High-Throughput Analysis of Cytochrome P450 Inhibition in Intact Human Hepatocytes

Application Note

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Abstract

Ultrafast methods of analysis were developed using the Agilent RapidFire High-Throughput Mass Spectrometry System in combination with an Agilent 6490 Triple Quadrupole Mass Spectrometer for cytochrome P450 (CYP) inhibition in human hepatocytes. The enzymatic activities of six major liver cytochrome P450 isoforms were evaluated using probe substrates under the application of 15 different model inhibitors. Dose-response curves and IC_{50} values were determined using seven inhibitor concentration points. Methods of analysis were all 13 seconds or less per sample, enabling ultrafast determination of CYP inhibition.

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Introduction

CYP inhibition is an important *in vitro* ADME assay for predicting potential adverse *in vivo* drug-drug interactions. The Agilent RapidFire High-Throughput Mass Spectrometry System has been successfully adopted for ultrafast analysis of CYP inhibition in human liver microsomes (HLMS) with comparable results to LC/MS/MS[1-3]. This Application Note investigates the application of the RapidFire system with an Agilent 6490 Triple Quadrupole Mass Spectrometer for analysis of CYP inhibition in another *in vitro* experimental system, human hepatocytes. The advantages of using hepatocytes over HLMs or recombinant CYP450s include the retention of an intact plasma membrane and uptake transporters. Multiple enzymatic pathways are also present with physiological concentrations of enzymes and cofactors. In the present study, the inhibition of CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2D6, and CYP3A4 activities were evaluated in the presence of 15 model inhibitors.

Experimental

The RapidFire/MS/MS system consisted of the following modules: an Agilent RapidFire 365, an Agilent 6490 Triple Quadrupole Mass Spectrometer, Agilent MassHunter Qualitative Analysis Software B.05.00, and Agilent MassHunter Quantitative Analysis Software B.05.00.

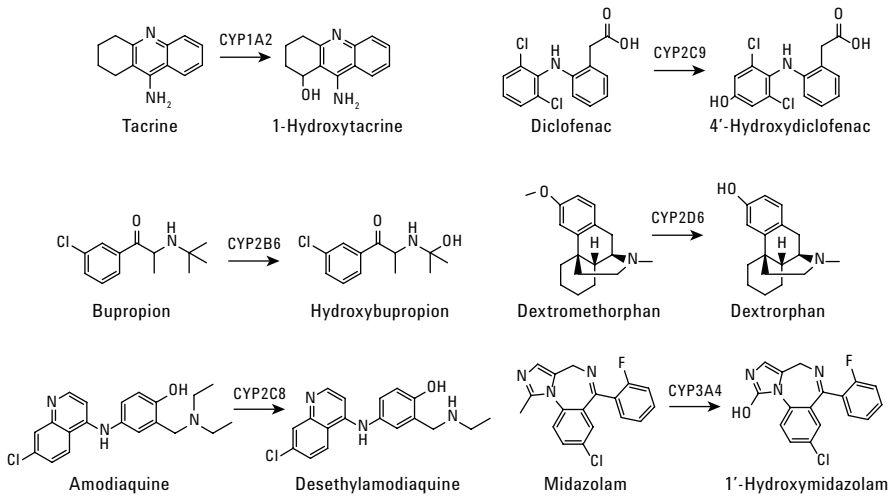

Figure 1. CYP substrate probe and metabolite structures.

Table 1. Agilent RapidFire/MS/MS conditions.

	CYP1A2	CYP2B6	CYP2C8	CYP2C9	CYP2D6	CYP3A4
Agilent RapidFire conditions						
State 1, aspirate	Sip sensor	Sip sensor	Sip sensor	Sip sensor	Sip sensor	Sip sensor
State 2, load/wash (ms)	3,000	3,000	3,000	3,000	3,000	3,000
State 3, elute (ms)	6,000	6,000	3,000	4,000	4,000	4,000
State 4, re-equilibrate (ms)	1,000	1,500	1,500	1,500	1,500	1,000
Injection volume (µL)	10	10	10	10	10	10
Flow to triple quadrupole (mL/min)	0.8	0.8	1.25	1.25	1.25	1.25
Agilent 6490 Triple Quadrupole Mass Spectrometer conditions						
Polarity	Positive	Positive	Positive	Positive	Positive	Positive
Gas temperature (°C)	150	150	200	200	200	200
Drying gas flow (L/min)	17	15	17	17	17	17
Nebulizer (psi)	35	40	45	45	45	45
Sheath gas temperature (°C)	400	400	400	400	400	400
Sheath gas flow (L/min)	11	11	11	11	11	11
Capillary voltage (V)	3,000	3,500	3,000	3,000	3,000	4,000
Nozzle voltage (V)	0	0	1,500	1,500	1,500	500

Table 2. MRM parameters.

Compound	Q1	Q3	CE	CAV
1-Hydroxytacrine	215.3	182.1	15	4
1-Hydroxytacrine-d4	219.3	186.1	15	4
Hydroxybupropion	256.1	184	8	7
Hydroxybupropion-d6	261.1	184	8	7
Desethylamodiaquine	328.1	283	10	7
Desethylamodiaquine-d3	331.1	283	10	7
4′-Hydroxydiclofenac	312	231.1	15	7
4′-Hydroxydiclofenac-[13C6]	318	237.1	15	7
Dextrorphan	258.1	201.1	15	7
Dextrorphan-d3	261.1	201.1	15	7
1′-Hydroxymidazolam	342.1	203	27	2
1′-Hydroxymidazolam-[13C3]	345.1	206	27	2

Chemicals and reagents

The isoform-selective P450 substrates: tacrine, bupropion, amodiaquine, diclofenac, dextromethorphan, and midazolam were obtained from Sigma-Aldrich, St. Louis, MO. Metabolite standards and internal standards were obtained from the following sources: hydroxybupropion-[D6], desethylamodiaquine-[D3], 4′-hydroxydiclofenac-[13C6], dextrorphan-[D3], 1′-hydroxymidazolam-[13C3], 1′-hydroxymidazolam (BD Biosciences, Franklin Lakes, NJ); 1′-hydroxytacrine (VWR, Radnor, PA); desethylamodiaquine, 4′-hydroxydiclofenac (Cerilliant, Round Rock, TX); 1′-hydroxytacrine-[D4] (TLC PharmaChem, Vaughan, Ontario, Canada); hydroxybupropion (Sigma Aldrich, St. Louis, MO). All inhibitors were purchased from Sigma-Aldrich, St. Louis, MO. LC/MS grade solvents were used for mass spectrometry.

Human hepatocytes

Cryopreserved human hepatocytes from five male and five female donors were used for the study. The human hepatocytes used in this study were isolated and cryopreserved at In Vitro ADMET Labortories, (Columbia, MD) using previously published methods for hepatocyte isolation and cryopreservation[4]. Hepatocytes were thawed once and pooled prior to use in the CYP inhibition assay.

CYP inhibition assay

To 384-well plates, 10 µL of protein-free Hepatocyte Incubation Medium (HIM; IVAL, Columbia, MD) was added, containing 4x dosing concentration of substrate (metabolism plates) or solvent control (viability plates). Final substrate concentrations were as follows: 5 µM tacrine, 400 µM bupropion, 7.5 µM amodiaquine, 25 µM diclofenac, 25 µM dextromethorphan, and 15 µM midazolam. Next, 10 µL of 4x dosing concentration of inhibitors in HIM was added. Seven different concentrations were used for each inhibitor with final concentrations ranging between 0 and 200 µM. Metabolism was initiated by the addition of 20 µL of human hepatocytes. Plates were incubated for 60 minutes and reactions were quenched with 40 µL of acetonitrile containing internal standards. Plates were centrifuged prior to analysis by RapidFire/MS/MS.

Data analysis

MassHunter Qualitative Analysis Software (B.05.00) and Quantitative Analysis Software (B.05.00) were used for data analysis.

Results and Discussion

RapidFire/MS/MS parameters were optimized for individual P450 probe substrates and corresponding metabolites (Tables 1 and 2). For each P450 isoform, 15 model inhibitors were evaluated at seven concentrations in triplicate. All samples were analyzed at a rate of 13 seconds or less, enabling a throughput of more than 275 samples/hour. Isoform-selective and dose-dependent inhibition was observed, with results consistent with the known properties of the model inhibitors (Figure 2). Percentage relative activity for the dose-response plots was determined using the following equation:

100 × (Activity corrected for viability of inhibitor-treated hepatocytes)/(Activity corrected for viability of solvent control hepatocytes)

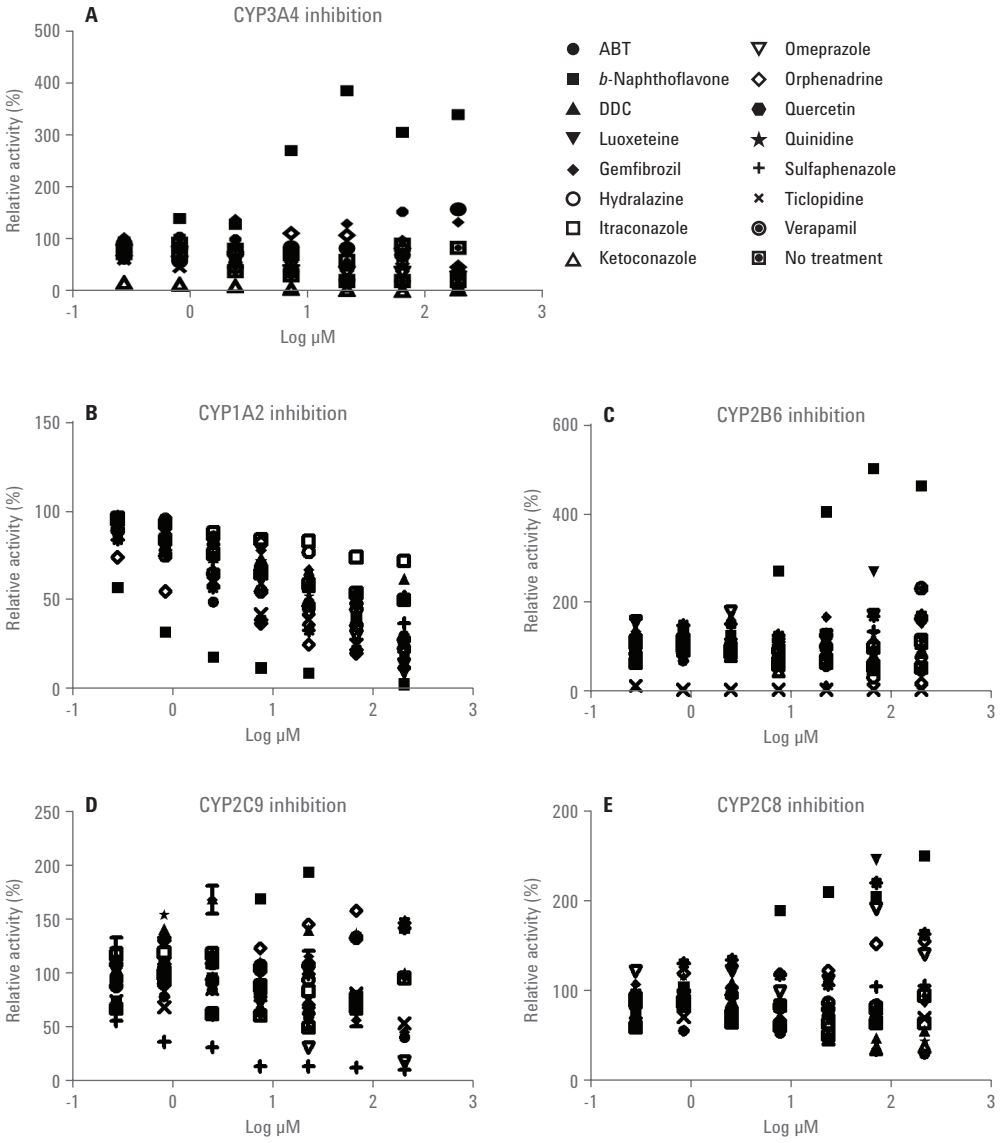

Figure 2. Dose-response curves.

One inconsistency found was the lack of CYP2C8 inhibition by gemfibrozil. A prolonged pre-incubation period to allow accumulation of the inhibitory glucuronide metabolite may be required. Another interesting finding was the lack of inhibition of CYP2C9 and CYP2D6 by 1-aminobenzotriazole. One example of relatively potent inhibition was inhibition of CYP3A4 by quinidine. IC_{50} values were calculated from the seven point curves for each inhibitor (Table 3).

Conclusions

A high-throughput method of analysis was developed for evaluation of isoform-specific P450 inhibition in cryopreserved human hepatocytes. Isoform-selective and dose-dependent inhibition was observed for CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2D6, and CYP3A4. Rates of analysis were ultrafast at less than 13 seconds per sample. This ultrafast system may also be useful for similar *in vitro* ADME assays.

Table 3. IC_{50} values (µM).

Chemical	CYP1A2	CYP2B6	CYP2C8	CYP2C9	CYP2D6	CYP3A4
1-Aminobenzotriazole	12.1	57.2	7.9	> 200	> 200	2.0
b-Naphthoflavone	< 0.27	> 200	> 200	> 200	> 200	> 200
Diethyldithiocarbamate	> 200	> 200	> 200	> 200	> 200	> 200
Fluoxetine	> 200	> 200	> 200	> 200	> 200	31.8
Gemfibrozil	> 200	> 200	> 200	> 200	> 200	> 200
Hydralazine	> 200	> 200	> 200	> 200	> 200	> 200
Itraconazole	> 200	78.2	> 200	> 200	> 200	2.9
Ketoconazole	> 200	62.4	35.4	> 200	> 200	< 0.27
Omeprazole	37.0	> 200	> 200	35.5	> 200	5.3
Orphenadrine	3.8	> 200	> 200	> 200	16.8	> 200
Quercetin	31.2	> 200	> 200	> 200	> 200	> 200
Quinidine	53.5	> 200	> 200	> 200	< 0.27	1.8
Sulfaphenazole	> 200	> 200	> 200	< 0.27	> 200	89.3
Ticoplidine	3.1	< 0.27	> 200	> 200	> 200	22.1
Verapamil	> 200	> 200	> 200	> 200	> 200	2.5

> 200
100–200
> 10–< 100
> 1–< 10
< 1

References

1. Miller, V. High-Throughput In Vitro ADME Analysis with Agilent RapidFire/MS Systems: Cytochrome P450 Inhibition, *Agilent Technologies Application Note*, publication number 5990-9184EN, **2011**.

2. Danker, K., and Schlicht, K.E. Optimization of Ultrafast CYP3A4 Inhibition and Multiplexed CYP Inhibition Assays Using the RapidFire High-Throughput Mass Spectrometry System, *Agilent Technologies Application Note*, publication number 5991-3693EN, **2013**.

3. Miller, V. SPE-MS analysis of absorption, distribution, metabolism and excretion assays: a tool to increase throughput and steamline workflow. *Bioanalysis* **2012**, *4(9)* pp 1111–1121.

4. Li AP. Human hepatocytes: isolation, cryopreservation and applications in drug development. *Chem. Biol. Interact*. **2007**, *168(1)* pp 16-29.

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