Automated Online Card Extraction LC/MS System for the Determination of Clozapine and its Metabolites in Rat Blood

Application Note

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Introduction

The use of dried blood spot (DBS) technology for clinical and pharmacokinetics studies has advantages compared to conventional plasma sampling because it allows sampling of small blood volumes, simplifies sample shipping and storage, and removes many concerns related the handling of biohazardous materials.[1-4] Compared with offline hole punching and extraction methods,[5-9] the fully integrated Agilent Automated Card Extraction (AACE) LC/MS system enables the automated flow-through analysis of DBS cards. This system greatly reduces analysis time and manual experimental errors.

This application note describes the development and validation of a method on the AACE LC/MS system for the online analysis of DBS cards for the quantitation of clozapine and two of its metabolites, norclozapine and clozapine-N-oxide (Figure 1), in rat whole blood. The quantitative performance of the online extraction method was evaluated and compared to that of a conventional offline extraction hole punching method. Excellent sensitivity, linearity, dynamic range, precision, accuracy, and reproducibility were demonstrated by the online extraction method. This approach can be readily applied to clozapine pharmacokinetic and metabolic profiling studies.

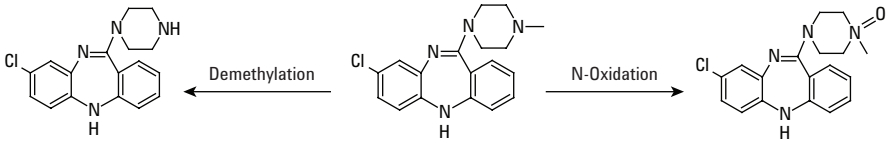

Figure 1. Clozapine and two of its metabolites, norclozapine and clozapine-N-oxide.

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Experimental

Sample Preparation

Calibration standards for clozapine and its metabolites were prepared by spiking clozapine, norclozapine, and clozapine-N-oxide at different concentrations (0.5 – 10,000 ng/mL) into rat whole blood. Calibration standards (20 µL) were spotted onto Prolab DBS Type 2B cards in triplicate and dried overnight before analysis. DBS cards spotted with rat whole blood were used as blank samples. Deuterated clozapine(D4) was used as internal standard for quantitation of clozapine and its metabolites.

System Configuration

The AACE LC/MS system (Figure 2) consists of the AACE instrument[10] for automated flow through analysis of DBS cards, two Agilent 1260 Infinity Binary LC pumps (one for online sample extraction and cleaning and the other for analytical LC separation), an Agilent 1260 Infinity Isocratic LC pump for sample dilution, and an Agilent 6460 Triple Quadrupole LC/MS System for analyte detection.

A single software program is used to control all components of the system, and the entire process of DBS card extraction, sample trapping, elution, and LC/MS analysis (Figure 3).

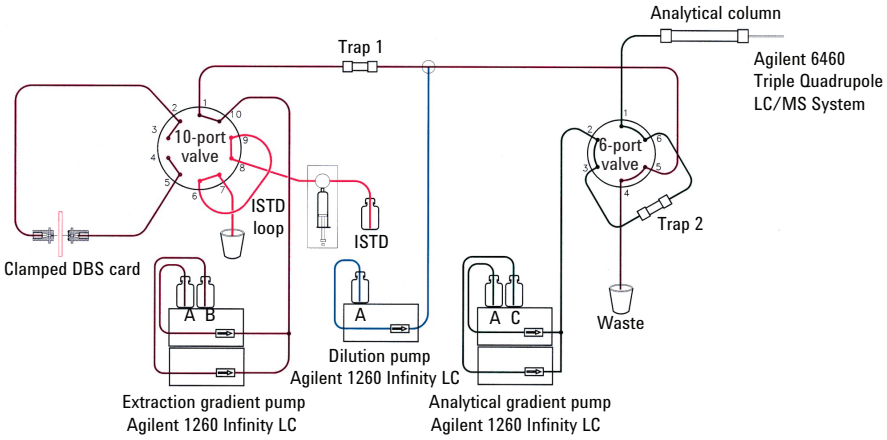


Figure 2. Agilent Automated Card Extraction system configuration.

Figure 3. Integrated software controls LC pumps and Agilent 6460 Triple Quadrupole LC/MS System.

DBS sample information is entered into a sample list and DBS cards are analyzed according to the sample list. A build-in camera in the card extraction unit captures a card image and records the barcode, allowing multiple extractions on the same blood spot and ensuring unambiguous assignment of analytical results with the extracted card and spot.

Table1. LC analysis conditions.

Solvents		
Extraction solvent A1	0.1 % formic acid in water	
Extraction solvent B1	Acetonitrile	
Diluting solvent	0.1 % formic acid in water	
Analytical solvent A2	0.1 % formic acid in water	
Analytical solvent B2	0.1 % formic acid in acetonitrile	
Columns		
Trap 1 column	CC 8/4.6 mm	Nucleosil 100-5 C6H5ec
Trap 2 column	CC 8/4 mm	Nucleosil 100-5 C18 HD
Analytical column	75 × 2.1 mm	Agilent Poroshell 300SB-C18 5 µm
Trap 1 Column temperature	Ambient	
Trap 2 Column temperature	Ambient	
Analytical Column temperature	55 °C	
Pump programs		
Extraction pump program (gradient)		
Time (min)	Solvent B (%)	Flow rate (mL/min)
0.00	5	0.50
2.00	5	0.50
3.00	85	0.50
3.80	85	0.50
3.90	100	0.75
6.00	100	0.75
6.30	5	0.75
6.50	5	0.50
9.50	5	0.50 TE 1 ON SCAP DBS Restart
Dilution pump program (isocratic)		
Time (min)	Flow rate (mL/min)	
0.00	0.05	
2.00	0.05	
2.20	2.50	
3.80	2.50	
4.00	0.05	
Analytical pump program (gradient)		
Time (min)	Solvent B (%)	Flow rate (mL/min)
0.00	5	0.40
1.00	5	0.40
2.50	50	0.40
3.50	50	0.40
3.60	95	1.00
6.50	95	1.00
6.80	5	1.00
7.00	5	0.40
8.50	5	0.40 STOP

Table 2. Mass spectrometer conditions.

MS conditions	
Gas temperature	300 °C
Drying gas flow	8 L/min
Nebulizer gas	45 psi
Sheath gas temperature	350 °C
Sheath gas flow	11 L/min
Capillary voltage	3250 V
Nozzle voltage	0 V
Delta EMV	250

Mass Spectrometer Settings

Mass spectrometry analysis was performed using a 6460 Triple Quadrupole LC/MS System equipped with an Agilent Jet Stream source operated in positive ionization mode. Source conditions and Multiple Reaction Monitoring (MRM) method parameters (Table 3) were optimized for clozapine and its metabolites.

DBS Method Timers

Timer 1	Extraction from DBS to Trap 1	90 s
Timer 2	Trap 1 sample wash	70 s
Timer 3	Sample transfer from Trap 1 to Trap 2	30 s
Timer 4	Analytical run and Trap 1 wash and condition	240 s

The DBS analysis method includes an initial preparation phase for sample loading, and four optimized timers (Timer 1 – Timer 4) with different valve positions and pump programs to enable online sample extraction, trapping and cleaning, and LC/MS analysis. As demonstrated by Figure 4, Timer 1 starts the extraction pump program and the dilution pump program, while Timer 4 starts the analytical pump program and 6460 Triple Quadrupole LC/MS System. The restart for the next DBS extraction is performed from the extraction pump program while the analytical LC gradient is still running. Thus, the extraction step overlaps the analytical run and achieves a cycle time (time duration from one DBS extraction until next DBS extraction) of less than 10 minutes.

Table 3. MRM method parameters for clozapine, norclozapine, clozapine-N-oxide, and internal standard, clozapine (D4).

Compound name	Precursor ion	MS1 resolution	Product ion	MS2 resolution	Dwell (ms)	Fragmentor (V)	CE (V)
Clozapine	327	Unit	192.1	Unit	40	150	51
	327	Unit	270.1	Unit	40	150	24
Norclozapine	313	Unit	192.2	Unit	40	150	51
	313	Unit	270.2	Unit	40	150	25
Clozapine-N-oxide	343	Unit	192.2	Unit	40	150	51
	343	Unit	256.2	Unit	40	150	19
Clozapine(D4) (IS)	331	Unit	192.2	Unit	40	150	51
	331	Unit	272.2	Unit	40	150	24

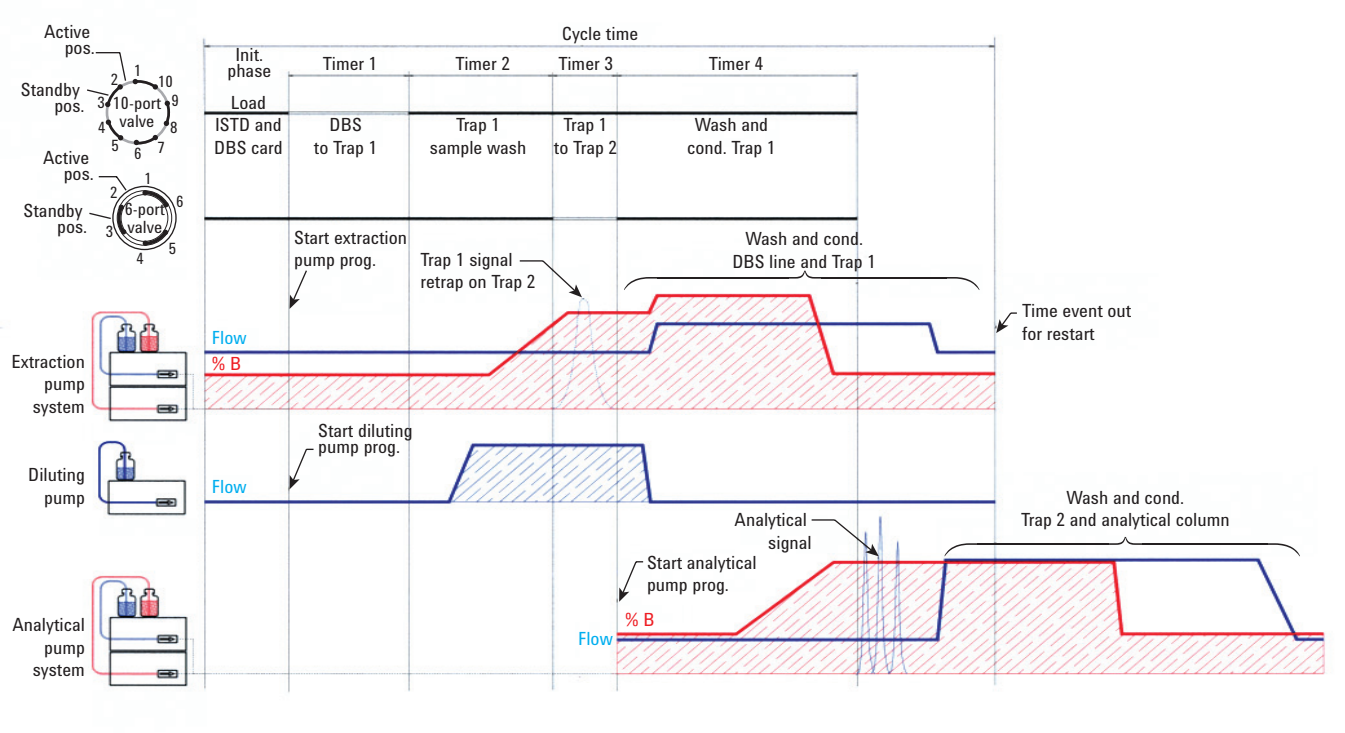


Figure 4. Extraction method timers and relevant valve positions and pump programs.

Method Development

The automated online extraction and analysis of DBS samples, is applicable to the analysis of a wide range of analytes. The separation of matrix components and the washing of the trapping columns is the key to the development of a sensitive and robust method. This was performed in two phases to establish the optimal parameters for the online extraction and the analytical LC/MS detection.

Extraction Components

In the initial phase of the analytical process (Figure 4), the internal standard solution was loaded to the internal standard sample loop. Next, a DBS card was selected from the sample tray and positioned into the clamp for online extraction. During the Timer 1 period (90 seconds), clozapine and its metabolites were extracted from the DBS cards, mixed with the internal standard, clozapine(D4), and trapped on the Trap 1 column. The analytes were then separated from the endogenous matrix components in rat blood during the Timer 2 period (70 seconds), using the optimized solvents and extraction pump gradient. The duration of the Timer 1 and Timer 2 periods were carefully chosen to ensure efficient DBS extraction and sample wash before the elution of clozapine and its metabolites from the Trap 1 column (Figure 5).

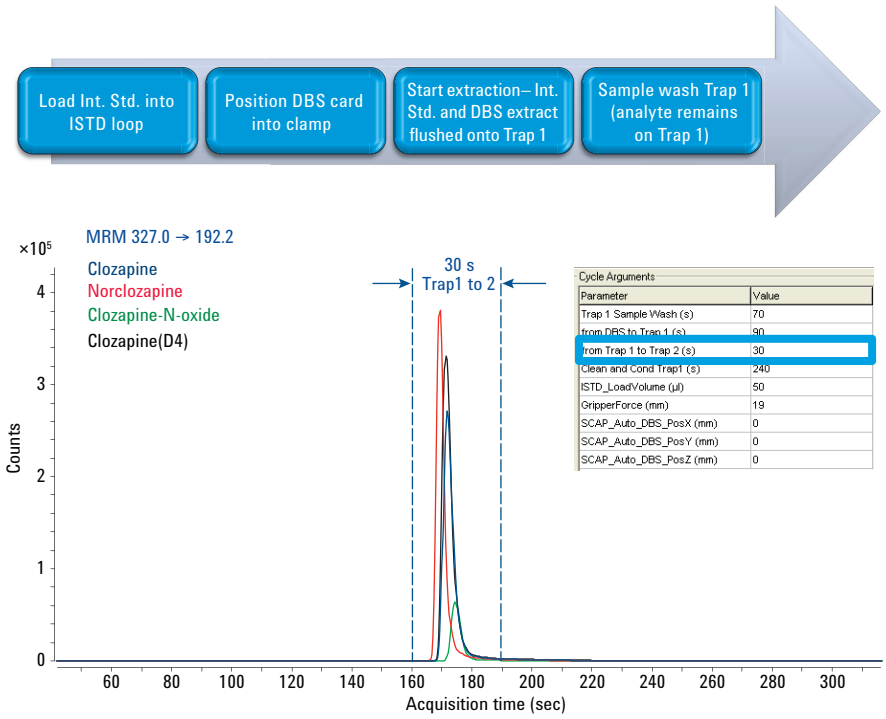


Figure 5. Clozapine and its metabolites were eluted from the Trap 1 column during the Timer 3 period.

Analytical Components

During the Timer 3 period (30 seconds), the Trap 1 column was connected to the Trap 2 column. Clozapine and its metabolites were eluted from the Trap 1 column during the 30-second window (Figure 5) and simultaneously diluted with water to enable their retention on the Trap 2 column prior to the analytical LC/MS run. The duration of the Timer 3 period was optimized to achieve sufficient transfer of the analytes from the Trap 1 to the Trap 2 column, while minimizing the matrix interference. The LC/MS analysis was performed on clozapine and its metabolites during the Timer 4 period (240 seconds), when the Trap 1 column was washed and conditioned for the next DBS extraction. As demonstrated by Figure 6, norclozapine, clozapine, and clozapine-N-oxide were eluted from the analytical column at 2.75, 2.81, and 2.87 min, respectively, and quantified in relation to the internal standard, clozapine(D4), using the optimized MRM transitions.

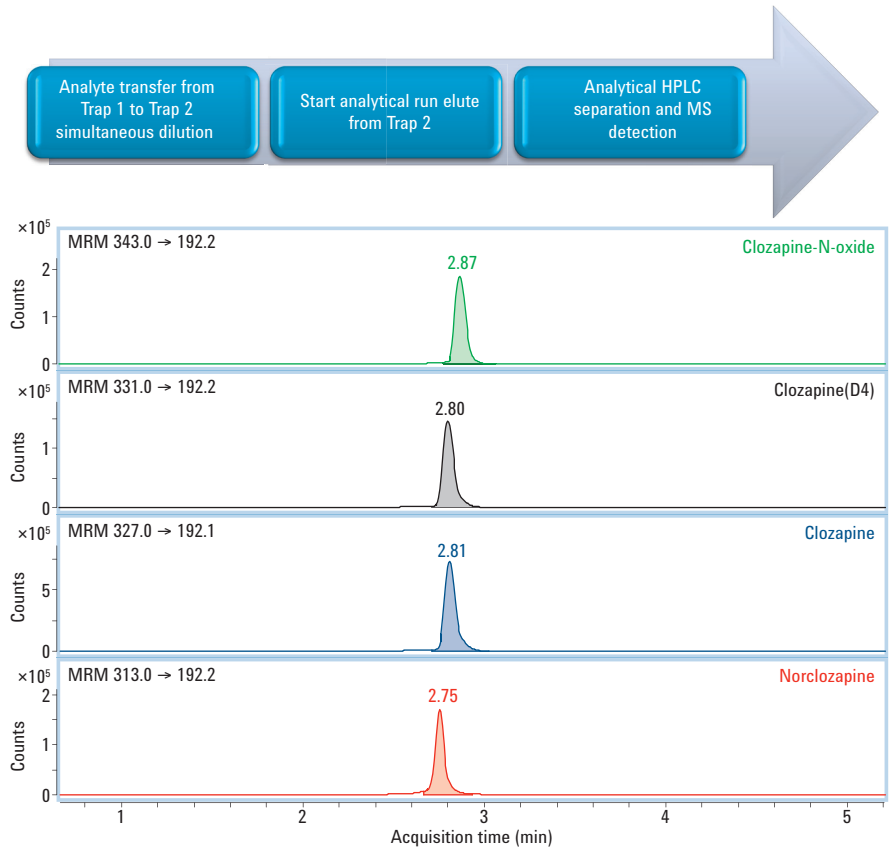


Figure 6. MRM chromatograms of clozapine, norclozapine, clozapine-N-oxide, and internal standard, d4-clozapine.

Data Processing and Reporting

MassHunter Software was used for data processing and reporting. The two most abundant MRM transitions were selected for each analyte as quantifier and qualifier transitions, and the ratio of these was used to confirm the presence of the target analyte in rat whole blood. Once the analysis is complete, a report can be generated. The report includes the MRM chromatograms, the quantitation results, and before and after photographic images for each DBS sample. This provides an easy way to review each analysis and to reveal any anomalies regarding sampling integrity (Figure 7).

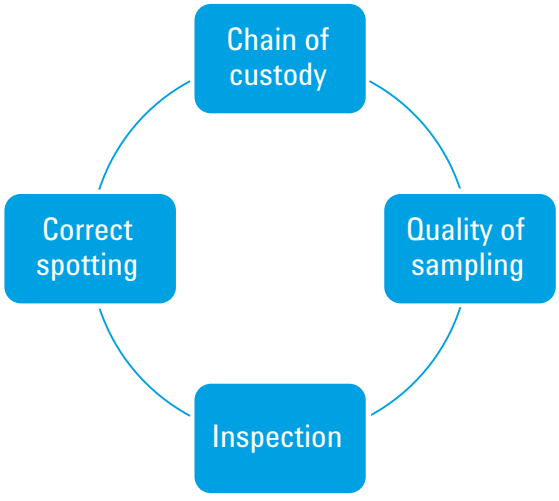


Figure 7. Data reporting and review workflow.

Results and Discussion

Sensitivity

The limit of quantitation (LOQ) is 0.5 ng/mL for clozapine, norclozapine, and clozapine-N-oxide, in rat whole blood, with a S/N ratio > 30:1 (Figure 8). Excellent accuracy (85 – 115 %) and reproducibility (% RSD < 10 from triplicate results) of both retention time and peak area response were obtained at the LOQ level for all three analytes.

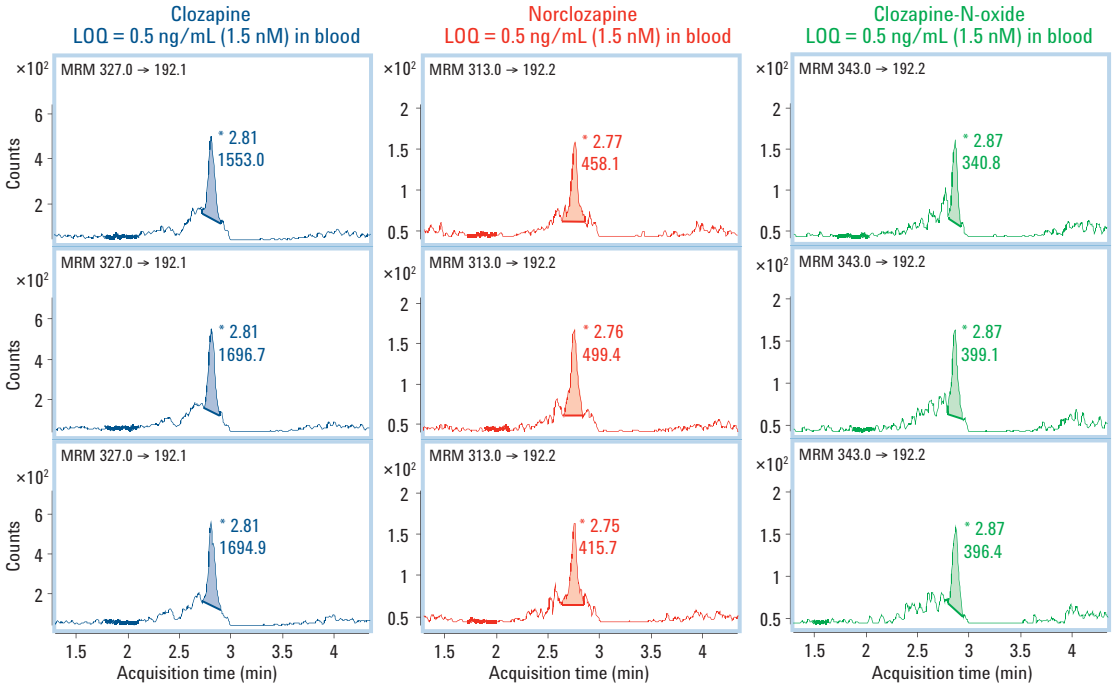


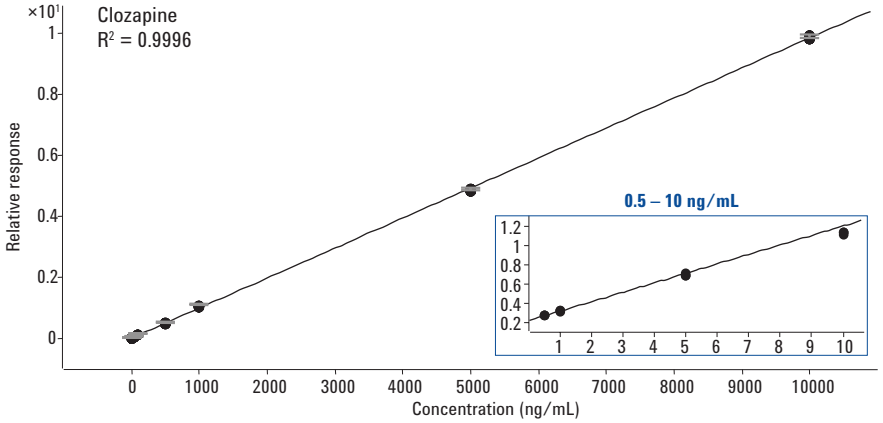
Figure 8. MRM chromatograms of clozapine, norclozapine, and clozapine-N-oxide at the LOQ levels.

Calibration Curve Linearity and Dynamic Range

As demonstrated by Figures 9 – 11, The calibration curves for clozapine, norclozapine, and clozapine-N-oxide showed excellent linearity (Average $R^2 > 0.999$) and wide dynamic range (≥ 4 orders of magnitude). Notably, the dynamic range for clozapine is over 4 orders of magnitude (0.5 – 10000 ng/mL) with an R^2 value of 0.9996. The figure insets demonstrate the detection accuracy (85 – 115 %) and reproducibility (% RSD < 10) at low concentration levels.

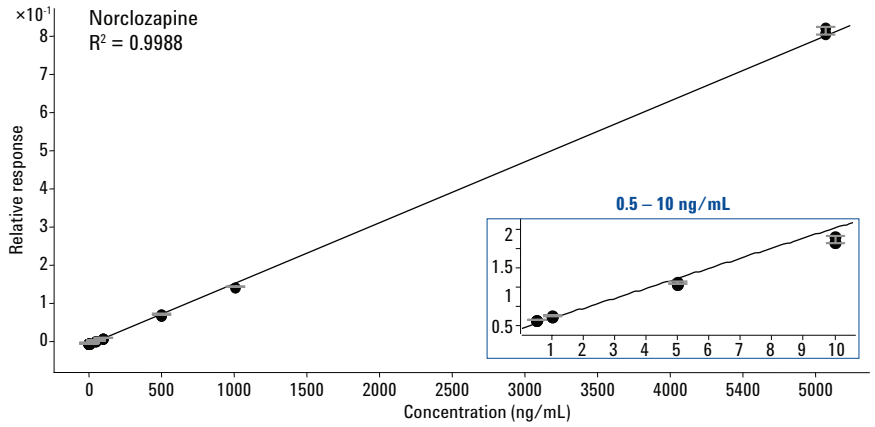
Accuracy, Reproducibility and Precision

The accuracy, reproducibility, and precision were evaluated at 10 standard concentrations for clozapine and its metabolites. The results are summarized in the tables of Figures 9 – 11. The accuracy, precision and reproducibility of the automated flow-through DBS method all meet the bioanalytical acceptance criteria.



	Calibration standards									
Concentration (ng/mL)	**0.5**	**1.0**	**5.0**	**10**	**50**	**100**	**500**	**1000**	**5000**	**10000**
% Accuracy	95.5	101.7	88.0	98.1	101.2	103.2	93.5	103.2	98.8	100.6
Reproducibility (% RSD, n = 3)	8.92	4.93	2.07	2.73	2.33	0.61	2.40	0.62	1.01	0.69
Response factor	0.61	0.65	0.57	0.63	0.65	0.66	0.60	0.66	0.63	0.65
Precision (% RSD, n = 10)	4.89									

Figure 9. Calibration curve of clozapine (0.5 – 10000 ng/mL), and accuracy, responsibility and precision of the quantitative analysis at 10 standard concentrations.

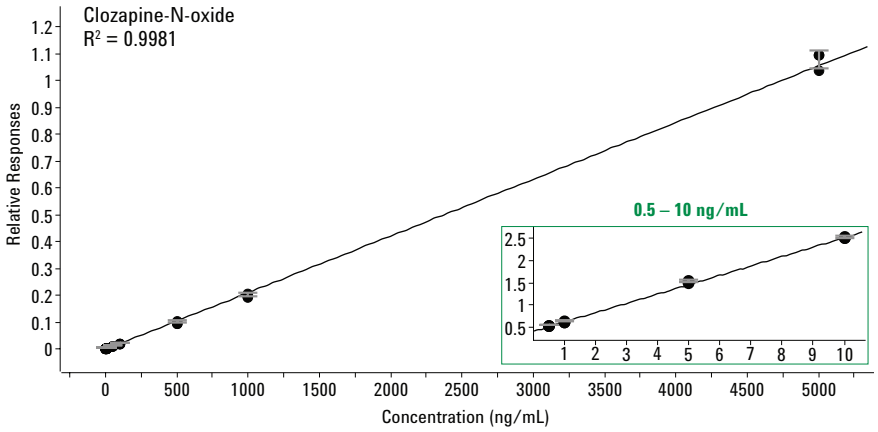


	Calibration standards									
Concentration (ng/mL)	**0.5**	**1.0**	**5.0**	**10**	**50**	**100**	**500**	**1000**	**5000**	**10000**
%Accuracy	95.2	98.9	91.2	104.9	107.1	110.5	116.3	89.3	100.7	100.5
Reproducibility (%RSD, n = 3)	5.69	8.98	3.54	4.26	1.58	1.74	7.92	0.08	0.18	0.32
Response factor	0.25	0.26	0.24	0.27	0.28	0.29	0.30	0.23	0.26	0.26
Precision (%RSD, n = 9)	8.36									

Figure 10. Calibration curve of norclozapine (0.5 – 5000 ng/mL), and accuracy, reproducibility, and precision of the quantitative analysis at 10 standard concentrations.

Comparison of Online Extraction and Offline Extraction Methods

The quantitation performance of the AACE LC/MS system was compared to that of an offline extraction hole punching method and consistent results were observed (Table 4). Comparable quantitative performance capabilities of the online and offline extraction methods demonstrated the validity of using the AACE LC/MS system for automated DBS analysis.



	Calibration standards									
Concentration (ng/mL)	**0.5**	**1.0**	**5.0**	**10**	**50**	**100**	**500**	**1000**	**5000**	**10000**
% Accuracy	105.1	108.4	93.6	102.0	96.0	109.2	92.1	91.8	101.5	100.4
Reproducibility (% RSD, n = 3)	1.99	2.55	8.66	5.38	6.88	4.16	6.57	2.01	0.77	0.25
Response factor	0.06	0.06	0.05	0.06	0.05	0.06	0.05	0.05	0.06	0.06
Precision (% RSD, n = 9)	6.64									

Figure 11. Calibration curve of clozapine-N-oxide (0.5 – 5000 ng/mL), and accuracy, reproducibility, and precision of the quantitative analysis at 10 standard concentrations.

Table 4. Quantitation performance comparison: the online extraction method versus the offline extraction method.

	Automated online card extraction method			Offline extraction hole punching method		
Compound name	LOQ (ng/mL)	Linear range (ng/mL)	Linearity correlation	LOQ (ng/mL)	Linear range (ng/mL)	Linearity correlation
Clozapine	0.5	0.5 – 10000	0.9996	0.5	0.5 – 10000	0.9997
Norclozapine	0.5	0.5 – 5000	0.9988	0.5	0.5 – 10000	0.9991
Clozapine-N-oxide	0.5	0.5 – 5000	0.9981	0.5	0.5 – 10000	0.9991
	Automated online card extraction method			Offline extraction hole punching method		
Compound name	Accuracy (%)	Reproducibility (% RSD, n = 3)	Precision (% RSD, n = 10)	Accuracy (%)	Reproducibility (% RSD, n = 3)	Precision (% RSD, n =10)
Clozapine	92.1 – 106.8	0.61 – 5.61	4.82	88.0 – 103.2	0.61 – 8.92	4.89
Norclozapine	85.2 – 109.9	0.84 – 6.05	8.94	89.3 – 107.1	0.08 – 8.98	8.36
Clozapine-N-oxide	93.2 – 104.1	0.68 – 6.15	3.82	91.8 – 108.4	0.25 – 8.66	6.64

Conclusions

We have demonstrated the use of an automated online card extraction system for the analysis of clozapine and two of its metabolites in rat whole blood directly from DBS cards using LC/MS. The system delivers excellent sensitivity with LOQ of 0.5 ng/mL and excellent linearity (average R^2 >0.999) over 4 orders of dynamic range. The accuracy (85 – 109 %), reproducibility (% RSD < 6.2 %) and precision (% RSD < 8.9 %) of the quantitative analysis are well within accepted bioanalytical criteria. Furthermore, the automated online card extraction system provides quantitative performance capabilities comparable to offline extraction hole punching methodologies.

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