Ultrafast Analysis of Clozapine and Norclozapine in Serum Using the Agilent RapidFire High-Throughput Mass Spectrometry System

Application Note

Authors

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Abstract

The steadily increasing need for greater analytical capacity and throughput have placed high demands on traditional technologies in the clinical research laboratory. The Agilent RapidFire High-throughput Mass Spectrometry System is an ultrafast SPE/MS/MS system capable of analyzing samples with cycle times less than 15 seconds. The quantitative capability of the Agilent RapidFire/MS/MS system to analyze clozapine and its metabolite, norclozapine, was evaluated in human serum. This analytical method employs protein precipitation followed by dilute and shoot on the SPE-MS/MS system, enabling analysis of these analytes at 14.5 seconds per sample, producing > 10x savings in analysis time and solvent consumption compared to typical LC/MS/MS methods.

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Introduction

Clozapine is a tricyclic dibenzodiazepine drug metabolized to numerous metabolites through *N*-demethylation, *N*-oxidation, and aromatic hydroxylation by CYP1A2 (Figure 1)[1,2]. Traditionally, clozapine and norclozapine analyses involve off-line liquid-liquid extraction followed by a high pressure liquid chromatography-ultra violet (HPLC/UV) or high pressure liquid chromatography-tandem mass spectrometry (HPLC/MS/MS) analysis[3]. LC/MS/MS provides a highly selective, sensitive, flexible methodology, but often lacks the throughput and speed required by many clinical research laboratories. The ability to develop a flexible, ultrafast, and selective analysis using an online solid phase extraction (SPE) tandem mass spectrometry (SPE/MS/MS) system was investigated.

Experimental

The Agilent RapidFire/MS/MS system consisted of the following modules: an Agilent RapidFire 360, an Agilent 6460 Triple Quadrupole Mass Spectrometer, Agilent MassHunter Qualitative Analysis B.05.00, and Agilent MassHunter Quantitative Analysis B.05.00. Samples were analyzed at an average rate of 14.5 seconds per sample. Analyte and internal standard ions were monitored simultaneously in all experiments.

Chemicals and reagents

Clozapine (1.0 mg/mL in methanol), norclozapine (1 mg/mL in methanol), and clozapine-d4 (0.1 mg/mL in methanol) were purchased from Cerilliant, Round Rock, TX. Blank control human serum was purchased from UTAK, Valencia, CA. All other solvents and reagents were purchased from Sigma-Aldrich, St. Louis, MO.

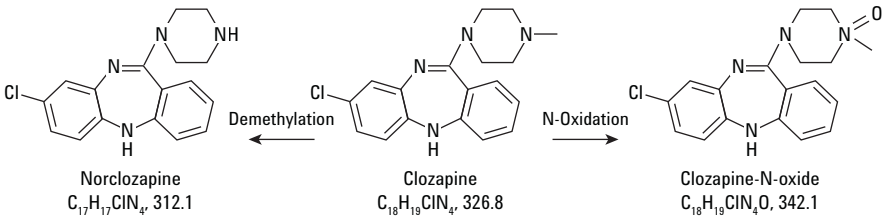

Figure 1. Chemical structures of clozapine and its major metabolites.

Table 1. Agilent RapidFire/MS/MS conditions.

Agilent RapidFire conditions	
Buffer A	Water with 2 mM ammonium acetate and 0.1 % formic acid
Buffer B	20 % Acetonitrile with 10 mM ammonium acetate
Buffer C	85 % Ethyl acetate and 15 % methanol with 10 mM ammonium acetate
Injection volume	10 µL
Back chimney wash	Water (aqueous), acetonitrile (organic)
SPE cartridge	Agilent RapidFire cartridge Type A (reversed-phase C4, G9203A)
RF State 1	Sip sensor
RF State 2	1,500 ms
RF State 3	1,000 ms
RF State 4	8,500 ms
RF State 5	500 ms
Agilent 6460 Triple Quadrupole conditions	
Gas temperature	350 °C
Gas flow	13 L/min
Nebulizer	55 psi
Sheath gas temperature	350 °C
Sheath gas flow	11 L/min
Nozzle voltage	500 V
Capillary voltage	3,500 V
Time filtering peak width	0.03 minutes

Table 2. MRM transitions.

Analyte	Q1	Q3	Dwell	Fragmentor	CE	CAV
Clozapine	327.0	270.1	40	159	22	6
Clozapine	327.0	192.1	40	159	50	6
Norclozapine	313.0	192.1	40	169	46	6
Norclozapine	313.0	270.1	40	169	22	6
Clozapine-d4 (IS)	331.0	192.2	20	150	54	4

Sample preparation

Standard calibrators were prepared by spiking drug-free control serum with 1,500 ng/mL of each analyte. Serial dilutions were used to achieve the remaining standard calibrator concentrations. All samples (200 µL) were precipitated with 400 µL acetonitrile containing 0.1 % formic acid and 100 µL methanol containing 200 ng/mL internal standard, clozapine-d4. Samples were vortexed and centrifuged at 2,000 xg for 10 minutes. Samples were transferred to a 96-well plate and diluted 1:10 with water. The sample plate was sealed with an Agilent PlateLoc Thermal Microplate Sealer, then analyzed by an Agilent RapidFire/MS/MS system.

Data analysis

MassHunter Qualitative Analysis (B.05.00) and Quantitative Analysis (B.05.00) were used for data analysis. A 1/x weighing factor was applied during linear regression of the calibration curves. The quantitation, using MassHunter Quantitative software, was performed by spectral peak area ratio to a known concentration of the internal standard.

Results and Discussion

Samples were prepared by spiking clozapine and norclozapine into drug-free control human serum, and then precipitating with acetonitrile containing 0.1 % formic acid. The supernatant was diluted 1:10 with water. Samples were then analyzed through SPE/MS/MS using the RapidFire/MS/MS system with a C4 cartridge at ~14.5 seconds per sample (Figure 2). This RapidFire/MS/MS methodology is capable of throughputs greater than 240 samples per hour, providing a high-throughput and very efficient mode of analysis.

Prepared calibration standards were run four times a day, over a course of four days to establish both intra- and inter-day precision and accuracy (Tables 3 and 4). All analytes had intra- and inter-day accuracies within 7 % and coefficient of variation values less than 10 % for all concentrations within the linear range.

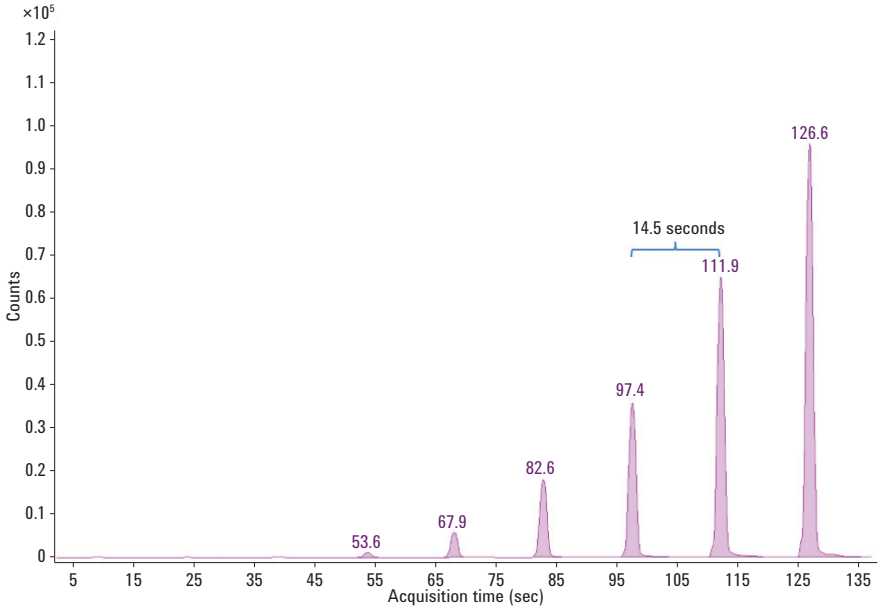

Figure 2. Representative clozapine standard curve showing injection cycle times of ~14.5 seconds.

Table 3. Intraday and interday precision and accuracy for Agilent RapidFire/MS/MS analysis of clozapine in serum.

(ng/mL)	Intraday % accuracy (n = 4)	Intraday % precision (n = 4)	Interday % accuracy (n = 4)	Interday % precision (n = 4)
20	100.32	0.79	105.31	1.24
100	99.20	4.02	96.56	1.92
300	97.15	2.40	97.72	1.28
600	99.32	2.71	99.21	3.57
1,000	99.61	5.50	100.81	2.62
1,500	104.40	7.90	100.39	2.51

Table 4. Intraday and interday precision and accuracy for Agilent RapidFire/MS/MS analysis of norclozapine in serum.

(ng/mL)	Intraday % accuracy (n = 4)	Intraday % precision (n = 4)	Interday % accuracy (n = 4)	Interday % precision (n = 4)
20	101.17	1.76	109.72	0.99
100	94.05	9.59	93.44	1.12
300	99.18	0.90	97.09	1.52
600	99.21	3.77	97.72	1.62
1,000	102.38	3.68	100.69	1.28
1,500	104.01	2.89	101.34	1.38

This method had excellent linearity within the measured range of 20–1,500 ng/mL with an R^2 value greater than 0.999 (Figure 3) for each analyte.

The reproducibility of the method was tested by measuring 2,000 sequential injections of both analytes spiked into control human plasma at 250 ng/mL. The same cartridge was used for all 2,000 injections without deviation in pump pressures or peak shape. The instrument response was stable for both analytes. Clozapine, for example, had a coefficient of variation of 5.6 % and accuracy within 1 %.

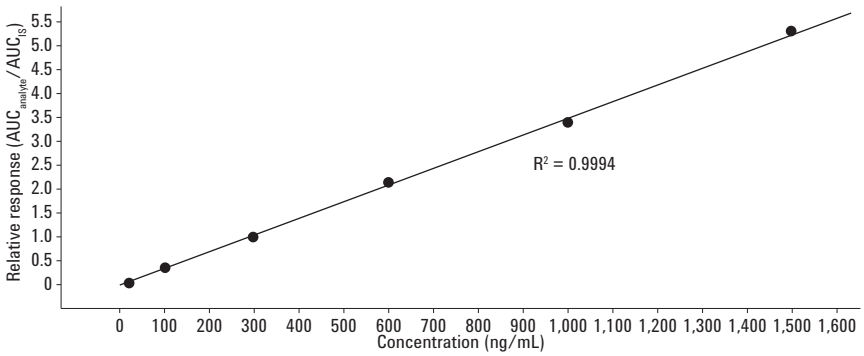

Figure 3. Representative standard curve for norclozapine.

Conclusions

Clozapine, a tricyclic dibenzodiazepine drug, and its metabolite, norclozapine, were accurately and precisely measured in serum using a simple precipitation and dilution method with the Agilent RapidFire/MS/MS system. Moreover, there is the potential to incorporate similar compounds into this method, thus providing a flexible analytical method. At a peak-to-peak injection cycle time of under 15 seconds, this analytical method is capable of analyzing more than 240 samples per hour. Using this SPE/MS/MS methodology, enhanced sensitivity and specificity were achieved compared to traditional HPLC/UV or HPLC/MS/MS methods without compromising throughput and speed.

References

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