Optimization of Ultrafast CYP3A4 Inhibition and Multiplexed CYP Inhibition Assays Using the RapidFire High-Throughput Mass Spectrometry System

Application Note

Authors

Kerstin Danker
Evotec AG
Essener Bogen 7
22419 Hamburg, Germany

Kari E. Schlicht
Agilent Technologies Inc.
Wakefield, MA

Abstract

The MRM parameters of an Agilent 6460 Triple Quadrupole LC/MS System were optimized for enzymatic activity analysis of cytochrome P450 isoform 3A4 (CYP3A4) under the application of testosterone as a probe substrate and an Agilent RapidFire high-throughput mass spectrometry system. Detection of the reaction product, 6β-hydroxytestosterone, and the internal standard, 6β-hydroxytestosterone-D7, by the presented method is linear over a range of 0.05–25 µM and appropriate for ultrafast assessment of CYP3A4 activity at 8.6 s/sample. Similar optimization was followed to develop the simultaneous analysis of the enzymatic activity of cytochrome P450 isoforms CYP3A4, CYP2C9, and CYP2D6. The presented multiplex method is linear over the range of 0.1–100 µM and possess a lower limit of quantitation (LLOQ) of 0.1 µM for OH-diclofenac and dextrorphan, and 0.5 µM for OH-testosterone. At a sampling rate of 10 seconds per sample, this method is suitable for ultrafast determination of IC50 curves of potentially inhibiting compounds of CYP3A4, CYP2C9, and CYP2D6.

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Introduction

Pharmacokinetic interaction between drug compounds often leads to severe alterations of the therapeutic effect of drugs and are mainly responsible for their severe side effects. Consequently, drug-drug interaction (DDI) studies are conducted during the whole drug discovery and drug development process to investigate the inhibition or activation potential of new drug candidates with existing xenobiotics. In humans, the cytochrome P450 enzyme isoforms CYP1, CYP2, and CYP3 are predominately responsible for the metabolism of a large number of therapeutic agents and xenobiotics. To characterize DDIs for new compounds, CYP-isoform selective substrates have been approved[1]. Testosterone is a commonly used probe substrate for assessing the CYP3A4 inhibition potential of drug candidates by monitoring the formation of the metabolite 6β-hydroxytestosterone[1]. Diclofenac is a CYP2C9-specific substrate biotransformed to 4'-hydroxydiclofenac[1]. Dextromethorphan is metabolized to dextrorphan exclusively by CYP2D6 (Table 1)[1]. In the past, LC/MS/MS detection methods have been shown to be highly selective and sensitive for inhibition analysis. However, long duty cycles led to low throughput and made LC/MS/MS unfavorable for high throughput screening in drug discovery and drug development. The Agilent RapidFire High-Throughput Mass Spectrometry System is a reliable tool for fast and accurate analysis of CYP450 enzyme inhibition[2,6,7]. This Application Note describes optimized parameters for the determination of CYP3A4 activity and an optimized method for the simultaneous determination of CYP3A4, CYP2C9, and CYP2D6 activity using a RapidFire high throughput mass spectrometry system interfaced to an Agilent 6460 Triple Quadrupole LC/MS System.

Table 1. Isoform-specificity of FDA recommended drug probes and recommended inhibitors for CYP P450 assays[1].

CYP-isoform	Substrate	Product	Inhibitor
CYP3A4	Testosterone	6β-Hydroxytestosterone	Ketoconazole
CYP2C9	Diclofenac	4'-Hydroxydiclofenac	Sulfaphenazole
CYP2D6	Dextromethorphan	Dextrorphan	Quinidine

Experimental

CYP3A4 assay

CYP3A4 inhibition assays were performed similar to described procedures[3]. Testosterone was used as substrate and OH-testosterone-D7 was spiked as internal standard (IS).

Multiplexed CYP inhibition assay

Multiplexed CYP inhibition assays were performed as described previously[2] except that testosterone, dextromethorphan, and diclofenac were used as substrates and 6β-OH-testosterone-D7, dextrorphan-D3, and 4'-hydroxydiclofenac-D4 were spiked as internal standards (IS).

Systems

The RapidFire/MS/MS system consisted of the following modules:

- Agilent RapidFire 300 High Throughput Mass Spectrometry System (p/n 5990-7966EN)
- Agilent 6460 Triple Quadrupole LC/MS System (G6460A)

Software

- Agilent MassHunter Triple Quadrupole Acquisition Software B.04.01 with Qualitative Analysis B.05.00
- RapidFire Integrator 3.4.0 for peak integration

RapidFire/MS/MS conditions

Table 2. Agilent RapidFire instrument conditions, previously optimized[4].

RapidFire conditions	
Pump 1	Water with 0.09 % formic acid and 0.01 % TFA at a flow rate of 1.5 mL/min
Pumps 2 and 3	Acetonitrile with 0.1 % formic acid at a flow rate of 1.25 mL/min
SPE cartridge	Agilent RapidFire cartridge A: reversed phase C4 chemistry (p/n G9203A)
Injection volume	10-µL injection loop
Plate format	Costar 384-well PP plates

Table 3. Agilent RapidFire SPE cycle timing for CYP inhibition assays.

CYP3A4 assay		Multiplex CYP assay	
RF State 1 (aspirate)	600 ms	RF State 1 (aspirate)	600 ms
RF State 2 (load/wash)	3,000 ms	RF State 2 (load/wash)	3,000 ms
RF State 3 (elute)	2,000 ms	RF State 3 (elute)	3,600 ms
RF State 4 (re-equilibrate)	500 ms	RF State 4 (reequilibrate)	500 ms

MRM conditions were optimized with matrix-free samples of the individual analytes through continuous infusion of 75 µM solutions at a flow rate of 25 µL/min using an external syringe pump. Various fragmentor voltages, collision energies, and cell accelerator voltages were tested for optimized detection of each precursor and product ion.

For the multiplex CYP inhibition assay, MRM conditions were optimized with matrix-free samples of the individual compounds through continuous infusion of the tuning solution (10 µM solution for each IS, 100 µM solution for each product) at 10 µL/min using an external syringe. Various fragmentor voltages, collision energies, and cell accelerator voltages were tested for optimized detection of precursor and product ion. Q1 and Q3 resolution for OH-testosterone and OH-testerone-D7 were wide and widest respectfully as part of the multiplex method with 4′-OH-diclofenac and dextrorphan.

Source conditions were optimized under final assay and RapidFire analysis conditions. For this purpose, control incubation samples containing OH-testosterone at low (full CYP3A4 inhibition) and high (no CYP3A4 inhibition) concentration were analyzed in alternating manner. The resulting 6β-OH-testosterone signal areas were used for calculation of statistical parameters as criteria for optimization. Resolution, gas temperature, gas flow, nebulizer, sheath gas temperature, sheath gas flow, capillary voltage, and nozzle voltage were varied to identify best readout parameters. All selectivity combinations of quadrupole 1 (Q1) and 3 (Q3) were tested and evaluated by the resulting Z′-value (Figure 1)[5].

Table 4. Optimized MRM conditions for CYP3A4 inhibition.

MRM conditions	OH-Testosterone	OH-Testosterone-D7
Chemical formula	$C_{19}H_{28}O_3$	$C_{19}H_{21}O_3D_7$
Ionization mode	Positive ESI	Positive ESI
Parent ion	$[M+H]^+$	$[M+H]^+$
Q1 (*m/z*)	305.1	312.1
Q3 (*m/z*)	269.1	276.1
Resolution Q1	Unit	Unit
Resolution Q3	Widest	Widest
Fragmentor	150	150
Collision energy (CE)	8	8
Cell accelerator voltage (CAV)	7	7

Table 5. Optimized MRM conditions for CYP2C9 and CYP2D6 inhibition.

MRM conditions	4′-OH-Diclofenac	4′-OH-Diclofenac-D4	Dextrorphan	Dextrorphan-D3
Chemical formula	$C_{14}H_{11}Cl_2NO_3$	$C_{14}H_7Cl_2NO_3D_4$	$C_{17}H_{23}NOO$	$C_{17}H_{20}NOD_3$
Ionization mode	Positive ESI	Positive ESI	Positive ESI	Positive ESI
Parent ion	$[M+H]^+$	$[M+H]^+$	$[M+H]^+$	$[M+H]^+$
Q1 (*m/z*)	312.0	316.1	258.2	261.2
Q3 (*m/z*)	230.0	234.0	157.0	157.0
Resolution Q1	Wide	Wide	Wide	Wide
Resolution Q3	Widest	Widest	Widest	Widest
Fragmentor	135	135	175	175
CE	20	20	40	40
CAV	6	6	1	1
Dwell time (ms)	50	50	65	65

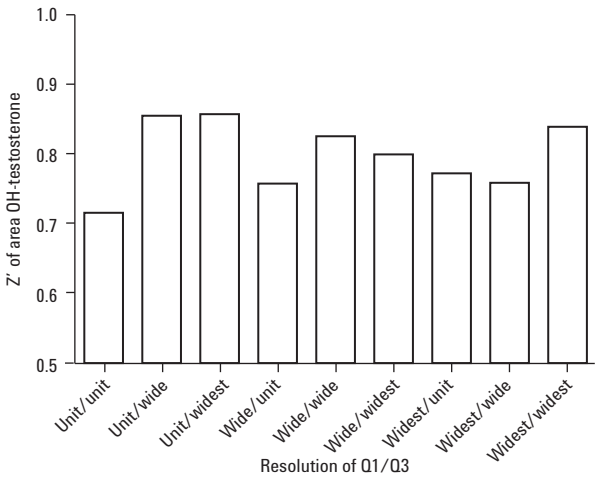

Figure 1. Effect of resolution settings for quadrupole Q1 and Q3 (Q1/Q3) on resulting assay Z′-values (n = 24).

The highest Z'-value was achieved by setting highest selectivity for Q1 (unit) and lowest selectivity for Q3 (widest). Moreover, sheath gas temperature and gas temperature were optimized regarding highest Z'-value and highest system robustness. Examination of highest Z'-value in combination with highest system stability showed the most favorable results at 250 °C for sheath gas temperature and 200 °C for gas temperature.

ΔEMV was optimized by analysis of matrix samples (assay buffer and stop solution) containing increasing concentrations of OH-testosterone (0–25 µM). Lowest limit of detection (LLOD) and highest signal-to-noise (S/N) ratio was detected at ΔEMV of 100 (Figure 2).

Source conditions for the multiplex CYP inhibition assay were similarly optimized with matrix-free samples containing 10 µM 6β-OH-testosterone-D7, dextrorphan-D3, 4'-hydroxydiclofenac-D4, 6β-OH-testosterone, OH-diclofenac, and dextrorphan by varying gas flow, nebulizer, sheath gas flow, capillary voltage, and nozzle voltages. Resolution, gas temperature, and sheath gas temperature were optimized using matrix samples at top, bottom, and median concentration. The optimal source conditions for both the single CYP3A4 inhbition and multiplex CYP inhibition assays are shown in Table 6.

Data analysis

RapidFire Integrator software was used for data acquisition, mass extraction, and peak integration. Agilent MassHunter Workstation Software with Qualitative Analysis was used for calculation of S/N ratio.

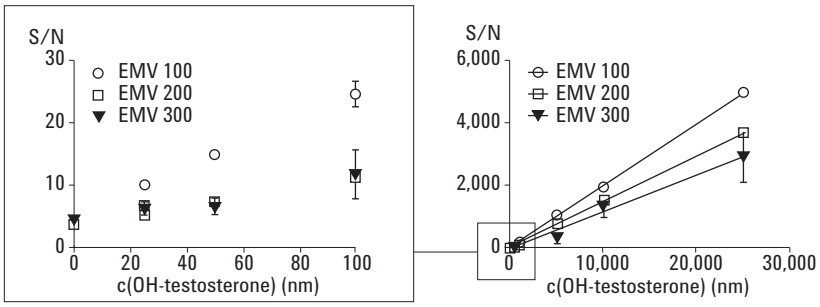

Figure 2. Effect of ΔEMV on the S/N ratio and the LLOD (n = 3).

Table 6. Optimized Agilent 6460 Triple Quadrupole LC/MS System source conditions.

CYP3A4 assay		Multiplex CYP assay	
Gas temperature and flow	200 °C, 11 L/min	Gas temperature and flow	300 °C, 8 L/min
Nebulizer	60 psi	Nebulizer	50 psi
Sheath gas temperature and flow	250 °C, 12 L/min	Sheath gas temperature and flow	400 °C, 12 L/min
Capillary voltage	3,500 V	Capillary voltage	3,000 V
Nozzle voltage	1,000 V	Nozzle voltage	2,000 V
ΔEMV	100	ΔEMV	100

Results and Discussion

After integration of all optimized parameters for the detection of 6b-OH-testosterone(-D7) by the 6460 Triple Quadrupole LC/MS System, concentration response curves were acquired by analysis of analyte samples in matrix spiked with a fixed concentration of deuterated internal standard. Data was processed by RapidFire Integrator software. The concentration response curve had excellent linearity over the range of 50 nM to 25 µM of 6β-OH-testosterone with a R^2 value of 0.9968 (Figure 3). The limit of detection (LOD) was 50 nM and the LLOQ was 100 nM. Total cycle time from injection to injection was 8.6 seconds, providing a throughput of greater than 400 samples/hour.

For the multiplex assay, concentration response curves of 6β-OH-testosterone, OH-diclofenac, and dextrorphan were acquired analyzing matrix samples spiked with a fixed concentration of each deuterated internal standard and increasing concentrations of the corresponding product (25 nM–100 µM). Samples were analyzed at a rate of 10 seconds/sample. The concentration response curve was linear over the range of 100 nM to 100 µM with a LOD of 100 nM for all three analytes. The LLOQ were 100 nM for dextrorphan and OH-diclofenac and 500 nM for OH-testosterone, respectively.

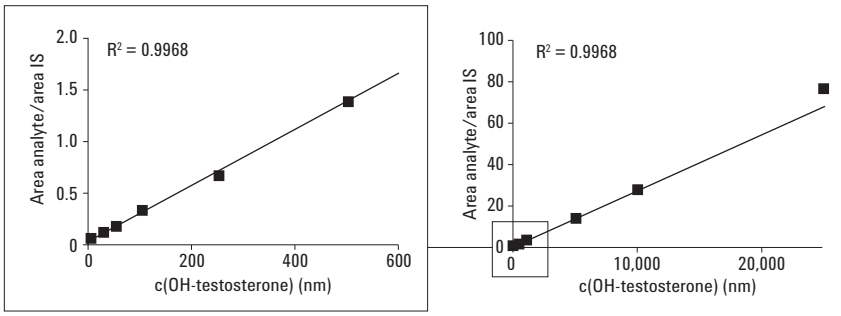

Figure 3. Linear detection range for the enzymatic product of CYP3A4 assays, OH-testosterone (25 nM–25 µM) (n = 3). The product area was normalized by calculation of the ratio to the deuterated internal standard for each data point.

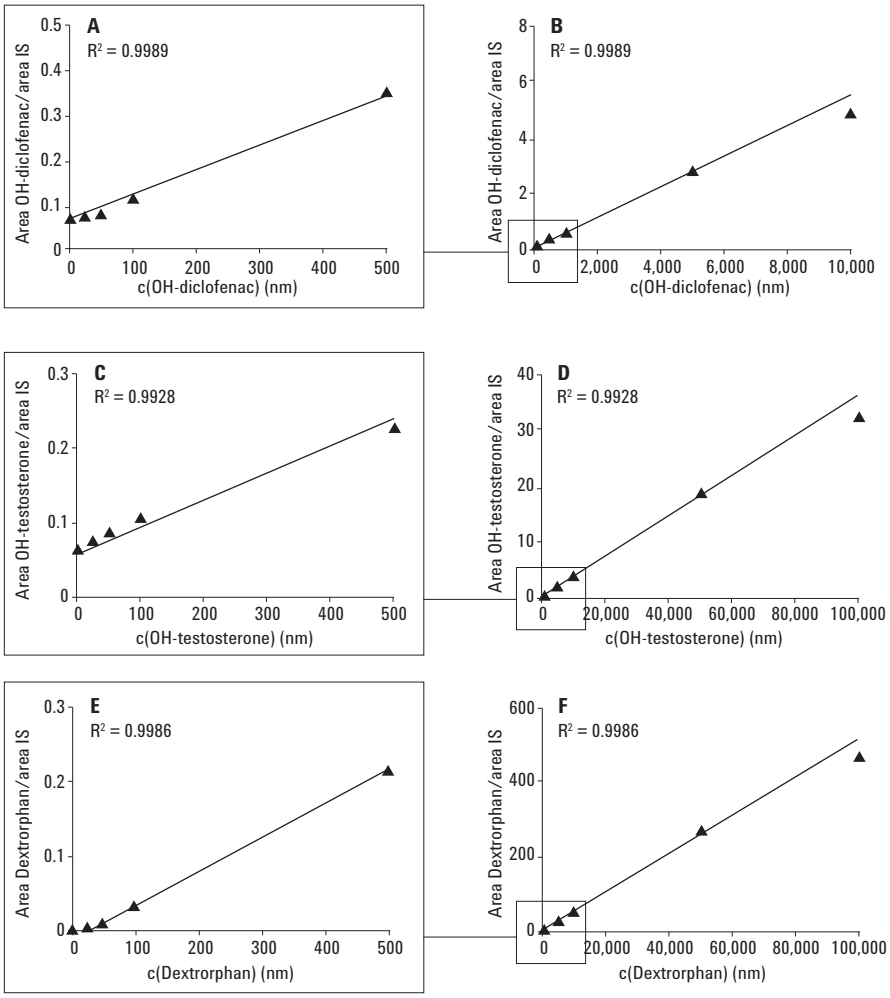

Figure 4. Linear detection range for the enzymatic products of multiplex CYP inhibition assays analyzing concentrations between 25 nM and 100 µM of OH-diclofenac (A, B), OH-testosterone (C, D), and dextrorphan (E, F) (n = 3). The product area was normalized by calculation of the ratio to the specific deuterated internal standard for each data point.

Conclusions

The presented methods illustrate that the RapidFire high throughput mass spectrometry system coupled to an Agilent 6460 Triple Quadrupole LC/MS System provides an efficient and precise tool for characterization of CYP inhibition. High-throughput methods were developed for CYP3A4 inhibition as well as the multiplexed enzymatic characterization of CYP3A4, CYP2C9, and CYP2D6 inhibition. Both singleton and multiplex methods provide an ultrafast rate of analysis at 10 seconds or less per sample. This methodology provides a combination of high data quality and fast performance that may be useful for other early drug discovery and drug development applications.

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