High Content Automated Metabolic Stability and CYP Inhibition Cocktail Screening Assays for Early Drug Development

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Overview

- The purpose of this study was to develop higher throughput methods to determine metabolic stability and CYP inhibition for drug discovery groups.
- Automated liquid handlers, substrate cocktails, and generic LC/MS/MS methods were used to determine metabolic stability and CYP inhibition endpoints.
- The metabolic stability and CYP inhibition profiles of the compounds studied were consistent with known data, indicating the utility of both screening assays.

Introduction

Metabolic stability and cytochrome P450 (CYP) inhibition are important parameters to determine earlier in the drug development process and are now increasingly assessed during early drug discovery. The evaluation of metabolic stability is important because the parameters of half-life ($t_{1/2}$) and the elimination rate constant (k_{el}) can be used to determine *in vitro* intrinsic clearance (CL_{int}) which can then be scaled to estimate *in vivo* intrinsic clearance. This information is important since the goal of the pharmaceutical industry is to develop drugs that require minimal dosing for maximum therapeutic exposure. Conversely, the assessment of CYP inhibition is important because CYP enzymes are typically the primary biotransformation mediators for the majority of drugs. As many drugs are co administered simultaneously, the inhibition of any given CYP enzyme by a perpetrator drug may result in the impaired clearance of a victim drug, leading to elevated exposures that may be toxic (i.e. drug-drug interactions; DDIs).[1] In the present study, we developed high content screens using automated methods on an automated liquid handler to provide metabolic stability endpoints (i.e. $t_{1/2}$, k_{el}, CL_{int}) and CYP inhibition endpoints (i.e. IC_{50} values).

Materials and Methods

Chemicals and test system
Alprazolam, 1-aminobenzotriazole (1-ABT), diltiazem, diclofenac, 7-ethoxycoumarin (7-EC), 7-hydroxycoumarin (7-HC), propranolol, testosterone, UDP-glucuronic acid (UDPGA) and verapamil were purchased from Sigma-Aldrich (St. Louis, MO). Human liver microsomes (HLM) and *S9* fractions were prepared from non-transplantable livers at XenoTech, LLC (Lenexa, KS). All other reagents were of analytical grade and purchased from commercial sources as described previously.[2, 3]

In vitro screens to determine metabolic stability
The metabolic stability of several well-known compounds was evaluated with a method developed with EVOWare® v2.5 (Tecan Group Ltd., Mannëdorf, Switzerland) for use on a Tecan Freedom EVO liquid handling instrument (Tecan Group Ltd., Mannëdorf, Switzerland) utilizing a 96-channel multi-channel arm (MCA-96). Briefly, up to 16 compounds at a single concentration and four time points may be screened for metabolic stability in up to three matrices (typically subcellular fractions from different species); or 8 compounds at a single concentration in up to six matrices. In this study, the model compounds alprazolam, diltiazem, diclofenac, 7-EC, 7-HC, propranolol, testosterone and verapamil, all at 1 µM, were incubated at 37°C with 0.5 mg/mL liver microsomes or 0.5 mg/mL liver *S9* from three species (human, rat and dog) with an NADPH regenerating system[2] in the presence or absence of UDPGA (to incorporate UGT metabolism) at four time points (0, 5, 30, 120 min). Samples were quenched with organic solvent (at ¾ ratio relative to the incubation volume), containing internal standards and analyzed for parent loss by LC/MS/MS as described below, followed by data processing (i.e., $t_{1/2}$, k_{el}, CL_{int} determination) with Galileo LIMS (Thermo Scientific, Waltham, MA), GraFit (Erithacus Software Ltd., Guildford, UK) and Microsoft Excel (Microsoft, Remond, WA).

In vitro screens to determine CYP inhibition potential
The *in vitro* CYP inhibition potential of known direct and metabolism-dependent inhibitors (MDI) was evaluated as described previously,[2, 3] with adjustments to incorporate throughput improvements. Briefly, a new method was developed with EVOWare® v2.5 (Tecan Group Ltd., Mannëdorf, Switzerland) for use on a Tecan Freedom EVO liquid handling instrument (Tecan Group Ltd., Mannëdorf, Switzerland) utilizing a 96-channel multi-channel arm (MCA-96), to screen up to 12 compounds each at seven concentrations (including solvent control). Samples with inhibitor were incubated with 0.1 mg/mL NADPH-fortified HLM at 37°C, with or without a 30 min pre-incubation for the assessment of MDI, followed by a 5 min seven-substrate cocktail incubation (with probe substrates specific for seven CYP enzymes, namely phenacetin [40 µM] - CYP1A2; bupropion [50 µM] - 2B6; paclitaxel [5 µM] - 2C8; diclofenac [6 µM] 2C9; S-mephenytoin [40 µM] - 2C19; dextromethorphan [7.5 µM]- 2D6 and midazolam [4 µM] 3A4/5). Samples were then quenched with an equal volume of organic solvent containing deuterated internal standards and analyzed for metabolite formation by LC/MS/MS as described below, followed by data processing (i.e., IC_{50} determination) with Galileo LIMS (Thermo Scientific, Waltham, MA). The panel of inhibitors screened included the direct CYP inhibitors a naphthoflavone (0.0005-0.5 µM), orphenadrine (0.75-750 µM), montelukast (0.03-30 µM), sulfaphenazole (0.003-3 µM), modafinil (0.5-500 µM), quinidine (0.003-3 µM), ketoconazole (0.0003-0.3 µM); and the MDI CYP inhibitors furafylline (0.003-3 µM), phencyclidine (0.1-100 µM), gemfibrozil glucuronide (0.001-1 µM), tienilic acid (0.0008-0.8 µM), *S*-fluoxetine (0.086-86 µM), paroxetine (0.001-1 µM), troleandomycin (0.0225-22.5 µM) and 1 ABT (10-1000 µM).

Analytical Methods
Samples were analyzed by liquid chromatography (LC) with tandem mass spectrometric detection (MS/MS) on an AB Sciex 4000 or 5500 QTrap mass spectrometer (Foster City, CA) with a Shimadzu Prominence or Nexera LC system (Columbia, MD) interfaced by electrospray ionization (ESI). Relative quantitation of each individual analyte was performed using Analyst v.1.6.2 or more recent (AB Sciex, Foster City, CA).

CYP inhibition: A cocktail method for the marker analytes, with commercially-available deuterated internal standards (IS), employed a Phenomenex Gemini C18 column (Torrance, CA) with formic acid modified water or acetonitrile as mobile phases. A gradient method over 3.5 min was used. Specific multiple reaction monitoring (MRM) transitions were tuned for optimal fragmentation parameters for each analyte/IS pair.

Metabolic stability: Ballistic gradients comprising formic acid modified water and acetonitrile mobile phases ramping up to 98% organic over approximately 1 min (early eluters) or 2 min (late eluters) were employed with a Phenomenex Gemini NX C18 column (Torrance, CA). For MS detection, a custom partial-tuning approach was developed in-house. Briefly, solutions of the analytes were evaluated to establish the de-/protonated molecule *m/z* and appropriate fragment ion to build MRM transitions for the analytes. Rudimentary optimization of fragmentation parameters was performed. Several ionization parameters were kept at generic values appropriate for the specific MS employed. A proprietary IS pool was employed with the appropriate IS for each analyte selected at the data processing stage.

Results

Metabolic stability screen
As shown in **Table 1** and **Table 2**, a panel of eight compounds was tested *in vitro* for metabolic stability with the newly developed method. **Table 1** shows the data with liver microsomes whereas **Table 2** shows the data with liver *S9* fractions, both from three species (rat, dog and human) with NADPH or with NADPH+UDPGA. Verapamil clearance was within a factor of two within each test system, indicating minimal species differences in clearance, and little or no change in clearance occurred with the addition of UDPGA. As expected, there was little to no clearance of alprazolam, with the exception of rat subcellular fractions where alprazolam clearance was measurable (suggesting a species difference in alprazolam metabolism). Within species, 7-EC was highly consistent with little difference in clearance with or without UDPGA. However, the clearance of 7-HC substantially increased with UDPGA, consistent with the rapid glucuronidation of this compound. The increase in clearance with UDGPA was also true for diclofenac, which is consistent with its conversion to an acyl-glucuronide metabolite.[4] Testosterone was rapidly cleared by most species and that clearance was enhanced with UDPGA (also consistent with its reported metabolism). For HLM with NADPH (as shown in **Figure 1**), the compounds could be classified as follows: 7-EC, verapamil, diclofenac and testosterone were high CL_{int}; propranolol and diltiazem were intermediate CL_{int}; and 7-HC and alprazolam were low CL_{int} which is consistent with the known clearance profiles of these compounds.[5] In human liver *S9*, the increase in half-life of all compounds can be attributed to the fact that *S9* contains a lower level (approximately 1/4th) of microsomal protein relative to isolated microsomes. Overall, for certain compounds there were several differences in clearance between species and also for certain compounds differences in metabolism with the addition of UDPGA, all of which can be accounted for by the metabolism of these compounds.

CYP inhibition
As shown in **Table 3** and **Table 4**, panels of direct and metabolism-dependent inhibitors (MDIs) were screened with the newly developed method utilizing a seven substrate cocktail. Of the direct inhibitors (shown in **Table 3**), at the concentrations tested, α-napthoflavone selectively inhibited CYP1A2; orphenadrine showed marked inhibition of CYP2D6 and minor inhibition of CYP2B6; montelukast inhibited CYP2C8 at least an order of magnitude more than the other CYP enzymes as expected;[6] sulfaphenazole selectively inhibited CYP2C9; modafinil selectively inhibited CYP2C19; quinidine selectively inhibited CYP2D6; and ketoconazole selectively inhibited CYP3A4/5. For the MDIs (shown in **Table 4**), furafylline selectively inactivated CYP1A2; phencyclidine selectively inactivated CYP2B6 and was a direct inhibitor of CYP2D6; gemfibrozil glucuronide selectively inactivated CYP2C8; tienilic acid selectively inactivated CYP2C9; *S*-fluoxetine inactivated CYP2C19, caused slight MDI of CYP2B6 and was a direct inhibitor of CYP2D6; paroxetine caused marked MDI of CYP2D6 and direct inhibition of CYP2B6; troleandomycin selectively inactivated CYP3A4/5; and 1-ABT caused both direct and marked MDI of all CYP enzymes (consistent with it being a non-specific MDI of all CYP enzymes [see **Figure 2** for example data]). Overall, the inhibitors performed as expected, consistent with the known specificity towards each CYP enzyme.[2]

Conclusions

- Metabolic stability of eight compounds was determined with an *in vitro* screening assay yielding results consistent with the known metabolic profiles of the compounds.
- CYP inhibition screening of direct and MDIs demonstrated specificity of inhibitors towards specific enzymes with a cocktail substrate method.
- Overall, both high content and throughput assays are ideal for use by drug discovery groups to assist in lead identification and optimization.

Tables and Figures

Table 1. Summary of metabolic stability endpoints in liver microsomes from three species

Compound	Rat				Dog				Human			
	NADPH		NADPH+UDPGA		NADPH		NADPH+UDPGA		NADPH		NADPH+UDPGA	
	$t_{1/2}$ (min)	CL_{int} (µl/min/mg protein)	$t_{1/2}$ (min)	CL_{int} (µl/min/mg protein)	$t_{1/2}$ (min)	CL_{int} (µl/min/mg protein)	$t_{1/2}$ (min)	CL_{int} (µl/min/mg protein)	$t_{1/2}$ (min)	CL_{int} (µl/min/mg protein)	$t_{1/2}$ (min)	CL_{int} (µl/min/mg protein)
7-HC	>120	ND	19.6	70.8	>120	ND	3.70	376	>120	ND	2.73	508
7-EC	14.1	98.2	14.7	94.0	15.6	89.0	15.6	89.0	13.5	103	14.9	92.8
Verapamil	10.1	137	9.36	148	12.2	113	7.20	193	9.12	152	7.62	182
Propranolol	1.65	842	1.35	1020	14.9	92.8	11.1	125	59.2	23.4	56.8	24.4
Alprazolam	51.3	27.0	55.4	25.0	>120	ND	>120	ND	>120	ND	>120	ND
Diltiazem	1.67	828	1.44	964	27.7	50.0	23.7	58.6	48.8	28.4	37.5	37.0
Diclofenac	15.3	90.8	5.16	268	>120	ND	9.04	153	6.88	202	3.39	408
Testosterone	0.880	1580	0.765	1810	18.0	76.8	4.05	342	13.3	105	5.81	238

ND: Not determined

Table 2. Summary of metabolic stability endpoints in liver *S9* fractions from three species

Compound	Rat				Dog				Human			
	NADPH		NADPH+UDPGA		NADPH		NADPH+UDPGA		NADPH		NADPH+UDPGA	
	$t_{1/2}$ (min)	CL_{int} (µl/min/mg protein)	$t_{1/2}$ (min)	CL_{int} (µl/min/mg protein)	$t_{1/2}$ (min)	CL_{int} (µl/min/mg protein)	$t_{1/2}$ (min)	CL_{int} (µl/min/mg protein)	$t_{1/2}$ (min)	CL_{int} (µl/min/mg protein)	$t_{1/2}$ (min)	CL_{int} (µl/min/mg protein)
7-HC	>120	ND	21.7	63.8	>120	ND	18.7	74.4	>120	ND	12.7	109
7-EC	26.2	52.8	42.6	32.6	14.1	98.0	15.4	89.8	49.2	28.2	57.2	24.2
Verapamil	26.6	52.2	22.7	61.0	52.1	26.6	41.5	33.4	48.5	28.6	47.8	29.0
Propranolol	6.38	218	5.72	242	52.9	26.2	42.8	32.4	>120	ND	>120	ND
Alprazolam	117	11.8	108	12.8	>120	ND	>120	ND	>120	ND	>120	ND
Diltiazem	2.69	514	2.73	508	>120	ND	>120	ND	>120	ND	>120	ND
Diclofenac	35.4	39.2	17.9	77.4	>120	ND	23.3	59.4	33.8	41.0	15.2	91.0
Testosterone	1.69	818	1.52	910	31.5	44.0	9.79	142	28.5	48.6	16.7	83.0

ND: Not determined

Table 3. Summary of IC_{50} values for direct CYP inhibitors

Inhibitor	IC_{50} (µM)						
	CYP1A2	CYP2B6	CYP2C8	CYP2C9	CYP2C19	CYP2D6	CYP3A4/5
α-Napthoflavone	0.0024	>0.500	>0.500	>0.500	>0.500	>0.500	>0.500
Orphenadrine	>750	460	710	>750	>750	250	720
Montelukast	27	15	0.09	3.8	>30	>30	27
Sulfaphenazole	>3.00	>3.00	>3.00	0.71	>3.00	>3.00	>3.00
Modafinil	>500	>500	>500	>500	290	>500	>500
Quinidine	>3.00	>3.00	>3.00	>3.00	>3.00	0.28	>3.00
Ketoconazole	>0.300	>0.300	>0.300	>0.300	>0.300	>0.300	0.009

Table 4. Summary of IC_{50} values for metabolism-dependent CYP inhibitors

Inhibitor	IC_{50} (µM)													
	CYP1A2		CYP2B6		CYP2C8		CYP2C9		CYP2C19		CYP2D6		CYP3A4/5	
	0 min	30 min	0 min	30 min	0 min	30 min	0 min	30 min	0 min	30 min	0 min	30 min	0 min	30 min
Furafylline	>3.00	0.36	>3.00	>3.00	>3.00	>3.00	>3.00	>3.00	>3.00	>3.00	>3.00	>3.00	>3.00	>3.00
Phencyclidine	>100	>100	>100	2.6	>100	>100	>100	>100	>100	>100	33	25	>100	>100
Gemfibrozil glucuronide	>20.0	>20.0	>20.0	>20.0	>20.0	3.3	>20.0	>20.0	>20.0	>20.0	>20.0	>20.0	>20.0	>20.0
Tienilic acid	>0.800	>0.800	>0.800	>0.800	>0.800	>0.800	>0.800	0.068	>0.800	>0.800	>0.800	>0.800	>0.800	>0.800
S-Fluoxetine	>86.0	>86.0	>86.0	73	>86.0	>86.0	>86.0	>86.0	>86.0	2.9	1.5	1	>86.0	52
Paroxetine	>1.00	>1.00	0.32	0.61	>1.00	>1.00	>1.00	>1.00	>1.00	>1.00	>1.00	0.047	>1.00	>1.00
Troleandomycin	>22.5	>22.5	>22.5	>22.5	>22.5	>22.5	>22.5	>22.5	>22.5	>22.5	>22.5	>22.5	4.5	0.11
1-ABT	2200	65	5800	130	500	37	>10000	350	2400	370	1000	180	100	38

Figure 1. Metabolic stability of various compounds (1 µM) in incubations with HLM (0.5 mg/mL) with NADPH for 120 min

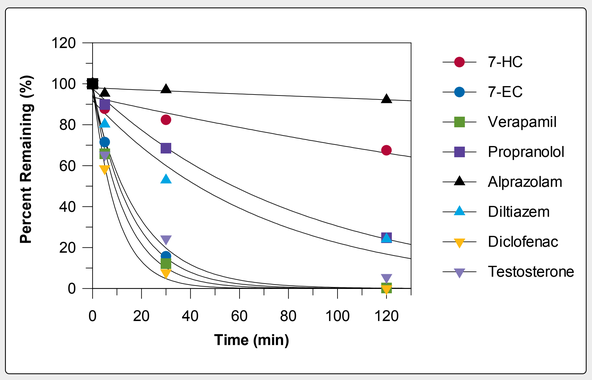
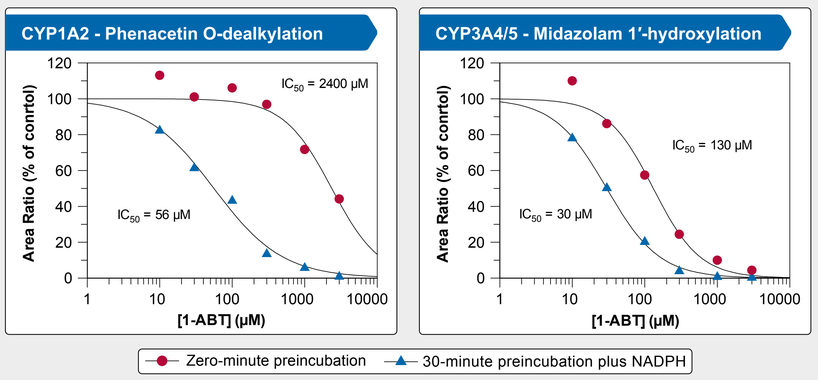


Figure 2. CYP1A2 and CYP3A4/5 inhibition by 1-ABT in HLM (0.1 mg/mL) with and without a 30 min preincubation



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