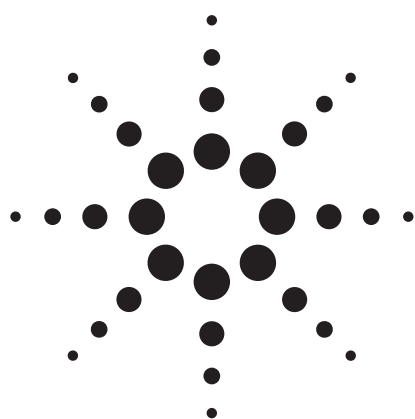


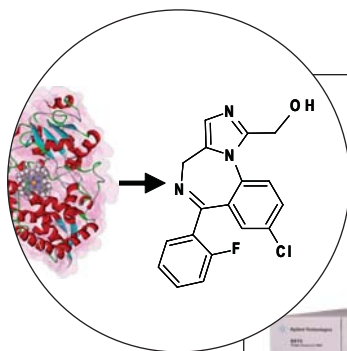


Ultra-high throughput determination of CYP450 enzyme inhibition through mass spectrometry

Approaching the throughput of fluorescence-based assays by combining an Agilent 6410 triple quadrupole mass spectrometer with a RapidFire RF-MS inlet system for CYP450 inhibition assays using microsomal enzyme preparations

Application Note

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Abstract

This Application Note describes how the BioTrove RapidFire high-throughput mass spectrometry (RF-MS) system interfaced to an Agilent 6410 triple quadrupole mass spectrometer was used for the characterization of the enzymatic reaction of CYP450 3A4/5 enzyme in a preparation of human liver microsomes and for the determination of IC₅₀ curves of potentially inhibiting drug compounds. The major features of this solution are:

- Ultra high throughput – mass spectrometry data is obtained at speeds approaching those of fluorescent assays.
- Fully compatible with liver microsomes – biologically relevant data is generated, as recommended by the September 2006 FDA Draft Guidance to Industry¹.
- Drug probes screened – the need for fluorogenic substrates is eliminated.
- Improved data quality – time-course data is obtained in the time required for end-point analysis by LC-MS.

Agilent Equipment

- 6410 triple quadrupole mass spectrometer
- MassHunter workstation software

BioTrove Equipment

- RapidFire RF-MS system
- RapidFire Integrator software

Application Area

- Drug Discovery
- Drug Development
- *In vitro* ADMETox



Introduction

Drug-drug-interactions (DDI) that cause inhibition of cytochrome P450 (CYP450) enzymes are a potentially serious threat for patients. This is because CYP450 enzymes are responsible for the metabolism of xenobiotics in the human body and a compound that is a substrate or an inhibitor of a specific CYP450 enzyme may alter the expected metabolic profile of another co-administered drug compound. For example, treatment of patients with a combination of terfenadine and ketoconazole has inadvertently caused hundreds of life threatening ventricular arrhythmias in patients because ketoconazole inhibits the CYP450 subfamily CYP 3A4/5, which is also responsible for the metabolism of terfenadine – resulting in a toxic accumulation of the latter².

In the past, testing for DDI activity in candidate drug compounds was performed using fluorescence-based assays in order to achieve the throughput required to screen large numbers of samples. The metabolic pathways of most fluorogenic probes are not specific to a single CYP450 isoform and so these fluorescent assays require the use of recombinant enzymes. In order to obtain more biologically relevant data, it is far preferable to use liver extracts or microsomal preparations rather than recombinant enzymes and for this analysis LC/MS has been the traditional approach.

Analysis by LC/MS is a serial method which, whilst considered the gold standard with regard to data quality, has not proven appropriate for high throughput analysis of large numbers of samples due to lengthy analysis times. Approaches to increase the throughput of LC/MS-based CYP450 inhibition assays include the use of pooled samples but, even with the pooling of eight individual assays and reasonably fast LC separation, the throughput of fluorescence assays or RF-MS assays (described here) can not be approached using this traditional method³.

In RapidFire DDI applications, the RF-MS system is used to analyze the competitive drug activity of test compounds as determined by the attenuation of the CYP450 metabolism of an unlabelled, isoform-specific native drug probe. The throughput of the system facilitates very fast enzymatic characterization, optimization of assay parameters for individual batches of liver microsome preparations (or recombinant enzymes), and screening of large numbers of test compounds.

Experimental

Equipment

- Agilent 6410 triple quadrupole mass spectrometer
- Agilent MassHunter workstation software (revision B.01.03) for data acquisition
- BioTrove RapidFire system
- BioTrove RapidFire integrator software (revision 2.0) for data analysis

RapidFire RF-MS method

Cartridge: Cartridge A – C4 material
Wash solution: water (0.09 % formic acid + 0.01 % trifluoroacetic acid)
Wash flow: 1.5 mL/min
Wash time: 3 s
Elution solution: acetonitrile:water (9:1, 0.09 % formic acid + 0.01 % trifluoroacetic acid)
Elution flow: 1.0 mL/min
Elution time: 3 s
Column equilibration: 500 ms
Injection volume: 10 µL
Total sample to sample time, including autosampler overhead, averages 7 seconds, with approximately 1 minute required for changing plates.

MS/MS (MRM) method

MRM conditions were optimized with matrix-free samples of the individual compounds, using flow injection analysis with varying fragmentor voltages for optimization of precursor ion, product ion and collision energy.
Ion source: ESI
Time filter: 0.1 min
DeltaEMV: 1800
Temperature: 350 °C
Gas flow: 13 L/min
Nebulizer pressure: 60 psi
Capillary voltage: 4000 V
MS1Res: wide
MS2Res: widest

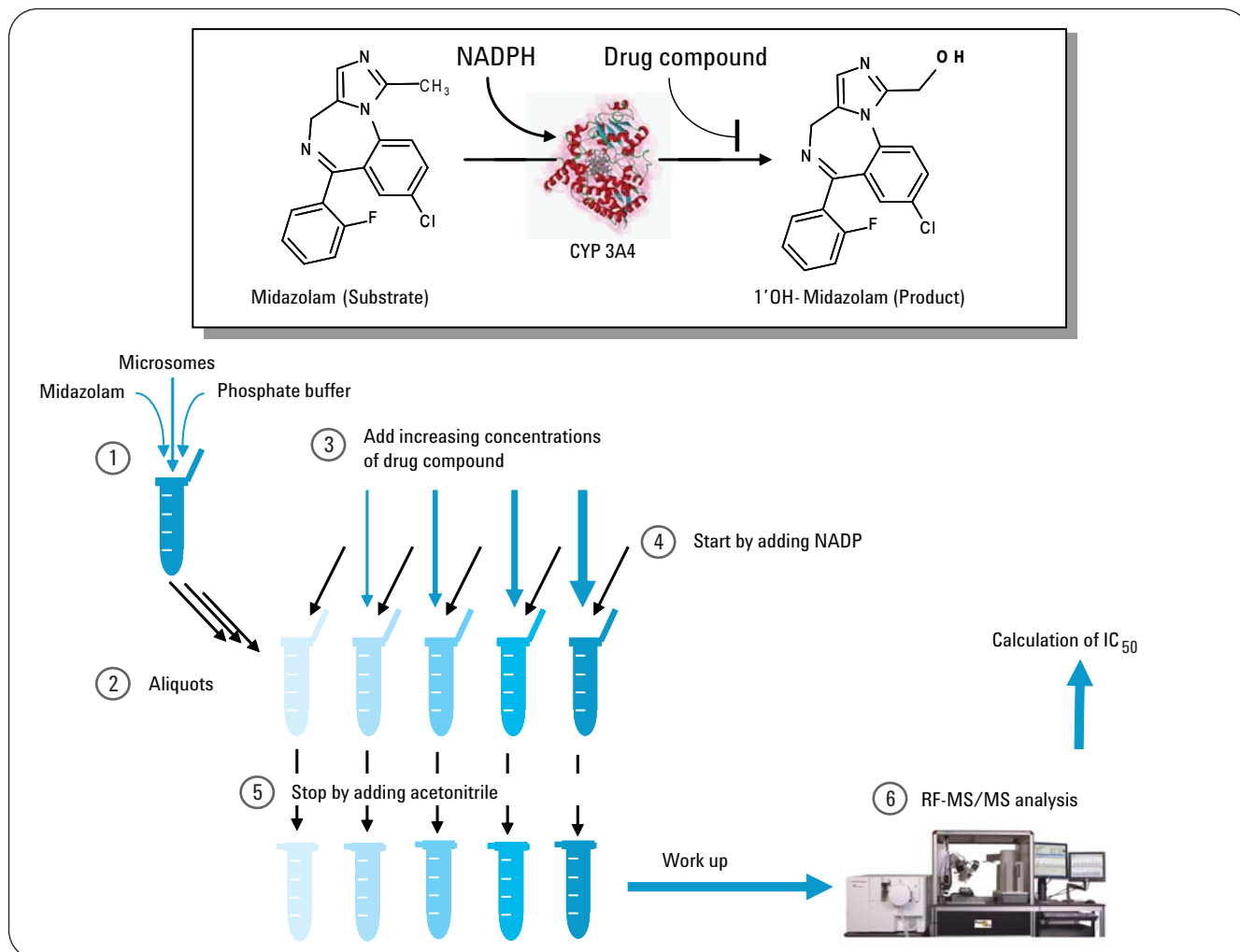


Figure 1
General CYP 3A4/5 enzymatic reaction converting midazolam substrate to 1'OH-midazolam.
To determine the IC_{50} values for an inhibiting drug compound, the assay is performed as in the scheme described above.

Human liver microsome CYP450 inhibition assay – protocol summary

CYP 3A4/5 substrate (midazolam) and the test compounds were pre-incubated with human microsomes in 100 mM phosphate buffer for 10 minutes (figure 1, step 1-3). The metabolic reactions were initiated by adding NADPH as the enzyme co-factor (step 4). The reactions were stopped by adding acetonitrile that contained

Compound	Polarity	Precursor ion [m/z]	Product ion [m/z]	Dwell time [ms]	Fragmentor voltage [V]	Collision energy [V]
1'OH Midazolam	pos	342.1	203.2	50	120	15
d4-1'OH Midazolam	pos	346.1	207.2	50	120	15

Table 1
MRM settings.

the internal standard for subsequent quantification (step 5). After a simple centrifugation, the samples were analyzed by RapidFire-

MS/MS analysis and the data evaluated (step 6). The general enzymatic reaction is shown at the top of figure 1.

Substrate
concentration: At stated concentrations (48.8 nM – 50 μ M)

Internal
standard (ISTD): D4-1'OH-midazolam
Final ISTD

concentration: 0.25 μ M

Microsomes: Human, pooled male and female from Xenotech (KS)

Microsome
concentration: At stated concentrations (25 – 150 μ g/mL)

NADPH solution: 1.3 mM, made fresh

Phosphate buffer: 100 mM, pH=7.4

Incubation

temperature: 37 °C

Final incubation

volume: 40 μ L in 384 well plate (Agilent)

Quench solution: 40 μ L acetonitrile (1 % formic acid, 0.5 μ M ISTD)

Final volume: 80 μ L

Sample

work-up: Reactions were stopped by adding 40 μ L of the quench solution containing the ISTD. Incubation plates were centrifuged at 1500 rpm for 5 min. and immediately read by the RF-MS system.

Results and discussion

System Description

The BioTrove RapidFire mass spectrometry (RF-MS) system uses an innovative microfluidic technology to aspirate an aliquot of sample directly from quenched assay plates and perform a solid-phase extraction for sample desalting/pre-processing. This allows the use of native, unlabelled drug-probes with microsomal liver preparations for CYP450 screening in a fraction of the time (< 8 seconds per sample) required for traditional LC/MS techniques

and approaching the throughput of fluorogenic assays. The RF-MS system comprises a plate stacker for up to 18 microtiter plates (using 384-well plates gives a sample capacity of 6912 samples), an xy-sampling stage, solvent pumps and a sampling head mounted to the z-axis of the stage holding the sample tip as well as the proprietary μ SPE cartridge and three unique high speed valves. The valves are used for solvent switching for washing the μ SPE cartridge, eluting the sample and re-equilibration of the μ SPE cartridge (figure 2).

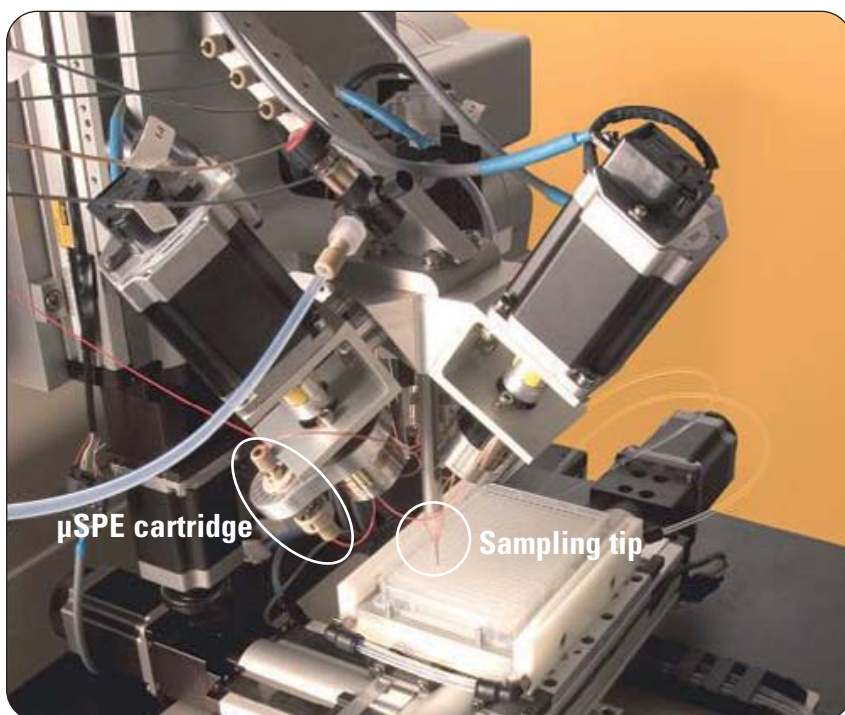


Figure 2
Close-up view of the RapidFire MS sampling stage. The assay plate is located on a xy-stage which also holds wash troughs containing aqueous and/or organic solvents for washing the aspirator and minimizing sample carryover. The sampling head is mounted on the z-axis and contains the sampling tip as well as the μ SPE column and three high speed valves for switching of wash and elution solvents.

Data is acquired and may be viewed in real time using Agilent MassHunter workstation software (figure 3) and final data analysis is performed using BioTrove RapidFire integrator software.

By utilizing the Agilent 6410 triple quadrupole MS for detection of the common CYP450 enzyme reaction products, issues commonly associated with fluorogenic assays are eliminated. Liver preparations or microsomes can be used rather than recombinant enzymes, fluorescence quenching or auto fluorescence effects of drug compounds is eliminated and the use of MRM detection gives very high specificity to further minimize false results.

Assay description

The RF-MS system was initially used to characterize the kinetics of the CYP 3A4/5 enzyme in a microsomal preparation of human liver samples. Different concentrations of human liver microsomes (HLM) and different substrate concentrations were incubated for 3, 5 and 7 minutes. The resulting kinetic plots are shown in Figure 4 a,b,c.

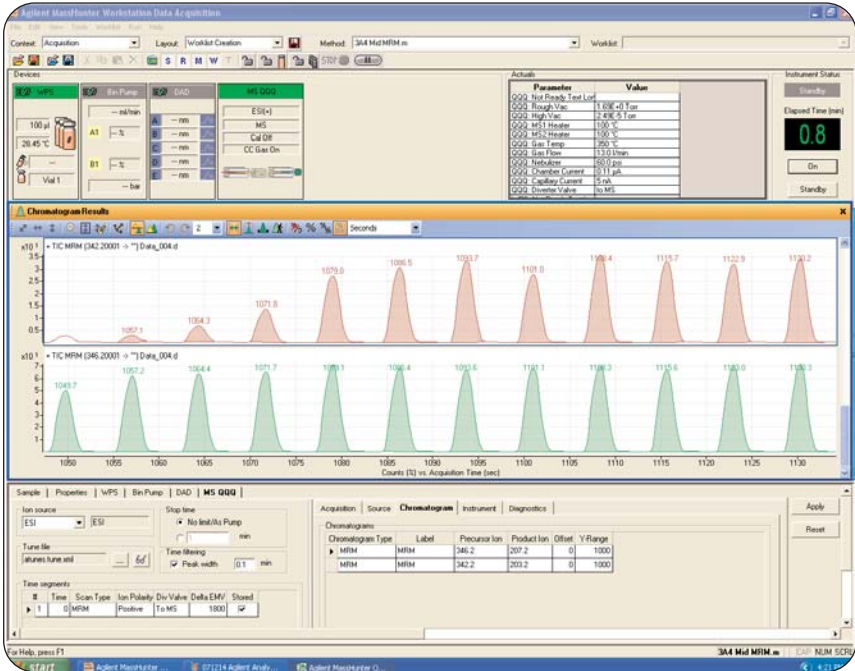


Figure 3
The Agilent MassHunter workstation for data acquisition showing the MRM chromatogram of the midazolam substrate and the ISTD (lower trace) during an IC₅₀ determination with a drug compound.

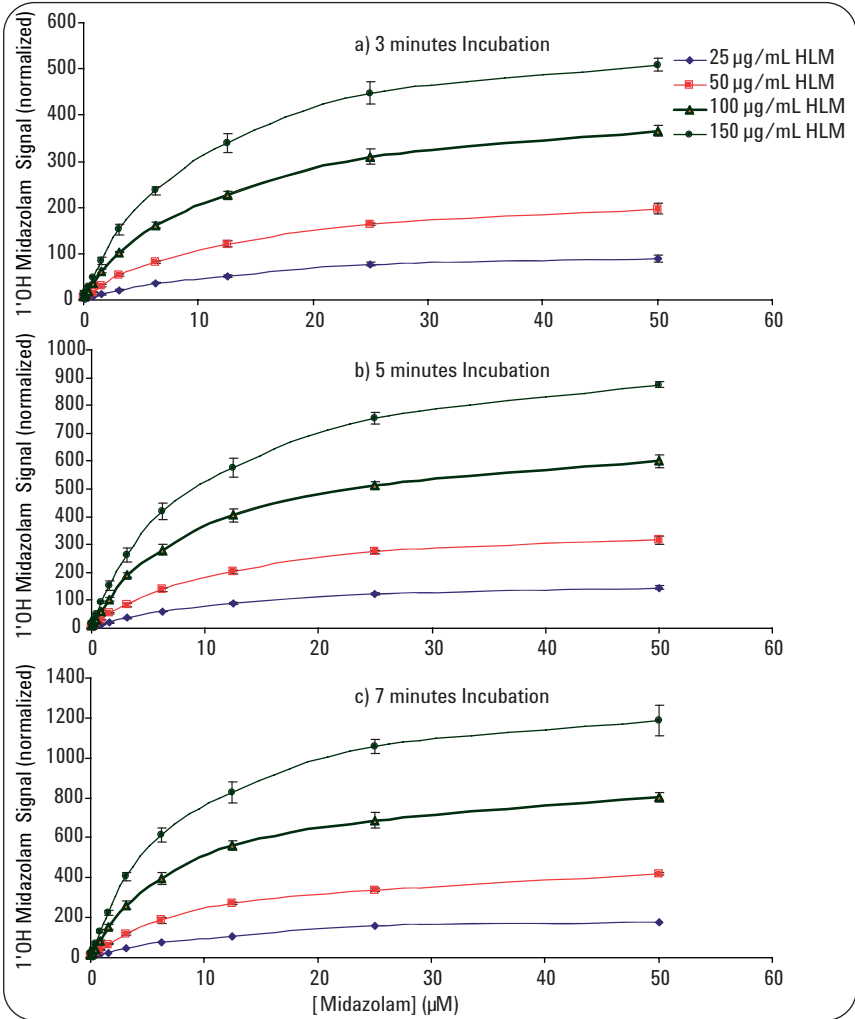


Figure 4
Enzyme kinetics of CYP 3A4/5 with 48 nM – 50 µM midazolam as substrate at 3, 5 and 7 minutes incubation time. Concentrations of human liver microsomes (HLM) ranged from 25 – 150 µg/mL.

The enzymatic time course at 50 μM midazolam substrate is shown in figure 5. The linearity of the enzyme reaction at increasing concentrations of HLM (at 50 μM midazolam concentration) is demonstrated in figure 6.

To measure the potential inhibition of a particular CYP450 isoform, test compounds are co-incubated with a known substrate that is specifically metabolized by that isoform of interest (the specific drug probe). In these experiments the probe midazolam was used as a specific probe for CYP 3A4/5 activity. In addition, other probes that are specific to CYP 3A4/5 have also been validated on the RF-MS system and include nifedipide, erythromycin, and testosterone (total hydroxylation). The appearance of the product (here 1'OH-midazolam) is monitored by triple quadrupole mass spectrometric analysis and normalized to an internal standard. IC_{50} curves are obtained by adding increasing amounts of the test drug compound and monitoring corresponding inhibition of the enzymatic reaction. Ketoconazole, a well-characterized and potent inhibitor of CYP 3A4/5 was used as an example (figure 7). During routine operations in the laboratories of the authors such CYP450 inhibition studies are performed using RF-MS at a throughput of approximately 4000 data points per 8-hour shift, which means that 10-point IC_{50} curves may be generated for over 400 test compounds per shift.

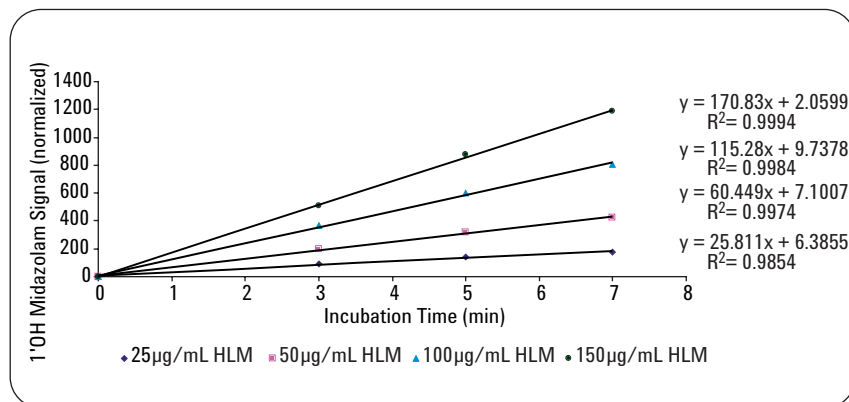


Figure 5
Enzyme CYP 3A4/5 time-course at 50 μM midazolam substrate.

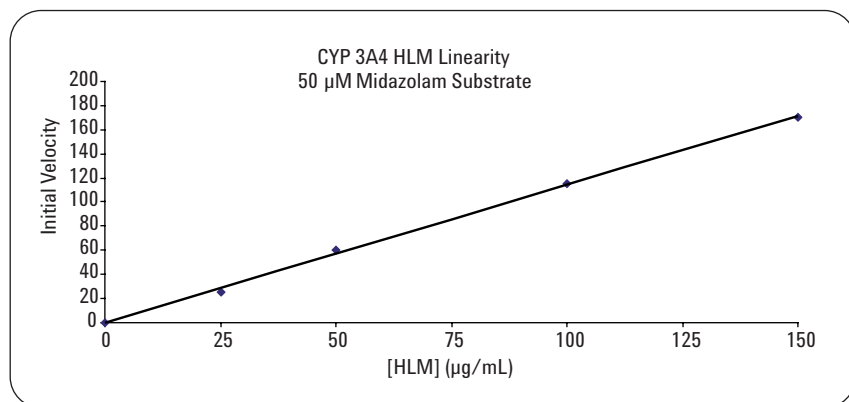


Figure 6
Linearity of the enzymatic reaction at increasing HLM concentrations (25 – 150 $\mu\text{g/mL}$) at 50 μM midazolam concentration.

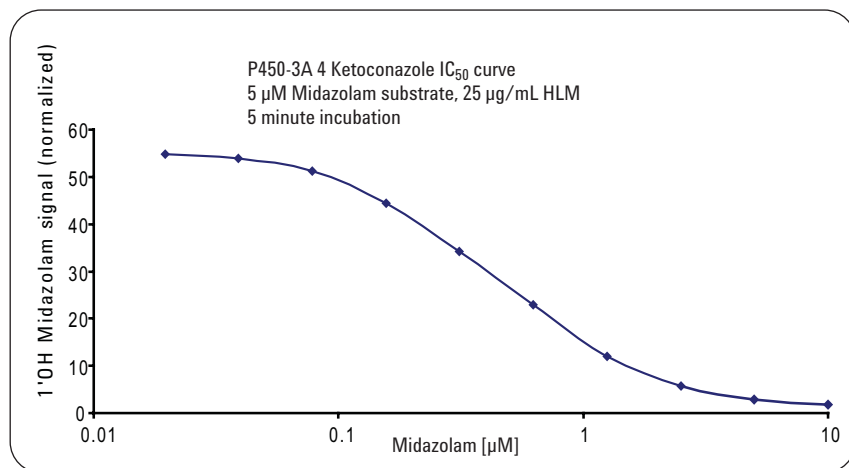


Figure 7
 IC_{50} curve of ketoconazole inhibition of oxidation of midazolam by CYP 3A4/5.
HLM concentration: 25 $\mu\text{g/mL}$, midazolam concentration: 5 μM , incubation time: 5 minutes.

Conclusion

RapidFire high-throughput mass spectrometry (RF-MS) system interfaced to an Agilent 6410 triple quadrupole mass spectrometer is a fully integrated and automated sample preparation and analysis system capable of cycle times from injection to injection of as little as 6 seconds per sample. The RF-MS system uses an online solid-phase extraction system to desalt samples. The workflow, data analysis, and throughput is similar to that of a fluorogenic assay using a 96/384-well plate reader, yet the system is fully compatible with non-fluorescent probes and microsomal enzyme preparations. Most important, no additional sample preparation other than required for fluorescence assays are required for the RF-MS system in contrast to other MS/MS-based detection systems achieving a similar throughput. The increased capacity created by the throughput of the RF-MS system affords customers the ability to perform CYP450 screening on more compounds at an earlier stage of drug development. RF-MS is routinely used in many applications including the high throughput screening of previously intractable drug targets, cytochrome P450 inhibition and other ADME assays and directed evolution studies.

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