Automated On-Line Card Extraction LC/MS System for the Determination of Clozapine and its Metabolties in Rat Blood

Na Pi Parra, Lester Taylor, Doug McIntyre, and Yuqin Dai, Agilent Technologies, Inc., Santa Clara, CA, USA.

ASMS 2012
MP18

Agilent Technologies

Introduction

The use of dried blood spots (DBS) technology for clinical and pharmacokinetics studies has advantages compared to conventional plasma sampling because it allows sampling of small blood volumes, easy sample shipping and storage, and removes many concerns related the handling of biohazardous materials. Compared with offline hole punching and manual extraction methods, the fully integrated Agilent Automated Card Extraction (AACE) LC/MS System (Figure 1) enables automated flow-through analysis of DBS cards.

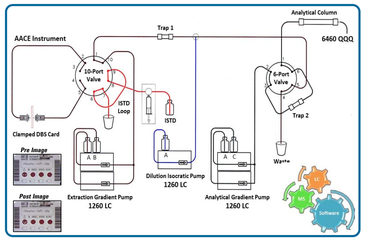


Figure 1. Agilent Automated Card Extraction (AACE) LC/MS System configuration.

This system greatly reduces analysis time and manual experimental errors. This work describes the development and validation of a method on the AACE LC/MS System for online analysis of DBS cards for the quantitation of clozapine and two of its metabolites, norclozapine and clozapine-N-oxide (Figure 2), in rat whole blood. Excellent sensitivity, linearity, dynamic range, precision, accuracy, and reproducibility, were demonstrated for the online extraction method. The quantitative performance capabilities of the online extraction method was evaluated and compared to that of a conventional offline extraction hole punching method and consistent results were observed. This DBS analysis approach using the AACE LC/MS system can be readily applied to clozapine pharmacokinetic and metabolic profiling studies.

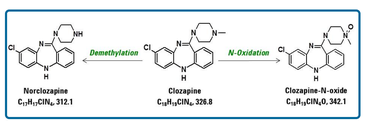


Figure 2. Clozapine and its metabolites, norclozapine and clozapine-N-oxide.

Experimental

Sample Preparation

Calibration standards were prepared by spiking clozapine, and its metabolites at 0.5 to 10000 ng/mL in rat whole blood. Calibration standards were spotted onto Prolab DBS cards in triplicate and dried overnight before analysis. The deuterated d4-clozapine was used as internal standard.

Instrumentation and Methods

The AACE system (Figure 1) consists of the AACE instrument for automated flow-through analysis of DBS cards, two Agilent 1260 Infinity Binary LC pumps (one for 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 QQQ for analyte detection. A single software (Figure 1) is used to control all components, and the entire process of DBS card extraction, sample trapping, elution, and LC/MS analysis. A build-in camera captures card images (Figure 1) and records the barcode, allowing multiple extractions on the same blood spot and ensuring unambiguous assignment of results.

Solvents and Columns	
Extraction solvent A	0.1% formic acid in water
Extraction solvent B	Acetonitrile
Dilution solvent	0.1% formic acid in water
Analytical solvent A	0.1% formic acid in water
Analytical solvent B	0.1% formic acid in acetonitrile
Trap 1 column	CC 8/4.6 mm, Nucleosil 100-5 C_6H_5ec, Ambient
Trap 2 column	CC 8/4 mm, Nucleosil 100-5 C18 HD, Ambient
Analytical column	75x2.1 mm, Agilent Poroshell 300SB-C18, 5 µm, 55 °C

Pump Programs

Extraction Pump			Dilution Pump		Analytical Pump		
Time (min)	B (%)	Flow rate (mL/min	Time (min)	Flow rate (mL/min)	Time (min)	B (%)	Flow rate (mL/min)
Initial	5	0.50	Initial	0.05	Initial	5	0.40
2.00	5	0.50	2.00	0.05	1.00	5	0.40
3.00	85	0.50	2.20	2.50	2.50	50	0.40
3.80	85	0.50	3.80	2.50	3.50	50	0.40
3.90	100	0.75	4.00	0.05	3.60	95	1.00
6.00	100	0.75			6.50	95	1.00
6.30	5	0.75			6.80	5	1.00
6.50	5	0.50			7.00	5	0.40
9.50	5	0.50			8.50	5	0.40

MS Source Conditions	
Gas temperature and flow	300 °C at 8 L/min
Sheath gas temperature and flow	350 °C at 11 L/min
Nebulizer pressure	45 psi
Capillary voltage	3250 V
Nozzle voltage	0 V

Compound Name	Precursor Ion	Product Ion	Dwell (ms)	Fragmentor (V)	CE (V)
Norclozapine	313.1215	270.0793	40	0.4	30
Clozapine	327.1371	270.0793	40	0.4	20
Clozapine-N-oxide	343.1320	256.0633	40	0.4	15
Clozapine (D4)	494.1511	369.0663	40	0.4	20

Results and Discussion

Method Development

The separation of matrix components and washing of the trapping columns are key to development of a sensitive method. Method development takes place in several phases to establish optimal parameters for online extraction (Figure 3) , LC/MS analysis (Figure 4), and DBS timers (Figure 5) .

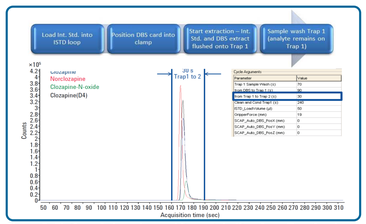


Figure 3. Elution of clozapine and its metabolites from Trap 1 column during analyte online extraction, trapping, and washing process.

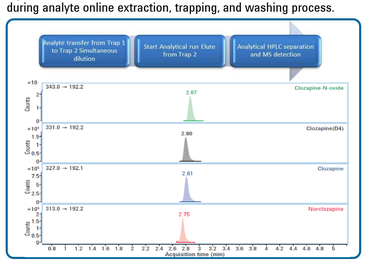


Figure 4. MRM chromatograms of clozapine, norclozapine, clozapine-N-oxide, and internal standard, d4-clozapine during LC/MS analysis.

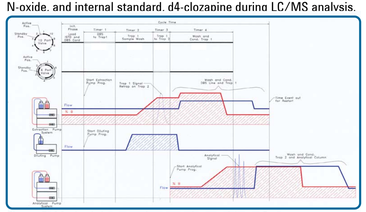


Figure 5. Method timers, relevant valve positions, & pump programs.

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 6). Excellent and reproducibility of both retention time and peak area response was obtained at the LOQ level.

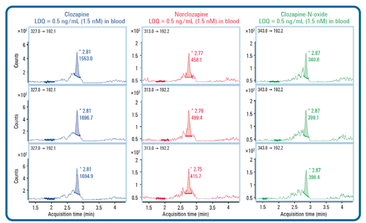
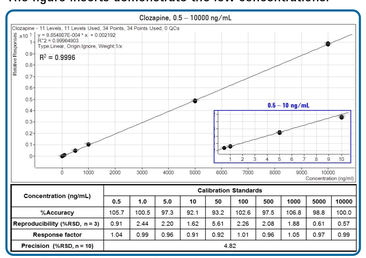


Figure 6. MRM chromatograms of clozapine, norclozapine, and clozapine-N-oxide at the LOQ levels.

Calibration Curve Linearity and Range

The calibration curves (Figure 7 – 9) show excellent linearity ($R^2 > 0.998$) and wide dynamic range (≥ 4 orders). The figure inserts demonstrate the low concentrations.



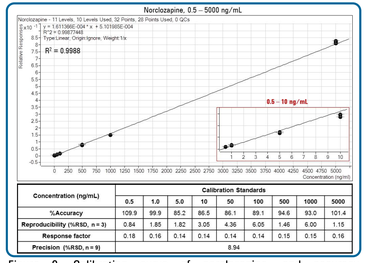
Concentration (ng/mL)	Calibration Standards									
	0.5	1.0	5.0	10	50	100	500	1000	5000	10000
%Accuracy	105.7	100.5	97.3	92.1	93.2	102.6	97.5	106.8	98.8	100.0
Reproducibility (%RSD, n = 3)	0.91	2.44	2.20	1.62	5.61	2.26	2.08	1.88	0.61	0.57
Response factor	1.04	0.99	0.96	0.91	0.92	1.01	0.96	1.05	0.97	0.99
Precision (%RSD, n = 10)	4.82									

Figure 7. Calibration curve of clozapine, and accuracy, responsibility and precision at 10 standard concentrations.

Accuracy, Reproducibility and Precision

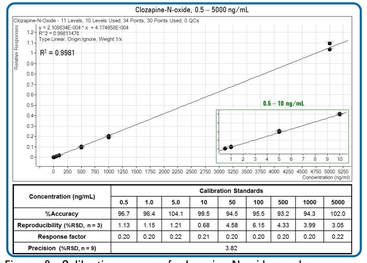
Accuracy, reproducibility, and precision were evaluated at 10 standard concentrations . The results are summarized in the tables of Figures 7 - 9.

Results and Discussion



Concentration (ng/mL)	Calibration Standards									
	0.5	1.0	5.0	10	50	100	500	1000	5000	
%Accuracy	109.9	99.9	85.2	86.5	89.1	94.6	93.0	101.4		
Reproducibility (%RSD, n = 3)	0.84	1.85	1.82	3.05	4.36	6.05	1.46	6.00	1.15	
Response factor	0.18	0.16	0.14	0.14	0.14	0.14	0.15	0.15	0.16	
Precision (%RSD, n = 9)	8.94									

Figure 8. Calibration curve of norclozapine, and accuracy, responsibility and precision at 10 standard concentrations.



Concentration (ng/mL)	Calibration Standards									
	0.5	1.0	5.0	10	50	100	500	1000	5000	
%Accuracy	96.7	96.4	104.1	99.5	94.5	95.5	93.2	94.3	102.0	
Reproducibility (%RSD, n = 3)	1.13	1.15	1.21	0.68	4.08	6.15	4.33	3.99	3.05	
Response factor	0.20	0.20	0.22	0.21	0.20	0.20	0.20	0.20	0.22	
Precision (%RSD, n = 9)	3.82									

Figure 9. Calibration curve of clozapine-N-oxide, and accuracy, responsibility and precision at 10 standard concentrations.

Comparison of Online Extraction and Offline Extraction Methods

The quantitation performance of the automated online card extraction method using the AACE LC/MS System was compared to that of an offline extraction hole punching method and consistent results were observed.

Compound Name	AACE Online Card Extraction Method			Offline Extraction Hole Punching Method		
	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

Compound Name	AACE Online Card Extraction Method			Offline Extraction Hole Punching Method		
	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

- Agilent Automated Card Extraction (AACE) LC/MS System offers automated flow-through DBS analysis with fully integrated software control, efficient method development, and data processing and reporting.
- This system was used for the DBS analysis of clozapine and its metabolites in rat whole blood.
- The AACE LC/MS system delivers excellent sensitivity with LOQ of 0.5 ng/mL.
- Calibration curves in rat blood show great linearity of >0.998 over 4 orders of dynamic range.
- Assay statistics of accuracy (85 - 109%), reproducibility (%RSD < 6.2%) and precision (%RSD < 8.9%) were well within accepted limits.
- Comparable quantitative performance capabilities of the online and offline extraction methods demonstrated the validity of using AACE for automated DBS analysis.