Simultaneous Quantitative and Qualitative Analysis of Clozapine and its Metabolites in Rat Plasma Using the Agilent 6540 Q-TOF LC/MS System

Application Note

Clinical Research

Authors

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Abstract

The employment of a high resolution accurate mass (HRAM) LC/MS approach for simultaneous quantitative and qualitative analysis was demonstrated for the bioanalysis and metabolite identifi cation of clozapine in rat plasma. Excellent assay performance was achieved for the quantitation of clozapine and metabolites based on the ultrahigh resolving power and mass accuracy of the Agilent 6540 Quadrupole Time-of-Flight (Q-TOF) LC/MS system. Two clozapine phase I metabolites were successfully identifi ed in rat plasma. The HRAM MS and MS/MS data acquired in this study can be retrospectively analyzed to search for potential metabolites, biomarkers, and endogenous components without sample re-injection.

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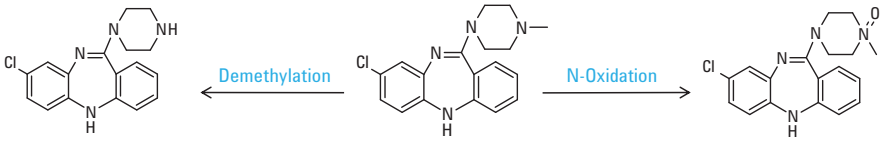
Introduction

Multiple reaction monitoring (MRM) by triple quadrupole mass spectrometry has been the most commonly used method for the quantitation of drugs and their metabolites in complex biological matrices.[1,2] With recent advances in Q-TOF HRAM mass spectrometry, there is a growing interest in using HRAM LC/MS and MS/MS methods for quantitative bioanalysis.[3-5] Q-TOF HRAM LC/MS and MS/MS methods offer advantages over triple quadrupole MRM methods by allowing rapid method development, and providing accurate mass and MS/MS fragmentation information for further metabolite identifi cation (ID) and structural characterization.[6] This application note presents HRAM LC/MS and MS/MS methods with great selectivity and mass accuracy for the simultaneous quantitative and qualitative analysis of clozapine and its metabolites, norclozapine and clozapine-N-oxide (Figure 1), in rat plasma. Excellent sensitivity, linearity, dynamic range, accuracy, reproducibility, and precision were demonstrated in the quantitative measurements. In addition, clozapine metabolites were identified using accurate mass MS and MS/MS data. A combined targeted and untargeted workflow is described for clozapine metabolite ID using Agilent MassHunter Qualitative analysis and Metabolite ID software tools.

Sample preparation

Calibration standards (1–10,000 ng/mL) and quality controls (QCs) (50 ng/mL) were prepared by spiking clozapine, norclozapine, and clozapine-N-oxide at varied concentrations into rat plasma. In the clozapine PK study, rats were dosed at 1 mg/kg through an intravenous (IV) route, and blood samples were taken over a time course of 5 minutes to 7 hours. PK samples, calibration standards, and QCs were spiked with glyburide (internal standard) at 50 ng/mL and extracted with ice-cold acetonitrile before LC/MS analysis. Blank plasma was used as a double blank and blank plasma with 50 ng/mL glyburide was used as a blank.



Norclozapine
$C_{17}H_{17}ClN_4$, 312.1142

Clozapine
$C_{18}H_{19}ClN_4$, 326.8233

Clozapine-N-oxide
$C_{18}H_{19}ClN_4O$, 342.1247

Figure 1. Clozapine and its metabolites, norclozapine and clozapine-N-oxide.

Instrumentation

Liquid chromatography (LC) was performed on the Agilent 1290 Infinity LC System, which consisted of a binary pump, a vacuum degasser, a high performance thermostatted autosampler, and a thermostatted column compartment. Full acquisition MS, auto MS/MS, and targeted MS/MS were performed on the 6540 Q-TOF Mass Spectrometer equipped with an Agilent Jet Stream source in positive ionization mode, and using the mass resolving power (MRP) of 20 K. LC method, ion source conditions, and acquisition method parameters were optimized for clozapine and its metabolites (Table 1).

Table 1. Liquid chromatography, Agilent Q-TOF MS source conditions, and acquisition method parameters.

LC conditions			
Column	Agilent Zorbax Eclipse Plus Rapid Resolution HT column, 2.1 × 50 mm, 1.8 µm (p/n: 959757-902)		
Column temperature	40 °C		
Injection volume	1 µL in MS mode and 2 µL in MS/MS modes		
Autosampler temperature	6 °C		
Needle wash	10 seconds in wash port		
Mobile phase	A = 0.1 % formic acid in water B = 0.1 % formic acid in acetonitrile		
Flow rate	0.75 mL/min		
Gradient program	Time (min)	A (%)	B (%)
	Initial	90	10
	0.70	90	10
	4.00	10	90
	4.50	90	10
	5.00	90	10
Post time	1 minute		
Agilent QTOF MS source conditions			
Drying gas temperature	300 °C		
Drying gas flow	7 L/min		
Sheath gas temperature	400 °C		
Sheath gas flow	11 L/min		
Nebulizer pressure	35 psi		
Capillary voltage	3,750 V		
Nozzle voltage	0 V		
Fragmentor voltage	200 V		
Reference delivery	Agilent 1200 Isocratic pump with 100:1 splitter (p/n: G1607-60000)		
Reference pump flow	0.5 mL/min for 5 µL/min to nebulizer		
Reference ions	121.050873 and 922.009798		
Instrument mass range	1,700 Da		
Instrument mode	Extended dynamic range		
Data storage	Centroid and profile		

The preferred precursor ions in auto MS/MS mode and the targeted precursor ions in targeted MS/MS mode are listed in Table 2.

Table 1. Liquid chromatography, Agilent Q-TOF MS source conditions and acquisition method parameters. (continued)

Agilent Q-TOF MS acquisition method parameters	
Mass range	100-1,000 *m/z*
Acquisition rate	2.5 Hz, 400 ms/scan
Agilent Q-TOF Auto MS/MS acquisition method parameters	
Mass range (MS)	100-1,000 *m/z*
Acquisition rate (MS)	5 Hz, 200 ms/scan
Mass range (MS/MS)	50-1,000 *m/z*
Acquisition rate (MS/MS)	3 Hz, 333.3 ms/scan
Quadrupole isolation Width	Medium
Collision energy	20 V
Maximum precursor ions/cycle	4
Precursor ion static exclusion	100-200 *m/z* and 500-1,000 *m/z*
Precursor ion active charge state	1 and Unknown
Agilent Q-TOF Targeted MS/MS acquisition method parameters	
Mass range (MS)	50-1,000 *m/z*
Acquisition rate (MS)	5 Hz, 200 ms/scan
Mass range (MS/MS)	25-1,000 *m/z*
Acquisition rate (MS/MS)	2.5 Hz, 400 ms/scan
Maximum time between MS1 spectra	3 seconds

Table 2. Auto MS/MS preferred precursor ion list and targeted MS/MS targeted precursor ion list.

Compound name	Precursor ion	Δ*m/z* (ppm)	Charge state	RT (min)	ΔRT (min)	Isolation width	CE (V)	Product ion
Norclozapine	313.1215	20	1	1.64	0.4	Medium	30	270.0793
Clozapine	327.1371	20	1	1.75	0.4	Medium	20	270.0793
Clozapine-N-oxide	343.1320	20	1	1.88	0.4	Medium	15	256.0633
Glyburide (IS)	494.1511	20	1	3.05	0.4	Medium	20	369.0663

Data acquisition and analysis

A MassHunter Workstation (version B.03.01) was used for data acquisition. MassHunter Quantitative (Quan) Analysis Software (version B.04.00) was used for quantitation. Extracted ion chromatograms (EICs) of *m/z* 327.1371, 313.1215, and 343.1320 in MS mode, and product ion EICs of *m/z* 327.1371 > 270.0793, 313.1215 > 270.0793, and 343.1320 > 256.0633 in targeted MS/MS mode, were employed for quantitation of clozapine, norclozapine, and clozapine-N-oxide, respectively. EIC of *m/z* 494.1511 and product ion EIC of *m/z* 494.1511 > 369.0663 were used for glyburide. The mass extraction window (MEW) was 10 ppm. MassHunter Qualitative (Qual) Analysis Software (version B.03.01) was used to find and confirm clozapine metabolites.

Results and Discussion

Quantitative analysis

Norclozapine, clozapine, clozapine-N-oxide, and glyburide were separated using UHPLC at retention times (RT) of 1.64, 1.75, and 1.88, 3.05 minutes, respectively (Figure 2 and Figure 3). The high MRP and narrow MEW employed in the HRAM LC/MS and MS/MS methods greatly decreased the endogenous interference from rat plasma, thus significantly improving the quantitation performance.

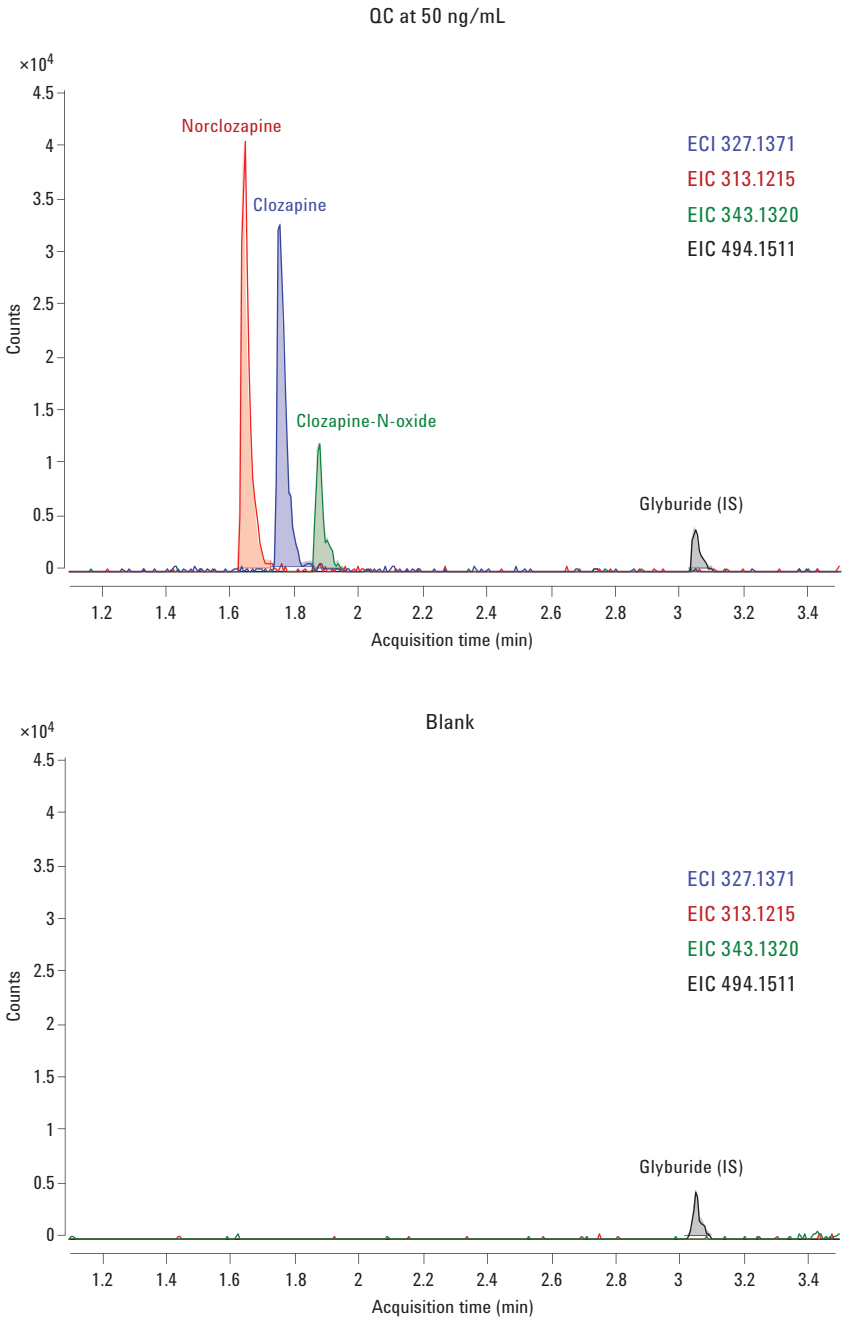


Figure 2. EICs of clozapine, norclozapine, clozapine-N-oxide, and glyburide in MS mode.

Sensitivity

The limit of quantitation (LOQ) is 1 ng/mL or 1 pg on-column for clozapine and its metabolites in rat plasma using the MS method. Using the targeted MS/MS method, the LOQ is 1, 1, 5 ng/mL or 2, 2, and 10 pg on-column for clozapine, norclozapine, and clozapine-N-oxide, respectively. The MS method is slightly more sensitive than the targeted MS/MS method. Based on our observations, the relative sensitivity of MS method versus targeted MS/MS method depends on the nature of the interference from the complex biological matrix. The MS method is highly selective based on the ultra-high MRP of Q-TOF. Alternatively, the targeted MS/MS method gains further selectivity based on fragmentation in the collision cell and selection of a specific product ion for quantitation; however, the ultimate signal intensity is sacrificed.

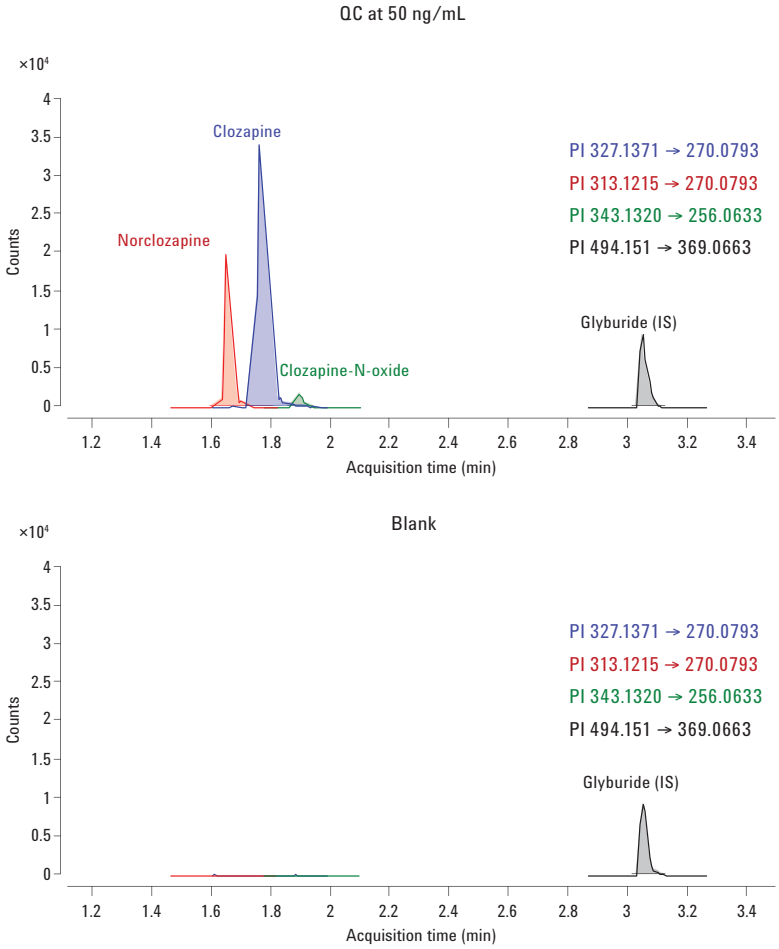


Figure 3. Product ion EICs of clozapine, norclozapine, clozapine-N-oxide, and glyburide in targeted MS/MS mode.

Calibration curve linearity and range

The calibration curves for clozapine, norclozapine, and clozapine-N-oxide in the MS and targeted MS/MS methods (Figure 4 and Figure 5) showed excellent linearity ($R^2 > 0.998$) and a wide dynamic range (≥ 3 orders).

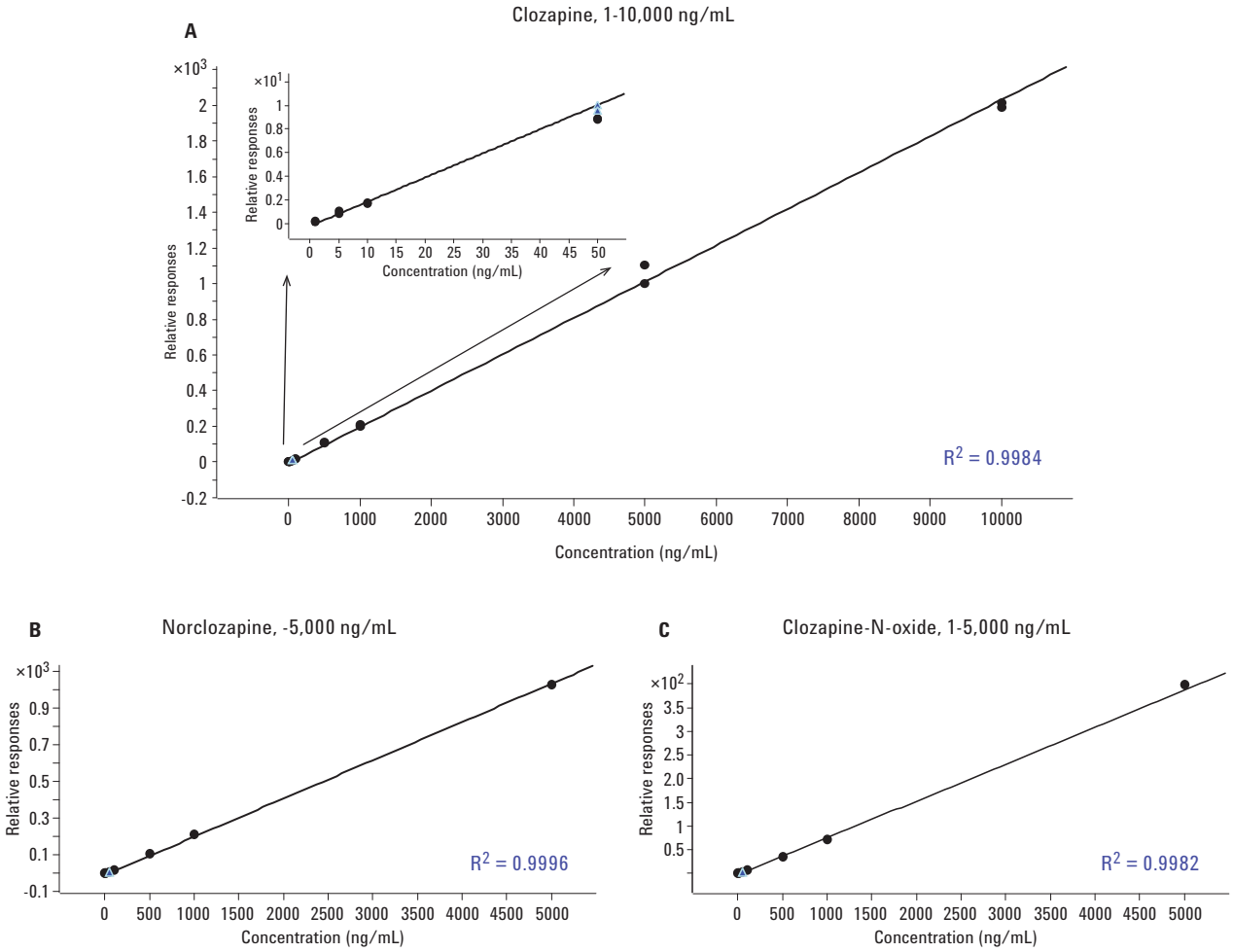


Figure 4. Calibration curves of clozapine (A), norclozapine (B), and clozapine-N-oxide (C) in rat plasma using the MS method. Inset graph (A) demonstrates the low concentration range for clozapine.

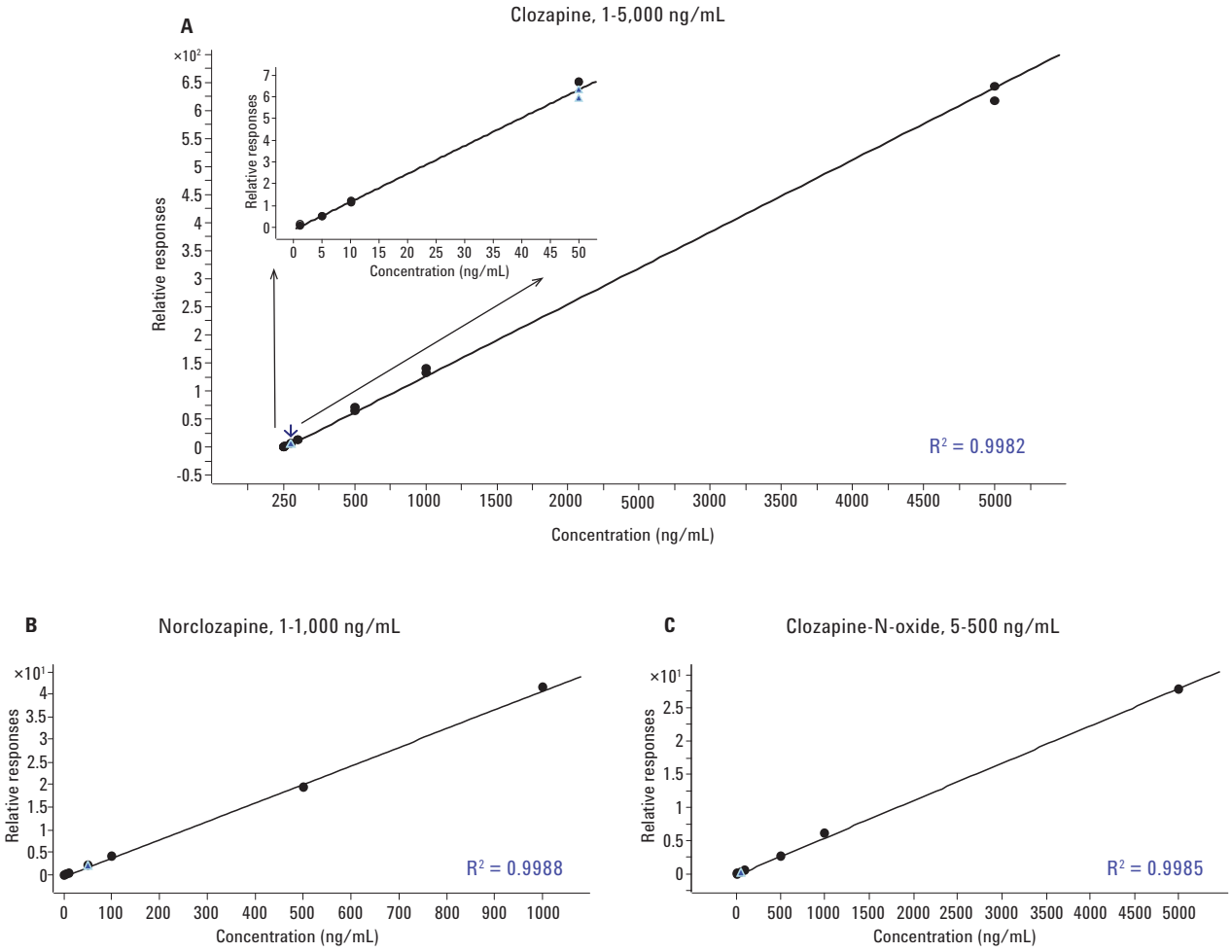


Figure 5. Calibration curves of clozapine (A), norclozapine (B), and clozapine-N-oxide (C) in rat plasma using the targeted MS/MS method. Inset graph (A) demonstrates the low concentration range for clozapine.

Accuracy, reproducibility, and precision

The accuracy, reproducibility, and precision were evaluated at up to nine standard concentrations, and the QC level for clozapine and its metabolites. The accuracy, precision and reproducibility all met the bioanalytical accepted criteria (Table 3).

PK analysis and metabolite profiles of clozapine

The concentrations of clozapine, norclozapine, and clozapine-N-oxide in rat plasma samples were successfully measured with excellent reproducibility (% RSD < 5 in triplicate) (Table 4). Consistent quantitation results were observed using the MS and targeted MS/MS methods. Figure 6 illustrates the concentration-time profiles of clozapine and its metabolites, norclozapine and clozapine-N-oxide, from which the clearance, area-under-curve (AUC), and half-life of clozapine in the rat PK study were determined to be 55 mL/min/kg, 300 ng/mL*h and 1 hour, respectively.

Table 3. Accuracy, reproducibility , and precision results using the MS and targeted MS/MS methods.

	MS method			Targeted MS/MS method		
Compound name	Accuracy (%)	Reproducibility (% RSD, n = 2)	Precision (% RSD, n = 9)	Accuracy (%)	Reproducibility (% RSD, n = 2)	Precision (% RSD, n =8)
Clozapine	89.6-109.5	0.37-9.03	7.24	87.2-106.1	0.31-6.05	6.51
Norclozapine	91.4-107.5	N/A	6.47	88.1-109.9	N/A	7.01
Clozapine-N-oxide	86.9-115.9	N/A	10.13	87.1-108.4	N/A	6.49

Table 4. Measured concentrations in rat plasma PK samples using the MS and targeted MS/MS methods.

Conc. (ng/mL)	Clozapine (ng/mL)		Norclozapine (ng/mL)		Clozapine-N-oxide (ng/mL)	
Time	MS	MS/MS	MS	MS/MS	MS	MS/MS
5 minutes	303	304	6.10	6.35	3.11	BLOQ
15 minutes	206	195	6.51	6.39	3.33	BLOQ
30 minutes	147	137	5.94	5.76	2.84	BLOQ
1 hour	78.1	81.5	4.50	4.88	1.61	BLOQ
2 hours	38.8	35.3	2.62	2.89	1.17	BLOQ
4 hours	9.74	10.6	BLOQ*	BLOQ	BLOQ	BLOQ
7 hours	1.81	1.51	BLOQ	BLOQ	BLOQ	BLOQ

*BLOQ = below limit of quantitation.

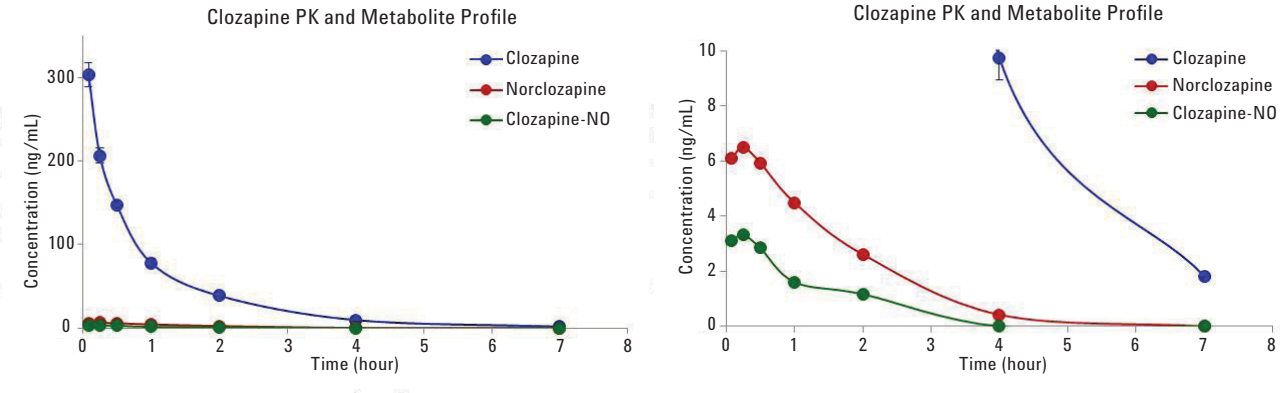


Figure 6. Pharmacokinetics time curve of clozapine and its metabolite profiles in rat plasma PK samples. Plot on the right is the zoom-in of the plot on the left at lower concentration range.

Qualitative analysis

Metabolite ID of clozapine was performed in the 5 minute rat plasma sample using MS, auto MS/MS, and targeted MS/MS data. In MassHunter Qualitative Analysis Software, metabolite ID of clozapine was achieved using both targeted data mining algorithms, find by formula (FbF) and find by targeted MS/MS (FbTMS2), and untargeted or naïve data mining algorithms, molecular feature extraction (MFE), and find by auto MS/MS (FbAMS2). FbF and MFE were used to process MS data, while FbTMS2 and FbAMS2 were used to process targeted and auto MS/MS data, respectively.

Metabolite ID of clozapine using MS data

In FbF, the MS data were searched against a personal compound database (PCD) for clozapine (Figure 7) to find matching peaks using accurate mass information. The database was created by entering the formulas of known clozapine metabolites previously published in literature. In MFE, multiple related ion clusters detected from the raw MS data are grouped into a list of qualified molecular features or compounds based on ion species, charge states, and dimer/trimer formation. The list of compounds found through untargeted MFE algorithm in the plasma sample was further identified using database (DB) search against the PCD of clozapine or MFG to generate formulas using accurate mass and isotope patterns. The triple criteria MFG score was based on accurate mass of the monoisotopic peak, isotope spacing, and isotope abundance pattern. Clozapine and the two metabolites were found in the plasma sample using FbF and MFE plus DB search and MFG with mass errors (MS) < 1 ppm and match scores > 90.

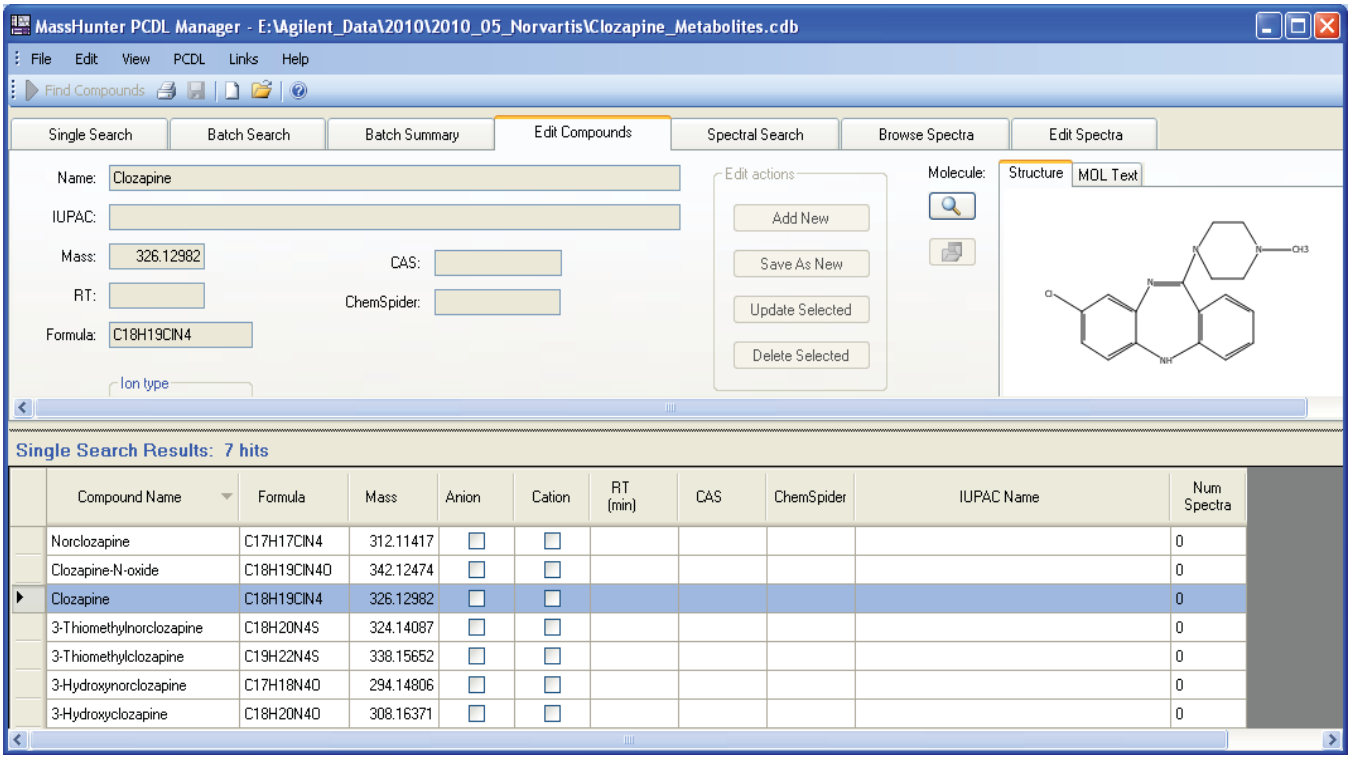


Figure 7. Clozapine personal compound database (PCD).

Figure 8 and Figure 9 illustrate the MS spectra, isotope patterns, and MFG results for clozapine and norclozapine. Notably, excellent mass accuracy with average mass errors < 1 ppm was observed for the isotopes (M+1, M+2, and M+3) of clozapine and the two metabolites, demonstrating the high sensitivity of the 6540 Q-TOF LC/MS System.

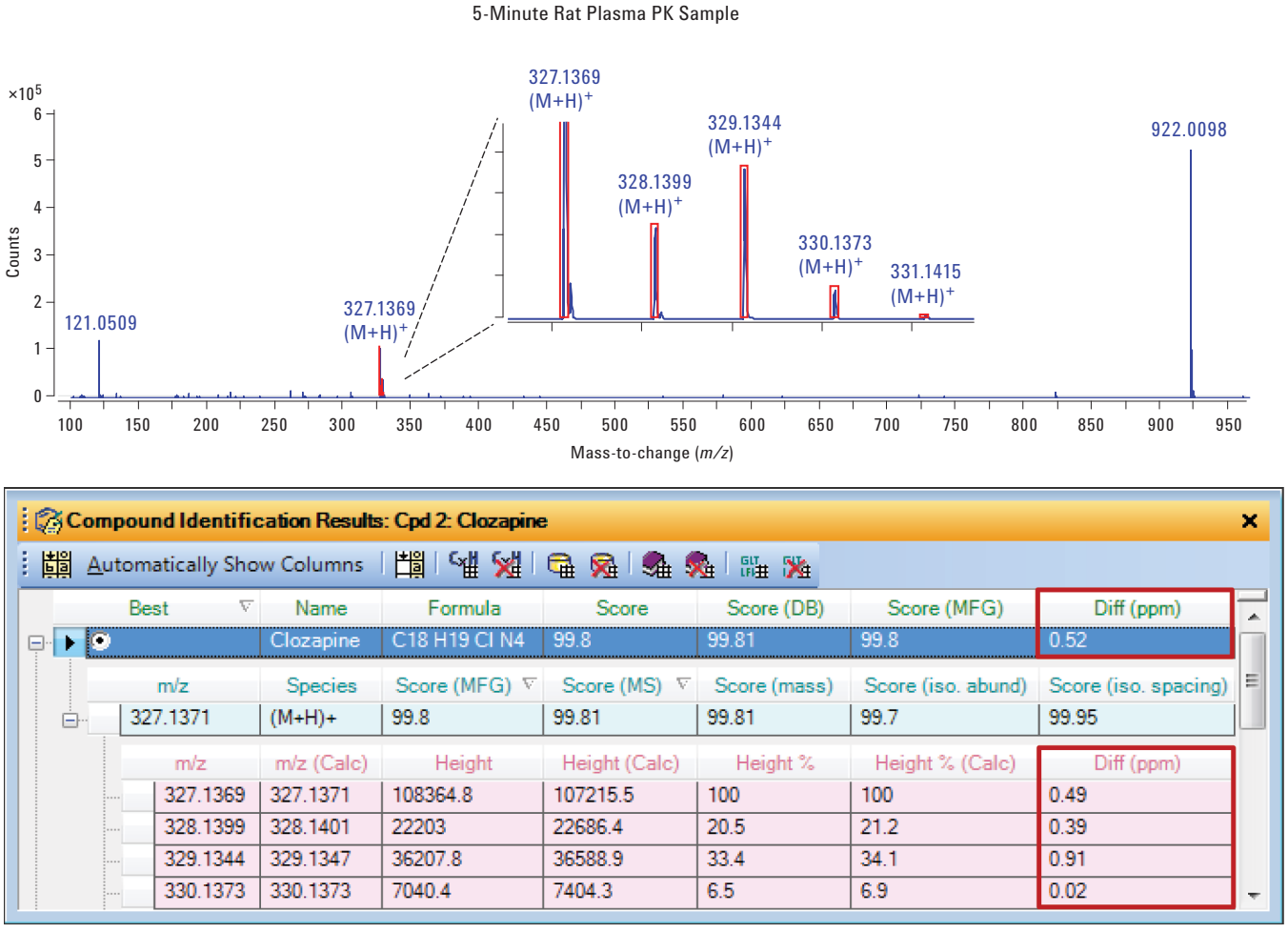


Figure 8. Clozapine MFG results using MS data in Agilent MassHunter Qualitative Analysis Software. The red boxes in the figure insert represent the theoretical isotope abundance and spacing of clozapine.

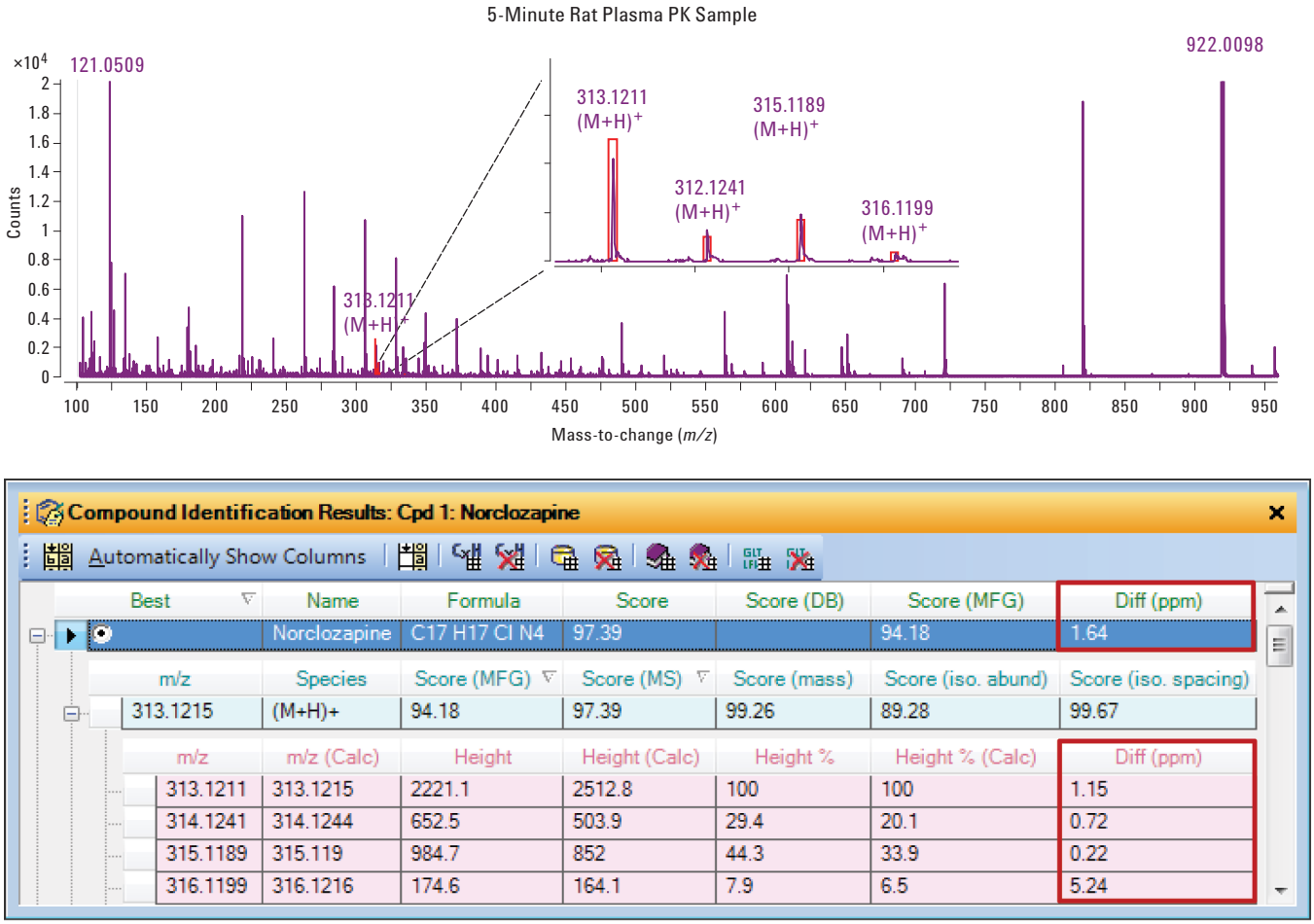


Figure 9. Norclozapine MFG results using MS data in Agilent MassHunter Qualitative Analysis Software. The red boxes in the figure insert represent the theoretical isotope abundance and spacing of norclozapine.

Metabolite ID of clozapine using MS/MS data

The MS/MS data were searched using FbAMS2 and FbTMS2 algorithms. The list of compounds found was subsequently identified using DB search or MFG to generate formulas for the compounds and their fragment ions. The MS/MS MFG scores were based on the coverage and mass errors of the fragment ions. Clozapine and norclozapine were found in the sample using FbTMS2 and FbAMS2 plus DB search and MFG with MS/MS mass errors < 2 ppm and MS/MS MFG scores > 90. Figure 10 and Figure 11 illustrate the auto MS/MS spectra and MFG results for clozapine and norclozapine. Clozapine-N-oxide was not found in rat plasma samples using FbTMS2 and FbAMS2 with either auto or targeted MS/MS data. This could be due to the low level (< LOQ) of clozapine-N-oxide in the PK samples.

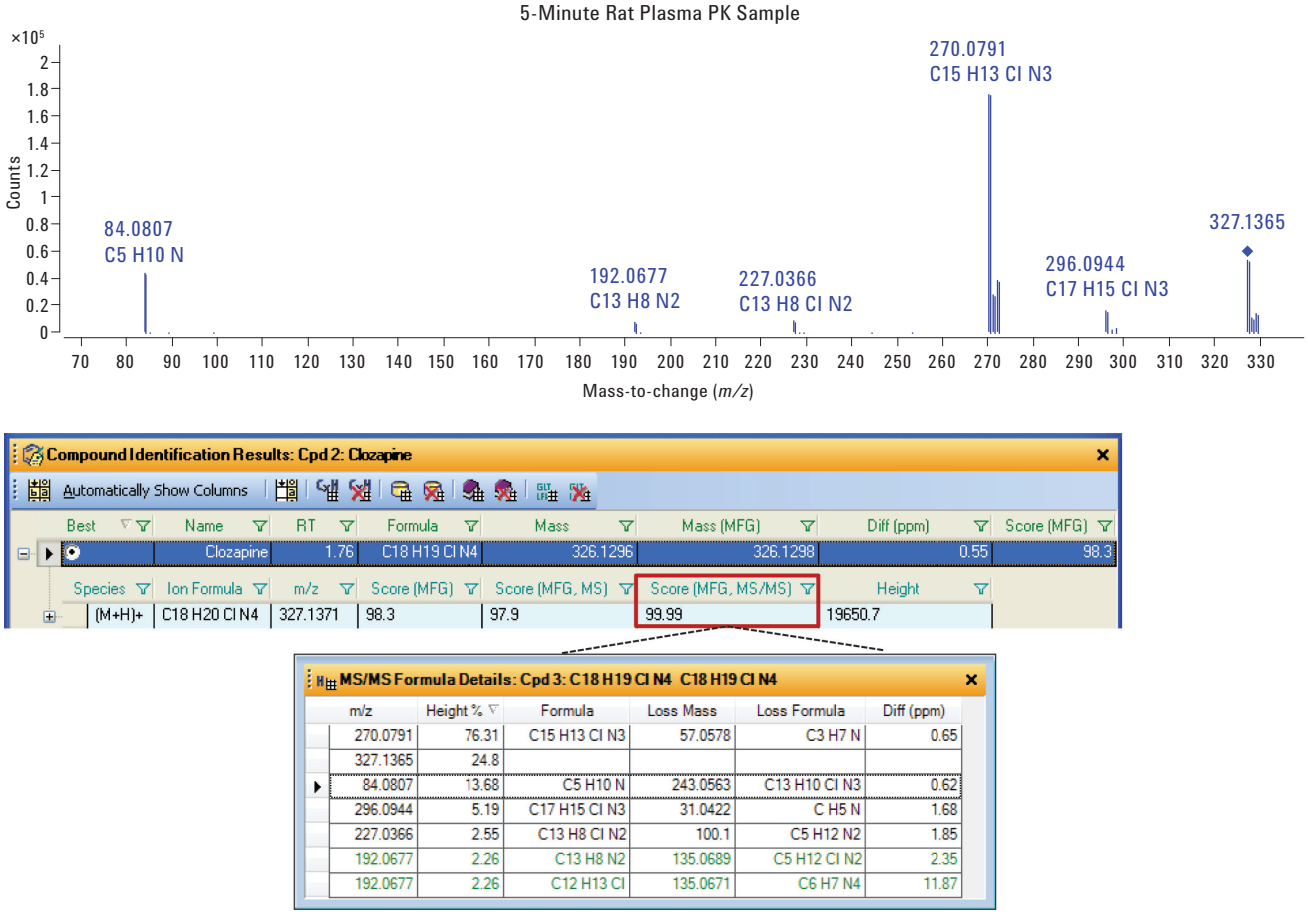


Figure 10. Clozapine MFG results using auto MS/MS data in Agilent MassHunter Qualitative Analysis Software.

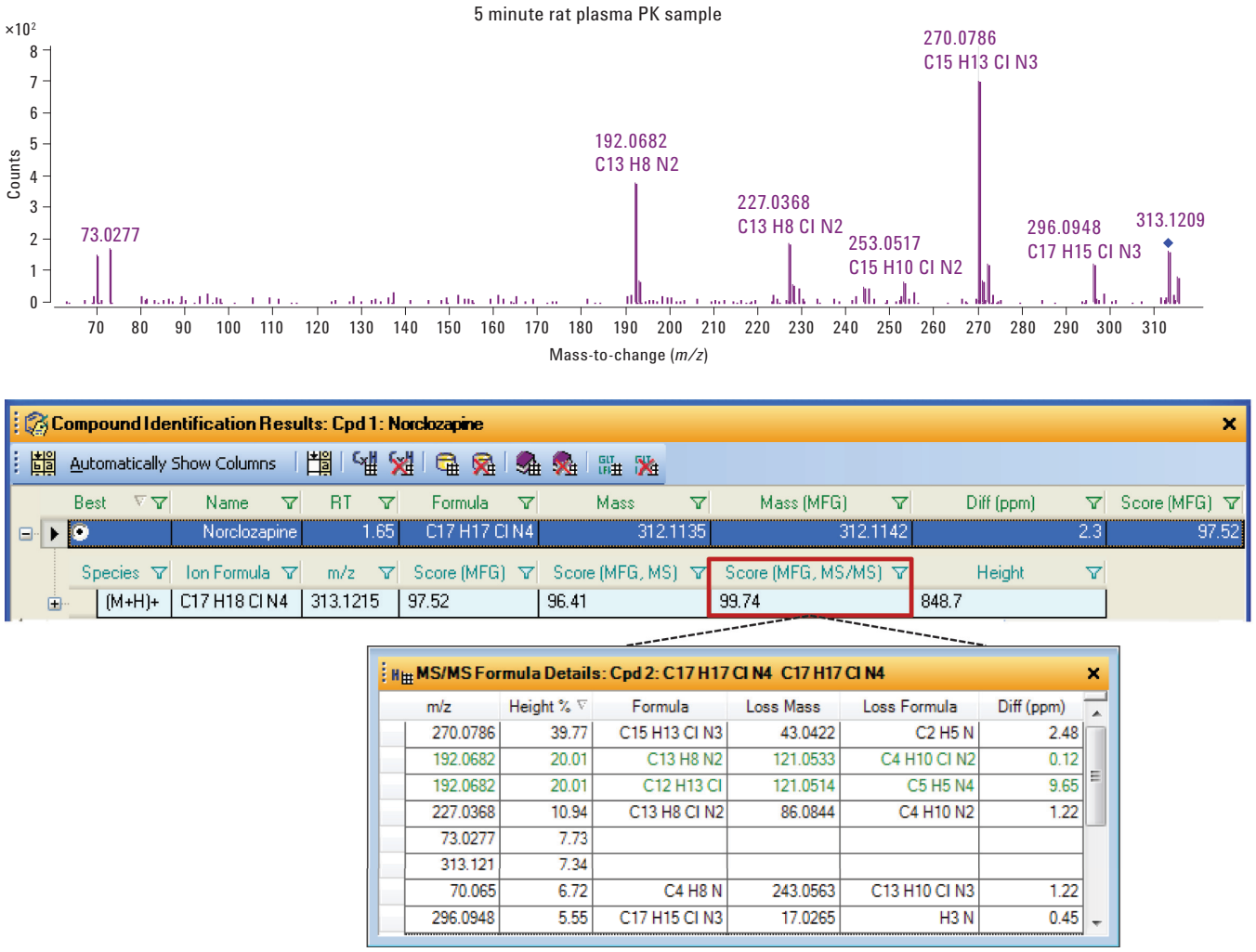


Figure 11. Norclozapine MFG results using auto MS/MS data in Agilent MassHunter Qualitative Analysis Software.

Conclusions

This application note describes high resolution accurate mass LC/MS and MS/MS methods with excellent sensitivity and mass accuracy for the simultaneous quantitative and qualitative analysis of clozapine and its metabolites in rat plasma samples.

- Excellent sensitivity with LOQ of 1 ng/mL or 1 pg on-column in rat plasma.
- Plasma calibration curves show the excellent linearity (> 0.995) with > 3 orders of dynamic range.
- Accuracy (87-116 %), precision (% RSD < 11 %), and reproducibility (% RSD < 10 %) of the assay were well within accepted bioanalytical criteria.
- Clozapine and its metabolites were identified in rat plasma samples with high scores (> 90) and average mass errors of < 1 ppm (MS) and < 2 ppm (MS/MS).
- Powerful software processing tools with sophisticated data mining and feature identification algorithms (FbF, MFE, FbTMS2, FbAMS2, DB search, and MFG) greatly facilitated metabolite identification.

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