

# Comparison of Various High Throughput Mass Spectrometry-based Technologies to Assess CYP Inhibitions



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## Introduction

Cytochrome P450 (CYP) enzymes comprise an important family of human drug metabolizing enzymes (DMEs) responsible for the biotransformation of many drugs. Inhibition of DMEs has the potential to result in significant drug-drug interactions (DDI), which may yield severe toxicities in patients undergoing multiple drug regimens [1-2]. There has been much effort to assess CYP inhibition liabilities early in drug discovery [4] to avoid attrition late in development, however, this requires screening large number of drug candidates. Methods utilizing fluorescent probes specific for various CYP isoforms have been employed to expedite the screening process due to the high through-out nature of the assay, but the artificial nature of the assay conditions may lead to misprediction of the DDI potential. Using common drug probe substrates together with human liver microsome (HLM) may be more advantageous [3], but this requires LC-MS/MS analysis of samples which traditional has been a low through-put process. Developing higher throughput mass spectrometer based methods has been challenging due to their constraints of limited sample cycle time. In this study, several advanced higher throughput sample introduction technologies were evaluated. Specifically, RapidFire™, Laser Diode Thermal Desorption (LDTD) and Ultra-high pressure liquid chromatography (UPLC)-MS/MS were utilized to monitor the conversions of drug probe to its specific metabolite. The evaluation criteria are the reproducibility (%CV), the cycle-time for each sample (speed), sample preparation needs, the specificity of metabolite quantification and sensitivity of each platform.

## Method and Procedures:

Competitive CYP inhibition assays are traditionally used to determine the effect a drug candidate may have on CYP-mediated metabolism of selected probe substrates. Table 1 shows the incubation conditions of the CYP probes and inhibitors used in this study. The protein concentration was 0.25 mg/mL of HLM prepared in a pH 7.4 phosphate buffer. Reactions were initiated by adding NADPH and all samples were incubated in 300-µL round bottom 96-well plates at 37 °C for 10 min. The incubations were quenched by adding equal volume of cold acetonitrile containing 50 ng/mL of buccetin as internal standard (IS). After vortexing and centrifuging, the supernatant were transferred to a 96-well injection plate. The resulting samples were then analyzed by various mass spectrometry and sample introduction platforms in vendor labs as well as in house.

## Instrumentations:

The technology platforms evaluated here were RapidFire™ of BioTrove, Laser Diode Thermal Desorption (LDTD) of Thermo Fisher Scientific and UPLC-MS/MS of Applied Biosystems. RapidFire™ system applies on-line SPE sample clean-up and is compatible with any mass spectrometer. The LDTD source uses an infrared laser to thermally desorb samples that have been dried onto stainless steel sample wells in 96-well plate format. In LDTD-APCI MS, the desorbed gas-phase molecules pass by a corona discharge needle which results in ionization prior to analysis in a mass spectrometer. UPLC is performed on a Shimadzu UPLC system. The same batch of samples (with 8 inhibitor concentrations and Buccetin as IS) were analyzed in house as well using LC-MS/MS (Sciex 4000). For the LDTD evaluation, stable isotope labeled metabolites were used as IS for midazolam and diclofenac.

## Results and Discussions:

All technologies evaluated here monitored the specific metabolite of the probe substrates as listed in Table 1. Data were fitted using XLfitting. Figure 1 showed the representative inhibition curves and Table 2 listed all the IC50s generated during this evaluation

Table 1. The concentrations of inhibitors and probe substrates in the study.

CYP	Inhibitors	Highest Conc. (µM)	Probe Substrates and Biotransformation	Conc. (µM)
1A2	Furafylline	10	Phenacetin →→ acetaminophen	30
			Diclofenac →→ 4-OH-diclofenac	10
			Tolbutamide →→ 4-OH-tolbutamide	100
2C9	Sulfaphenazole	10	S-Mephenytoin →→ 4-OH-mephenytoin	50
2C19	Ticlopidine	10	Bufuralol →→ 1'-OH-bufuralol	5
			Dextromethorphan →→ Dextrorphan	5
2D6	Quinidine	5	Midazolam →→ 1'-OH midazolam	2
			Testosterone →→ 6β-OH testosterone	50
			Nifedipine →→ OH-nifedipine	10
3A4	Ketoconazole	5		

Figure 1. Inhibition curves of some CYP isoforms.

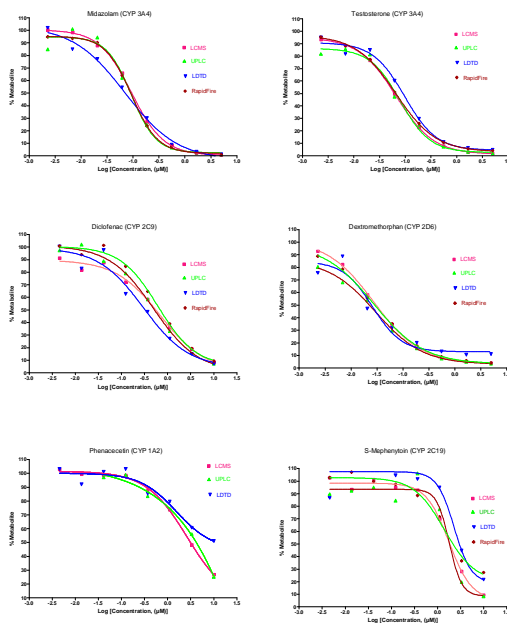


Table 2. Summary of IC50 values.

CYP Isoforms	Metabolites	LC-MS/MS	RapidFire™	LDTD	UPLC-MS/MS
1A2	Acetaminophen	3.14	Failed	N/A*	3.59
	OH-Diclofenac	0.555	0.693	0.585	0.520
2C9	OH-Tolbutamide	0.357	0.312	0.515	0.345
	OH-Mephenytoin	1.85	2.17	2.99	1.83
2D6	OH-Bufuralol	0.0170	0.0190	0.00800	0.0170
	Dextromethorphan	0.0310	0.0290	0.0250	0.0230
3A4	OH-Midazolam	0.0950	0.0910	0.0930	0.0900
	OH-Testosterone	0.0620	0.0650	0.0900	0.0660
	Oxidized Nifedipine	0.159	0.223	0.351	0.217

\* Data are not available.

## Results and Discussions

For the high throughput platforms which has no chromatographic separation, there might be issues with non-specificity associated with in-source fragmentation. An example is the conversion of CYP1A2 substrate phenacetin to acetaminophen illustrated in Figure 2. LC based technology has an advantage in overcome such issue by providing chromatographic separation between probe substrate and its metabolite of interest.

Figure 2. A representative chromatogram of acetaminophen and phenacetin in UPLC/MS/MS from Applied Biosystems

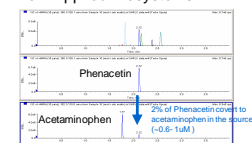


Table 3. A comparison of reproducibility, specificity, speed, sample preparation and price of all technologies evaluated.

	%CV	Speed	Sample Prep	Specificity
In-House LC/MS/MS	1-10%	252 sec	None	All probes
RapidFire	NA	3-4 sec	None	Select
LDTD	4-18%	4 sec dilution	All probes	All probes
ABI UPLC/MS/MS	1-14%	120 sec	None	All probes

## Conclusions

- A set of high-throughput mass spectrometry based platforms were evaluated for CYP inhibition assays
- These platforms significant decreases sample cycle times compared to traditional LC-MS/MS approaches
- The %CVs of all platforms evaluated were less than 20%, suggesting acceptable reproducibility
- The calculated IC50 values as well as inhibition curves were comparable to in-house data, suggesting acceptable performance
- These technologies will allow higher throughput mass spectrometry-based screening for CYP inhibition using drug probe substrates

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