessary, but are difficult due to the lack of a known steady state time frame. These experiments can be further complicated by cases in which product signal is low during analysis, incentivizing experimenters to use long incubations which may convolute K_m measurements if the incubation time exceeds the time over which product formation is linear. Commonly, steady state experiments are performed using the latest boundary time for linear product formation. The rationale for choosing the end of the steady state time frame is to increase the signal for product formation. This is especially important for substrates with low turnover rates. Once the linear region of the time-course plot is determined, a saturation curve should be developed by incubating several concentrations of the substrate for a specific amount of time to accurately determine kinetics parameters such as K_m , V_{max} and k_{cat} . Counterintuitively, steady state kinetics seek primarily to remove variable time from parameter calculations and are therefore strictly valid during the linear time range observed from progress curves. This has been a reasonable assumption historically but deserves rigorous scrutiny when attempting *in-vitro in-vivo* extrapolation. Drug regimens intended to keep plasma concentrations in a therapeutic concentration window for an extended time will inevitably exceed the steady state period, and thus necessitate additional *in-vitro* data treatment considerations.

Here, we have demonstrated the inaccuracy of the steady state assumption for enzymes with nonlinear kinetics such as AO using ES simulations. The simulation results clearly demonstrate that the concentration of the ES complex does not remain constant with time and is indeed dramatically changing during the first few minutes of the reaction where it is believed to remain constant (Fig. 3). In addition to

the steady state requirements, traditional methods necessitate a further simplification for computational analysis. The substrate concentration should remain much greater than that of the enzyme to maintain a pseudo-first-order relationship. This assumption cannot always be satisfied. For example, *in-vitro* experiments on very tight-binding molecules can necessitate substrate concentrations that approach the concentration of the enzyme itself, violating the pseudo-first order requirement.

Requirements such as these stem from a simple lack of computational resources during the advent of the field of steady state enzyme kinetics but may result in errors or limitations for experimental conditions and parameter outputs. Fortunately, advances in computational technology have rendered such restrictions unnecessary. Numerical parameterization strategies now allow separation from the steady state realm and instead permit a focus on realistically complex kinetic pathways. This strategy requires only the creation of a kinetic reaction scheme and a description of that scheme using ordinary differential equations, which can then be fit to a dataset.

One goal of this study is to introduce a method that simplifies the experimental aspects of enzyme kinetics by homogenizing the necessary protocols for kinetic parameterization. With 3D Kinetics, a single experiment type can provide K_m and V_{max} estimates as well as the micro-rate constants that correspond to the customizable kinetic scheme. What we have done is simply reconcile the effect of time on saturation curves by performing a unique time-course experiment across a set of substrate concentrations. This is the basis of the third dimension in “3D Kinetics” (reaction rate vs. substrate concentration vs. time). This type of approach has been discussed in the literature historically by different scientists including Michalis and Menten^{38–40}. Experiments here output a saturation curve for each time point, all of which are expected to give similar K_m values. A range of time-dependent saturation curves also eliminates the need to perform replicate experiments due to the repetition inherent to the protocol. Furthermore, numerical fitting of each substrate-concentration time-course plot at once is more favorable than fitting the time-course of a single substrate concentration since numerical fittings become more precise as larger datasets increase the reliability of the differential equation solutions. Also, the k_{cat} values from each saturation curve can be compared with the differential-equation-output micro-rate constant(s) associated with product formation (e.g. with k_3 in a MM scheme).

There are several factors to be considered for the numerical modeling type of analysis. First, a semi-mechanistic understanding of enzyme kinetics is essential. While it is clear that one may not know each step of the enzyme's mechanism of action, it is necessary to have the ability or knowledge to develop and evaluate a model that represents the observed kinetic behavior. For example, if the product formation progress curve is not linear, one could speculate right away that the kinetic model must contain a pathway that decreases enzyme activity, such as an inhibition pathway or a non-specific enzyme death. Statistical methods should always be used in model analysis. There are numerous statistical criteria to assess the goodness of fit and/or model validity. For the present work, we have used R^2 values to assess the goodness of fit, AIC to assess model selection while penalizing overparameterization, p-value to assess the significance of the estimated individual micro-rate constants, and the plot of residuals to

make sure the errors in the estimated values are random and void of any obvious trends. The third consideration is to avoid overparameterization. Overparameterization mostly occurs when a large number of parameters have to be solved for using a limited amount of data ⁴¹. This could result in an overfitted model that appears to perform well since the developed model is not necessarily fitting the data trend but the errors in the dataset. This can usually be identified by high p-values for parameters and highly correlated parameters (observed in the correlation matrix). It should also be noted that random error affects both the p-value and correlation matrix. By chance, random error can result in choosing the wrong model, particularly for small datasets. Large datasets and duplicate experiments can prevent this. Lastly, to have an optimized model, we have also tested different weighing schemes. Our results indicated that weighing with respect to $[P]_t$ and $[S]_0$ had the most optimal outcome (Table 1).

To compare the performance of the 3D Kinetics method with traditional MM analysis, we have performed four different case studies portraying various kinetic scenarios. For our first case study, we have chosen the simplest case of having a linear rate of product formation. This type of reaction is in line with the steady state assumption and is considered an ideal type of kinetic reaction to analyze via the MM method. As expected, the k_{cat} and K_m values derived from fitting the data from monitoring the CZN and CYP2E1 reaction to the MM equation (Fig. 5) were consistent with the k_3 and the average K_m that was derived from numerical, non-steady state fitting to the MM model (Table 2). This type of comparison has given us a way to cross-validate both methods. However, what the 3D Kinetics method offers for this type of reaction is not necessarily a more accurate or different result, but yet a simpler, all in one experimental design.

The performance of 3D Kinetics on the previously established nonlinear kinetics of AO ³⁰ has been investigated for our second case study. The 3D dataset was fitted to all of the models previously developed for studying the kinetics of AO and several of its substrates including O6BG, namely MM, DEM, and MAM ³⁰. Comparing the AIC values, MAM was the best performing model among the three models tested as it best describes the biphasic kinetics of O6BG and AO encompassing both the fast initial oxidation phase as well as the slower reduction phase of this enzyme ^{30,31}. In comparing 3D Kinetics with MM analysis, we have observed a consistency between k_{cat} and k_3 in MAM which means that MM analysis completely disregards the second phase of enzymatic reaction captured by k_5 through numerical modeling (Table 2, Fig. 6).

A case of substrate inhibition was demonstrated through the reaction between DACA and AO. Substrate inhibition occurs when the substrate concentration in the reaction exceeds a certain amount and two substrate molecules could bind to the active site and/or a non-catalytic site and reduce the enzyme activity ^{42,43}. For the case of DACA interaction with AO, for substrate concentrations higher than 40 mM, the enzyme activity started to decrease. Therefore, if similar to other substrates of AO, MAM was fitted to the dataset, the model would not be able to fit the higher concentration points as it does not have any pathways to account for this type of reaction. Substrate inhibition models (MM-SIM, SIM1, and SIM2) were developed for this reaction instead (Fig. 7). SIM1 and SIM2 outperformed MM-SIM dramatically

since they were both nested with MAM as the biphasic kinetics of AO still needs to be accounted for. For this type of reaction, MM k_{cat} does not correspond to k_3 , or k_5 (Table 2). That is because enzyme inhibition is occurring as time progresses and therefore the selected time point for MM analysis is only peering into a window of time somewhere closer to the beginning of the reaction without informing on the decreasing trend of k_{cat} if the bigger 3D picture was considered.

For our final case study, we have decided to look into the utility of 3D Kinetics in investigating time-dependent inhibition (TDI). A recent numerical analysis of the interaction between MDMA and CYP2D6 has identified this reaction as a reversible TDI ²⁹. The experimental design for TDI reactions usually involves the use of a probe substrate to measure the remaining activity of the enzyme after being preincubated with the inhibitor. Currently, whether or not to use dilution when adding the probe substrate is not perfectly agreed upon ^{44,45}. In the case of 3D Kinetics analysis, we have removed the unnecessary use of a probe substrate as a TDI interaction can simply be modeled the same way as any other type of kinetic reaction. Therefore, the reversible TDI model in this study (Fig. 8) has been adopted from the previously developed numerical model for MDMA and 2D6 interaction ²⁹ with minor adjustments to remove the unnecessary use of a probe substrate for 3D Kinetics analysis (RevTDI). In this model, enzyme inactivation rate is represented by $k_4 = 0.44 \pm 0.05$ which is consistent with what was previously reported as $k_{inact} = 0.309 \pm 0.008 \text{ min}^{-1}$ using a probe substrate and the traditional replot method ²⁹ (Table 2).

Overall, this work was intended to underline the shortcomings of classical MM analysis and present 3D Kinetics as a customizable and comprehensive alternative to study enzyme kinetics. 3D Kinetics could also be considered partly an enhanced extension of MM method as it takes advantage of developing multiple saturation curves for each time point in the reaction allowing us to monitor the changes in MM parameters with time and to compare the results of the 3D Kinetics analysis with the traditional methods and to accommodate a smoother transition into numerical analysis in the future. A single set of experiments can be used regardless of the kinetic model that will eventually describe the reaction. Many experimental problems, such as substrate depletion, enzyme loss or modification, non-steady-state kinetics, etc., do not obviate the validity of the data.

Declarations

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Author Contributions

A. A., and J. T. R. participated in research design, conducted experiments, performed data analysis; A. A., J. T. R., and J. P. J. wrote the manuscript; J. P. J. supervised the project.

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Figures

Figure 1.

Figure 1

Schematic representation of 3D Kinetics assay development and analysis. This assay requires incubation of various substrate concentrations, usually in the range of $0.1-10 \times K_m$, under favorable conditions and quenching the reaction over time. Here, we have incubated all of our assays at 37°C to mimic biological conditions and quenched the reaction at several time points from 0.1-60 minutes. Product formation has been monitored and measured using HPLC/MS/MS and the resulting dataset was modeled through

solving differential equations specific to the kinetic scheme that best describes the kinetic behavior of the reaction such as the model described below. The third dimension (substrate concentration versus rate also known as saturation curve) can be developed for each time point during the course of reaction such as the one demonstrated by the red rectangles.

Figure 2

Kinetic scheme development. Different kinetic schemes were developed to model different enzymatic reactions in this study based on the previous knowledge of the reactions and statistical evaluations. These models are used to fit the kinetic trends in the enzymatic reaction and should not be confused with physiologically based or mechanistic models.

a **b** **c** **d** **Figure 3**

ES-complex simulation for nonlinear kinetic reactions. The simulated results of the changes in the concentration of the ES complex during the course of interaction between hAO and several of its substrates namely, A) O6BG, B) DACA, C) zaleplon, and D) phthalazine, as an example of non-steady state enzyme kinetics reaction. These simulations were obtained subsequent to solving the differential equations fitting the rate of product formation for each of these interactions

Figure 4

A comparison between different weighting schemes of DACA for the SIM1 model. This comparison was presented as an example of using different weighting schemes and the fit outputs are provided in Table 1. As the data suggests, the absence of weighting yields similar outputs as weighing with respect to $[P]_t$ or $[S]_0$ in most cases, and therefore either could be used. However, checking for the best fit is always recommended.

Figure 5

The enzymatic reaction between CZN and CYP2E1 as an example of a linear kinetic reaction. 0.1-10* Km of the substrate was incubated with the enzyme and quenched at different time points to develop the time course plot below. The linear MM model was used to fit the product formation for this dataset.

Figure 6

The adaptability of the 3D Kinetics to the nonlinear kinetic reaction between O6BG and hAO. The product formation during the course of reaction between O6BG (0.1-10* Km) and hAO was fitted using three different models namely, A) linear MM model (AIC= 217), B) MAM (AIC= 176), and C) DEM (AIC= 184).

Figure 7

The application of 3D Kinetics to a case of substrate inhibition between DACA and hAO. The product formation and the saturation curves developed from the interaction between DACA ($0.1-10 \times K_m$) and hAO pointed towards the presence of substrate inhibition. Three different substrate inhibition models A) MM-SIM (AIC= 155), B) SIM1 (AIC= -19.1), and C) SIM2 (AIC= -11.7) were fitted to the dataset and compared with the model fits D) MAM (AIC= 14.6) which does not include enzymatic pathways to account for substrate inhibition and thus missing the higher concentration points above 40 μM shown in magenta, green and red.

Figure 8

Confirming the applicability of 3D Kinetics for TDI studies. The RevTDI kinetic scheme was developed to model the previously established reversible TDI of the interaction between MDMA (0.1-10* K_m) and CYP2D6. In this model, the reversible ES-complex inactivation is modeled by the k5-k6 pathway and the irreversible death pathway for unbound protein is modeled by k4.

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