Application Note

Drug Discovery

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Ultrafast Analysis of Metabolic Stability Assays using an Agilent Ultivo Triple Quadrupole LC/MS

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Abstract

A previous Application Note[1] highlighted the advantages of using the Agilent RapidFire 365 high-throughput MS to alleviate bottlenecks in the analysis of *in vitro* ADME samples. This Application Note builds on the previous work by showing that equivalent results can be achieved using the RapidFire integrated with an Agilent Ultivo triple quadrupole LC/MS, while significantly reducing the instrument's footprint. Eight compounds were compared across LC/MS/MS, RapidFire with Q-TOF LC/MS, and RapidFire with Ultivo LC/TQ, with excellent agreement observed between all three configurations (R^2 >0.95). Furthermore, the cycle time of 9.5 seconds per sample using the RapidFire was maintained from the previous experiment.

Introduction

Metabolic stability studies are an important part of the drug discovery process, and an ever-increasing volume of samples to analyze is a daunting challenge to face. A previous study[1] explored the possibility of removing the analysis bottleneck using the RapidFire 365 high-throughput MS system. Significant gains in throughput were seen by achieving analysis times of 9.5 seconds per sample. This Application Note looks to maintain the ultrafast analysis time obtained in the previous study while reducing the instrument's footprint using the Ultivo triple quadrupole LC/MS.

Experimental

Reagents and Standards

Human liver microsomes (HLM), chemicals, reagents, and standards were purchased from Sigma-Aldrich, St. Louis, MO.

Instrumentation

The Agilent RapidFire-MS system consisted of a RapidFire 365 high-throughput MS coupled to an Agilent Ultivo triple quadrupole LC/MS. Software used included Agilent MassHunter Acquisition software Ultivo 1.1 and RapidFire Analyzer software.

RapidFire 365 Parameters

Parameter	Value
SPE Cartridge	Cartridge A (reversed-phase C4)
Buffer A	Water + 0.09% formic acid, 0.01% trifluoroacetic acid
Buffer A Flow Rate	1.5 mL/min
Buffer B	Acetonitrile + 0.09% formic acid, 0.01% trifluoroacetic acid
Buffer B Flow Rate	1.25 mL/min
Injection Volume	10 µL

MS Parameters

Parameter	Value
Ion Mode	AJS ESI+
Gas Temperature	200 °C
Drying Gas (Nitrogen)	11 L/min
Nebulizer Gas (Nitrogen)	35 psi
Sheath Gas (Nitrogen)	400 °C
Sheath Flow	12 L/min
Capillary Voltage	2,750 V
Nozzle Voltage	0 V
Q1/Q3 Resolution	Unit/Unit
Dwell Time	10 msec

Name	ISTD	Precursor Ion	Product Ion	Fragmentor	CE
Nicardipine		480.2	315.1	140	22
Nicardipine		480.2	90.9	140	66
Haloperidol		376.2	165.0	140	26
Haloperidol		376.2	122.8	140	46
Thioridazine		371.2	126.1	140	22
Thioridazine		371.2	98.0	140	38
Propafenone		342.2	116.1	140	22
Propafenone		342.2	71.9	140	34
Fluconazole		307.1	238.1	110	14
Fluconazole		307.1	220.1	110	18
Bupivacaine	X	289.2	140.1	110	22
Promethazine		285.1	86.0	110	14
Promethazine		285.1	71.0	110	50
Amitriptyline		278.2	117.0	110	26
Amitriptyline		278.2	91.0	110	34
Ticlopidine		264.1	154.0	110	14
Ticlopidine		264.1	125.0	110	34

Sample preparation

Samples were prepared in 1.5 mL microfuge tubes containing:

- Substrate (1 µM)
- HLM (0.5 mg/mL)
- Magnesium chloride (5 mM) in potassium phosphate buffer (50 mM, pH 7.4)

A quenching solution was prepared with acetonitrile containing 0.1% formic acid and 0.5 µM bupivacaine (internal standard).

Reactions were initiated with the addition of NADPH (1.3 mM final concentration) and heating the samples to 37 °C while shaken. An equal volume of quenching solution was used to terminate the reactions at 0, 5, 10, 20, 30, and 60 minutes. Samples were centrifuged at 4,000 rpm for 10 minutes, and the supernatant was transferred to a 96-well plate for analysis.

Data analysis

Data were acquired at a rate of 9.5 seconds per sample using the RapidFire 356 coupled to the Ultivo LC/MS in multiple-reaction monitoring (MRM) mode. Peak area for each compound was extracted from the data files using RapidFire Analyzer software (Figure 1), which quickly parses data and returns results in a convenient format.

While observing the peak area for compounds over time, it is possible to measure how quickly each compound is metabolized by determining the percent remaining, and comparing the results to t_0 (t_0 = 100%). Samples were prepared and analyzed in triplicate, and results for each compound were averaged.

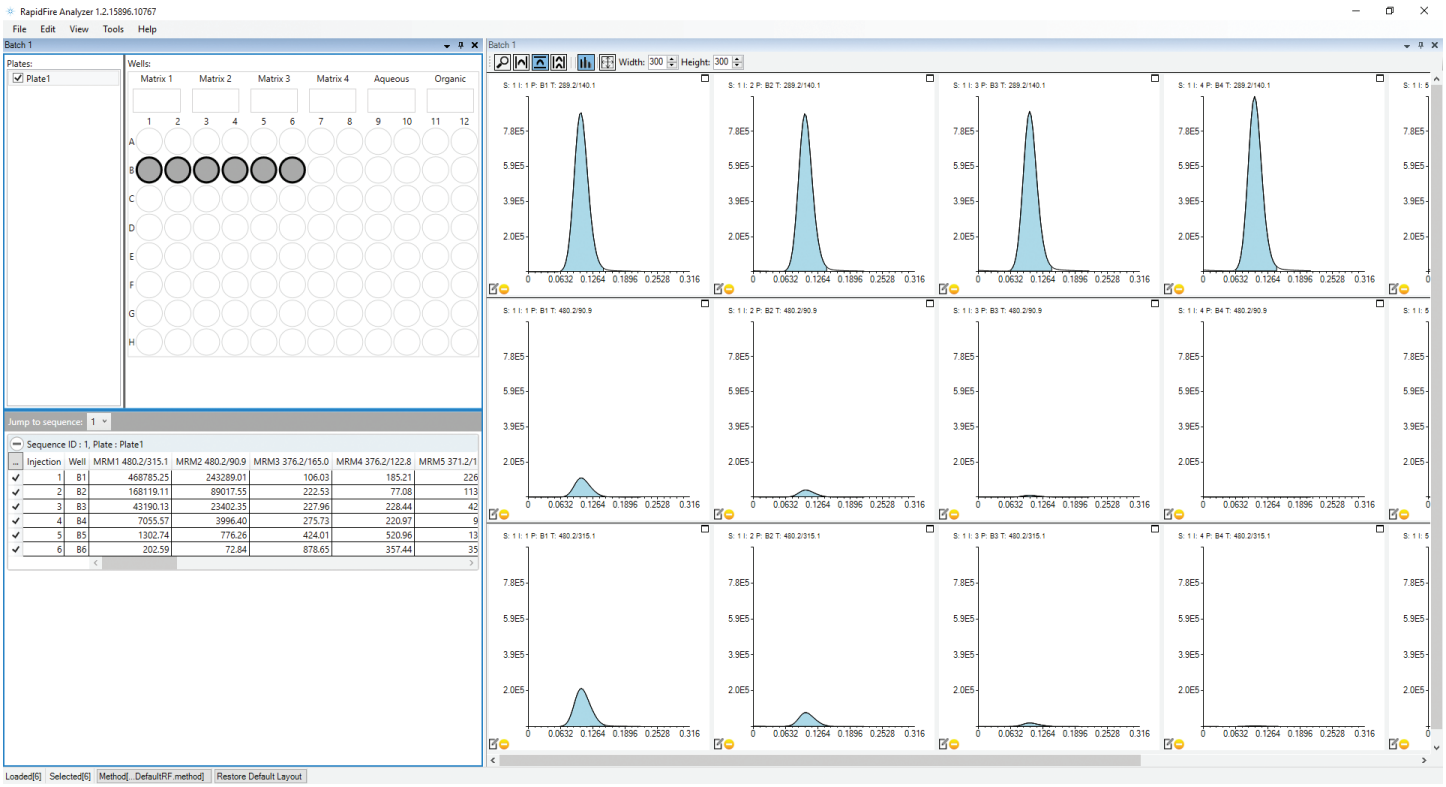

Figure 1. Agilent RapidFire Analyzer software.

Results and discussion

Substrate depletion can be visualized by plotting the natural log of the percent remaining of each compound against time and performing a linear regression. From there, the metabolic half-life values ($t_{1/2}$) of each compound can be calculated as −0.693/slope.[2,3] Figure 2 plots the substrate depletion for compounds with fast and intermediate $t_{1/2}$.

Once half-life was determined for each of the compounds, they were classified as fast ($t_{1/2}$ <20), intermediate ($t_{1/2}$ 20–60), or slow ($t_{1/2}$ >60) clearance. This binning is typical of these types of experiments due to the level of biological variation and the screening nature of the experiment.

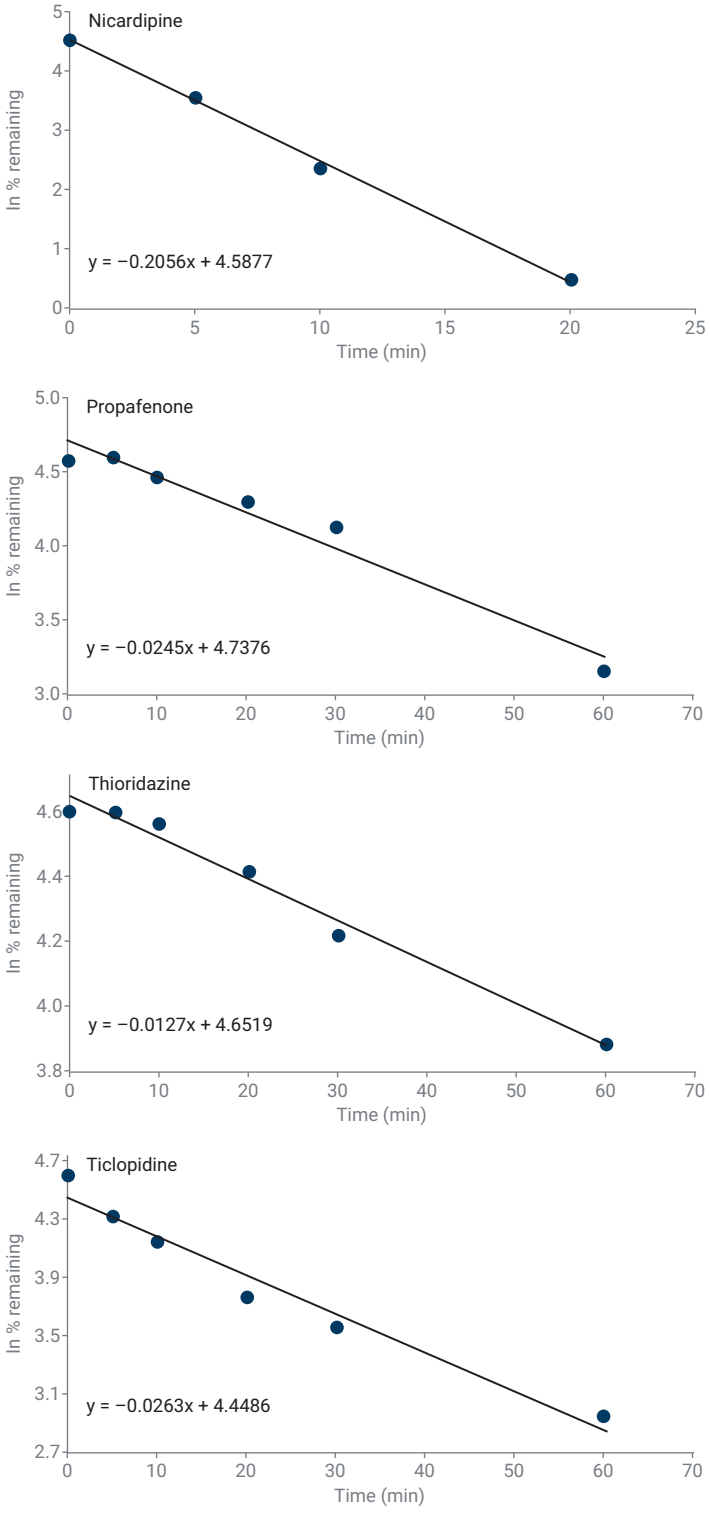

Figure 2. Substrate depletion for compounds with fast and intermediate half-life values.

Table 1 compiles the results for each compound. Data from previous experiments using the Agilent 6460 triple quadrupole LC/MS and the Agilent 6530 Q-TOF LC/MS have also been included for comparison. Actual $t_{1/2}$ values have not been calculated for slow clearance compounds, as those values exceeded the time periods of the experiment, and results would have a high degree of variation.

Previous experiments[1] have shown excellent correlation between the results achieved on an Agilent LC/MS and RapidFire-Q-TOF. This experiment uses a subset of those compounds to demonstrate that the RapidFire-Ultivo can achieve results equivalent to the Agilent 1260 Infinity II LC with the 6460 triple quadrupole LC/MS (LC/6460 MS) and the RapidFire with the 6530 Q-TOF LC/MS (RapidFire-Q-TOF) (Figures 3 and 4, respectively).

There is a strong agreement between all three approaches to this analysis, both in the binning of compound $t_{1/2}$, as well as the calculated results for the fast and intermediate $t_{1/2}$ compounds.

Table 1. Binned and calculated $t_{1/2}$ results for Agilent RapidFire-Ultivo, LC/6460 MS, and RapidFire-Q-TOF.

Compound	Binned Data			Calculated $t_{1/2}$		
	RF-Ultivo	LC-6460	RF-Q-TOF	RF-Ultivo	LC-6460	RF-Q-TOF
Amitriptyline	>60	>60	>60	>60	>60	>60
Fluconazole	>60	>60	20–60	>60	>60	58
Haloperidol	>60	>60	>60	>60	>60	>60
Nicardipine	<20	<20	<20	3.2	2.7	3.6
Promethazine	>60	>60	20–60	>60	>60	56
Propafenone	20–60	20–60	20–60	31.0	39.1	35.7
Thioridazine	20–60	20–60	20–60	49.5	55	54.9
Ticlopidine	20–60	20–60	20–60	26.4	26.2	30.1

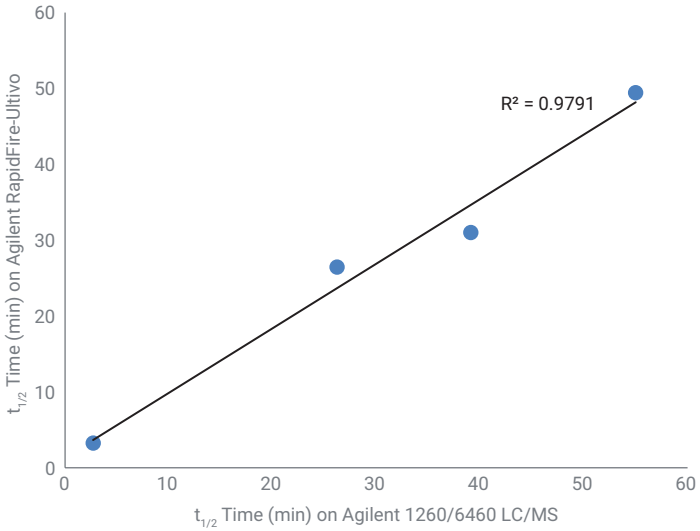

Figure 3. Correlation of results for fast and intermediate $t_{1/2}$ between the Agilent 1260 Infinity II LC with the Agilent 6460 triple quadrupole LC/MS and the Agilent RapidFire with the Agilent Ultivo LC/TQ.

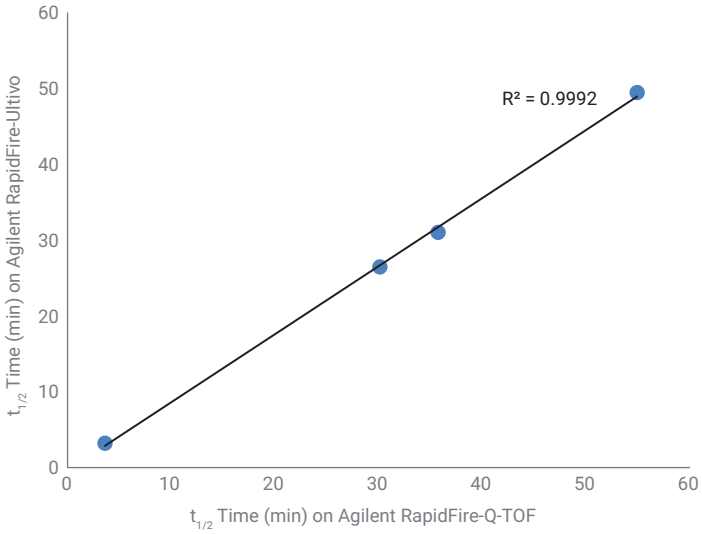

Figure 4. Correlation of results for fast and intermediate $t_{1/2}$ between the Agilent RapidFire with the Agilent 6530 Q-TOF LC/MS and Agilent RapidFire with the Agilent Ultivo LC/TQ.

Conclusion

Agilent instrumentation provides robust and dependable data across a wide portfolio of analytical instrumentation. When determining the proper instrumentation for a laboratory, it is important to understand the big picture. Whether an instrument will be dedicated to an analytical method or used for multiple types of analyses is important to consider. When chromatographic separations are critical, Agilent's LC/MS systems offer reliable and reproducible results. If sample throughput is the priority, the Agilent RapidFire delivers answers at a rate of 5 to 10 seconds per sample. When pairing the RapidFire with Q-TOF instrumentation, the system can provide an untargeted approach to data acquisition that allows for further workflow efficiencies. Alternatively, by connecting the RapidFire to an Agilent Ultivo LC/TQ, highly sensitive and accurate quantitation can be achieved in an unbeatably small footprint.

Regardless of your approach, you can be confident in the results. Excellent agreement is observed between all metabolic stability studies performed on the RapidFire with Ultivo LC/TQ, the RapidFire with 6530 Q-TOF LC/MS, and the Agilent 1260 Infinity II LC with the Agilent 6460 LC/MS.

References

1. Romm, M. V.; *et al.* Ultrafast Analysis of Metabolic Stability Assays Using Agilent RapidFire High-Resolution MS. *Agilent Technologies Application Note*, publication number 5990-8344EN, **2019**.
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3. 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 Metabolism and Disposition **1999**, *27(11)*, 1350–1359.

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