Ultrafast Analysis of Metabolic Stability Assays Using Agilent RapidFire High-resolution MS

Application Note

Drug Discovery

Authors

Michelle V. Romm,
Nikunj Parikh and
Vaughn P. Miller
Agilent Technologies
Wakefield, MA

Yuqin Dai
Agilent Technologies
Santa Clara, CA

Abstract

The analysis of *in vitro* ADME assays like metabolic stability is often a throughput bottleneck in the drug discovery process. Assay results generated by LC/MS/MS were compared to those of the Agilent RapidFire 360 High-throughput Mass Spectrometry System integrated with a high resolution accurate mass Q-TOF MS. The two systems provided equivalent assay results, but the Agilent RapidFire 360 offered a more efficient workflow and higher throughput. For LC/MS/MS, specific MRM methods for 39 compounds were optimized using Agilent MassHunter Optimizer software. *In vitro* samples were analyzed using an Agilent 1260 Infinity LC interfaced to an Agilent 6460 Triple Quadrupole MS with cycle times of approximately 2.2 minutes per sample. For RapidFire 360 MS, *in vitro* samples were analyzed utilizing generic MS source parameters and exact mass extraction on an Agilent 6530 Q-TOF interfaced to a RapidFire 360 with cycle times of 9.5 seconds per sample. The metabolic half-life values as determined by substrate depletion for a chemically diverse set of 39 compounds were essentially equivalent by the two platforms (R^2 greater than 0.95). In addition to the greater than 13-fold decrease in analysis cycle time of the RapidFire-MS system, these results indicate that the MRM method development can be eliminated for the metabolic stability assay, thus providing additional workflow efficiency.

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Introduction

The metabolic half-life or stability of a drug discovery compound has important pharmacokinetic and clinical significance, because it influences both oral bioavailability and plasma concentration of a compound, ultimately affecting efficacy. Large compound libraries and advancements in liquid handling have placed demands on the throughput of *in vitro* metabolic stability assays. Analysis of assay samples, typically accomplished by LC/MS/MS, is a bottleneck in the process due to MRM method development and sample analysis time. We evaluated the ability of an Agilent RapidFire High-throughput System integrated with an Agilent Q-TOF MS to provide equivalent assay results to LC/MS/MS, but with a much more efficient workflow and higher throughput.

Experimental

Systems

The Agilent LC/MS/MS system consisted of the following modules:

- Software: MassHunter Workstation Triple Quadrupole Acquisition Software B.04.01 with Quantitative Analysis B.04.00 SP2 and Qualitative Analysis B.04.00, Mass Hunter Study Manager B.04.01 were used for study submission, MRM optimization, data acquisition, quantitation, and final report generation.
- Agilent 1260 Infinity Binary LC System, comprising binary pump (G1312B), degasser (G1379B), high performance autosampler (G1367D) and Agilent thermostat (G1316B)
- Agilent 6460 Triple Quadrupole mass spectrometer (G6460A)

The RapidFire 360-MS system consisted of the following modules:

- Agilent RapidFire 360 (G9214AA)
- Agilent 6530 Q-TOF mass spectrometer (G6530AA)
- Software: MassHunter Acquisition Software B.02.01 with Qualitative Analysis B.03.01, RapidFire Integrator

LC/MS/MS Conditions

Column: Agilent ZORBAX SB-C18, 2.1x 30 mm, 3.5 µm (873700-902)
Mobile phase: A = 0.1% formic acid in water, B = 0.1% formic acid in acetonitrile
Gradient (%B): 10% from 0 to 0.2 minutes, 95% at 1 minute until 1.6 minutes, 10% at 1.7 minutes, Post time: 0.5 minutes
Flow rate: 0.6 mL/minute
Injection volume: 6.0 µL
Ionization mode: positive electrospray, Agilent Jet Stream enabled
Capillary voltage: 3500 V
Drying gas : 300 °C at 5 L/minute
Sheath gas: 250 °C at 11 L/minute
Nozzle voltage: 500 V
Acquisition mode: MRM
Dwell time: 100 milliseconds
Fragmentor, MRM transitions, and Collision energy: determined by Optimizer software

RapidFire 360-MS Conditions

Samples were analyzed at a rate of 9.5 seconds per sample.
Buffer A = Water with 0.09% formic acid, 0.01% trifluoroacetic acid; 1.5 mL/minute flow rate
Buffer B = Acetonitrile with 0.09% formic acid, 0.01% trifluoroacetic acid; 1.25 mL/minute flow rate
Injection volume: 10 µL
SPE Cartridge: Agilent RapidFire cartridge A (reversed-phase C4 chemistry, G9203A)
Each compound and an internal standard (bupivicaine) were monitored simultaneously in all experiments.

Chemicals and reagents
Human liver microsomes (HLM) were purchased from BD Biosciences, Billerica, MA. All other chemicals, reagents and solvents were purchased from Sigma-Aldrich, St. Louis, MO.

Sample preparation
Metabolic stability assays were prepared in 96-well plates. Experiments were conducted in triplicate. Incubations were performed at 37 °C by incubating on a plate shaker in a controlled environment. Substrate stock solutions (10 mM) were prepared in DMSO. A quenching solution containing 0.5 µM internal standard (bupivicaine) in 0.1% formic acid was prepared in acetonitrile.

Incubation mixtures in each well contained substrate (1 µM), HLM (0.5 mg/mL) and magnesium chloride (5 mM) in 50 mM potassium phosphate buffer (pH 7.4) in a total volume of 200 µL/well. Reactions were initiated by the addition of NADPH (final concentration = 1.3 mM) and terminated at time periods 0, 5, 10, 20, 30, and 60 minutes by the addition of an equal volume of quenching solution. Following quenching of the incubations, the plates were centrifuged at 4000 rpm for 10 minutes, the supernatant transferred to a new 96 well plate and the plates frozen overnight at -80˚C prior to analysis.

Data analysis
Data from samples run on the RapidFire 360-MS System were acquired in full scan high resolution MS mode (not MS/MS) using generic MS source and SPE conditions. Following data acquisition, exact mass extraction and peak integration were performed using RapidFire Integrator software.

The metabolic stability of each compound was determined by measurement of the change in peak area over time. The triplicate values for each compound were then averaged. A % remaining value was calculated by comparing to the t_0 value (t_0 = 100%). The natural log (Ln) of % remaining was plotted versus time and a $t_{1/2}$ value was calculated from a linear regression of this plot using the following equation[1,2]: $t_{1/2}$ = - 0.693/slope.

Carryover
A subset (30) of the 39 compounds used for the metabolic stability incubations was assessed for carryover on the Agilent RapidFire-MS System. Samples from t_0 were subjected to RapidFire-MS analysis and carryover into a subsequent blank injection was monitored. Results were recorded as relative % of compound in the initial injection.

Results

A diverse set of 39 commercially available drugs and drug-like compounds was chosen in order to provide a broad spectrum of physical-chemical properties. As shown in Table 1, this set of compounds had a molecular weight range of 218 to 734 and XlogP3 values from 0.4 to 7.1. XlogP3 values are *in silico* model calculations based on the structure of each compound and are closely correlated to the aqueous solubility and the octanol/water partition coefficient. XLogP3 values are from the PubChem database (http://pubchem.ncbi.nlm.nih.gov/). These database values were calculated using XLogP3 software[3]. For LC/MS/MS analysis, specific MRM methods for each compound were optimized using Agilent Optimizer software. Samples were then analyzed using a generic LC method with cycle times of 2.2 minutes per sample. RapidFire 360-MS samples were analyzed utilizing generic SPE and MS source parameters with cycle times of 9.5 seconds per sample on an Agilent 6530 Q-TOF interfaced to a RapidFire 360. Following data acquisition, exact mass extraction and peak integration were performed using RapidFire Integrator software. Carryover using the generic RapidFire 360-MS method was assessed on a large subset of these compounds. No significant carryover was seen for any of the compounds tested, as seen in Table 1. All carryover values were less than 0.4%.
The compounds were incubated with human liver microsomes over a time course of 0-60 minutes and analyzed using LC/MS/MS and RapidFire 360-MS systems. The metabolic half-life values ($t_{1/2}$) were determined by substrate depletion[1,2]. Early in drug discovery, half-life values are usually binned into groups due to the variability inherent in this biological assay (especially for $t_{1/2}$ greater than 60 minutes) and for ease of data interpretation. Half-life values were binned into 3 groups: fast (less than 20 minutes), intermediate (20-60 minutes) and slow (greater than 60 minutes) as described by McNaney et al.[3]. Results are shown in Table 1. All compounds were binned in the same manner using either analysis system except for four compounds whose RapidFire 360-MS values were just below the 60 minute cutoff. A correlation of all $t_{1/2}$ values whose RapidFire 360-MS values were less than 60 minutes is shown in Figure 1. The correlation coefficient (R^2) for these fast and intermediate half-lives was 0.965 indicating that the two assay analysis systems produced equivalent results.
In addition to the greater than 13-fold decrease in cycle time of the RapidFire 360-MS System, these results indicate that the MRM method development required for LC/MS/MS analysis can be eliminated for the metabolic stability assay, thus providing additional workflow efficiency.

Table 1

Compound	RapidFire-Q-TOF	LC-MS/MS	Mol Formula	MW	XLogP3	%Carryover
Nicardipine	<20	<20	$C_{26}H_{29}N_3O_6$	479.5250	3.8	0
Nefazadone	<20	<20	$C_{25}H_{32}ClN_5O_2$	470.0069	4.3	0
Midazolam	<20	<20	$C_{18}H_{13}ClFN_3$	325.7673	2.5	0.21
Nimodipine	<20	<20	$C_{21}H_{26}N_2O_7$	418.4403	3.1	0.09
Dicolfenac	<20	<20	$C_{14}H_{11}C_{l2}NO_2$	296.1486	4.4	ND
Pyrilamine	<20	<20	$C_{17}H_{23}N_3O$	285.3840	3.3	ND
Propafenone	<20	<20	$C_{21}H_{27}NO_3$	341.4440	3.3	0
Ticlopidine	20-60	20-60	$C_{14}H_{14}ClNS$	263.7857	3.6	0
Verapamil	20-60	20-60	$C_{27}H_{38}N_2O_4$	454.6016	3.8	0.06
Terfenadine	20-60	20-60	$C_{32}H_{41}NO_2$	471.6734	6.6	0
Buspirone	20-60	20-60	$C_{21}H_{31}N_5O_2$	385.5031	2.6	0.04
Chlorpromazine	20-60	20-60	$C_{17}H_{19}ClN_2S$	318.8642	5.2	0.5
Fluphenazine	20-60	20-60	$C_{22}H_{26}F_3N_3OS$	437.5216	4.4	ND
Promazine	20-60 (53)	>60	$C_{17}H_{20}N_2S$	284.4191	4.5	0
Thioridazine	20-60	20-60	$C_{21}H_{26}N_2S_2$	370.5745	5.9	0
Promethazine	20-60 (56)	>60	$C_{17}H_{20}N_2S$	284.4191	4.8	0
Dextromethorphan	20-60 (59)	>60	$C_{18}H_{25}NO$	271.3972	3.4	0
Cinnarizine	>60	>60	$C_{26}H_{28}N_2$	368.5139	5.8	0
Fluconazole	20-60 (58)	>60	$C_{13}H_{12}F_2N_6O$	306.2708	0.4	ND
S-mephenytoin	>60	>60	$C_{12}H_{14}N_2O_2$	218.2518	1.5	ND
Haloperidol	>60	>60	$C_{21}H_{23}ClFNO_2$	375.8642	3.2	0
Amoxapine	>60	>60	$C_{17}H_{16}ClN_3O$	313.7814	2.6	0
Amitriptyline	>60	>60	$C_{20}H_{23}N$	277.4033	5	0.02
Tamoxifen	>60	>60	$C_{26}H_{29}NO$	371.5146	7.1	0
Propanolol	>60	>60	$C_{16}H_{21}NO_2$	259.3434	3	0
Bufuralol	>60	>60	$C_{16}H_{23}NO_2$	261.3593	3.5	ND
Fluvoxamine	>60	>60	$C_{15}H_{21}F_3N_2O_2$	318.3347	2.6	0.02
Clozapine	>60	>60	$C_{18}H_{19}ClN_4$	326.8233	3.2	0
Imipramine	>60	>60	$C_{19}H_{24}N_2$	280.4073	4.8	0
Tripolidine	>60	>60	$C_{19}H_{22}N_2$	278.3914	3.9	0
Diphenhydramine	>60	>60	$C_{17}H_{21}NO$	255.3547	3.3	0
Desipramine	>60	>60	$C_{18}H_{22}N_2$	266.3807	4.9	0
Chlorpheniramine	>60	>60	$C_{16}H_{19}ClN_2$	274.7885	3.4	0
Metoprolol	>60	>60	$C_{15}H_{25}NO_3$	267.3639	1.9	0
S-warfarin	>60	>60	$C_{19}H_{16}O_4$	308.3279	2.7	ND
Diltiazem	>60	>60	$C_{22}H_{26}N_2O_4S$	414.5178	3.1	0.01
Erthyromycin	>60	>60	$C_{37}H_{67}NO_{13}$	733.9268	2.7	ND
Clomipramine	>60	>60	$C_{19}H_{23}ClN_2$	314.8523	5.2	ND
Tolbutamide	>60	>60	$C_{12}H_{18}N_2O_3S$	270.3479	2.3	0.36

Note: Actual values for RapidFire-Q-TOF half-lives are shown in parentheses for those compounds that did not bin the same for each analysis method. ND= Not Done

Conclusions

Metabolic stability experiments using human liver microsomal incubations with a diverse set of 39 drug compounds were performed and the results analyzed by both an Agilent RapidFire 360-MS System and an Agilent LC/MS/MS system. The results presented here illustrate that the use of the Agilent RapidFire 360-MS system for this assay results in a greater than 13-fold increase in throughput while maintaining equivalent results to LC/MS/MS. In addition to the decrease in cycle time of the Agilent RapidFire system, these results indicate that the MRM method development can be eliminated for the metabolic stability assay, thus providing additional workflow efficiency. This ultrafast system may also be useful for the analysis of similar *in vitro* ADME assays.

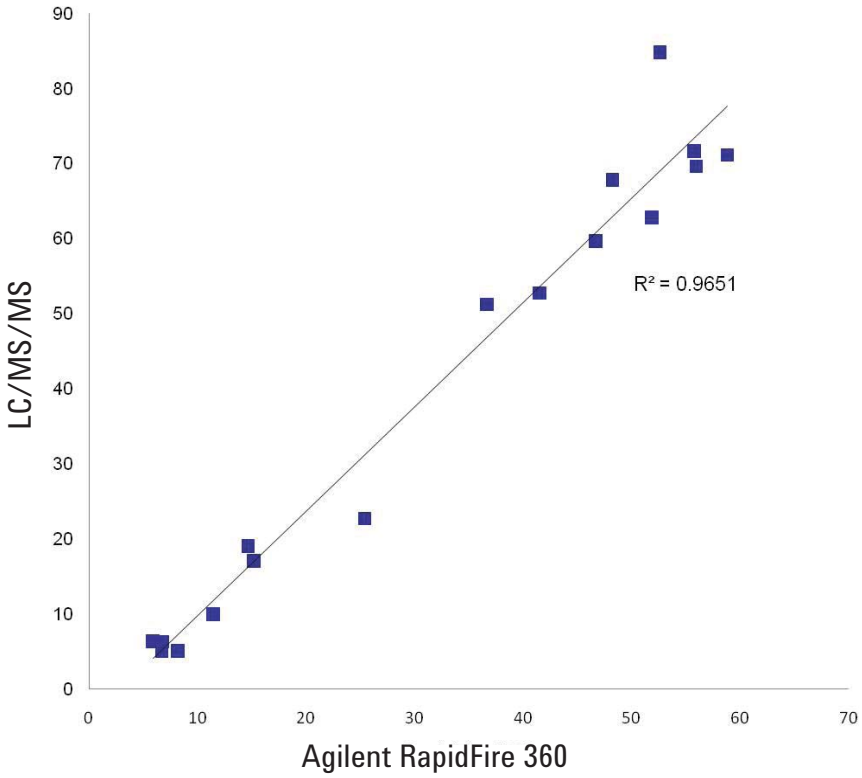

Figure 1. Correlation of results for fast and intermediate half-lives.

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