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Research Article

FLUORESCENT PROBE BASED CYP INHIBITION ASSAY: A HIGH THROUGHPUT TOOL FOR EARLY DRUG DISCOVERY SCREENING

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ABSTRACT

Inhibition of cytochrome P450 (CYP450) enzymes can result in potential clinical drug-drug interaction liabilities. Because of the safety and economic concern leading to clinical failure or product withdrawal, screening of lead molecules for CYP450 inhibition potential is nowadays conducted much earlier in the drug development process. At this early phase, a large number of lead molecules are in need of evaluation. Thus, safety, economic, and throughput pressures have forced the development of assays that are rapid throughput but have high predictive value. Fluorometric assays using recombinant human enzymes are ideally suited for this purpose.

Objective: This study was mainly designed with an objective to address the reliability of fluorogenic probes for CYP450 inhibition studies.

Methods: Individual cDNA-expressed CYP isoforms (1A2, 2D6, 3A4, 2C9 and 2C19) were applied to microtitre plate assays. Standard inhibitors were co-incubated with fluorogenic substrates and their inhibitory potential (IC_{50}) was determined. Assay conditions in terms of CYP450 protein concentration and time of incubation were standardized, enzyme kinetics parameters of each fluorescent probe substrate were estimated and IC_{50} values of inhibitors were determined and validated on different days to check reproducibility.

Results: The fluorescent probe based CYP Inhibition assay methods were found to be robust, efficient and highly reproducible.

Conclusion: These assays can be quite useful in preliminary high throughput screening of inhibitory potential of new drugs on individual P450 enzymes and predicting clinical drug-drug interactions.

Keywords: cDNA-expressed CYPs, Fluorometric probes, Microtitre plate assays, CYP450 inhibition, High throughput.

INTRODUCTION

The drug discovery and development process is rapidly evolving due to the technological developments in target identification along with automation of combinatorial synthesis and high throughput screening (HTS); however it still remains a time and resource intensive process. On an average, it takes approximately 12 years and costs about 1 billion US dollars to register a new drug, and attrition rate of the process is high. Several factors contribute to this high attrition. The primary being the lack of efficacy and adverse effects in clinical studies which are responsible for approximately 60% of all failures [1].

Another major contributor to high attrition rates of new chemical entities (NCE's) in preclinical and clinical development is the undesirable or poor pharmacokinetics. Hence, it is imperative to realize the role of drug metabolism and pharmacokinetics (DMPK), which has become an indispensable discipline in drug discovery and development process. In humans, five Cytochrome P450 enzymes namely 3A4, 1A2, 2D6, 2C9 & 2C19 contribute to the metabolism of greater than 90% of marketed drugs and inhibition of catalytic activity of these enzymes is a principle mechanism for adverse pharmacokinetic drug-drug interactions (DDI). A noteworthy example of drug-drug interaction is cardiac toxicity caused by co-administering the anti-histamine terfenadine and antifungal Ketoconazole or the antibiotic erythromycin where inhibition of CYP3A4 by ketoconazole or erythromycin results in elevated terfenadine levels and eventually prolongation of QTc interval [2]. Thus, the knowledge of the drug-drug interaction potential of the early leads and their possible metabolites is essential for successful drug discovery and planning human clinical studies.

Cytochrome P450 inhibition studies are therefore performed routinely in pharmaceutical industry to find out candidates with potential clinical drug-drug interaction liabilities [3-5].These inhibition screens previously relied on time consuming HPLC assays, which were convenient for evaluating small numbers of compounds. Since screening for enzyme inhibition is moving to the discovery stage, these HPLC assays are becoming outdated. The large number of chemicals to be tested has created a need for higher-throughput methods of analyzing cytochrome P450 inhibition. In recent years, advances in LC/MS/MS have allowed increased throughput, offering great promise for the implementation of high-quality, high-throughput approaches at the earliest phase of compound assessment [6-9]. However, it requires very expensive instrumentations along with skilled workforce and may be beyond the reach of many small sized laboratories.

Another high-throughput assay approach such as using the Supersomes (derived from recombinant baculovirus-infected insect cells expressing a single human CYP450 isoforms) allows the simultaneous measurement of 96 samples within 10 minutes, which is much faster than conventional HPLC and high-throughput LC-MS/MS methods using pooled human liver microsomes (HLM) [10]. Fluorescence-based assays that do not require end-stage metabolite separation allow for parallel monitoring of large reaction arrays on plate readers, thus enhancing sample throughput.

The current study was based on a high throughput fluorescent assay which utilizes individual recombinantly expressed CYP isozymes (rCYPs), individual fluorogenic substrates and fluorescent detection of metabolites. The inhibitory potentials of the standard inhibitors were measured in terms of their IC_{50} values and validated on different days to check the reproducibility.

MATERIALS AND METHODS

Fluorogenic substrates viz., 3-cyano-7-ethoxycoumarin (CEC) for CYP1A2 and CYP2C19, 7-methoxy-4-trifluoromethylcoumarin (7-MFC) for CYP2C9, 3-[2-(NN diethyl-N-methyl amino)-ethyl]-7-methoxy-4-methylcoumarin (AMMC) for CYP2D6, and 7-benzyloxy-trifluoromethyl coumarin (7-BFC) for CYP3A4 were purchased from BD GENTEST Corporation (Woburn, MA). Standard CYP inhibitors such as α-Naphthoflavone, Furafylline, Sulfaphenazole, Ticlopidine, Quinidine, Ketoconazole, Itraconazole and Verapamil were purchased from Sigma Aldrich (St. Louis, MO). Potassium phosphate (KH_2PO_4) (pH 7.4) was used as incubation buffer. The laboratory water was purified through a Milli-Q system. 96 well black polystyrene plates were obtained from Nunc™ A/S (Roskilde, Denmark). Supersomes and control proteins were purchased from BD Biosciences Discovery Labware (Woburn, MA). All other reagents were of analytical grade and obtained from commercial suppliers.

Incubations for fluorescence based assays were performed in 96-well plates and the final metabolite formation was quantified fluorometrically using TECAN Infinite®200 PRO series micro plate reader (Männedorf, Switzerland).

Standardization of assay conditions for CYP450 assays using fluorogenic probes

Optimization of protein concentration

Supersome mixtures containing different concentration of proteins **(**ranging from 0.3-20 pmoles/ml (CYP1A2); 0.1-10 pmoles/ml (CYP3A4); 0.3-20 pmoles/ml (CYP2C9); 0.39-20 pmoles/ml (CYP2C19); (CYP2D6) 2.5-20 pmoles/ml**)** were prepared in incubation buffer followed by addition of respective fluorogenic probe substrates. The substrate concentrations selected were approximately equal to or less than their Km values [11]. A 100µL of each individual supersome-buffer mixture was spiked to a 96-well microtitre plate and pre-incubated at 37˚C for 10 minutes. Reaction was initiated by adding 100µl of co-factor-buffer mixture to the wells and the plate was incubated for 30 minutes (45 minutes for CYP2C19) at 37˚C. The co-factor-buffer mixture consisted of KH_2PO_4 buffer (pH 7.4), 1mM Nicotinamide Adenine Di-nucleotide Phosphate, reduced tetra-sodium salt (NADPH) (0.015mM for CYP2D6), 3mM MgCl2; (0.3 mM for CYP2D6). After the stipulated time period, the reaction was stopped by addition of 75µl ice cold acetonitrile containing 0.1% acetic acid and fluorescence was measured using the excitation and emission wavelengths to quantify the metabolite formation. The wavelengths used for analysis are shown in Table 2.

Optimization of incubation time

Supersome mixtures containing optimized concentrations of CYP protein (1.25pmoles/ml (CYP1A2); 5pmoles/ml (CYP3A4); 5pmoles/ml (CYP2C9); 3pmoles/ml (CYP2C19); 5pmoles/ml: CYP2D6) and respective probe substrates (CEC: 3µM, BFC: 10µM, MFC: 30µM, CEC: 10µM and AMMC: 1.5 µM) was prepared. About 100µl of protein-substrate mix was added in 96 wells plate and pre-incubated for 10 minutes at 37˚C. The reaction was initiated by adding 100µl of co-factor-buffer mix in the respective wells containing protein-substrate mix as described above. The plate was incubated and reaction was quenched by adding 75µl ice cold acetonitrile containing 0.1% acetic acid each at different time intervals i.e. 15, 30, 45, 60 minutes. The fluorescence was measured to quantify the metabolite formation.

Km Determination

Supersome mixtures containing optimized concentrations of CYP protein [1.25pmoles/ml (CYP1A2); 5pmoles/ml (CYP3A4); 5pmoles/ml (CYP2C9); 3pmoles/ml (CYP2C19); 5pmoles/ml (CYP2D6)] and different concentrations of respective probe substrates (0.63-40µM, CEC; 3.91-250µM, BFC; 3.91-125µM, MFC; 1.6-100µM, CEC; 0.03-25µM, AMMC). A 100µl of respective protein-substrate mixture was added into 96-well plate and the plate was pre-incubated for 10 minutes at 37˚C. The reaction was initiated by adding 100µl of co-factor-buffer mix and incubated for 30 minutes (45 minutes for CYP2C19). The reaction was quenched by adding 75µl of ice cold acetonitrile containing 0.1% acetic acid and the fluorescence was measured using the excitation and emission wavelengths to quantify the metabolite formation. The Km values were obtained by curve fitting using GraphPad Prism software.

Determination of NADPH concentration for CYP2D6 enzyme

A co-factor-buffer mixtures containing different concentrations of NADPH (10, 15, 25, 50µM), 0.3mM $MgCl_2$ and KH_2PO_4 buffer (pH 7.4) were prepared. Enzyme-substrate mixture (ES) containing 5pmoles/ml of CYP2D6 enzyme and 1.5µM of AMMC (substrate) was also prepared. The reaction was initiated by adding 100µl of co-factor-buffer premix. The plate was incubated for 30 minutes at 37˚C in an incubator. After the stipulated time period, the reaction was quenched by adding 75µl ice cold acetonitrile containing 0.1% acetic acid. Fluorescence was measured using the excitation and emission wavelengths to quantify the metabolite formation. The final DMSO concentration in all incubations was ≤1%.

CYP450 inhibition assay

Incubations were carried out in a 96-well microtitre plate in which 100µl of co-factor-buffer mix containing NADPH, $MgCl_2$, and potassium phosphate buffer (pH 7.4) was added. A 100µl of protein-substrate mixture was added into the reactions followed by pre-incubation at 37˚C for 30 minutes. The reactions were initiated by adding different concentrations of inhibitors. The various compounds used as inhibitors of different CYP isoforms are listed as below (Table 1):

Table 1: CYP inhibitors and the concentration ranges used in IC_{50} estimation

CYP isoform	Inhibitor	Concentration range
1A2	α-Naphthoflavone	0.00002-1 µM
	Furafylline	0.0007-40 µM
2D6	Quinidine	0.0001-5 µM
3A4	Ketoconazole	0.00004-2.5 µM
	Itraconazole	0.0002-10 µM
	Verapamil	0.0017-100 µM
2C9	Sulfaphenazole	0.0003-20 µM
2C19	Ticlopidine	0.0007-40 µM

The samples were incubated for 30 minutes (45 minutes for CYP2C19) and then the reaction was quenched by adding 75µl ice cold acetonitrile containing 0.1% acetic acid. Fluorescence was measured using the excitation and emission wavelengths (Table 2) to quantify the metabolite formation. The fluorescence signals were used to calculate the percent inhibition of the respective CYP isoform. The IC_{50} was calculated by using non-linear regression analysis with GraphPad Prism.

The following equation was used to estimate the IC_{50} value of the inhibitor:

Y=Bottom + (Top-Bottom) / (1+10^ (LogEC50-X)

Here, Y= the response; X = the logarithm of concentration

In all the above experimental procedures, background fluorescence was corrected with a reading from control blank samples. The inhibition experiments were performed on three different days to assess the reproducibility.

RESULTS

Optimal assay conditions for CYP450 assays using fluorogenic substrates

A series of studies was conducted to define optimal assay conditions for fluorescence-based methods. The final supersome concentration, time of incubation, Km of fluorogenic probe substrates and the co-factor concentration (for CYP2D6) were optimized for different CYP isoforms. The summary of these optimized parameters is shown in Table 2 and Figure 1.

Table 2: Summary of optimal assay conditions for different CYP450 enzymes

CYP isoform	Substrates	Optimized protein conc. (pmoles/ml)	Optimized time of incubation (min)	Km (µM)	Excitation/Emission (nm)
1A2	CEC	1.25	30	3	410/460
3A4	BFC	5	30	10	410/530
2C9	MFC	5	30	60	410/530
2C19	CEC	3	45	10	410/460
2D6	AMMC	5	30	2.4	405/460

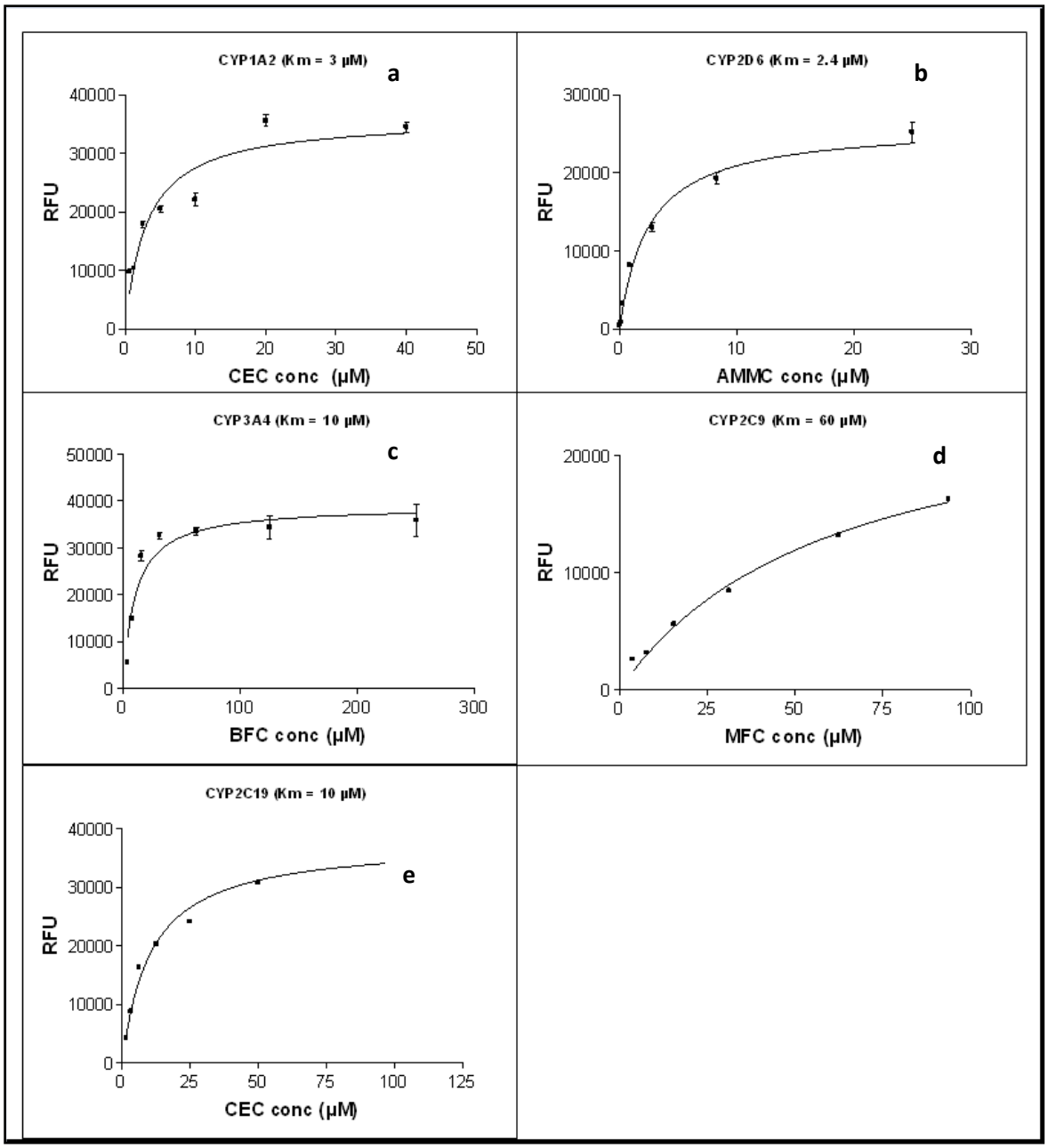

Fig. 1: Km values for CYP1A2 (a), CYP2D6 (b), CYP3A4 (c), CYP2C9 (d) and CYP2C19 (e).

CYP450 inhibition assays

Table 1 and Figures 2-4. For CYP1A2, α-naphthoflavone was found to be at least 20-fold more potent than furafylline. For CYP3A4, the

The IC_{50} values of different standard inhibitors were estimated on three different days. The results and GraphPad Prism fittings obtained are presented in inhibitory potency was found to be in the order ketoconazole > itraconazole > verapamil. The IC_{50} values obtained on three different days were found to be consistent and within two to three fold differences.

Table 1: IC_{50} values obtained for standard CYP450 inhibitors using fluorogenic substrates on different days

CYP isoform	Inhibitor	IC_{50} (µM)		
		Day-1	Day-2	Day-3
CYP1A2	α-naphthoflavone	0.28±0.04	0.13±0.03	0.23±0.04
	Furafylline	5.22±0.38	4.78±0.57	4.63±0.31
CYP3A4	Ketoconazole	0.01±0.0003	0.03±0.0004	0.01±0.002
	Itraconazole	0.06±0.003	0.06±0.003	0.06±0.008
	Verapamil	3.94±0.63	4.06±0.44	3.29±0.25
CYP2C9	Sulphaphenazole	0.64±0.12	0.34±0.20	0.33±0.11
CYP2C19	Ticlopidine	0.04±0.002	0.08±0.01	0.11±0.01
CYP2D6	Quinidine	0.004±0.001	0.004±0.001	0.008±0.003

IC_{50} values are reported as Mean ± SD, n=3

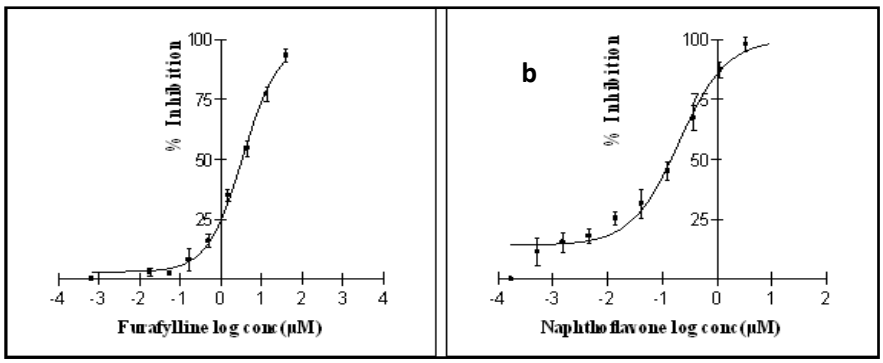

Fig. 2: Inhibition of CYP1A2 by Furafylline (a) and α-Naphthoflavone (b)

Values plotted are mean±SD of three days

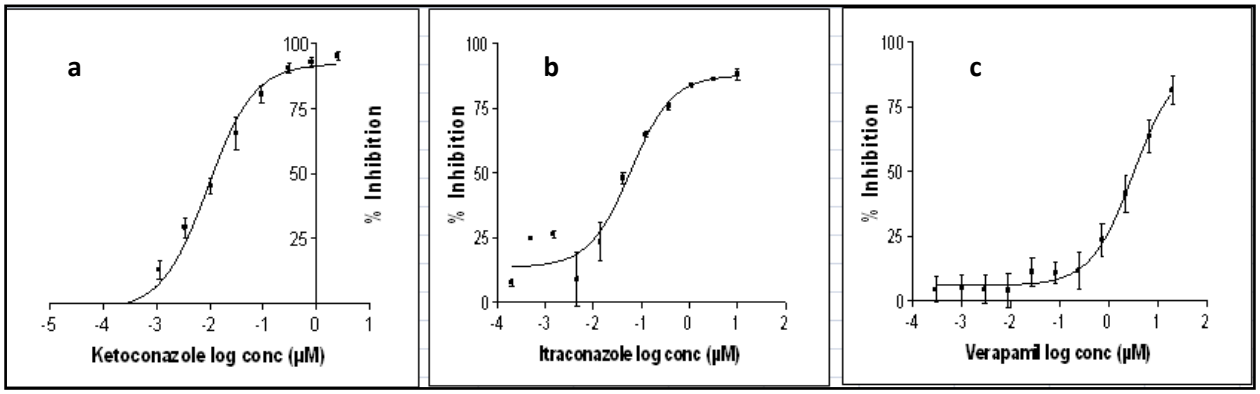

Fig. 3: Inhibition of CYP3A4 by Ketoconazole (a), Itraconazole (b) and Verapamil (c)

Values plotted are mean±SD of three days

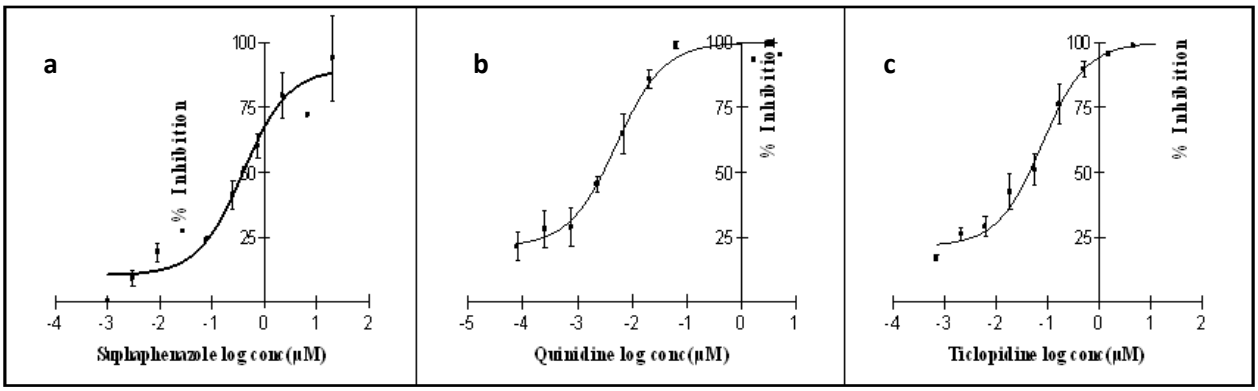

Fig. 4: Inhibition of CYP2C9 by Sulfaphenazole (a), CYP2D6 by Quinidine (b) and CYP2C19 by Ticlopidine (c)

Values plotted are mean±SD of three days

DISCUSSION

The high cost associated with developing new NCE's into drugs that may fail due to adverse drug-drug interactions makes it highly desirable to predict and avoid this liability early in the drug discovery process. Since the inhibition of hepatic CYP450 enzymes are recognized as the major cause of these drug-drug interactions, screening of NCE's to determine if they inhibit the major CYP450 isoforms has become routine practice in drug discovery.

In the present study, the application and reliability of CYP inhibition assay using fluorescence-based high throughput method was assessed. This was accomplished by using five major recombinant CYPs (CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4) and their respective fluorogenic substrates. The inhibition assay was standardized and validated using various known inhibitors of the CYP enzymes.

Initially, the incubation conditions with respect to incubation time, protein content and substrate concentrations for each of the CYP isoforms were optimized, so as to follow linear kinetics for metabolites formation. The Km values of fluorogenic substrates for various isoforms were found to be consistent with the reported literatures. In brief, the Km values for CEC (1A2), BFC (3A4), MFC (2C9), CEC (2C19) and AMMC (2D6) were found to be 3, 10, 60, 10 and 2.4 µM, respectively.

In some cases, fluorescence interference was observed in CYP inhibition assay after using high NADPH concentration. In the current study, in addition to optimizing the various experimental parameters, NADPH concentration was also assessed for optimal

turnover of all the fluorogenic substrates. No fluorescence interference was observed in CYP3A4, 1A2, 2C9 and 2C19 incubations at NADPH final concentration of 1mM. However, significant fluorescence interference was observed in CYP2D6 incubations at 1mM final NADPH concentration. Subsequently, it was optimized and set to a final concentration of 15µM for 2D6 incubations.

The important *in vitro* enzyme kinetic parameter used to evaluate inhibition is the IC_{50} (inhibitor concentration causing a reduction of enzyme activity by half). The various standard inhibitors used and their IC_{50} values obtained are given in Table 3. The IC_{50} values were highly reproducible on different occasions and found to be consistent with reported literatures [11-12].

Although, LC/MS/MS and HLM based assays offer the real potential for studying DDI as HLM contains different CYP isoforms and would be more relevant in the context of clinical DDI, they offer significantly less throughput than fluorescence-based methods. The cocktail HLM based LC/MS/MS assays have been developed for better throughput but considerable efforts need to be undertaken on the bioanalysis and still less cost effective as compared to fluorometric assay. The fluorometric assays can monitor metabolites of probe substrates in real time. The main advantage of these assays is cost-effectiveness, time and labour saving. Easy miniaturization allows high throughput. With the ease of use and availability of fluorescence based method, the large number of compounds with CYP inhibition potential can be quickly triaged. However, the recombinant systems may not represent the clinically relevant concentrations of CYP enzymes and can be only used as a first tier screening of NCE's. Subsequently, the smaller number of compounds showing positive signal in fluorescence assay can be confirmed for their CYP inhibition potential in a more definitive HLM and LC/MS/MS based assays.

CONCLUSION

In this study, a high throughput *in vitro* fluorometric method was developed and validated for assessing the CYP inhibitory potential of a compound. The different CYP isoforms used in this study were CYP3A4, CYP2C9, CYP2C19, CYP2D6 and CYP1A2. The data obtained in this study was found to be reliable, reproducible and of sufficient quality and thus this assay can be used for routine and rapid CYP inhibition screening of NCE's.

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