Application Note

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Ultrafast Analysis of *In Vitro* Microsomal Metabolic Stability using RapidFire Coupled to the Ultivo Triple Quadrupole Mass Spectrometer

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Abstract

Metabolic stability studies are important steps in the initial drug discovery process. During the investigation of phase one drug metabolism, large quantities of samples are analyzed, creating the need for fast and reliable analytical methods. The Agilent RapidFire is an ultrafast, integrated mass spectrometry autosampler capable of automated solid phase extraction (SPE) sample cleanup. With cycle times ranging from 2 to 15 seconds, RapidFire dramatically reduces analysis times compared to traditional LC/MS without compromising data quality. This study compared the results of *in vitro* microsomal metabolic stability (MMS) assays analyzed by RapidFire/TQ and LC/TQ. Agilent MassHunter Optimizer software was used to automatically determine multiple reaction monitoring (MRM) transitions for 72 compounds. The results for the two systems correlated well (R^2 = 0.94), and RapidFire required only 10 seconds per sample, providing 10-times faster throughput than LC/TQ.

Introduction

Pharmacokinetic analysis is an important early process of drug discovery that aims to quantify absorption, distribution, metabolism, and excretion (ADME) of compounds over time. These initial analyses include a large set of samples, so high-throughput analytical methodology is desirable. The Agilent RapidFire harnesses the power of traditional liquid chromatography mass spectrometry (LC/MS) analysis but allows for a 10-times increase in throughput by replacing chromatography with on-line solid phase extraction (SPE). Also, with a large sample capacity of over 130,000 samples and integrated automated sample-handling robotics, RapidFire allows longer unattended operation than LC/MS, further improving productivity.

An *in vitro* microsomal metabolic stability (MMS) assay is one type of ADME experiment used to evaluate compounds of interest. It is widely used in early drug discovery studies because it is an *in vivo* stability indicator. When considering the pharmacokinetic properties of a drug candidate, the stability of a compound ultimately affects its efficacy as a drug.

In this study, MMS assays were performed on various drug candidates using the RapidFire 400 coupled to an Agilent Ultivo Triple Quadrupole Mass Spectrometer (TQ). This RapidFire/TQ system can produce analytical results that are equivalent to traditional liquid chromatography triple quadrupole mass spectrometry (LC/TQ) in just 10 seconds per sample. The findings in this study demonstrate that RapidFire/TQ is a suitable replacement for LC/TQ in these types of ADME assays.

Experimental

Sample preparation

Standard stock solutions for 72 compounds of interest were dissolved in acetonitrile. To assess the linearity and reproducibility of the method, a serial dilution of the stock solution was prepared using water containing 0.1% formic acid.

MMS assays were carried out in 96-well plates where target compounds were incubated with human liver microsomes (HLM; Corning). After incubation, the samples were transferred to a new plate where the reaction was quenched with an acetonitrile solution containing tolterodine as the internal standard (ISTD). Samples were centrifuged at 3,000 rpm at 4 °C for 10 minutes. The supernatant was transferred to a new plate, then diluted 1:2 with water containing 0.1% formic acid, before being injected for analysis.

Instrumentation

The RapidFire/TQ system consisted of a RapidFire 400 coupled to an Ultivo TQ. An Agilent RapidFire C4 (Type A) cartridge was used for SPE.

The LC/TQ system consisted of an Agilent 1290 Infinity II LC coupled to an Agilent 6470 TQ. Chromatography was performed using an Agilent ZORBAX Eclipse Plus C18, 2.1 x 50 mm, 1.8 µm column.

Data was acquired with Agilent MassHunter Acquisition (version 10.1) and analyzed with MassHunter Qualitative Analysis (version 10.1) and MassHunter Quantitative Analysis (version 10.1) software.

Instrument operating conditions are given in Tables 1 to 4.

Table 1. Agilent RapidFire parameters.

Parameter	Value
Pump 1	Water with 0.1% formic acid 1.5 mL/min flow rate
Pump 2	Acetonitrile 1 mL/min flow rate
Pump 3	60% acetonitrile with 0.1% formic acid 1 mL/min flow rate
Injection Volume	5 µL
SPE Cartridge	C4 (Type A; part number G9203A)
Aspiration	600 ms
Load/Wash	3,000 ms
Extra Wash	0
Elute	3,000 ms
Re-equilibrium	500 ms

Table 2. Agilent Ultivo TQ parameters.

Parameter	Value
Ion Source	ESI with Agilent Jet Stream
Acquisition Mode	MRM
Gas Temperature	350 °C
Gas Flow	12 L/min
Nebulizer	30 psi
Sheath Gas Temperature	350 °C
Sheath Gas Flow	11 L/min
Capillary	(+)3000, (-)4,000 V
Nozzle voltage	(+)0, (-)1,500 V
Polarity	Positive/Negative

Table 3. Agilent 1290 Infinity II LC parameters.

Parameter	Value
Column	ZORBAX Eclipse Plus C18, 2.1 × 50 mm, 1.8 µm
Column Flow	0.4 mL/min
Injection Volume	5 µL
Mobile Phase	A: 0.1% formic acid in water B: 0.1% formic acid in acetonitrile
Gradient (%B)	60% isocratic for 2 min

Table 4. Agilent 6470 LC/TQ parameters.

Parameter	Value
Ion Source	ESI with Agilent Jet Stream
Acquisition Mode	MRM
Gas Temperature	350 °C
Gas Flow	12 L/min
Nebulizer	30 psi
Sheath Gas Temperature	350 °C
Sheath Gas Flow	11 L/min
Capillary	(+)3,000, (-)4,000 V
Nozzle Voltage	(+)0, (-)1,500 V
Polarity	Positive/Negative

Results and discussion

To assess the accuracy and reliability of the RapidFire/TQ method, a complete MMS study of 72 compounds of interest was analyzed by RapidFire/TQ and LC/TQ. Tolterodine was used as an ISTD for all analyses. Warfarin was used as a target compound to verify linearity and reproducibility data on each system; it was also used as a reference compound in the MMS assay.

To ensure a direct comparison of RapidFire and traditional LC analysis, the same Ultivo TQ instrument was used for the linearity and reproducibility studies.

Correlation data was collected using the RapidFire/Ultivo and LC/6470.

A comparison of linearity

Warfarin was used to create a 7-point calibration curve ranging from 0.5 to 50 ng/mL. The results for RF/TQ (Figure 1A) and LC/TQ (Figure 1B) were equivalent, both showing excellent linearity (R^2 ≥0.999) and accuracy (between 90 and 110%).

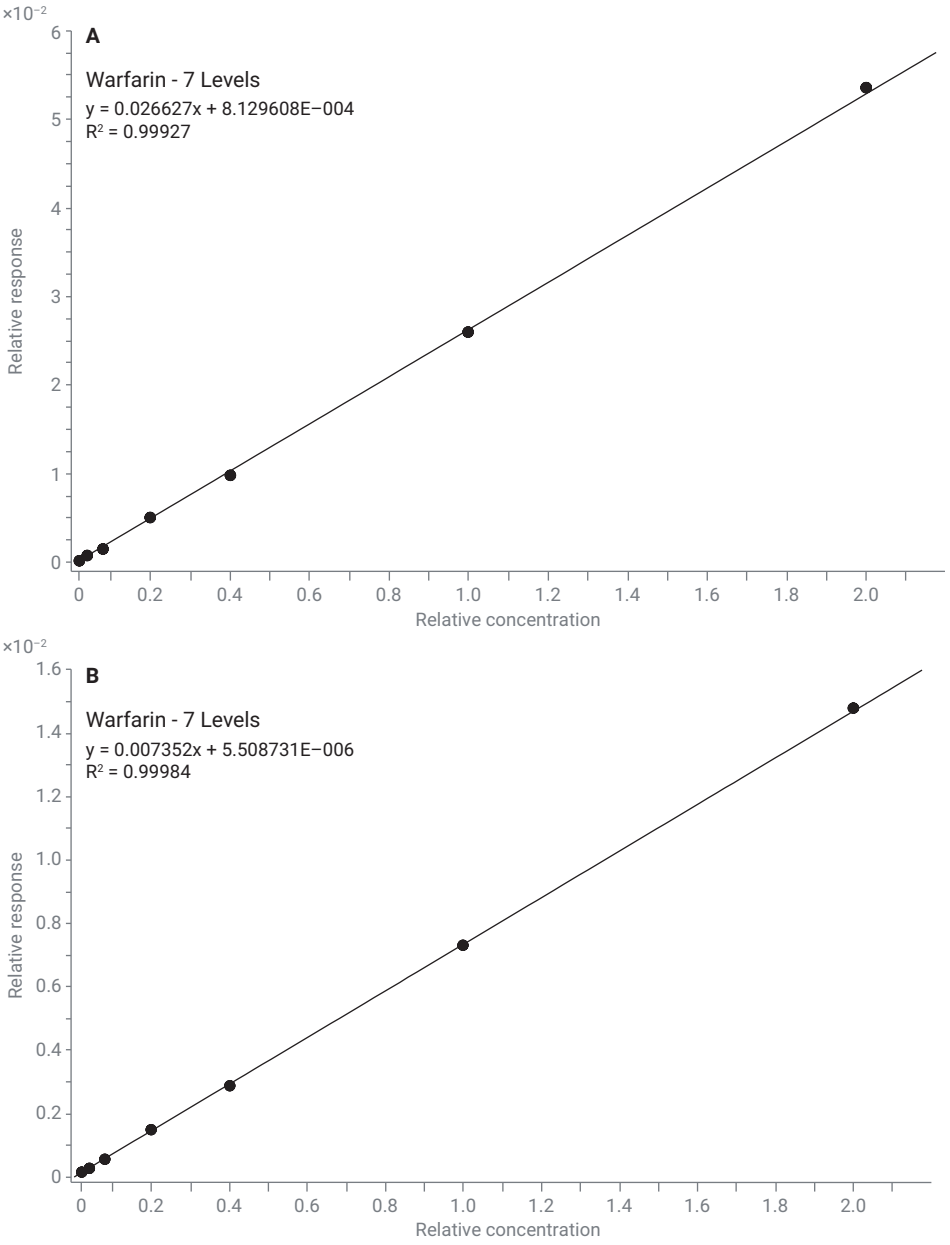

Figure 1. Calibration curves for warfarin obtained using (A) RapidFire/TQ and (B) LC/TQ.

A comparison of reproducibility

A 1 ng/mL warfarin standard was measured five times to assess the precision of each system. The relative standard deviation (%RSD) was calculated for the ratio of warfarin to tolterodine (ISTD). As shown in Table 5, both RapidFire/TQ and LC/TQ achieved highly reproducible results, with %RSDs of 4.87 and 2.06%, respectively.

Table 5. Reproducibility of measurement of 1 ng/mL warfarin standard using RapidFire/TQ and LC/TQ (n = 5).

Sample	Target/ISTD Ratio	
	RF/TQ	LC/TQ
1	0.00140	0.00030
2	0.00132	0.00030
3	0.00127	0.00031
4	0.00126	0.00031
5	0.00125	0.00030
Average	0.00130	0.00030
SD	0.00006	0.00001
%RSD	4.87	2.06

A comparison of correlation

To assess whether RapidFire/TQ can produce results equivalent to LC/TQ, identical MMS assays were analyzed by each system. The studies determined the amount of each compound remaining after the MMS assay and reported results as a percentage. A plot comparing RapidFire/TQ results to LC/TQ results (Figure 2) shows excellent correlation (R^2 = 0.9376), a slope of 1.0203, and a small y-intercept. The correlation data indicates that the systems produced equivalent MMS assay results, however, the RapidFire/TQ results were acquired 10 times faster than the LC/TQ data.

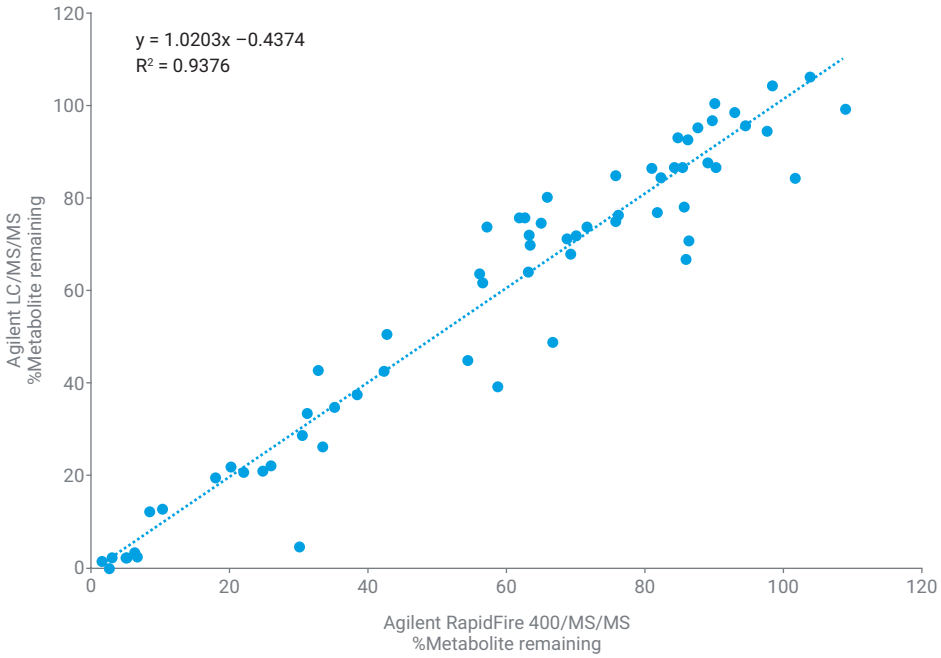

Figure 2. Correlation of RapidFire/TQ and LC/TQ results for percent of 72 compounds remaining at the end of MMS analysis.

Conclusion

A metabolic stability assessment of 72 different compounds was performed using an Agilent RapidFire coupled to an Agilent Ultivo triple quadrupole mass spectrometer (RapidFire/TQ). To determine if the RapidFire/TQ could produce equivalent results to traditional methods, the same samples were analyzed by liquid chromatography triple quadrupole mass spectrometry (LC/TQ). The comparison results showed that both sets of results were equivalent, but the RapidFire/TQ method, which uses solid phase extraction (SPE) rather than chromatography, was 10 times faster than LC/TQ. A comparison of the microsome metabolic stability (MMS) assay results obtained by RapidFire/TQ and LC/TQ showed excellent correlation between the methods.

The study has shown that RapidFire/TQ can improve the sample throughput, productivity, and efficiency of MMS assays and is potentially useful for other, similar *in vitro* ADME assays.

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RA44585.6442824074-RA

Printed in the USA, February 17, 2022
5994-4532EN

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