Chapter 5
Enzyme kinetics

5.1 Introduction

Determination of enzyme kinetics explains linear (first order kinetics) or non-linear metabolism (saturation of enzyme) of a drug. It has been observed that saturation of hepatic metabolism is an important factor contributing to non-linear pharmacokinetics [1-3]. Metabolism based non-linearity has been expressed as a balance between effective unbound plasma concentration determined from animal studies and Michaelis -Menten constant K_m value of hepatic drug metabolism [4].

The initial velocity v_0 of an enzyme catalyzed reaction is dependent on the substrate (drug) concentration $[S]$ and normally follows Michaelis –Menten relationship. However, allosteric kinetics has also been observed and reported in *in vitro* situations.

The Michaelis -Menten constant K_m (representing the substrate concentration at which v_0 is half the maximal velocity V_{max} for a particular concentration of enzyme) is a measure of affinity of an enzyme towards a substrate and under first order conditions the ratio V_{max}/K_m is equivalent to intrinsic clearance [5]. Low K_m denotes high affinity of an enzyme towards a drug (substrate) and the enzyme is saturated at low concentrations of the drug. Hence, drugs with low K_m value will exhibit non-linear, supraproportional dose-exposure relationship. Moreover, drugs with low therapeutic indices and K_m values will exhibit toxicity on a small increase in dose of the drug.

Two approaches have widely been discussed in the determination of enzyme kinetics. The first approach is conventional and it involves the monitoring of product at different concentrations of the drug [5]. However, the approach has limited applicability as metabolites are unidentified at preliminary stages of drug discovery process. The other approach involves the application of *in vitro* $t_{1/2}$ method. *In vitro* $t_{1/2}$ method has been used for estimation of intrinsic clearance and it involves the quantification of substrate consumption with respect to time at a single drug concentration below K_m [6, 7]. The demerit of *in vitro* $t_{1/2}$ method is its inability to quantify K_m. However, the concept has found applicability in the determination of enzyme kinetics by monitoring the depletion of substrate at multiple concentrations [8].

5.2 Experimental

5.2.1 Materials

HPLC-grade acetonitrile (ACN), tris (hydroxymethyl)aminomethane (tris base), KCl, $MgCl_2 \cdot 6H_2O$, and a reduced form of β-nicotinamide adenine dinucleotide phosphate (NADPH) were purchased from Sisco Research Laboratory (Mumbai, Maharashtra, India). Ethylenediaminetetraacetic acid disodium salt (EDTA-2Na) was obtained from USB Corporation (Cleveland, Oh., USA). Ammonium formate was purchased from Loba Chemie (Mumbai, Maharashtra, India). Male human liver microsomes were procured from Sigma (St Louis, MO, USA).

5.2.2. Assay using substrate depletion approach

The protein-ligand interaction was assessed by incubating the substrate at 37 ± 1°C in a shaking water bath at varying concentrations ranging from 0.2 µM to 30 µM in an optimized incubation milieu (0.4 mg/mL of rat liver microsomes and an incubation time of 5 min). Reaction was commenced with NADPH after a preincubation of 4 min. The experiment was performed in triplicate. A set of standards in the linearity range (0.1 µM to 30 µM) were simultaneously incubated with the test samples under identical composition (except active protein were substituted for heat denatured protein) and conditions. The purpose of standards was to generate a standard curve for determining the actual concentration of analyte in the samples. Test samples and standards were simultaneously quenched at time points 0 min (after commencement of the reaction) and at 5 min (termination of the reaction) with an equal volume of ice cold ACN spiked with IS. The time interval between samples was approximately less than a minute for commencement and quenching of the reaction. Samples were processed and supernatant was analyzed on a robust, sensitive and selective isocratic program developed in HPLC-PDA system to quantify the disappearance rate of the candidate drug (Table 1).

Table 1. Assay conditions for the evaluation of enzyme kinetics of CDRI 99/411 using substrate depletion approach.

Analytical conditions	
Analyte	CDRI 99/411
Internal Standard	CDRI 99/357
Column	Spheri-5 ODS Perkin Elmer Brownlee Column (5 µm, 220 × 4.6 mm)
Flow rate	1.5 mL/min
Injection volume	50 µL
Mobile phase	ACN , TDW
Isocratic	90% ACN , 10% TDW
Lambda Max	247 nm
Incubation conditions	
CDRI 99/411	0.2 µM to 30 µM
Rat liver microsomes	0.4 mg/mL
Incubation time	5 min
Sample Preparation	1:1 protein precipitation with ice cold ACN
Standard Curve	0.1 µM to 30 µM

Calculation

Initial velocity was calculated by the following method:

(v_0) (μM/min) =

(Calculated normalized concentration of the analyte at time (t_0) - Calculated normalized concentration of the analyte at time (t_t)) / time of incubation at time (t_t)

The processed data was analyzed through GraphPad Prism 4.0 and was fit to the equation Sigmoidal-dose response (variable slope)

$$Y = Bottom + (Top - Bottom)/[1 + 10\text{^}\{(Log\ EC_{50} - X) * Hill\ Slope\}]$$

Where, X is the logarithm of concentration. Y is the response and Y starts at *Bottom* and goes to *Top* with a sigmoid shape.

EC_{50} is the concentration of substrate that gives a response half way between *Bottom* and *Top*. **Equation 1**

5.2.3 Assay using product monitoring approach

The protein ligand interaction was also assessed by monitoring the formation of regioisomers (M1 and M2) with rat liver microsomes and M2 with human liver microsomes. CDRI 99/411 was incubated at 37 ± 1°C in a shaking water bath at varying concentrations ranging from 0.5 μM to 100 μM in an optimized incubation milieu (0.4 mg/mL and an incubation time of 20 min with rat liver microsomes and 0.4 mg/mL and an incubation time of 30 min with human liver microsomes, respectively). Reaction was commenced with NADPH after a preincubation of 4 min. The experiment was performed in triplicate. Samples were processed and supernatant was analyzed using a gradient program developed in LCMS/MS system to quantify the relative rate of metabolite formation. Table 2 summarizes the assay conditions.

Table 2. Assay conditions for the evaluation of enzyme kinetics of CDRI 99/411 using product monitoring approach.

Analytical conditions		
Analyte	CDRI 99/411	
Internal Standard	CDRI 99/357	
Column	Phenomenex Luna C18 (50 ×2.00 mm, 5µm)	
Flow rate	0.4 mL/min	
Injection volume	10 µL	
Mobile phase	ACN, 2 mM Ammonium formate (pH 3.0)	
Gradient , %B (min)	40 (0.01)- 40 (1)- 15 (6) – 5 (8) – 5 (14) – 40 (14.01) – 40 (18)	
Mass spectrometric conditions		
Polarity	Positive	
Ion Spray voltage	5500V	
Temperature	400°C	
Declustering Potential	30V	
Collision energy	20V	
MRM transitions	CDRI 99/411	412.5-185
	M1 & M2	428.5-201
	CDRI 99/357	406.6-179.3
Incubation conditions		
CDRI 99/411	0.5 µM to 100 µM	
Rat liver microsomes, Human liver microsomes	0.4 mg/mL(both species)	
Incubation time	20 min (with rat liver microsomes) & 30 min (with human liver microsomes)	
Sample preparation	1:1 protein precipitation with ice cold ACN	

Calculation

The relative rate of formation of metabolites was analyzed through GraphPad Prism 4.0 and was fit to the equation Sigmoidal-dose response (variable slope)

$$Y = Bottom + (Top - Bottom)/[1 + 10\text{^}\{(Log\ EC_{50} - X) * Hill\ Slope\}]$$

Where, X is the logarithm of concentration. Y is the response and Y starts at *Bottom* and goes to *Top* with a sigmoid shape. EC_{50} is the concentration of substrate that gives a response half way between *Bottom* and *Top*. **Equation 2**

5.3 Results & discussions

The cardinal feature of metabolism lies with the understanding of inherent behavior (protein–ligand interaction) of drug metabolizing enzymes involved in the biotransformation of drug. The protein-ligand interaction is manifested in terms of allosteric and non-allosteric kinetics. Allosteric or Michaelis-Menten kinetics can be assessed by monitoring product formation or by integrated approach.

5.3.1 Assay using substrate depletion and product monitoring approach

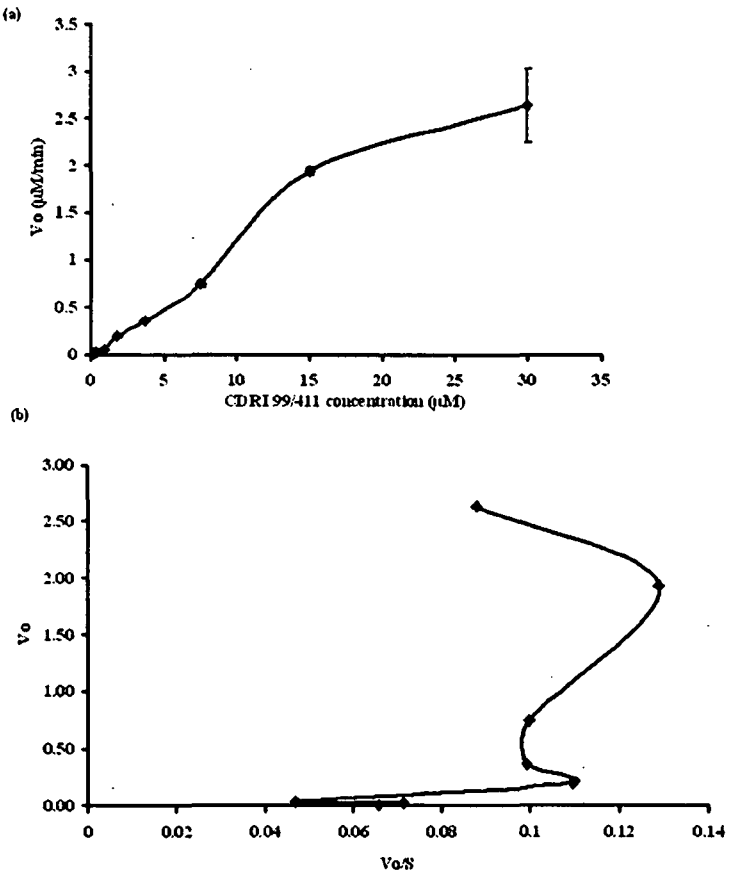
Metabolites are unidentified at preliminary stages of drug discovery process. Therefore, identification and quantification of product formation is time consuming and tedious. We applied integrated approach to assess enzyme-ligand interaction in our study.

Briefly, integrated approach ascertains the use of an optimized incubation milieu justifying incubation of the candidate drugs at low protein concentrations and short duration of time [9]. Low protein concentration minimizes protein binding. A short incubation period ensures depletion of substrate (C_0) at time (t_0) to a concentration at time (t_t). Conclusively, it reflects the transient loss of substrate and indirectly quantifies initial velocity (v_0) of the reaction.

The initial rate was calculated using normalized concentrations of CDRI 99/411 at (t = 0 min) and (t_t = 5 min) determined by the analytical method described previously. The data was fit to sigmoidal dose response variable slope with weighting *1/Y* (equation 1).

The plot illustrated in Figure 1 shows results, which is coherent with a kinetics following a non-Michaelis-Menten (allosteric) enzymatic reaction. The non-linear regression results $r \geq 0.99$ show that the observed EC_{50} for CDRI 99/411 was 17 μM and Hill slope was 1.50. Eadie-Hofstee plot resulted in a convex graph between v_0 and (v_0 / [S]) (Figure 1b).

Figure 1. (a) Enzyme kinetics (Hill Plot). Each point in the curve represents the mean ± S.E. of triplicate determinations (p < 0.05 between control and test) (b) Eadie-Hofstee Plot.



Several experimental artifacts may contribute to sigmoidicity. Therefore, it is essential to circumvent the effect of non-enzymatic processes that may cause false interpretation of data and conclusions. The turnover of substrate was below 10 % to comply with the initial rate conditions. Hence, it prevented substantial substrate depletion

and sequential metabolism of the drug. Futile binding of substrate to protein or lipid sites of microsomes was minimized with incubations at low concentrations of microsomes (0.4 mg/mL). Analytical (sensitive and robust bio-analytical method and saturation of response) and solubility issue (< 200 μM) was overcome by recording and analyzing a minimum of eight data points [10].

Enzyme kinetics of CDRI 99/411 using product monitoring approach was investigated with rat and human liver microsomes. In the absence of authentic standards of metabolites, the rate of formation of regioisomers was quantified using LCMS/MS and was expressed in relative units. Under isocratic conditions regioisomers eluted as a single (merged) peak. Hence, a gradient time program was developed to achieve baseline resolution of regioisomers (Figure 2).The assay conditions were found suitable for quantification of both regioisomers (M1 and M2) with rat liver microsomes. It was found that at non-linear regression ($r \geq 0.98$) the relationship between the rate of formation of M1 and M2 *versus* concentration of CDRI 99/411 was sigmoidal (Figure 3a & 3b). However, only M2 was quantifiable with human liver microsomes. At non-linear regression $r \geq 0.94$ the relationship between the rate of formation of M2 *versus* concentration of CDRI 99/411 was sigmoidal (Figure 3c).

Sigmoidal kinetics may be a consequence of single CYP possessing two binding sites in a large catalytic pocket (multiple ligand binding concept or space filling mechanism), ligand induced changes in the enzyme conformation, modulation of protein-protein interactions in the enzyme oligomers and multiple metabolites of a drug [11-13]. A CYP isoform possessing two binding sites in a catalytic pocket can generate non-cooperative (both sites are identical and independent and initial velocity (v_0) versus substrate concentration [*S*] follow Michaelis-Menten relationship) and co-operative kinetic (binding of one substrate molecule induces structural or electronic changes of the enzyme resulting in an altered affinity and or increase in the rate of product formation for a second substrate-binding site and initial velocity (v_0) versus substrate concentration [*S*] disobey Michaelis-Menten relationship) profiles [14].

Co-operativity can be positive or negative. Co-operativity is designated as allosterism and kinetic parameters for positive co-operativity are deduced by Hill equation. It has been found that Hill equation fits well into proposed three models for

explaining sigmoidal kinetics observed for one substrate – two binding site kinetics [1] [15]. Hence, we propose one substrate – two binding site kinetic theory to explain sigmoidal kinetics of CDRI 99/411.

5.4 Conclusion

Enzyme kinetics of CDRI 99/411 was found to be allosteric with rat and human liver microsomes. The *in vivo* significance of allosteric kinetics has not been known [16]. However, in one case enzyme activation has been reported to be responsible for the increased clearance of diclofenac in the presence of quinidine in monkeys [17, 18].

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1 $\frac{v}{E_t} = \frac{k_{24}([S]/(K_{m1})+k_{35}([S]^2/K_{m1}K_{m2})}{1+[S]/K_{m1}+[S]^2/K_{m1}K_{m2}}$.

The three models which fit well into Hill equation are –(1)singly bound substrate increases the affinity of the enzyme for second substrate and it occurs when $K_{m2} < K_{m1}$ and $V_{max1} = V_{max2}$,(2) there is an increase in V_{max} for enzyme-substrate-substrate complex and it occurs when $K_{m1} = K_{m2}$ and $V_{max2} > V_{max1}$, sigmoidicity is observed when $K_{m2} < K_{m1}$ and $V_{max2} > V_{max1}$.Since V_{max1} cannot be determined independently, it is not possible to determine whether sigmoidal kinetics are due to increased binding of enzyme-substrate or increased velocity from enzyme-substrate-substrate or both.

Figure 2. Typical MRM chromatogram of IS (m/z 406.6-m/z 179.3), metabolites (m/z 428.5-m/z 201) and CDRI 99/411 (m/z 412.5-m/z 185) in (a) rat blank liver microsome ,(b) CDRI 99/411 incubated with rat liver microsomes and (c) CDRI 99/411 incubated with human liver microsomes.

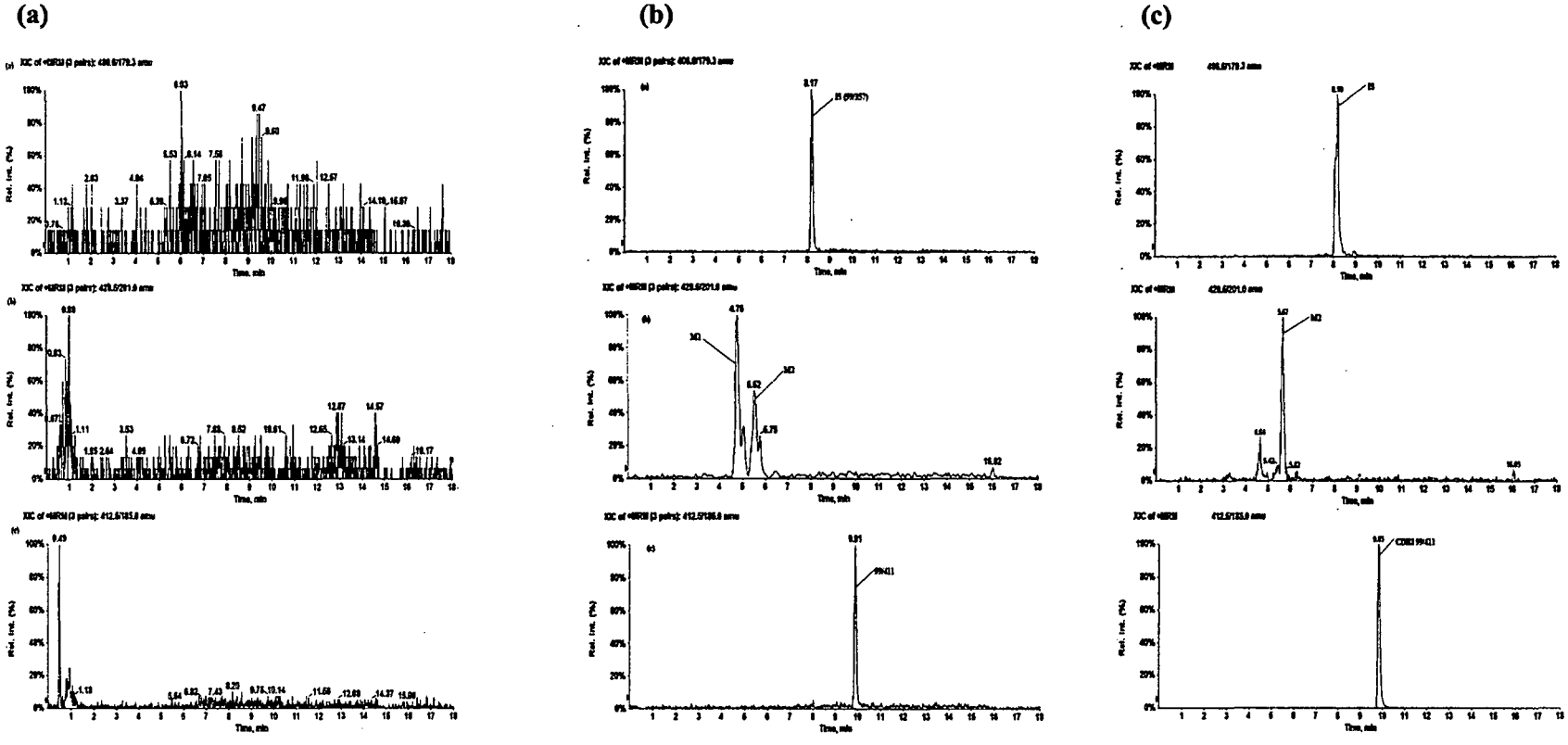
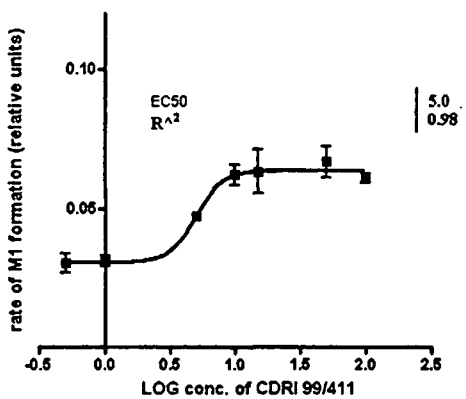
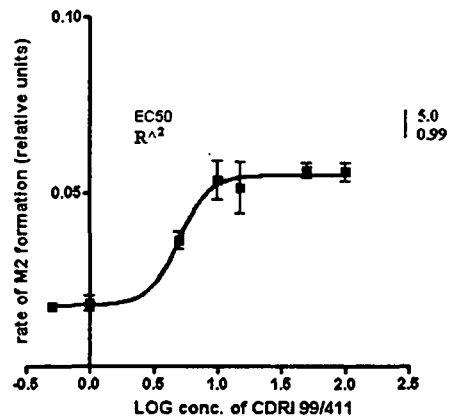


Figure 3. Enzyme kinetics of CDRI 99/411 with rat (a & b) and human (c) liver microsomes. Each point in the curve represents the mean ± S.E. of triplicate determinations.

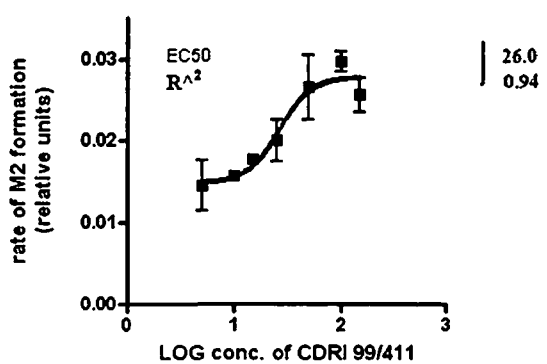
(a)



(b)



(c)



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