Ultra Fast Analysis of Hydroxymidazolam in Plasma Using the Agilent RapidFire High-Throughput Mass Spectrometry System

Application Note

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Introduction

The ever-increasing demand for mass spectrometry-based analysis has created opportunities for the development of high-throughput approaches in clinical research. We evaluated the ability of the Agilent RapidFire High-throughput Mass Spectrometry System to analyze small molecules in plasma with much faster sample cycle times (< 15 seconds) and comparable analytical results to LC/MS/MS methods. Critical bioanalytical parameters were systematically investigated using hydroxymidazolam spiked into plasma. Comparable accuracy, precision, linearity, and sensitivity were achieved at rates 20–30 times faster than LC/MS/MS.

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Experimental

The Agilent RapidFire/MS/MS system consisted of the following modules: an Agilent RapidFire 360 and an Agilent 6460 Triple Quadrupole Mass Spectrometer with RapidFire Integrator Software.

The Agilent LC/MS system consisted of the following modules: an Agilent 1200 Series Binary HPLC pump, an Agilent Degasser, an Agilent high performance Autosampler, an Agilent thermostat and an Agilent 6460 Triple Quadrupole Mass Spectrometer. Both systems used MassHunter Acquisition Software (B.04.00) with Qualitative Analysis (B.04.01).

RapidFire-triple quadrupole conditions

Samples were analyzed at a rate of 9 seconds per sample. Hydroxymidazolam and its internal standard (1′-hydroxymidazolam-[$^{13}C_3$]) were monitored simultaneously in all experiments.

Chemicals and reagents

1′-Hydroxymidazolam and all other chemicals, reagents and solvents were purchased from Sigma-Aldrich; St. Louis, MO. The stable-labeled isotope internal standard, 1′-hydroxymidazolam-[$^{13}C_3$], was purchased from BD Biosciences, Billerica, MA. Rat plasma was purchased from Xenotech, Lenexa, KS.

Sample preparation

Hydroxymidazolam was spiked into rat plasma at varying concentrations (10–1,000 ng/mL), either pre- or post protein precipitation. The rat plasma was protein precipitated by adding acetonitrile 4:1, vortexed and centrifuged at 5000 rpm for 10 minutes. The samples were then diluted 1:1 with water containing internal standard. Hydroxymidazolam was also spiked into 50 % acetonitrile/water.

Data analysis

Data generated on the RapidFire/MS/MS system was analyzed by the RapidFire Integrator software. Data generated on the LC/MS/MS system was analyzed by MassHunter software. The AUC of the hydroxymidazolam analyte was normalized by the AUC of the internal standard. The data was subjected to linear regression with 1/x weighting.

Table 1. RapidFire/MS/MS Conditions.

RapidFire conditions						
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, p/n: G9203A)					
RF state 1	sip sensor					
RF state 2	3,000 ms					
RF state 3	3,000 ms					
RF state 4	500 ms					
Triple quadrupole conditions						
Gas temp	350 °C					
Gas flow	8 L/min					
Nebulizer	45 psi					
Sheath gas temp	400 °C					
Sheath gas flow	11 L/min					
Nozzle voltage	300 V					
Capillary voltage	3,500 V					
	Q1	Q3	Dwell	Fragmentor	CE	CAV
IS	345.2	206.1	50	145	27	2
Quantifier	342.2	203	50	145	27	2

Results and Discussion

Robustness

To evaluate the robustness of the analytical method, hydroxymidazolam at a concentration of 30 ng/mL was spiked into rat plasma. This sample was then subjected to 1,500 sequential injections on the Agilent RapidFire/MS/MS System and the data analyzed. The accuracy was determined to be within 6 % while the coefficient of variation was 3.6 % (Figure 1). This data indicates that injection volume and instrument response were very stable over the course of this experiment.

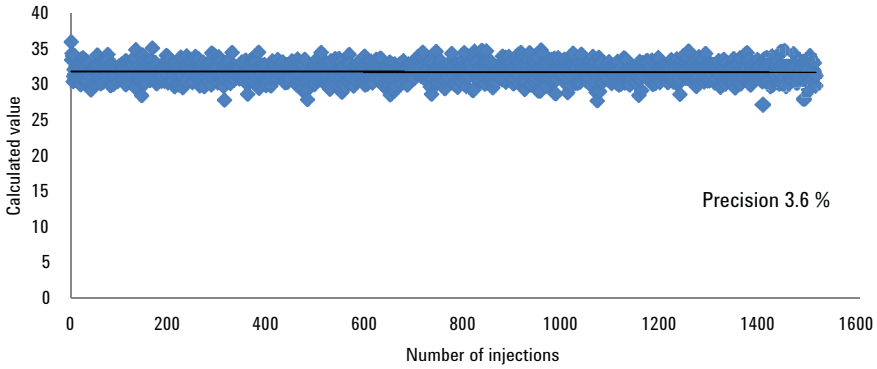

Figure 1. Robustness evaluation: sequential hydroxymidazolam injections.

Inter/Intraday variability

Standard curves and control samples were analyzed to obtain intra- and interday precision and accuracy values on both the Agilent RapidFire High-throughput Mass Spectrometry System and a LC/MS/MS system. The samples were analyzed in triplicate over a four day period. The results between both systems were found to be comparable. Standard curves in plasma had excellent linearity within the measured range (10–1,000 ng/mL) with an R^2 value greater than 0.995 (Figure 2). Intra- and interday accuracies determined were within 15 % and coefficient of variation values were all less than 5 % for concentrations within the measured range (Table 2).

Table 2. Intraday and interday precision and accuracy for RapidFire/MS/MS.

Hydroxymidazolam (ng/mL)	Intraday % accuracy (n=3)	Intraday % precision (n=3)	Interday % accuracy (n=4)	Interday % precision (n=4)
10	113.6	2.3	114.8	3.7
20	98.5	0.9	100.7	7
30	96.5	4.1	95.3	2.7
50	88.8	2.7	88.3	0.6
100	102.7	0.6	102.3	1.8
200	107.7	2.6	100.9	1.3
500	97.2	0.9	95.7	1.3
800	97.6	0.4	98.4	0.7
1,000	103.2	1	103.5	0.7
LQC (30)	97.9	3.8	97.5	3.2
MQC (500)	96.2	1.1	94	1.8
HQC (800)	98.8	3.8	98.6	0.7

Suppression

The signal intensities in the plasma samples were about a third of those in the neat solutions, demonstrating significant ion suppression. The matrix factor calculated by comparing the average peak area of 96 injections of post-spiked samples to that of the neat samples was 0.33 for hydroxymidazolam and 0.35 for the internal standard hydroxymidazolam-[$^{13}C_3$]. Because the stable isotope labeled compound exhibited an equivalent matrix factor as its nonlabeled analog, this internal standard can effectively compensate for ion suppression leading to acceptable assay performance even in the presence of strong matrix effects.

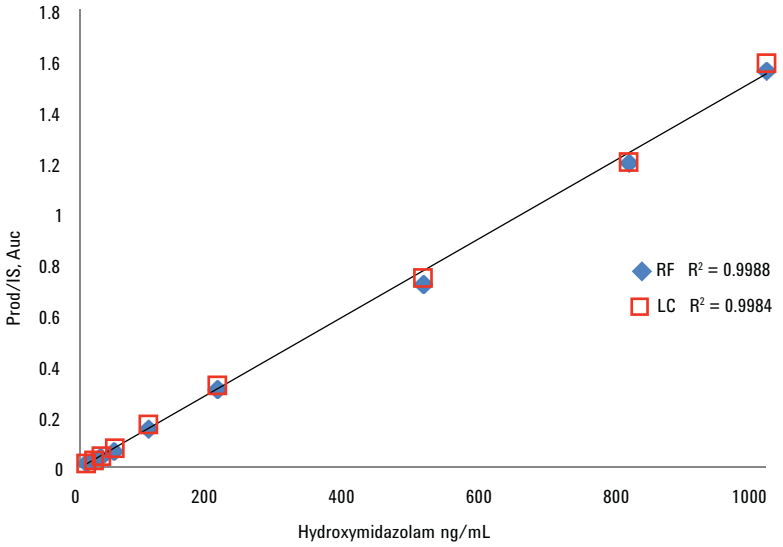

Figure 2. RapidFire/MS/MS versus LC/MS/MS standard curves.

Carryover

To evaluate carryover, 10 replicates of 10 ng/mL hydroxymidazolam were injected on the Agilent RapidFire/MS/MS system, followed by 10 replicates of 5,000 ng/mL hydroxymidazolam, and then 10 replicates of blank plasma (no spiked analyte present). The analyte peak area in each blank was calculated as the % of the mean peak area of the ten 10 ng/mL samples. The first blank had a calculated carryover of 10 % which was reduced to 0 % in the second blank injection. Carryover was therefore not significant (<20 %) within the concentration range used in this application note.

Conclusions

The Agilent RapidFire/MS/MS System was carefully evaluated for the analysis of a small molecule, hydroxymidazolam, in plasma samples for use in clinical research. Important aspects of bioanalytical assay performance including accuracy, precision, linearity, and sensitivity were evaluated for selected analytes. Potential analytical liabilities, such as ion suppression and carry-over, were systematically investigated. The assay results were found to correlate well with those from a conventional LC/MS/MS system — comparable accuracy, precision, linearity, and sensitivity were achieved at rates 20–30 times faster. This methodology is capable of throughputs greater than 360 samples per hour. The Agilent RapidFire/MS/MS System may be useful for the fast and efficient analysis of similar analytes in biological matrices.

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