Application Note

Drug Discovery

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

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Abstract

The analysis of *in vitro* ADME assays such as 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 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 using 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.

Introduction

The metabolic half-life or stability of a drug discovery compound has important pharmacokinetic and clinical research significance, because it influences 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 a 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.

Figure 1. Agilent RapidFire High-Throughput System.

Experimental

Systems

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

- **Software:** Agilent 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:** Agilent MassHunter Acquisition Software B.02.01 with Qualitative Analysis B.03.01, RapidFire Integrator

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 4,000 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 half-life value ($t_{1/2}$) was calculated from a linear regression of this plot using the following equation[1,2]: $t_{1/2} = -0.693/\text{slope}$.

Carryover

A subset (30) of the 39 compounds used for the metabolic stability incubations was assessed for carryover on the 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.

LC/MS/MS Conditions

Parameter	Value
Column	Agilent ZORBAX SB-C18, 2.1 × 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/min
Injection Volume	6.0 µL
Ionization Mode	Positive electrospray, Agilent Jet Stream enabled
Capillary Voltage	3,500 V
Drying Gas	300 °C at 5 L/min
Sheath Gas	250 °C at 11 L/min
Nozzle Voltage	500 V
Acquisition Mode	MRM
Dwell Time	100 ms
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. Each compound and an internal standard (bupivicaine) was monitored simultaneously in all experiments.

Pamameter	Value
Buffer A	Water with 0.09% formic acid, 0.01% trifluoroacetic acid; 1.5 mL/min flow rate
Buffer B	Acetonitrile with 0.09% formic acid, 0.01% trifluoroacetic acid; 1.25 mL/min flow rate
Injection Volume	10 µL
SPE Cartridge	Agilent RapidFire cartridge A (reversed-phase C4 chemistry, G9203A)

Results and discussion

A diverse set of 39 commercially available drugs and drug-like compounds was chosen to provide a broad spectrum of physical-chemical properties. Table 1 shows that 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 using generic SPE and MS source parameters with cycle times of 9.5 seconds per sample on a 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. Table 1 shows that no significant carryover was seen for any of the compounds tested. All carryover values were less than 0.4%. The compounds were incubated with human liver microsomes over 0 to 60 minutes, and analyzed using LC/MS/MS and RapidFire 360-MS systems. The metabolic $t_{1/2}$ values 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.

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	$C_{14}H_{11}Cl_2NO_2$	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 to 60 (53)	>60	$C_{17}H_{20}N_2S$	284.4191	4.5	0
Thioridazine	20 to 60	20-60	$C_{21}H_{26}N_2S_2$	370.5745	5.9	0
Promethazine	20 to 60 (56)	>60	$C_{17}H_{20}N_2S$	284.4191	4.8	0
Dextromethorphan	20 to 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 to 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 Agilent 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

Half-life values were binned into three groups: fast (less than 20 minutes), intermediate (20 to 60 minutes), and slow (greater than 60 minutes), as described by McNaney *et al.*[3] Table 1 shows the results. 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. Figure 2 shows a correlation of all $t_{1/2}$ values whose RapidFire 360-MS values were less than 60 minutes. 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.

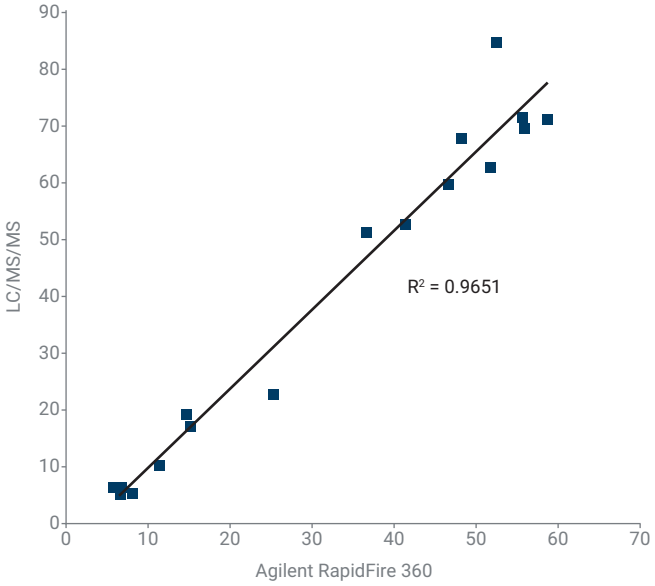

Figure 2. Correlation of results for fast and intermediate half-lives.

Conclusion

Metabolic stability experiments using human liver microsomal incubations with a diverse set of 39 drug compounds were performed, and the results were analyzed by an Agilent RapidFire 360-MS System and an Agilent LC/MS/MS system. The results presented illustrate that the use of the 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 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.

References

1. McNaney, C. A. *et al.* An Automated Liquid Chromatog-raphy-Mass Spectrometry Process to Determine Metabolic Stability Half-Life and Intrinsic Clearance of Drug Candidates by Substrate Depletion. *Assay and Drug Development Technologies* **2008**, *6(1)*, 121–129.
2. Obach, R. S. Prediction of Human Clearance of Twenty-Nine Drugs From Hepatic Microsomal Intrinsic Clearance Data: An Examination of In Vitro Half-Life Approach and Nonspecific Binding to Microsomes. *Drug Metab. Dispos.* **1999**, *27(11)*, 1350–1359.
3. Cheng, T. *et al.* Computation of Octanol-Water Partition Coefficients by Guiding an Additive Model with Knowledge. *J. Chem. Inf. Model*. **2007**, *47*, 2140–2148.

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