Using High-Resolution Mass Spectrometry and Software Aided Metabolite Assessment for Cytochrome P450 Reaction Phenotyping of Low Clearance Small Molecule Drugs

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Introduction

Understanding Cytochrome P450 (CYP) metabolism is an important (and FDA required) aspect of small molecule drug discovery for IND submission. CYP reaction phenotyping is routinely performed via measurement of in vitro metabolic clearance of potential substrates with liver microsomes and/or recombinant enzyme suspensions to estimation the percent (%) contribution to the total in vitro metabolic clearance of your drug candidate by one or more CYP isoforms. Low clearance compounds pose a major challenge for CYP phenotyping, as well as metabolic clearance determination. In addition, early and late metabolite identification studies may not necessarily provide sufficient information required to identify responsible enzymes and metabolic pathways for clearance and human metabolite profiles.

With many drug discovery programs aiming to reduce metabolic clearance to obtain longer half-life and lower dose drugs, characterizing metabolite profiles and potential drug-drug interactions (DDI) becomes a challenge. CYP phenotyping can be a labor intensive (and expensive) assay, therefore reliably identifying major enzymes involved in the metabolism of a drug candidate before investing in costly DDI studies during drug development is important. We offer the ability to measure and characterize major metabolites formed during CYP Phenotyping and other clearance related assays, useful for both high clearance and low clearance drug candidates. In this work we used high-resolution mass spectrometry (HRMS) to estimate the contribution of eight human cytochrome P450 enzymes (CYP 1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 3A4 and 3A5 isoforms) to the in vitro metabolism of various compounds in human liver microsomes, recombinant human CYP enzymes (rhCYPs), and long-term human hepatocyte co-cultures in the presence of selective CYP inhibitors. In addition to the metabolic clearance, metabolite formation was also monitored in the same sample. Data was acquired using liquid chromatography-high resolution mass spectrometry (LC-HRMS) and data-dependent MS/MS scans for metabolite identification and structure elucidation. Data is processed using Tracefinder or the MassMetasite and Webmetabase platform.

Methods

Cytochrome P450 (CYP) Reaction Phenotyping

The contribution of eight human CYP isoforms (1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 3A4 and 3A5) to the in vitro metabolism of various compounds evaluated in human liver microsomes (HLMs) and in recombinant human CYP isoforms (rhCYPs) in the presence and absence of the CYP cofactor NADPH. The disappearance of substrate was monitored at 37 °C over six timepoints (variable) to assess the NADPH-dependent metabolism.

Low Clearance Hepatocyte Stability and CYP3A4 Percent (%) Contribution to Metabolism

The intrinsic metabolic clearance for various compounds evaluated in human hepatocyte cocultures (Hepatopac). The metabolism of test articles were investigated over 168 hours using vendor specified conditions and metabolic clearance rates were obtained in the presence and absence of a CYP3A4 selective inactivator Erythromycin. The procedure was adapted from Chan, T.S. et al.[1]

Table 1: Matrices Used In this Work

Matrix	Protein/Cell Conc.
CYP1A2	10 pmol/mL
CYP2B6	10 pmol/mL
CYP2C8	10 pmol/mL
CYP2C9	10 pmol/mL
CYP2C19	10 pmol/mL
CYP2D6	10 pmol/mL
CYP3A4	10 pmol/mL
CYP3A5	10 pmol/mL
Human Liver Microsomes (HLM)	0.5 mg/mL
Human Hepatopac	~50000 cells/mL

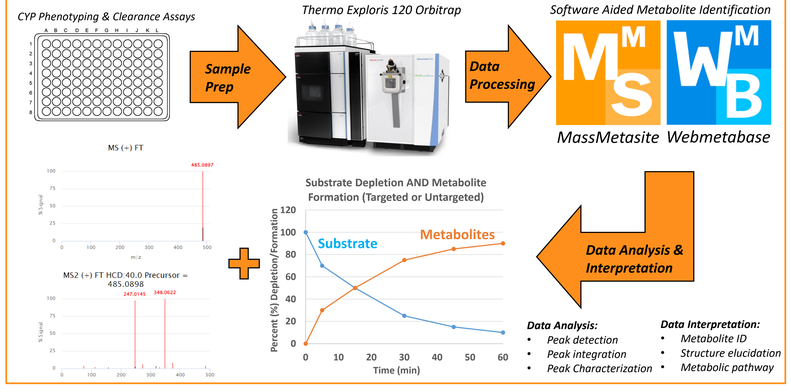
Table 2: Matrices Used In this Work

Substrates	Incubation Concentration	Major Known or Identified CYP Enzyme(s) Involved
Compound A	1 µM	CYP3A4/5, CYP1A2
Compound B	10 µM	CYP3A4, CYP3A5
Alprazolam[3,4]	1 µM	CYP3A4/5
Quinidine[4]	1 µM	CYP3A4/5
Furosemide[4]	1 µM	UGT (non-CYP)
Verapamil[5]	1 µM	CYP3A4/3A5, CYP2C8, and UGT (non-CYP)

Liquid Chromatography High Resolution Mass Spectrometry (LC-HRMS) Workflow for CYP Phenotyping via Analysis of Substrate and Metabolite

Data acquisition was performed using a Thermo Exploris 120 (Orbitrap) for in vitro matrix or media extracts at each time point and subsequent analysis of the rate of disappearance of test articles over time to determine the in vitro half-life and intrinsic clearance values. Additionally, targeted and untargeted metabolite analysis of the raw data was used to determined relative rate of formation of various metabolites in each matrix/condition. Data was processed using Molecular Discovery ONIRO/Webmetabase platform for parent depletion and UNTARGETED metabolite analysis. *Data obtained via Thermo Tracefinder for parent depletion and TARGETED metabolite analysis provided similar results and were not presented here.*

Figure 1: Metabolic Clearance and Metabolite Assessment Workflow Using LC-HRMS



Results

Figure 2: Software Aided In Vitro CYP Phenotyping of Alprazolam

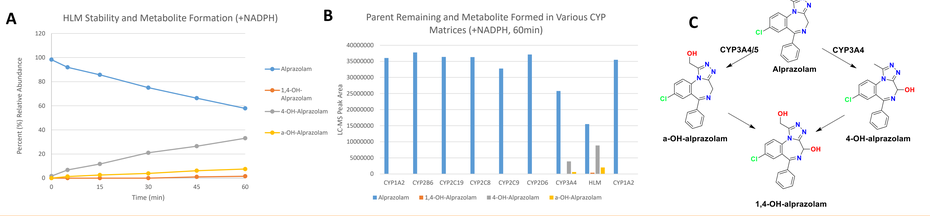


Figure 3: Software Aided In Vitro CYP Phenotyping of Low Clearance Compound A

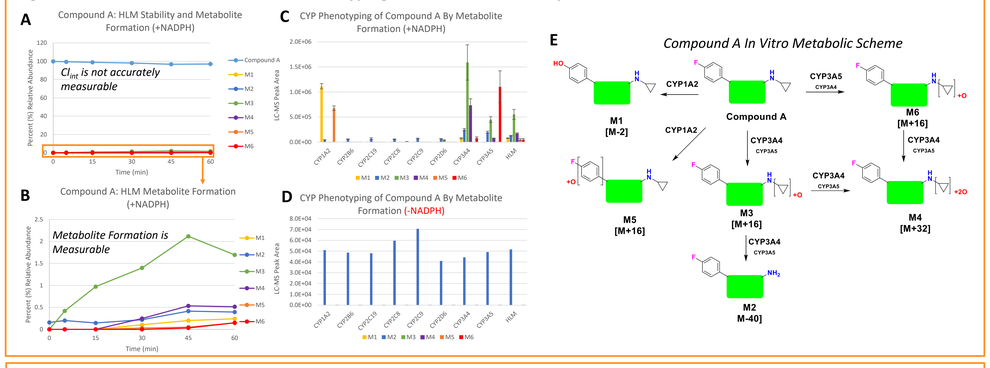
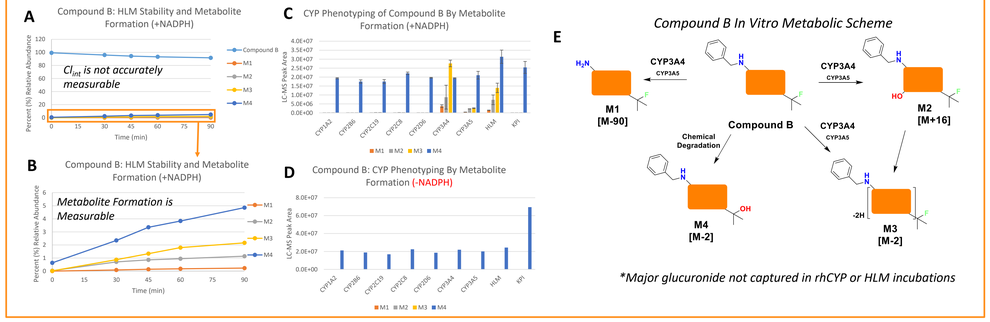
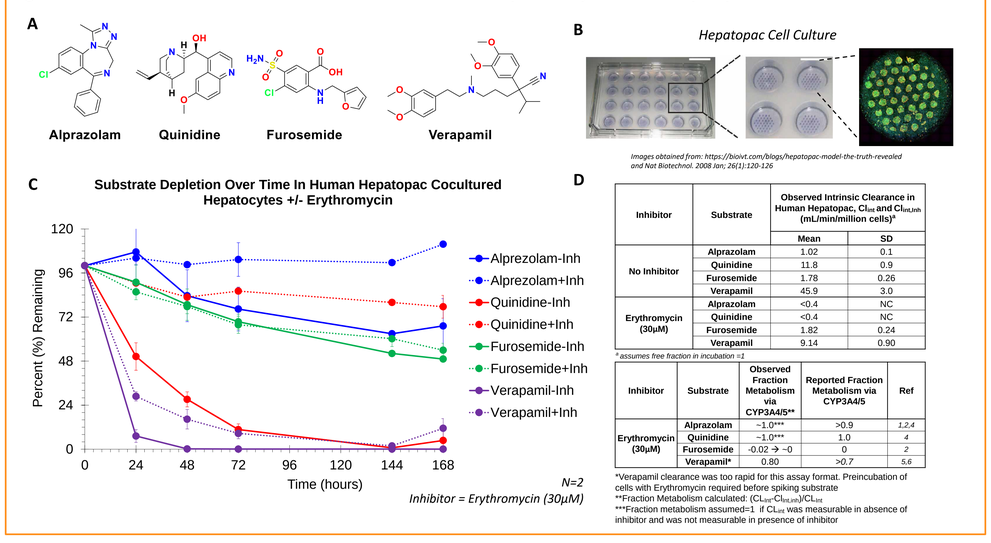


Figure 4: Software Aided In Vitro CYP Phenotyping of Low Clearance Compound B



*Major glucuronide not captured in rhCYP or HLM incubations

Figure 5: Estimation of In Vitro Metabolic Clearance of Various Compounds by CYP3A4 in Hepatopac



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Inhibitor	Substrate	Observed Intrinsic Clearance in Human Hepatopac, Cl_{int} and $Cl_{int,Inh}$ (mL/min/million cells)*	
		Mean	SD
No Inhibitor	Alprazolam	1.02	0.1
	Quinidine	11.8	0.9
	Furosemide	1.78	0.26
	Verapamil	45.9	3.0
Erythromycin (30µM)	Alprazolam	<0.4	NC
	Quinidine	<0.4	NC
	Furosemide	1.82	0.24
	Verapamil	9.14	0.90

*assumes free fraction in incubation =1

Inhibitor	Substrate	Observed Fraction Metabolism via CYP3A4/5**	Reported Fraction Metabolism via CYP3A4/5	Ref
Erythromycin (30µM)	Alprazolam	~1.0***	>0.9	*1,2,4*
	Quinidine	~1.0***	1.0	*4*
	Furosemide	-0.02 → ~0	0	*2*
	Verapamil*	0.80	>0.7	*5,6*

*Verapamil clearance was too rapid for this assay format. Preincubation of cells with Erythromycin required before spiking substrate
**Fraction Metabolism calculated: $(Cl_{int}-Cl_{int,Inh})/Cl_{int}$
***Fraction metabolism assumed=1 if Cl_{int} was measurable in absence of inhibitor and was not measurable in presence of inhibitor

Figure 6: Software Aided Estimation of In Vitro Clearance of Quinidine by CYP3A4 in Hepatopac

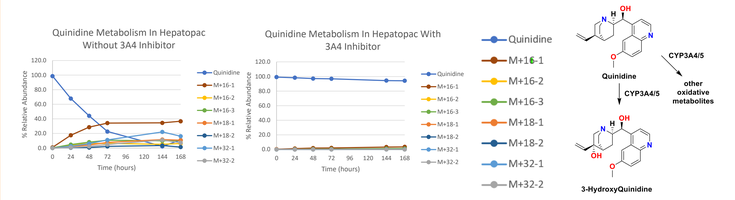
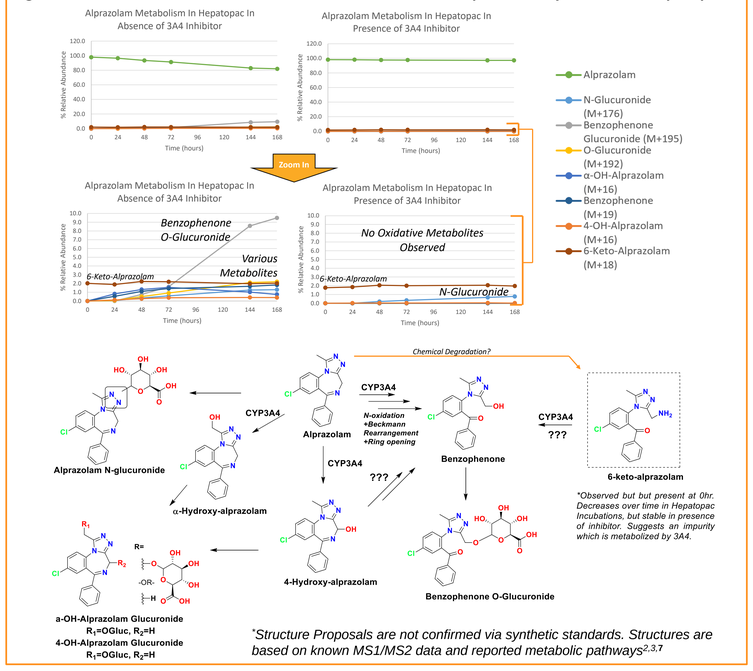


Figure 7: Software Aided Estimation of In Vitro Clearance of Alprazolam by CYP3A4 in Hepatopac



*Structure Proposals are not confirmed via synthetic standards. Structures are based on known MS1/MS2 data and reported metabolic pathways[2,3,7]

Summary of Conclusions

- Using LC-HRMS techniques, one may obtain metabolite specific (targeted or untargeted) phenotyping data without the need for synthetic standards. This allows scientists to assign metabolites and metabolic pathways to specific enzymes investigated, avoiding the need for sufficient activity to measure in vitro T1/2 of drug candidates.
- Coupling in vitro clearance data collected via LC-HRMS with metabolite identification software (Metasite/Webmetabase used here) streamlines data processing workflows for metabolite assessment and metabolite structure elucidation (using sample samples and raw data).
- Qualitative reaction phenotyping may be accomplished with low clearance compounds in traditional assays via metabolite formation (semi-quantitative and qualitative characterization) → *Software aided data processing by Metasite requires chemical structures*
- Analyzing clearance data via LC-HRMS enables structure identification of expected and unexpected metabolites in complex mixtures, enabling enzyme reaction phenotyping at different stages of programs.
- Enzyme phenotyping and contribution to in vitro metabolism for low clearance drugs can be assessed in long-term hepatocyte cocultures (Hepatopac used here) using selective inhibitors.[1]
- Reduction in clearance by inhibitors can be compared with changes in metabolite formation.

References

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