Development of a generic LC-MS/MS cocktail analysis method for the characterization of cytochrome P450 inhibition and phenotyping properties of new chemical entities (NCEs) using 3200 QTRAP system

Pauline L.Yeo, Md Khalid Pasha, Evelyn Goh, Venkatesh P and Kantharaj Ethirajulu.

Department of Preclinical Development, S*BIO Pte Ltd, 1 Science Park Road,# 05-09, The Capricorn, Singapore Science Park II, Singapore 117528. www.sbio.com

Abstract

Compounds that are potent inhibitors of one or more cytochrome P450 enzymes have a potential for drug-drug interactions. *In vitro* studies to determine IC_{50} or K_i values for inhibition of the major human drug-metabolizing enzymes are currently very time consuming using traditional methods (e.g., HPLC and fluorescent enzyme assays), which permit the evaluation of the activity of a single enzyme at one time. For most drugs, biotransformation is the major route of elimination, and oxidative metabolism by P450 enzymes is a common metabolic pathway. Therefore, it is important to assess the relative contribution of metabolic pathways to the overall elimination processes and to identify the P450 isoforms responsible for oxidative reactions. A generic LC-MS/MS method for evaluating both the inhibition and phenotyping properties of NCEs with respect to the major human drug-metabolizing P450 isoforms (3A4, 1A2, 2D6, 2C9 and 2C19) has been developed. The LC consisted of binary mobile phase, 0.1 % formic acid in water (A) and 0.1 % formic acid in acetonitrile (B). The mobile phase was pumped at a flow rate of 1 ml/min using eclipse XDB-C18 column (5μm, 4.6 X 150mm) with a gradient flow, 95% of A for 0.2 min and reaching to 5 % by 2.5 min and maintained till 2.7 min, then regaining back to 95% at 2.8 min and maintained till 6.0 min. The MS/MS was tuned to the MRM transitions of different compounds and the mass parameters were optimized for the analysis. For CYP inhibition studies, selective inhibitors towards specific enzymes were used separately in HLM incubations. For CYP phenotyping analysis, respective substrates were incubated with individual recombinant enzymes at pre-determined time points. The samples were processed and the supernatant from individual incubations was pooled for LC-MS/MS analysis. This cocktail method offers an efficient, robust way to determine the cytochrome P450 inhibition and phenotype profile of a large number of compounds.

Introduction

- High-throughput compound screening methods and combinatorial chemistry employed in the drug discovery has led to increased number of compounds to be evaluated rapidly in cytochrome P450 (CYP450) inhibition and phenotyping assays
- Cytochrome P450 (CYP) enzyme family comprises multiple isoforms with different substrate specificities and catalyzes the biotransformation of a vast number of drugs
- Knowledge of the specific CYP isoform responsible for the metabolism of a drug is critical for the prediction of drug-drug interactions
- CYP isoform-selective substrates have been identified and commonly used for probing the role of specific CYP isoforms in drug metabolism
- Evaluation of enzyme activity is traditionally performed for a single CYP isoform at a time, which is labor-intensive, time-consuming and cost-ineffective
- To increase the throughput, a mixture of CYP probe substrates or individual substrates can be incubated with liver microsomes and the activities of several CYP isoforms are simultaneously assessed by cocktail analysis
- Here, we report a LC-MS/MS generic method based on 3200 QTRAP, for rapid and quantitative determination of multiple probe substrates for CYP3A4, 2C9, 2C19, 2D6, and 1A2 isoforms in a single run, that could be applied for both CYP phenotyping and inhibition measurements

Materials and methods

Chemicals

Potassium phosphate, Nicotinamide adenine dinucleotide phosphate (NADPH), dimethylsulfoxide (DMSO), 6-β-hydroxytestosterone, phenacetin, acetaminophen, dextromethorphan, dextrorphan, diclofenac, s-mephenytoin, omeprazole ethoxycoumarin, ketoconazole, furafylline, quinidine, sulfaphenazole and ticlopidine hydrochloride were purchased from Sigma-Aldrich. Testosterone was purchased from Fluka, 4'-hydroxydiclofenac from Cayman Chemicals and 5-hydroxyomeprazole was purchased from I-DNA Biotechnology Pte Ltd. Recombinant CYP enzymes were purchased from Cypex Ltd and the human liver microsomes were purchased from BD Gentest.

LC-MS/MS measurements for cocktail analysis

Table 1: Analytical method

LC parameters	
System	Agilent
Column	Agilent, Zorbax Eclipse XDB-C18 column (5 μm, 4.6 X 150 mm)
Mobile phase	A: 0.1% HCOOH in water and B: 0.1 % HCOOH in acetonitrile
Flow rate	1 mL/min
Run time	6 min
Injection volume	10 μL (individual analysis) 50 μL (cocktail analysis)
Gradient	Time(min) %A %B 0.0 95 5 0.2 95 5 2.5 5 95 2.7 5 95 2.8 95 5 6.0 95 5

MS parameters	
System	AB 3200 QTRAP
Mode	ES+ and ES-
Ion spray voltage	5500 volts
Curtain gas (nitrogen flow)	10 psi
Temperature	650°C
Collisionally activated dissociation (CAD)	Medium
Ion spray gas 1	60 psi
Ion spray gas 2	65 psi

Table 2: MS/MS parameters of substrates used in CYP phenotyping studies

Compound	ESI mode	Transition pair $Q_1/Q3$	DP (V)	EP (V)	CE (V)
Ethoxycoumarin	Positive	191 / 163	52	10	24
Testosterone	Positive	289 / 109	76	5	33
Dextromethorphan	Positive	272 / 171	60	10	52
Diclofenac	Negative	294 / 250	-30	-3	-18
S-mephenytoin	Negative	217 / 188	-47	-10	-27

Table 3: MS/MS parameters of metabolites used in CYP inhibition studies

Compound	ESI mode	Transition pair $Q_1/Q3$	DP (V)	EP (V)	CE (V)
Acetaminophen	Positive	152 / 110	16	4	25
6-β-hydroxytestosterone	Positive	305 / 269	75	9.5	19
Dextrorphan	Positive	258 / 157	60	10	52
4'-hydroxydiclofenac	Negative	310 / 266	-30	-3	-18
5-hydroxyomeprazole	Positive	362 / 214	45	6	18

Table 4: CYP isoforms, substrates and their concentrations for CYP phenotyping

Enzyme	Enzyme conc (pmol/ml)	Probe substrate	Substrate conc (μM)
CYP1A2	150	Ethoxycoumarin	5
CYP3A4	50	Testosterone	5
CYP2D6	100	Dextromethorphan	5
CYP2C9	75	Diclofenac	5
CYP2C19	100	S-Mephenytoin	10

Table 5: Human liver microsomes, probe substrate concentrations and metabolites

HLM protein (mg/ml)	Probe substrate	Substrate conc (μM)	Metabolite
0.5	Phenacetin	20	Acetaminophen
0.25	Testosterone	50	6-β-hydroxytestosterone
0.5	Dextromethorphan	5	Dextrorphan
0.5	Diclofenac	5	4'-hydroxydiclofenac
0.5	Omeprazole	10	5-hydroxyomeprazole

Assay procedure

CYP phenotyping

The assay was done using recombinant CYP450 (CYP1A2, 3A4, 2D6, 2C9 and 2C19) along with the selective CYP probe substrate (Table 4). Concentration of all the substrates was fixed at 5 μM except for S-mephenytoin, which was used at 10 μM. The substrate was incubated at 0, 5, 15, 30 and 45 minutes. Percentage (%) remaining was calculated at each time point to determine the half life ($t_{1/2}$)

CYP inhibition

The assay was done using human liver microsomes (HLM) monitoring for the individual enzymes (CYP1A2, 3A4, 2D6, 2C9 and 2C19) using their selective probe substrates (Table 5). The concentration of the selective substrates was at their K_m values or less than K_m values[1]. Amount of metabolite formed was calculated using standard curve ranging from 0.01-10 μM for respective metabolites. Percent inhibition was measured in the presence of concentrations of test compounds ranging from 0.001 to 100 μM

Incubation mixture

The assay was done in a 96-well format. The incubation mixture consists of 100 mM potassium phosphate buffer (pH 7.4), optimized protein concentration of recombinant CYP enzyme or human liver microsomes (Tables 4 and 5), NADPH at final concentration of 1 mM in a total assay volume of 200 μL

Extraction procedure

The extraction procedure used was common for both phenotyping and inhibition assays. After quenching the reaction with dimethylsulfoxide and acetonitrile (20:80), the plate was shaken for 15 min at 500-600 rpm on a plate shaker. Subsequently, the sample plate was centrifuged at 2000 rpm at 4°C. An aliquot of 75 μL of the supernatant was transferred to the polypropylene LC-MS plate for analysis. The supernatant collected after extraction procedure was analyzed individually (single analysis) and was pooled for cocktail analysis

Data analysis

CYP phenotyping:

Half life ($t_{1/2}$) of the probe substrates was determined with the aid of Graph pad prism using non-linear regression.

CYP inhibition:

The IC_{50} values were calculated by non-linear regression,sigmoidal dose response curve using Graph pad prism software.

Results and discussion

- The generic LC-MS/MS method developed has a unique feature wherein, 3200 QTRAP is used in both positive and negative scan modes simultaneously and facilitates monitoring for probe substrates and metabolites having different polarities that require either negative or positive mode of detection
- MRM transitions have been optimized for the probe substrates [Fig 1(a)] and metabolites [Fig 1(b)] in a cocktail approach
- Exponential decay of probe substrates in the phenotyping assay is presented in Fig 2(a) individual analysis and 2(b) cocktail analysis
- Sigmoidal dose response curves for the measure of IC_{50} in the P450 inhibition assay Fig 3(a) individual and 3(b) cocktail analysis
- This method is applicable to resolve multiple substrates (CYP phenotyping) and metabolites (CYP inhibition) using cocktail approach
- CYP phenotyping results obtained using selective substrates for respective enzymes are in good correlation for both individual and cocktail analysis (Table 6)
- The IC_{50} values obtained for CYP inhibition are comparable with the literature values (Table 7)

Fig 1(a): MRM transitions of probe substrates in a cocktail mixture in the P450 phenotyping assay

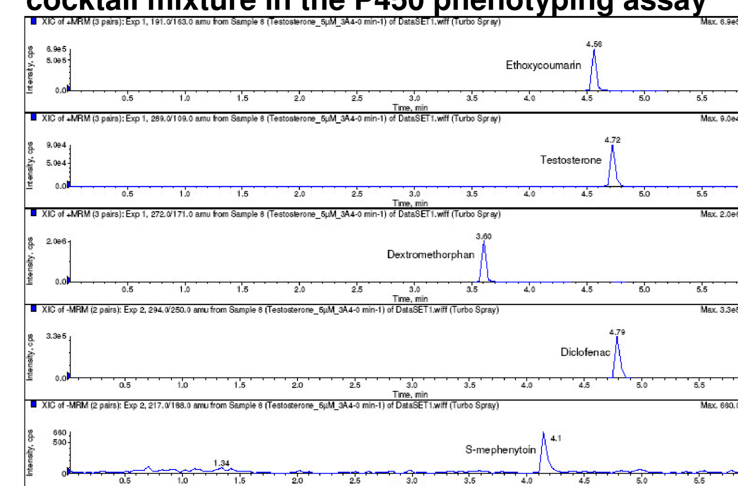

Fig 1(b): MRM transitions of metabolites in a cocktail mixture in the P450 inhibition assay

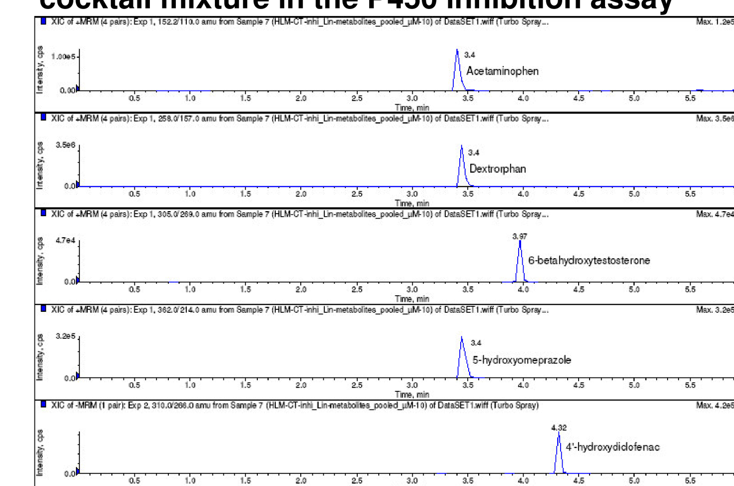

Fig 2(a):Exponential decay of probe substrates using individual analysis

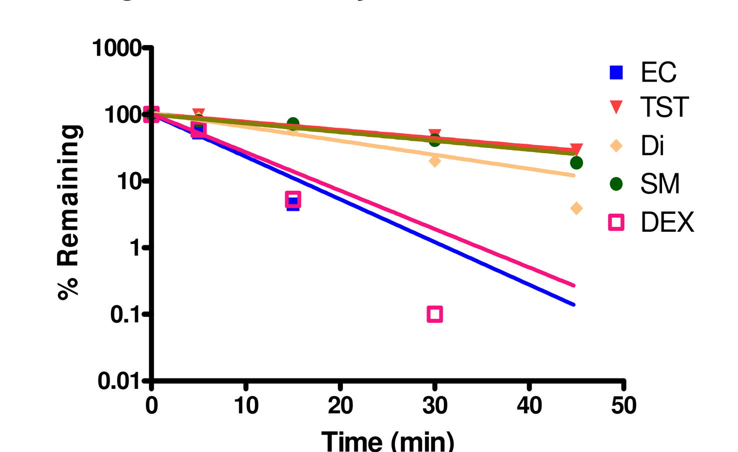

Fig 2(b):Exponential decay of probe substrates using cocktail analysis

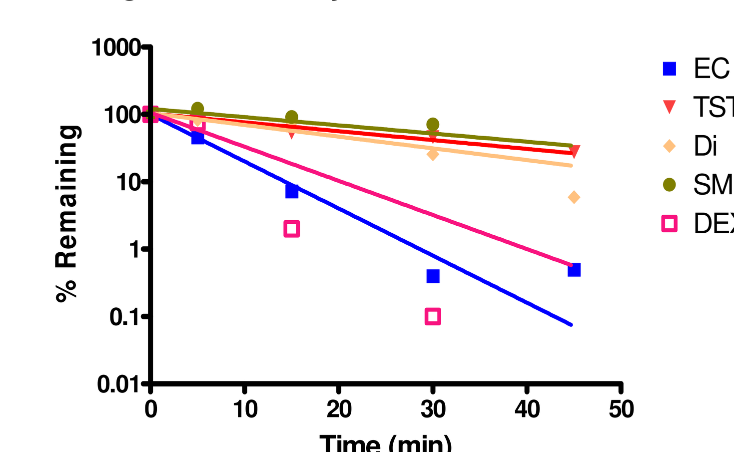

Table 6: Comparison of half life ($t_{1/2}$) between individual and cocktail analysis

Enzyme	Substrate	$t_{1/2}$ (min)	
		Individual analysis	Cocktail analysis
CYP1A2	Ethoxycoumarin (EC)	4.7	4.3
CYP3A4	Testosterone (TST)	24.5	22.9
CYP2D6	Dextromethorphan (DEX)	5.2	5.9
CYP2C9	Diclofenac (Di)	14.3	17.3
CYP2C19	S-mephenytoin (SM)	22.7	26.1

Fig 3(a): Sigmoidal dose response curve for IC_{50} determination using individual analysis

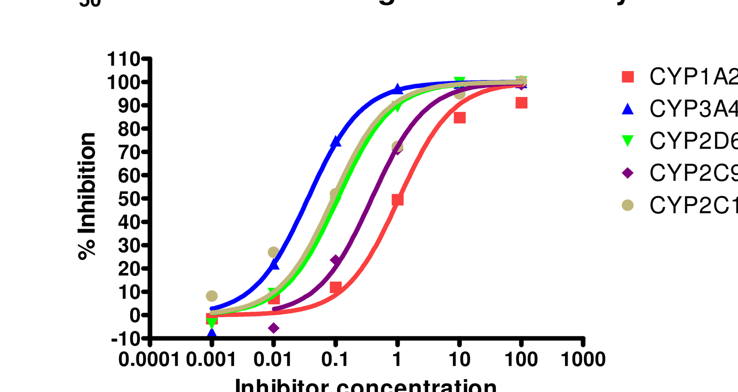

Fig 3(b): Sigmoidal dose response curve for IC_{50} determination using cocktail analysis

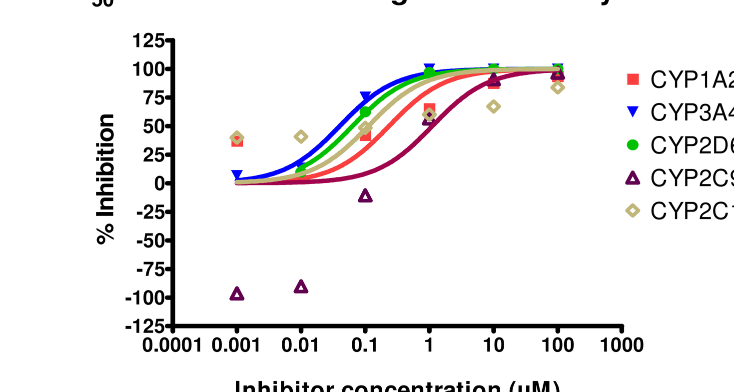

Table 7: Comparison of IC_{50} using individual and cocktail analysis

Inhibitor	Substrate	Enzyme	IC_{50} (μM)		Literature IC_{50} (μM)
			Single	Cocktail	
Furafylline	Phenacetin	CYP1A2	1.06	0.24	0.48 [2]
Ketoconazole	Testosterone	CYP3A4	0.04	0.04	0.1 [3]
Quinidine	Dextromethorphan	CYP2D6	0.11	0.06	<0.1 [3]
Sulphaphenazole	Diclofenac	CYP2C9	0.38	1.11	1.0 [4]
Ticlopidine	Omeprazole	CYP2C19	0.09	0.11	NA

NA:Not available

Summary

The current methodology

- Allows for fast and simple assessment of the potential effects which drug candidates may or may not have on the metabolism and inhibition of specific CYP450 probe substrates
- Takes advantage of fast gradient chromatography coupled to tandem mass spectrometry as a generic means of sample separation and analysis
- Provides early drug metabolism information with respect to drug-drug interactions that can be used to rationalize *in vivo* interaction studies required in the clinic

References

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