



Automation and Optimization of an On-Line Extraction System for Dried Blood Spot Analysis

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Na Parra Ph.D.
Doug McIntyre

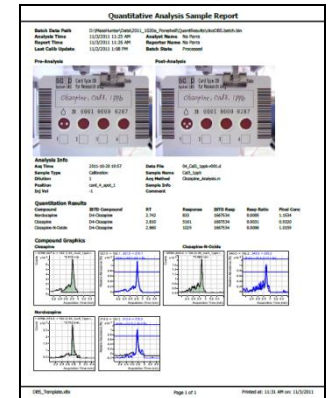
Agilent Technologies
Santa Clara, CA
USA



EBF Conference
Barcelona
Nov 17, 2011

Overview

- Dried Blood Spot Analysis
- Methodologies
 - Hole punch, offline extraction, LC/MS analysis
 - Automated flow through with LC/MS analysis
- SCAP System
- Software Control
- Data Processing & Reporting
- LC/MS Analysis
 - 1290 HPLC 6400 QQQ
 - Clozapine study



Dried Blood Spots

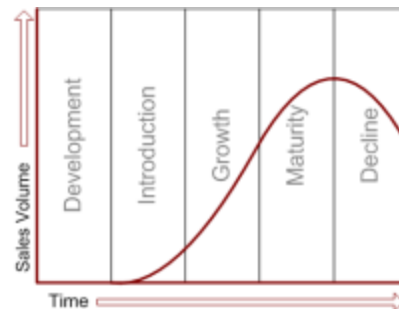
Article

Prev.

Dried Blood Spots as a Sample Collection Technique for the Determination of Pharmacokinetics in Clinical Studies: Considerations for the Validation of a Quantitative Bioanalytical Method

Neil Spooner*, Rakesh Lad and Matt Barfield
PreClinical Development Drug Metabolism and Pharmacokinetics, GlaxoSmithKline Research and Development, Ware, Hertfordshire SG12 0DP, U.K.

Anal. Chem., 2009, 81 (4), pp 1557-1563



- Less sample
- Stable on card at ambient temp
- Easy to ship
- Non-biohazardous
- Not yet FDA approved

- More sample
- Must store frozen
- Difficult to ship
- Biohazardous
- FDA approved

DBS Sampling Methodologies

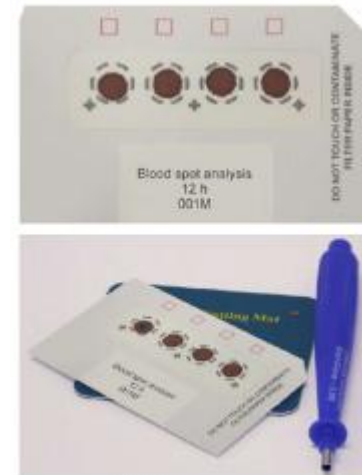
- **Sampling**

- Collect ~ 15 μL blood
- Spot card

- **Analysis**

1. Punch hole method
 - followed by extraction and LC/MS.
2. Clamp card
 - automated flow through LC/MS method.

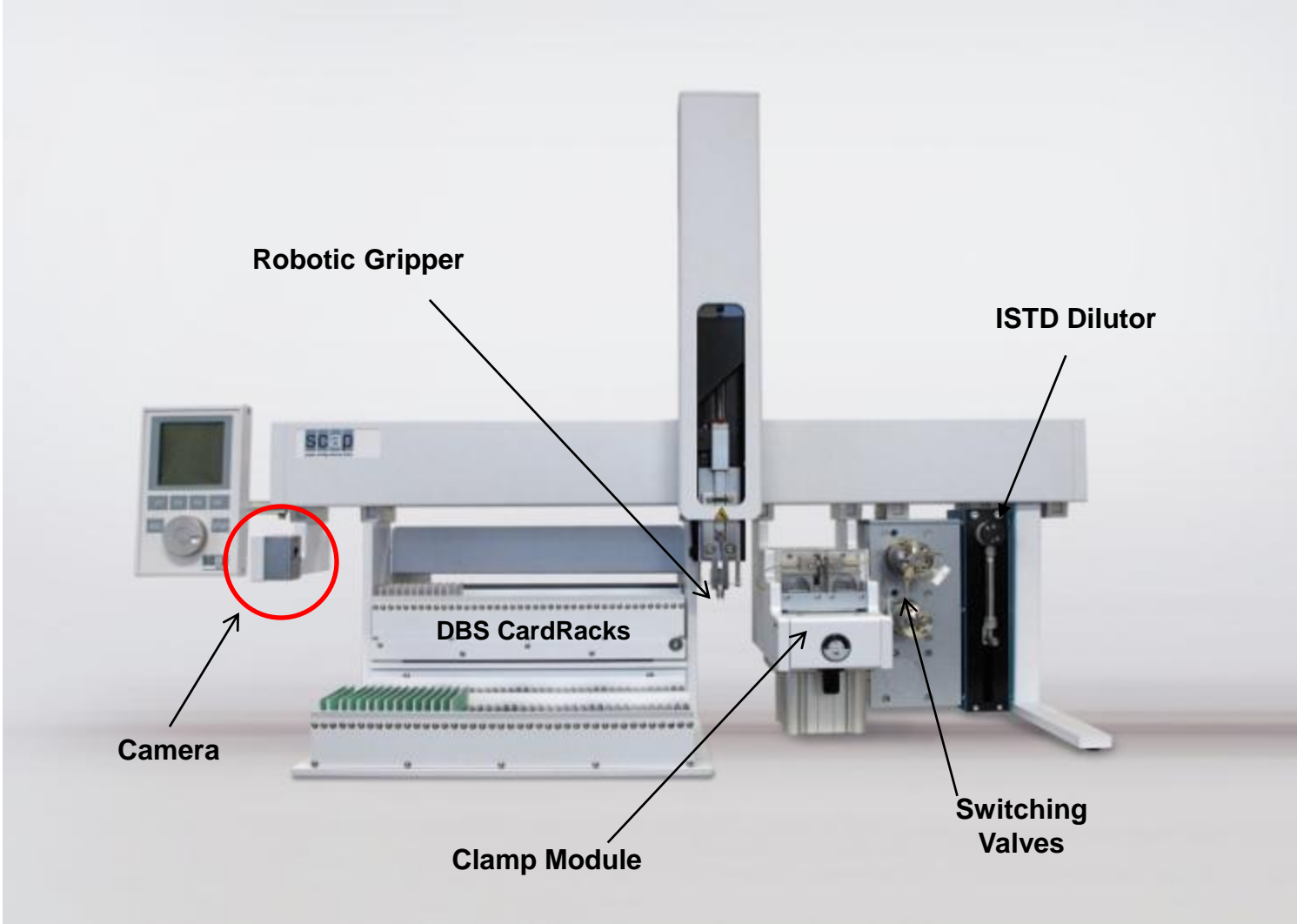
Hole Punch



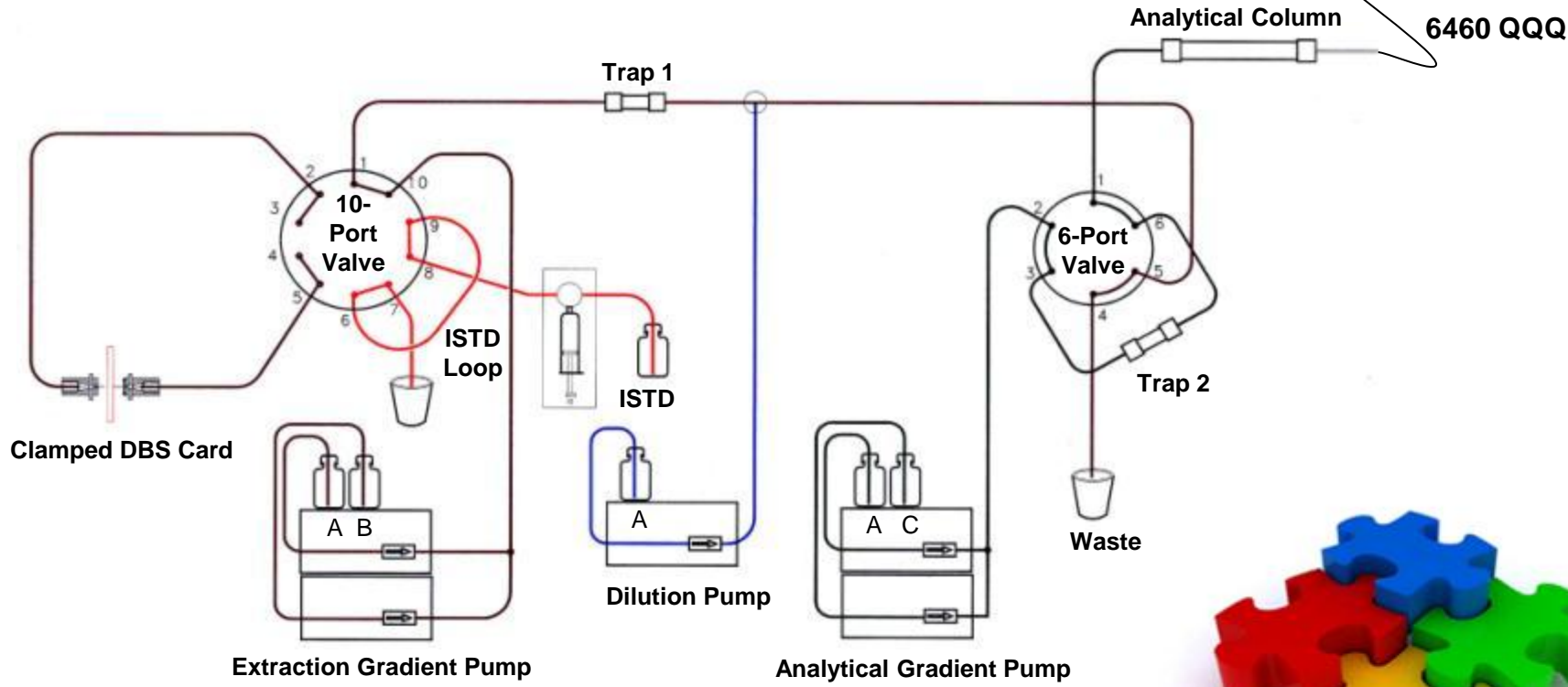
Automated Flow Through



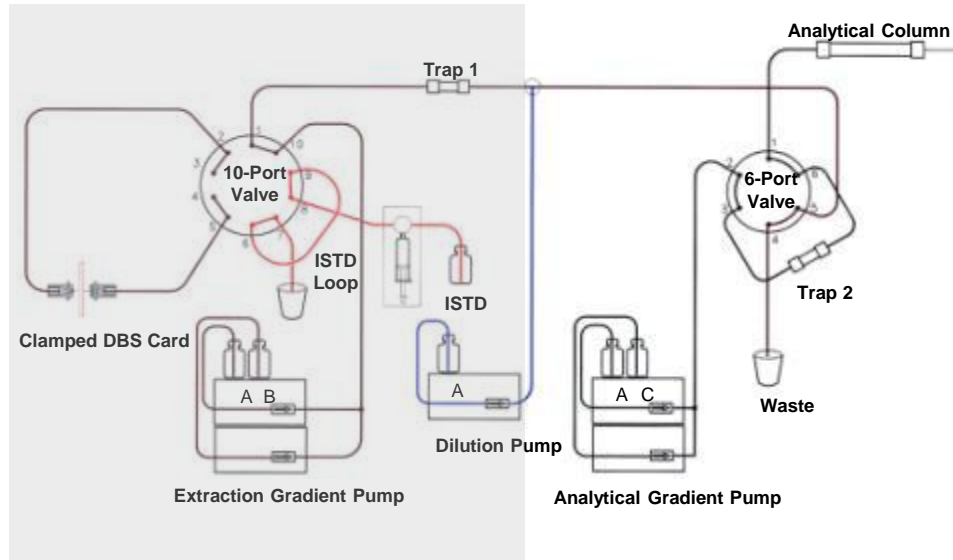
SCAP System from Prolab Instruments



SCAP LC/MS Flow Schematic



Extraction Components



6460 QQQ

Load Int. Std. into ISTD loop

Position DBS card into clamp

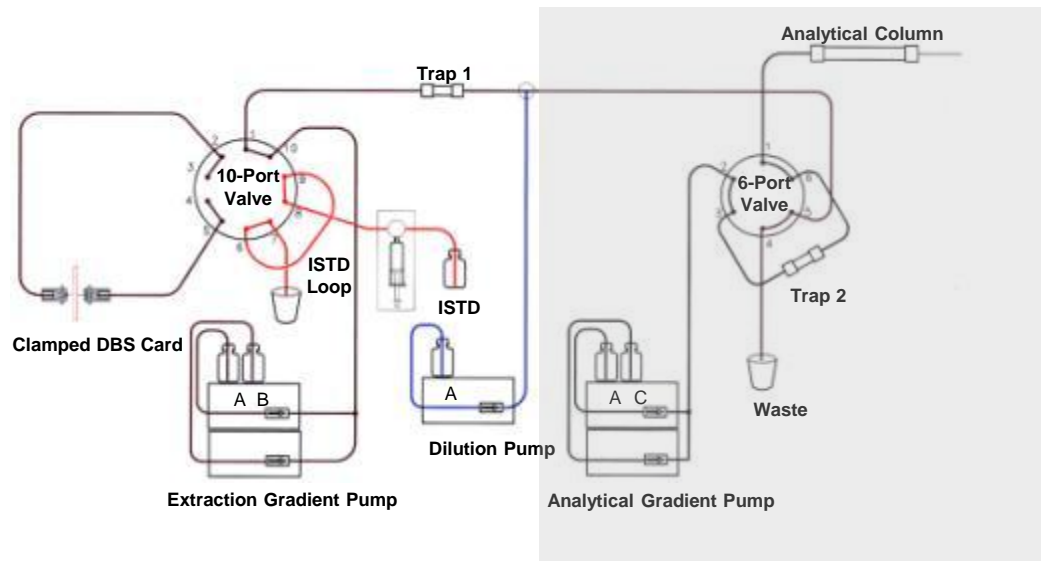
Start extraction – Int. Std. and DBS extract flushed onto Trap 1

Sample wash Trap 1 (analyte remains on Trap 1)

Analytical Components



6460 QQQ



Analyte transfer from
Trap 1 to Trap 2
Simultaneous dilution

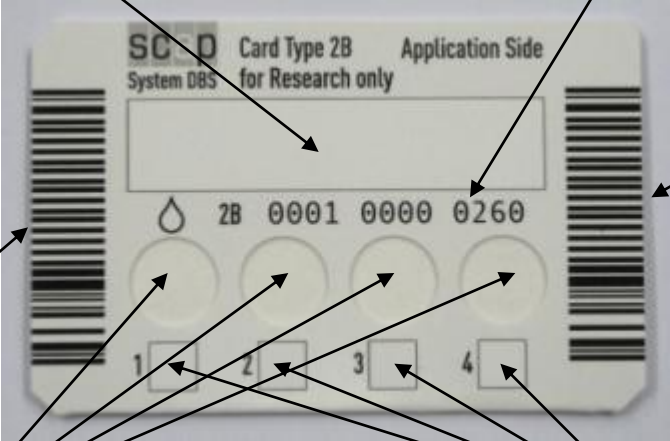
Start Analytical run
Elute from Trap 2

Analytical HPLC
separation and MS
detection

Dried Blood Spot Card

LABEL AREA FOR CUSTOMER
INCL. CUSTOMER BARCODE

CARD BARCODE NUMBER

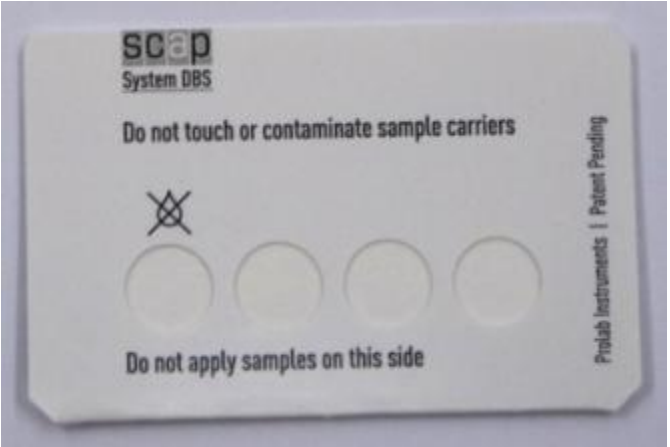


REDUNDANT CARD BARCODE

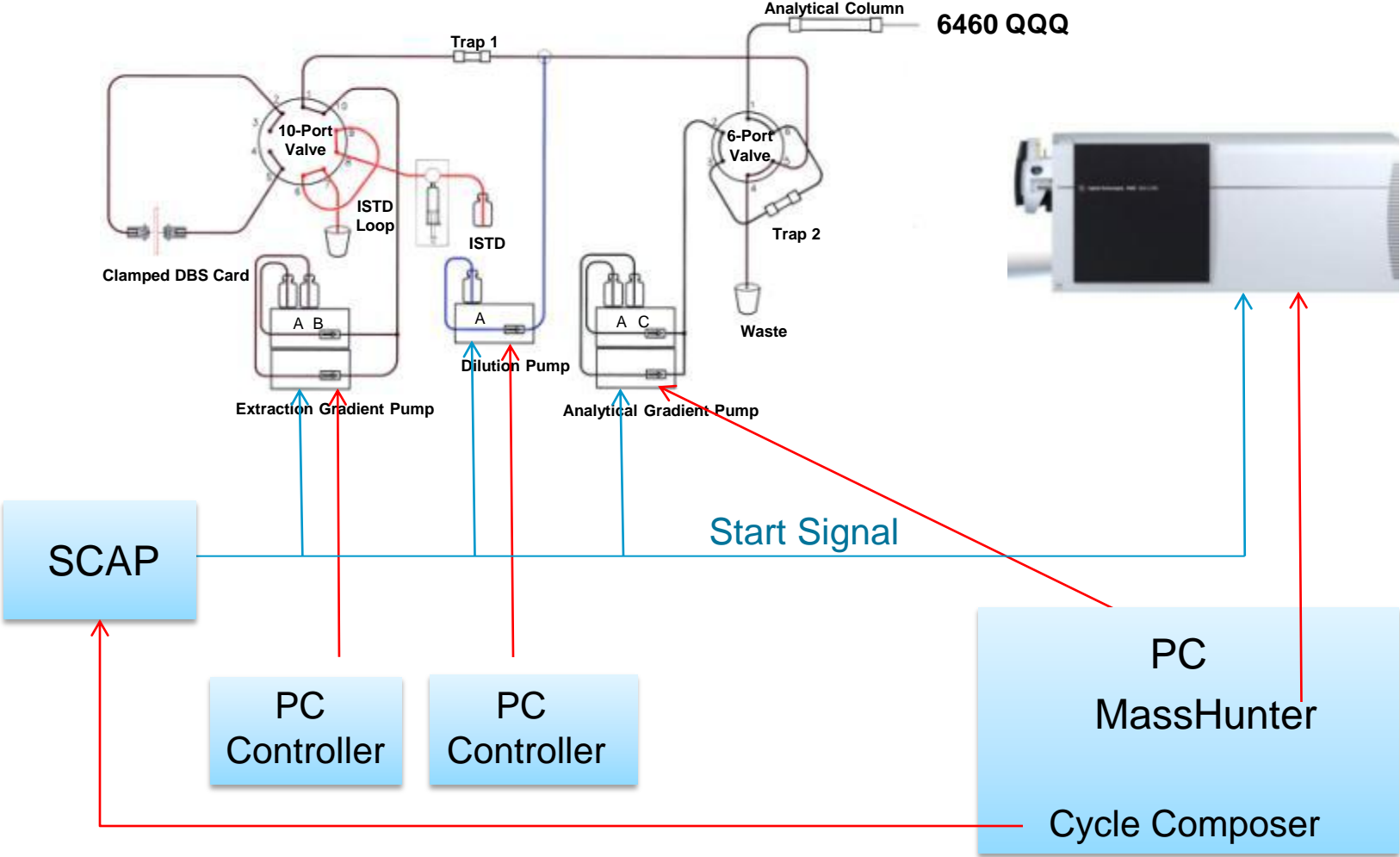
REDUNDANT CARD BARCODE

SUBSTRATE

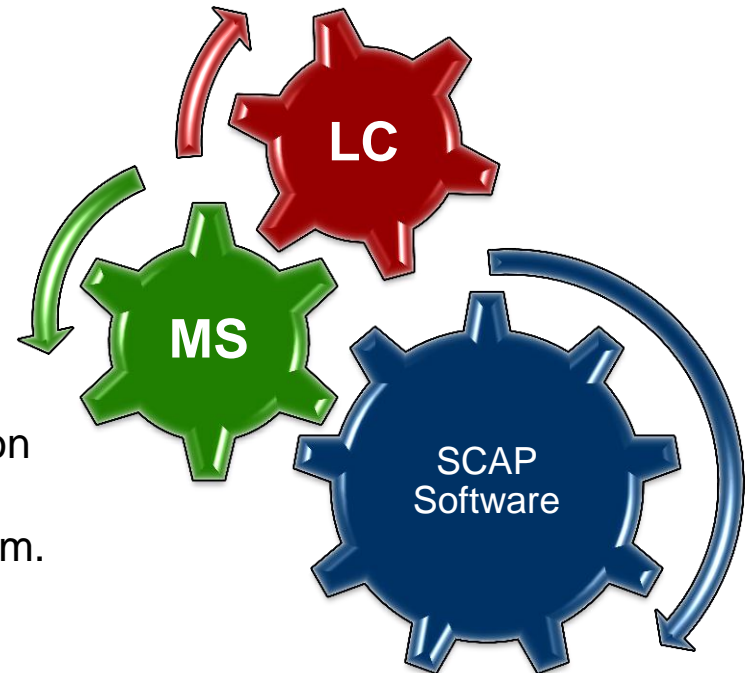
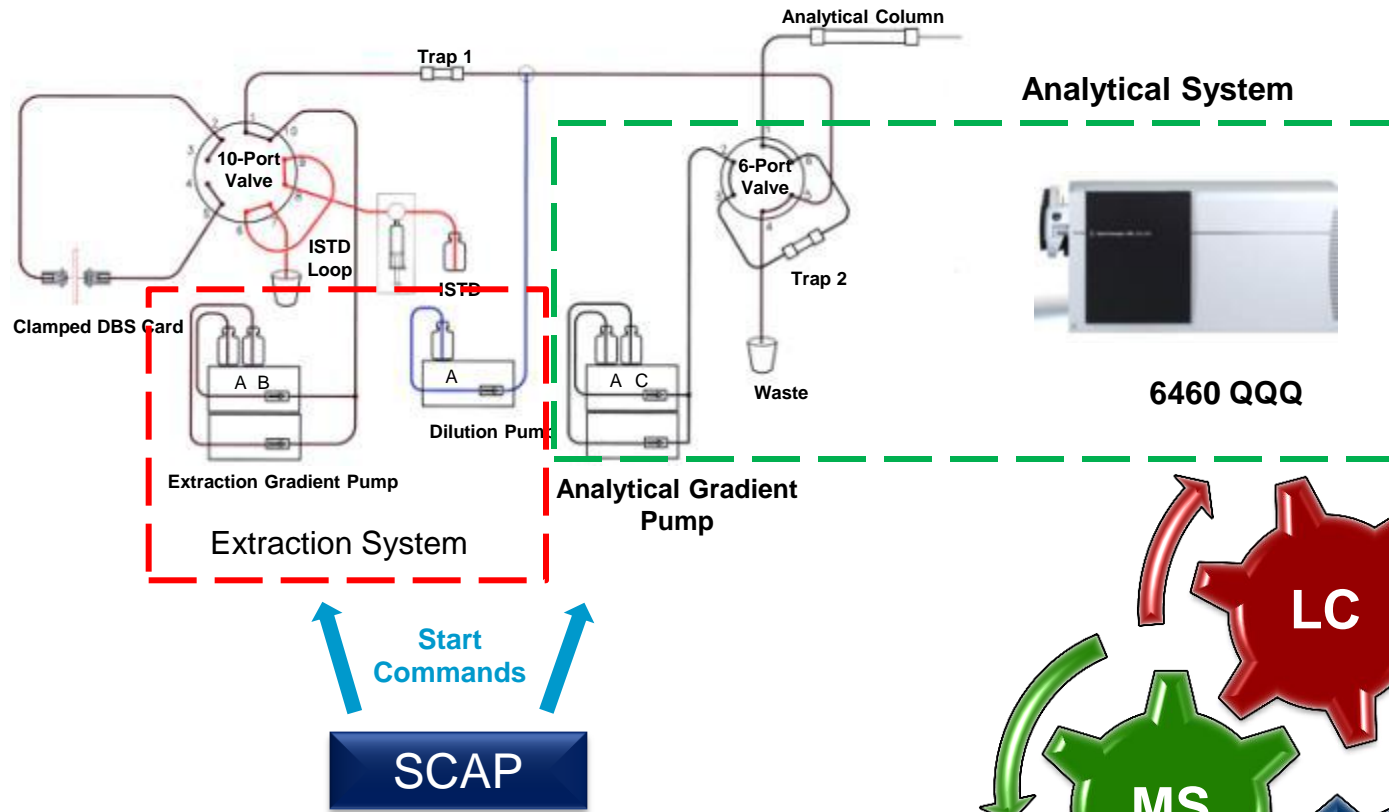
DBS SAMPLE NO. FIELD



Software Control - Initial Implementation

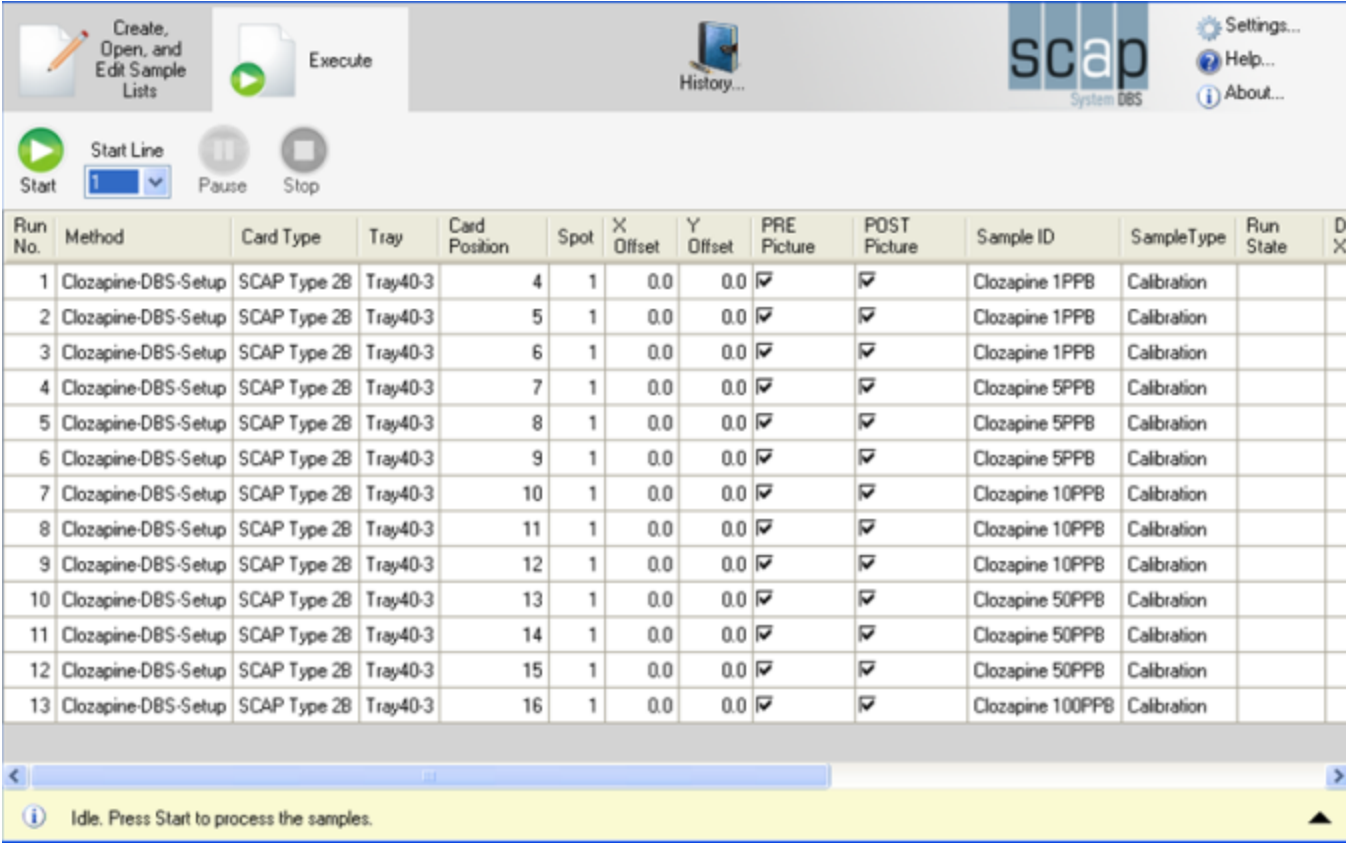


Software and Control (Current)



- Sample information imported/entered into single location
- Camera – captures card image and recorded barcode
- Dilution and Extraction pump controlled as single system.
- Analytical LC and MS controlled as single system

Sample Set Up and Run...



The screenshot displays the SCAP (System Calibration and Acquisition Platform) software interface. At the top, there are buttons for 'Create, Open, and Edit Sample Lists', 'Execute', and 'History...'. The 'scap System DBS' logo is visible in the top right corner, along with 'Settings...', 'Help...', and 'About...' options. Below these are control buttons for 'Start Line' (with a dropdown menu), 'Start', 'Pause', and 'Stop'. The main area contains a table with 13 rows of sample data. The table columns are: Run No., Method, Card Type, Tray, Card Position, Spot, X Offset, Y Offset, PRE Picture, POST Picture, Sample ID, SampleType, Run State, and De (partially visible). The status bar at the bottom indicates 'Idle. Press Start to process the samples.'

Run No.	Method	Card Type	Tray	Card Position	Spot	X Offset	Y Offset	PRE Picture	POST Picture	Sample ID	SampleType	Run State	De
1	Clozapine-DBS-Setup	SCAP Type 2B	Tray40-3	4	1	0.0	0.0	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	Clozapine 1PPB	Calibration		
2	Clozapine-DBS-Setup	SCAP Type 2B	Tray40-3	5	1	0.0	0.0	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	Clozapine 1PPB	Calibration		
3	Clozapine-DBS-Setup	SCAP Type 2B	Tray40-3	6	1	0.0	0.0	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	Clozapine 1PPB	Calibration		
4	Clozapine-DBS-Setup	SCAP Type 2B	Tray40-3	7	1	0.0	0.0	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	Clozapine 5PPB	Calibration		
5	Clozapine-DBS-Setup	SCAP Type 2B	Tray40-3	8	1	0.0	0.0	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	Clozapine 5PPB	Calibration		
6	Clozapine-DBS-Setup	SCAP Type 2B	Tray40-3	9	1	0.0	0.0	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	Clozapine 5PPB	Calibration		
7	Clozapine-DBS-Setup	SCAP Type 2B	Tray40-3	10	1	0.0	0.0	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	Clozapine 10PPB	Calibration		
8	Clozapine-DBS-Setup	SCAP Type 2B	Tray40-3	11	1	0.0	0.0	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	Clozapine 10PPB	Calibration		
9	Clozapine-DBS-Setup	SCAP Type 2B	Tray40-3	12	1	0.0	0.0	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	Clozapine 10PPB	Calibration		
10	Clozapine-DBS-Setup	SCAP Type 2B	Tray40-3	13	1	0.0	0.0	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	Clozapine 50PPB	Calibration		
11	Clozapine-DBS-Setup	SCAP Type 2B	Tray40-3	14	1	0.0	0.0	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	Clozapine 50PPB	Calibration		
12	Clozapine-DBS-Setup	SCAP Type 2B	Tray40-3	15	1	0.0	0.0	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	Clozapine 50PPB	Calibration		
13	Clozapine-DBS-Setup	SCAP Type 2B	Tray40-3	16	1	0.0	0.0	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	Clozapine 100PPB	Calibration		

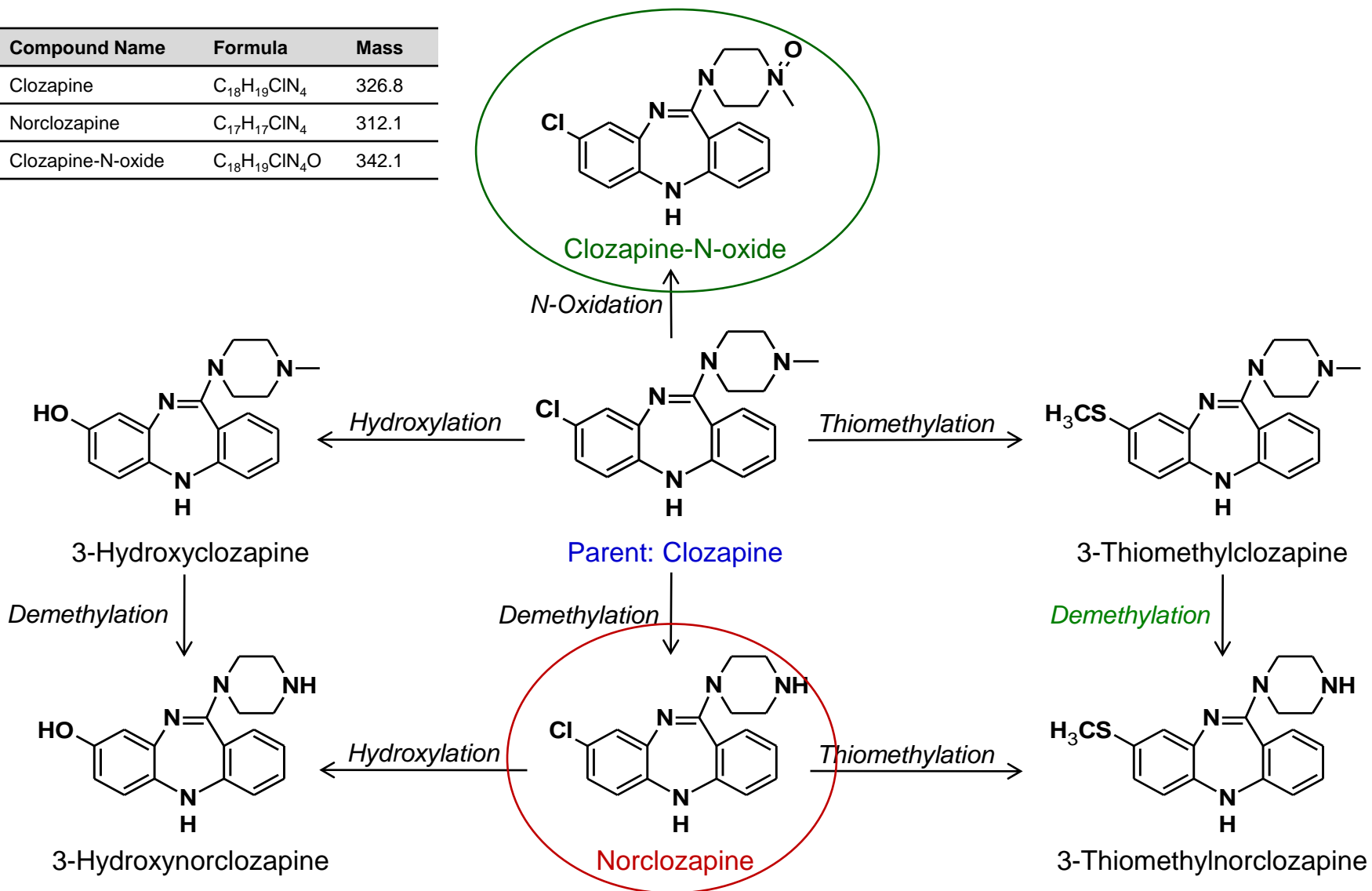
- Input sample information in SCAP software
- Specify acquisition details
- Pre and Post Images
- Execute run

Overview – Clozapine DBS Analysis

- Sample Information and Preparation
- Method Development: SCAP and LC/MS
- Quantitation of Clozapine and Metabolites using SCAP DBS
 - Sensitivity: LOQ
 - Calibration curve linearity and range
 - Accuracy, reproducibility and precision
- Quantitation of Clozapine and Metabolites using off-line DBS
 - Sensitivity: LOQ
 - Calibration curve linearity and range
 - Accuracy, reproducibility and precision
- Result Comparison and Summary

Clozapine PK and Metabolic Stability Study

Compound Name	Formula	Mass
Clozapine	C ₁₈ H ₁₉ ClN ₄	326.8
Norclozapine	C ₁₇ H ₁₇ ClN ₄	312.1
Clozapine-N-oxide	C ₁₈ H ₁₉ ClN ₄ O	342.1



Clozapine DBS Sample Preparation

Clozapine Calibration Samples in Rat Whole Blood

Level Name		1	2	3	4	5	6	7	8	9	10	11
Conc. (ng/mL)	Clozapine	0.1	0.5	1	5	10	50	100	500	1000	5000	10000
	Norclozapine	0.1	0.5	1	5	10	50	100	500	1000	5000	10000
	Clozapine-NO	0.1	0.5	1	5	10	50	100	500	1000	5000	10000

Note 1: Rat whole blood in sodium heparium, pooled, mixed gender, was used for sample preparation.

Note 2: D_4 -Clozapine was used as internal standard for quantitation of clozapine and metabolites.

- **Spot:** 15 μ L blood (diameter ~7.5 mm)
- **Dry:** Overnight at room temperature



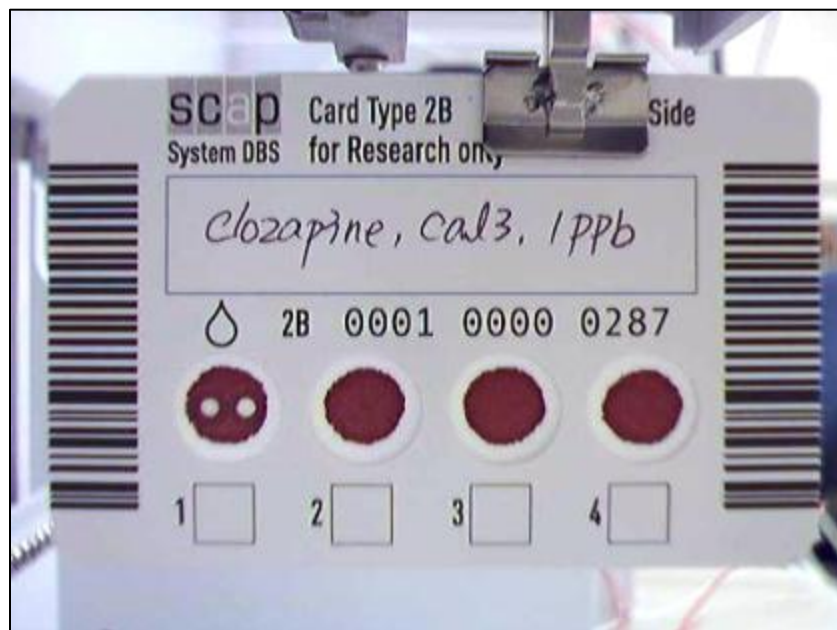
- **ISTD** (D_4 -Clozapine) mixed on-line

SCAP-6460

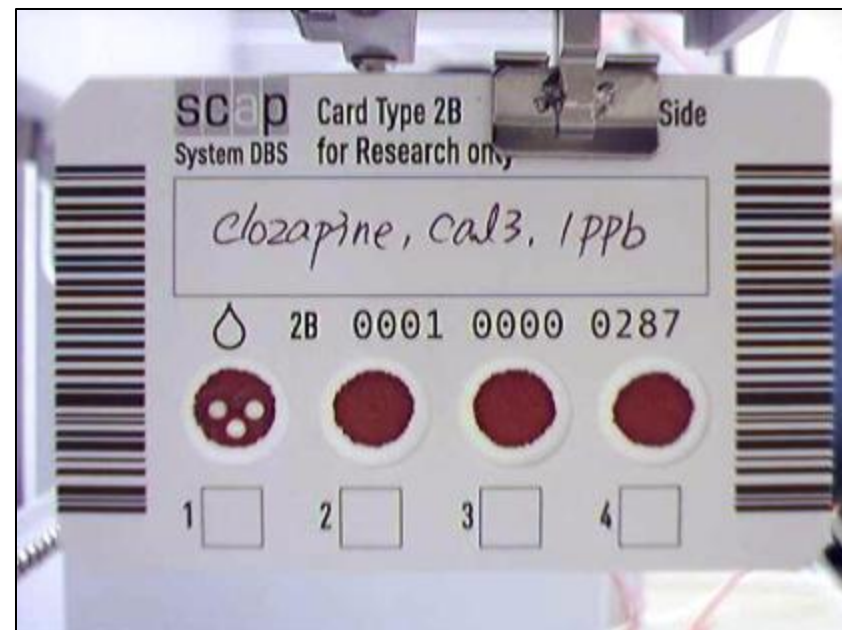
- **Punch:** 6mm (Harris Uni Core)
- **Transfer:** into 2mL Eppendorf tube
- **Dry:** Speed-vac (~20min)
- **Add 200 μ L** ISTD solution (80% ACN [1%FA])
- **Mix:** Vortex (few sec) + ultra sonic (10-15min)
- **Spin:** 15,000rpm (15min)
- **Transfer Supernatant:** into AS-vial (~150 μ L)

1290-6460

SCAP DBS Card



Before Sampling
1 ng/mL Cal
3rd replicate

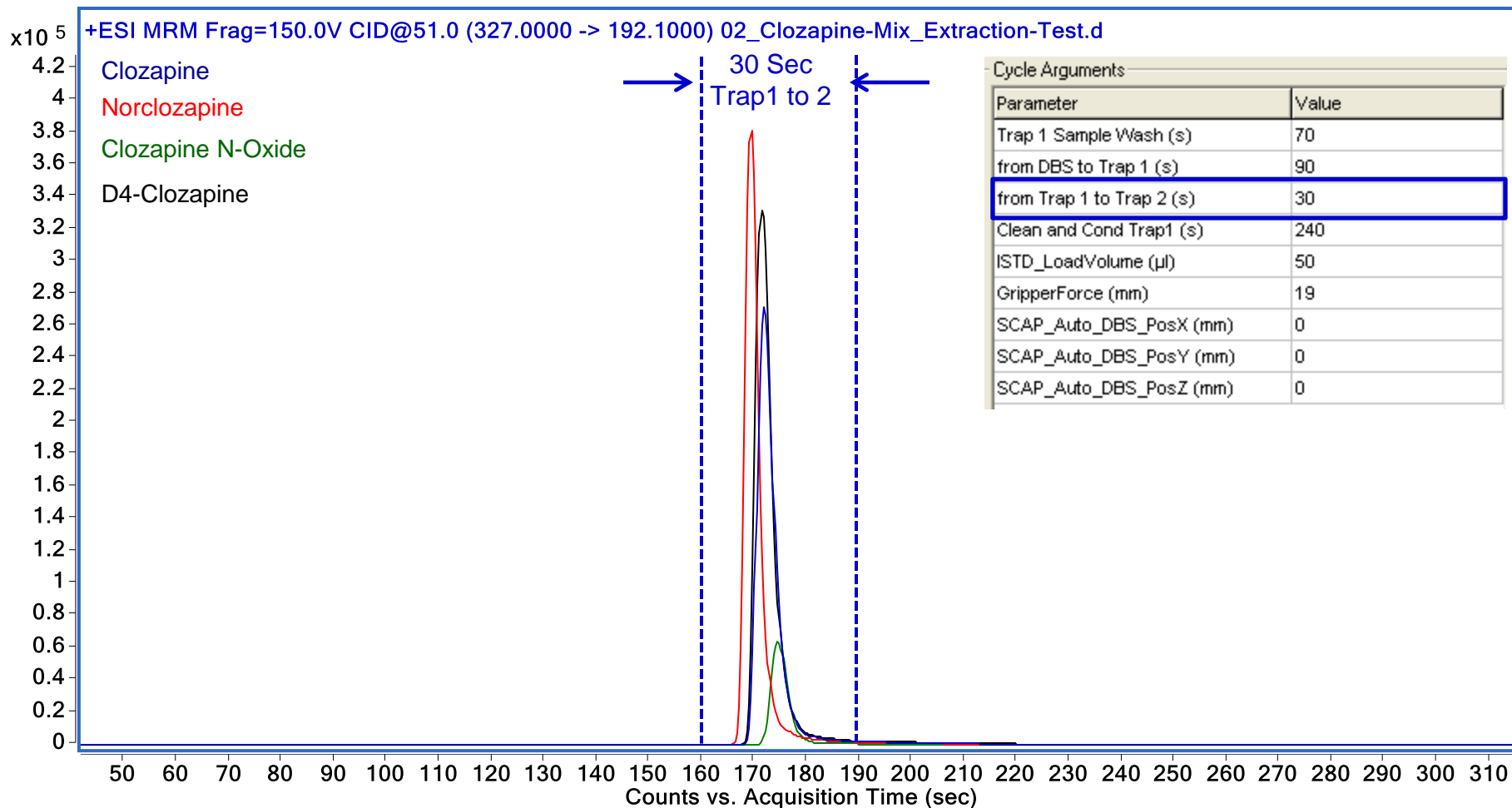


After Sampling
1 ng/mL Cal
3rd replicate

SCAP Timer Setup for Clozapine and Metabolites

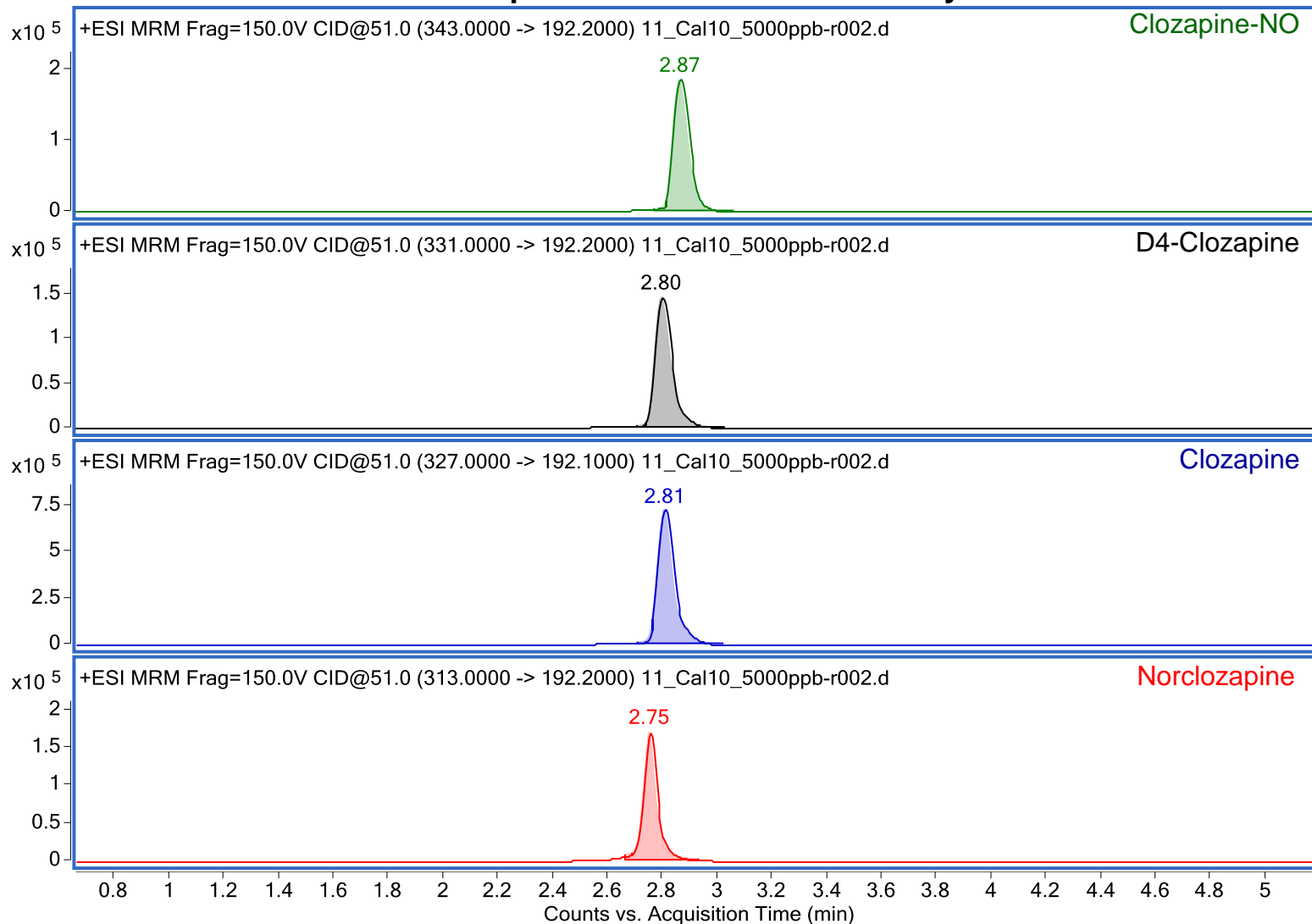
Method Development

Elution of Clozapine and Metabolites on Extraction Column (Trap1)



Clozapine and Metabolites, 5000 ng/mL Standard

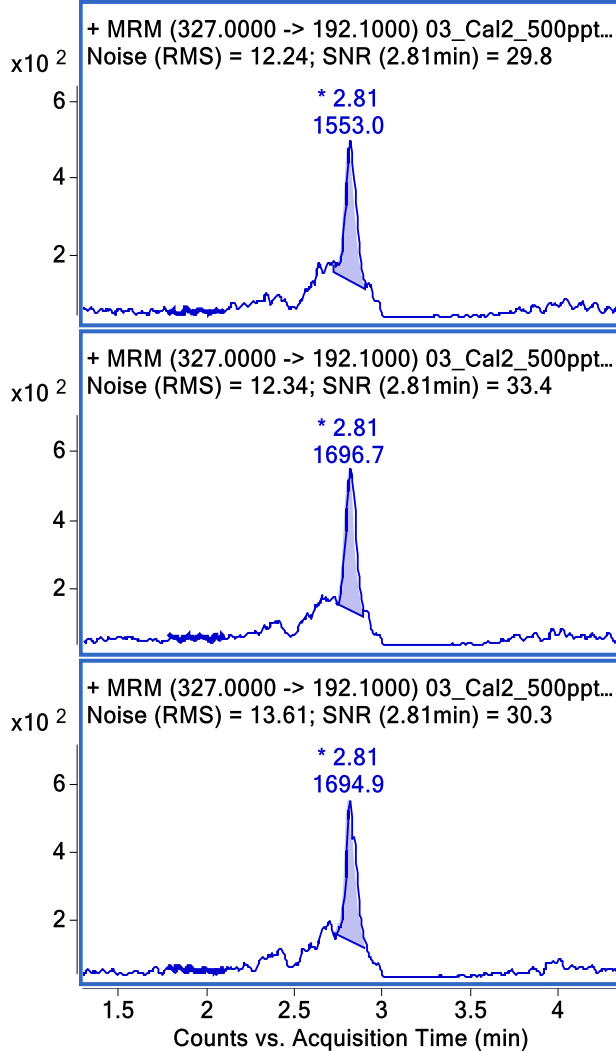
Elution of Clozapine and Metabolites on Analytical column



Sensitivity LLOQ: 0.5 ng/mL (1.5 nM) in Blood

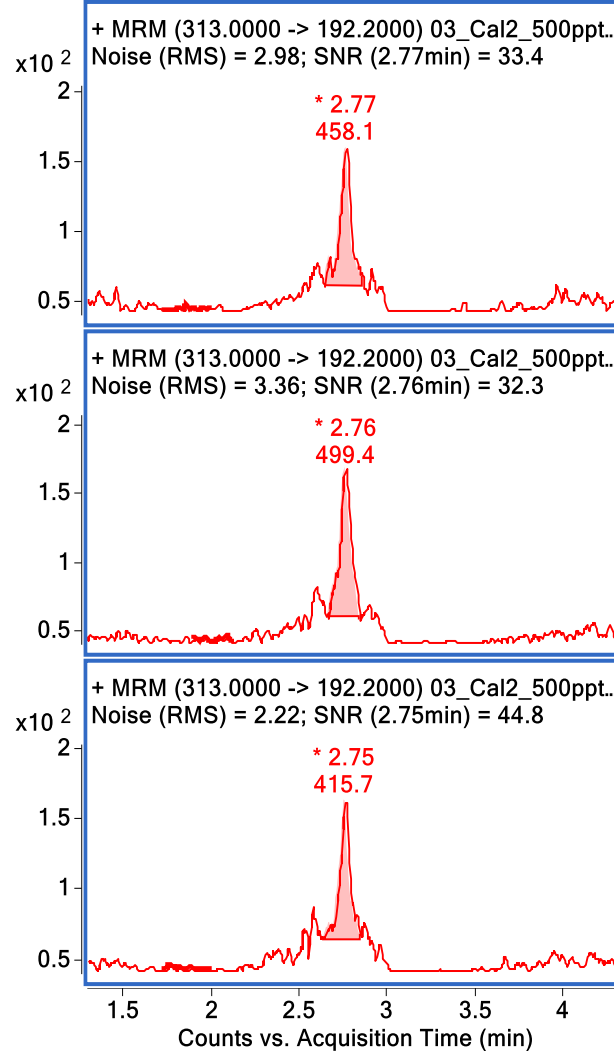
Clozapine

LLOQ = 0.5 ng/mL (1.5 nM) in blood



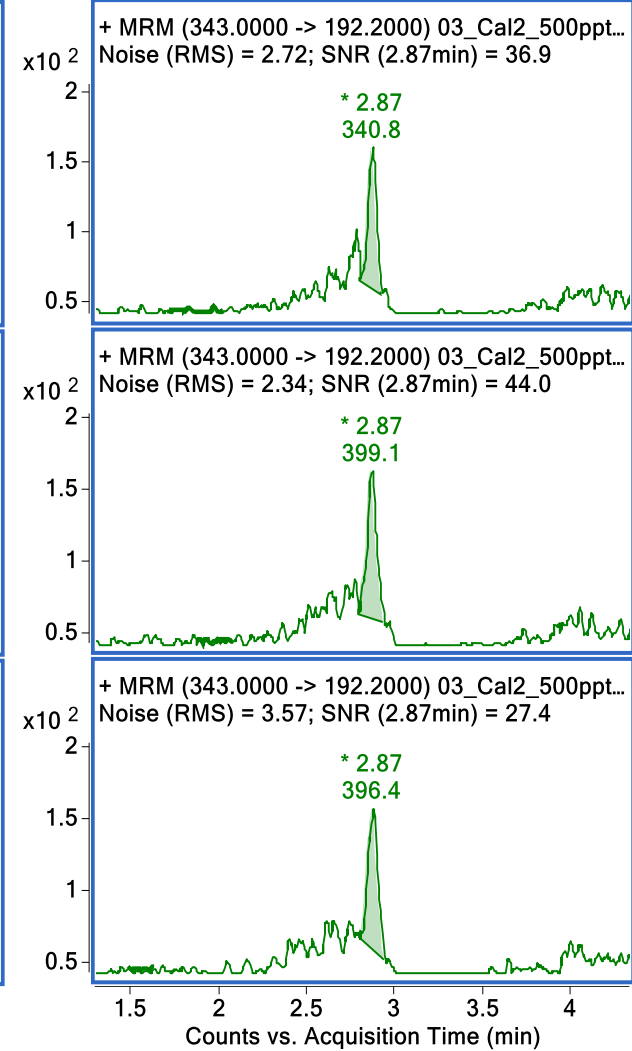
Norclozapine

LLOQ = 0.5 ng/mL (1.5 nM) in blood

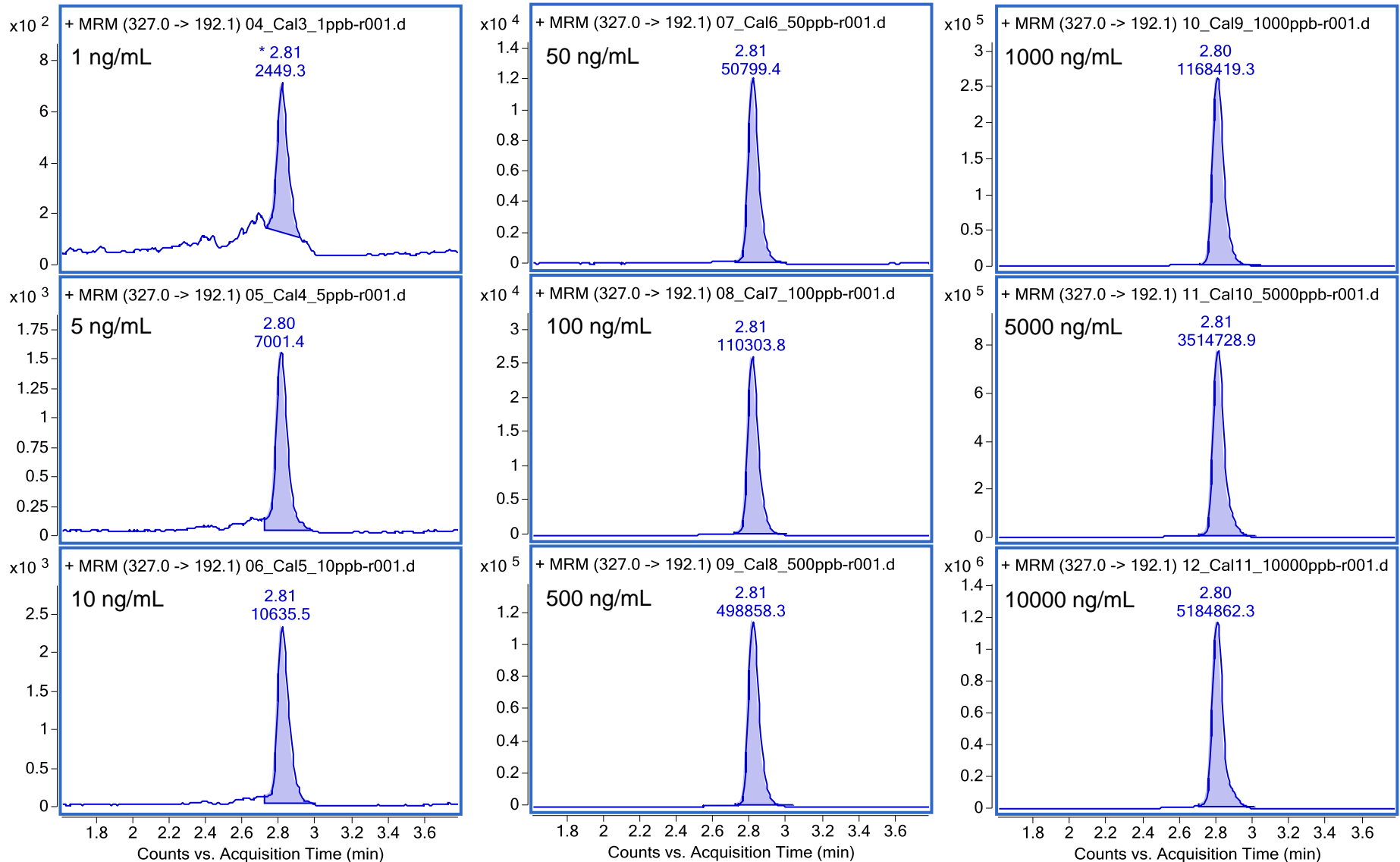


Clozapine-NO

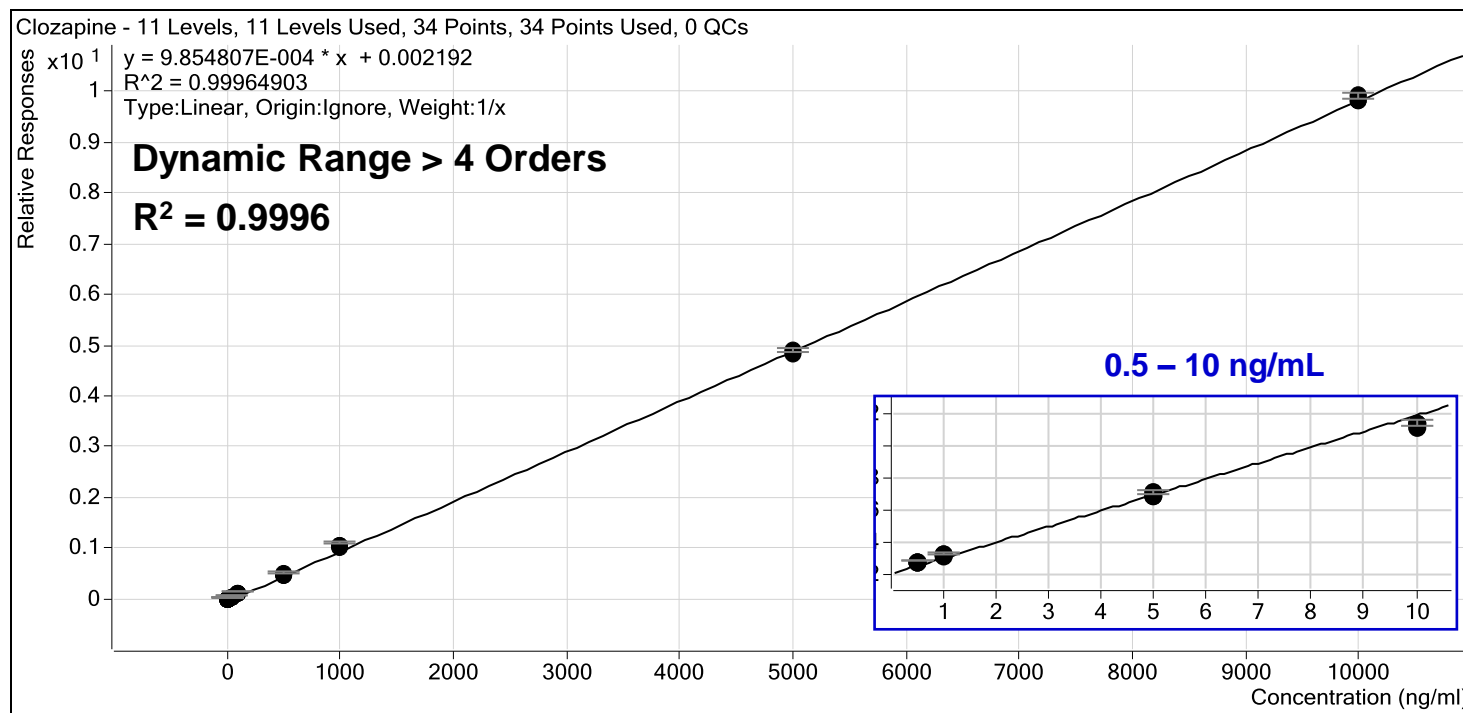
LLOQ = 0.5 ng/mL (1.5 nM) in blood



SCAP DBS: Clozapine, 0.5 - 10000 ng/mL in blood



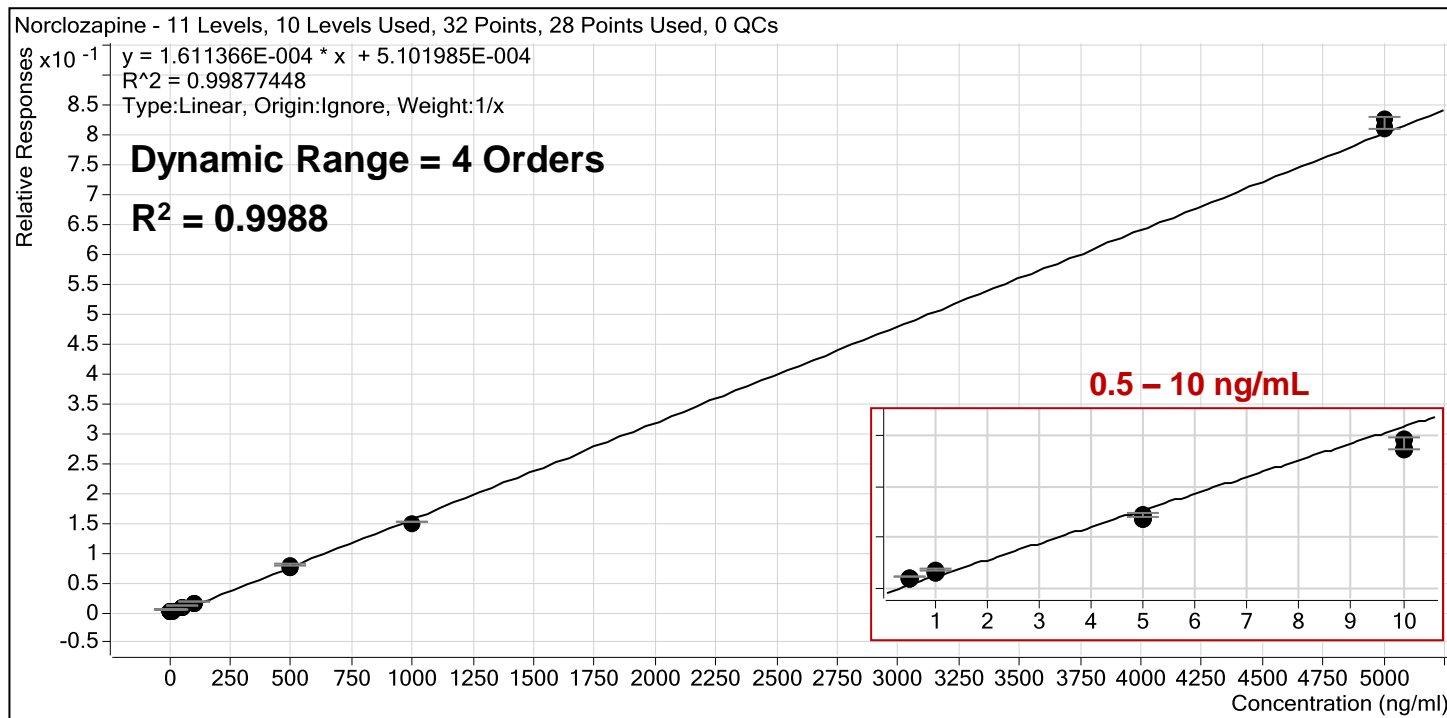
Calibration Curve: Clozapine, 0.5 - 10000 ng/mL in blood



Accuracy, Reproducibility and Precision

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									

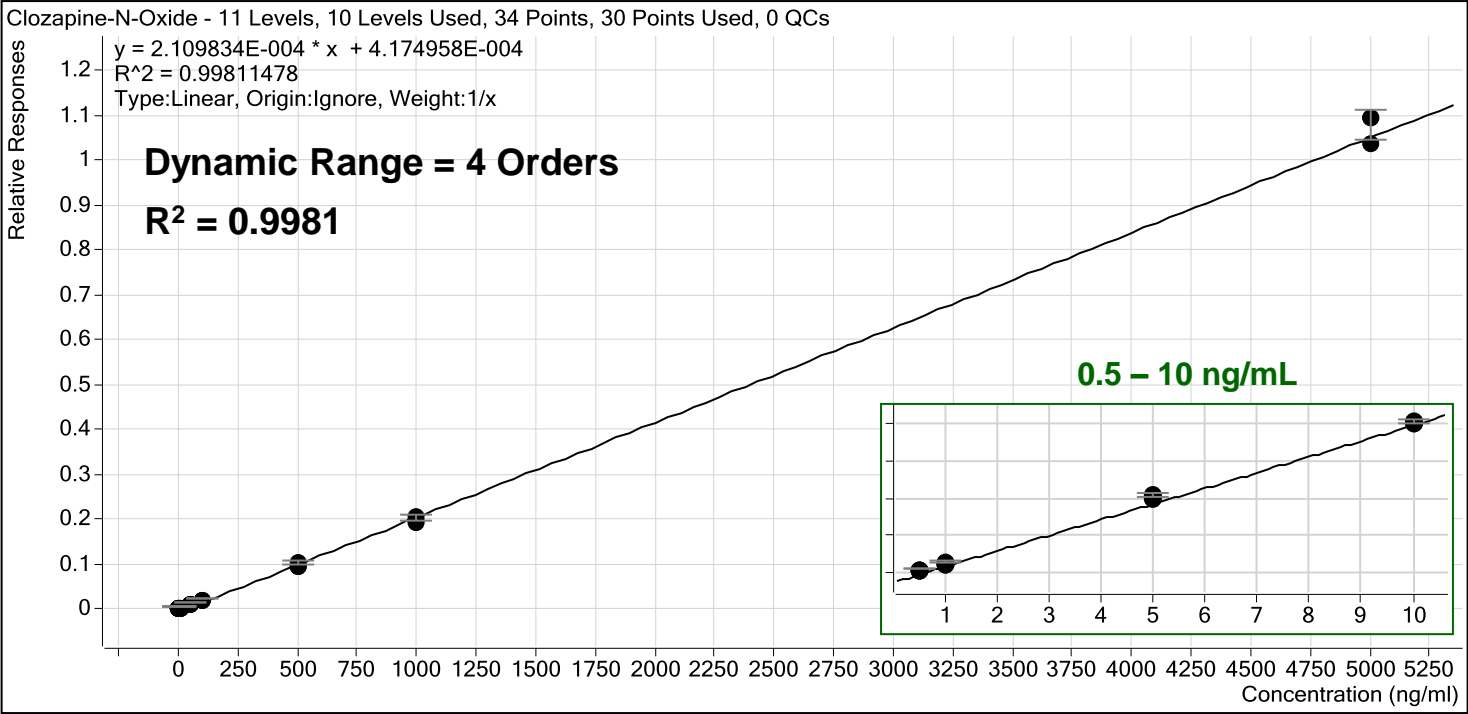
Calibration Curve: Norclozapine, 0.5 - 5000 ng/mL in blood



Accuracy, Reproducibility and Precision

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	86.1	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								

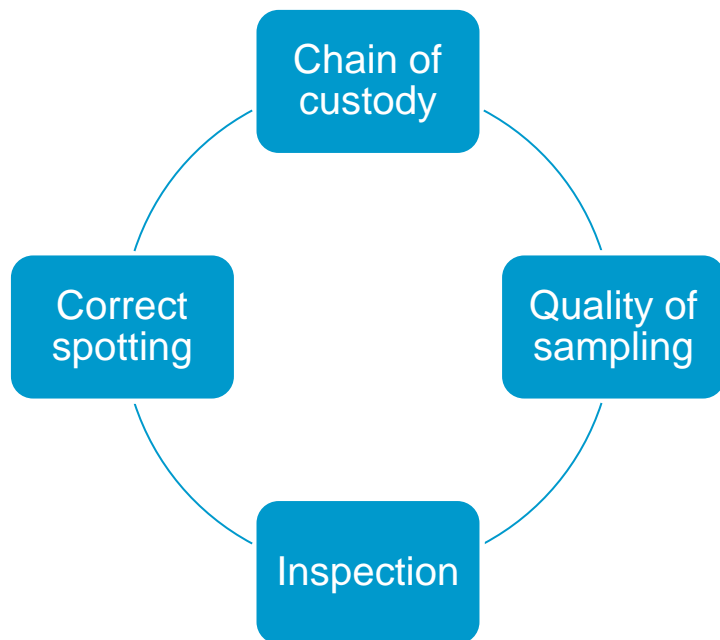
Calibration Curve: Clozapine NO, 0.5 - 5000 ng/mL in blood



Accuracy, Reproducibility and Precision

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	4.58	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								


SCAP 6460 Reporting




Quantitative Analysis Sample Report

Batch Data Path	D:\MassHunter\Data\2011_1020a_Poreshell\QuantResults\clozDBS.batch.bin		
Analysis Time	11/3/2011 11:25 AM	Analyst Name	Na Parra
Report Time	11/3/2011 11:25 AM	Reporter Name	Na Parra
Last Calib Update	11/2/2011 1:08 PM	Batch State	Processed

Pre-Analysis



Post-Analysis

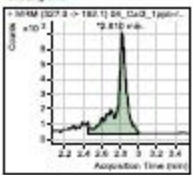
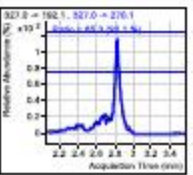


Analysis Info		Data File	04_Cal3_1ppb-r001.d			
Acq Time	2011-10-20 19:57	Sample Name	Cal3_1ppb			
Sample Type	Calibration	Acq Method	Clozapine_Analysis.m			
Dilution	1	Sample Info				
Position	card_4_spot_1	Comment				
Inj Vol	-1					

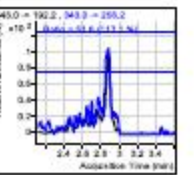
Quantitation Results		RT	Response	ISTD Resp	Resp Ratio	Final Conc
Compound	ISTD Compound					
Nordazapine	D4-Clozapine	2.742	833	1667534	0.0005	1.1534
Clozapine	D4-Clozapine	2.810	5161	1667534	0.0031	0.9320
Clozapine-N-Oxide	D4-Clozapine	2.860	1029	1667534	0.0006	1.0159

Compound Graphics

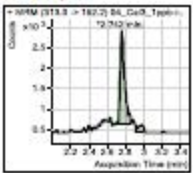
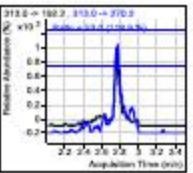
Clozapine

Clozapine-N-Oxide

Nordazapine

DBS_Template.txt
Page 1 of 1
Printed at: 11:31 AM on: 11/3/2011

Offline DBS LC/MS Analysis

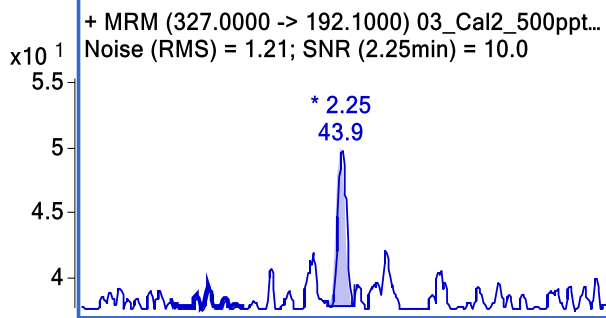
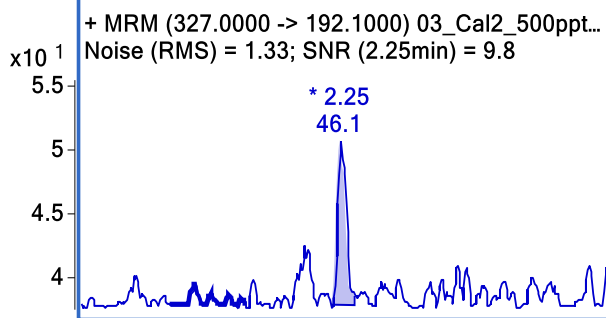
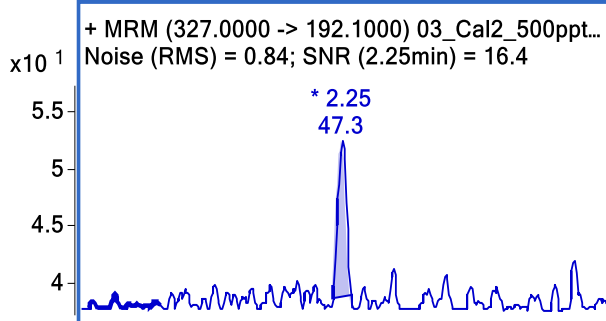
1290 UHPLC 6460QQQ

1. **Punch:** 6mm (Harris Uni Core)
2. **Transfer:** into 2mL Eppendorf tube
3. **Dry:** Speed-vac (~20min)
4. **Add 200 μ L** ISTD solution (80% ACN [1%FA])
5. **Mix:** Vortex (few sec) + ultra sonic (10-15min)
6. **Spin:** 15,000rpm (15min)
7. **Transfer Supernatant:** into AS-vial (~150 μ L)

Sensitivity LLOQ: 0.5 ng/mL (1.5 nM) in Blood

Clozapine

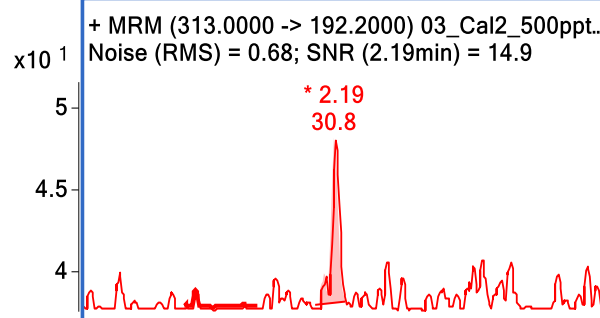
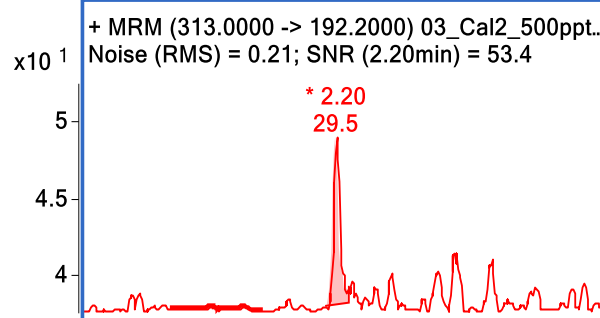
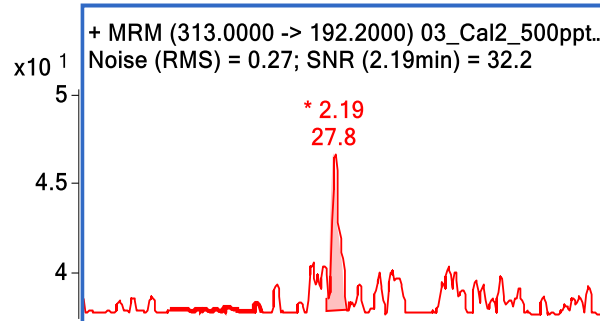
LLOQ = 0.5 ng/mL (1.5 nM) in blood



Counts vs. Acquisition Time (min)

Norclozapine

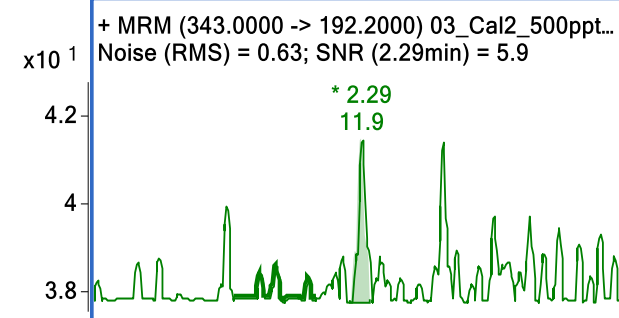
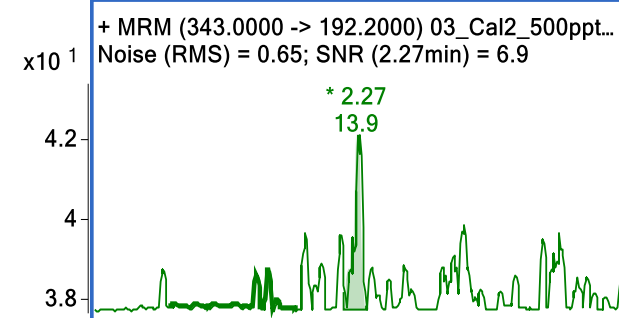
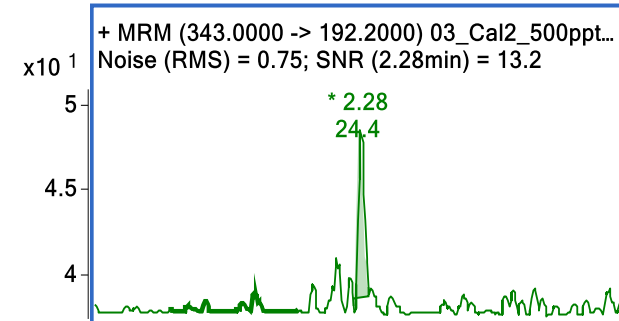
LLOQ = 0.5 ng/mL (1.5 nM) in blood



Counts vs. Acquisition Time (min)

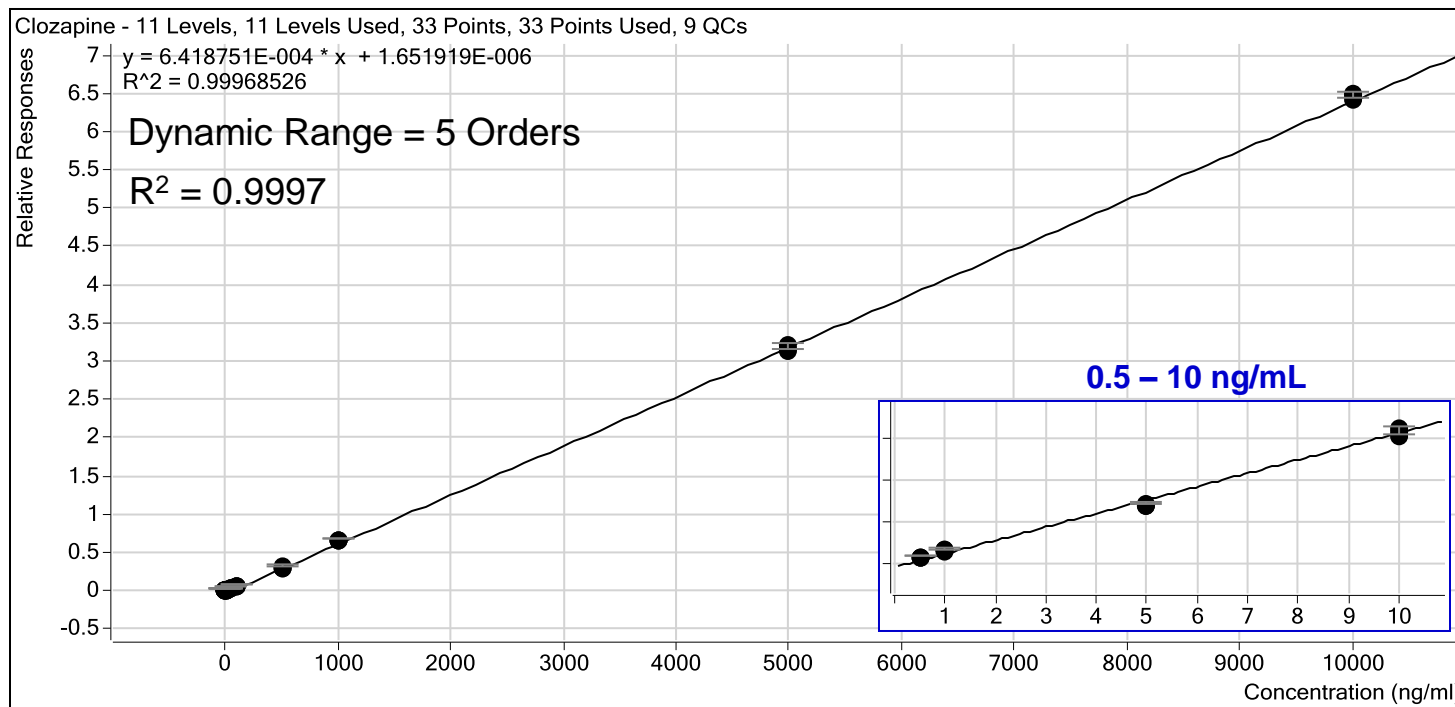
Clozapine N-Oxide

LLOQ = 0.5 ng/mL (1.5 nM) in blood



Counts vs. Acquisition Time (min)

Calibration Curve: Clozapine, 0.5 - 10000 ng/mL in blood



Accuracy, Reproducibility and Precision

Concentration (ng/mL)	Calibration Standards									
	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									

Summary: DBS SCAP vs. Offline DBS

Comparable Quantitation Performance Parameters

DBS SCAP

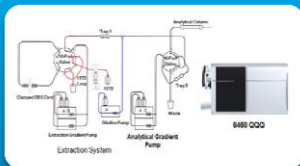
Compound	LOQ (ng/mL in blood)	Range (ng/mL)	Linearity (R ²)	Accuracy (%)	Reproducibility (%)	Precision (%)
Clozapine	0.5	0.5 – 10000	0.9996	92.1 – 106.8	0.61 – 5.61	4.82
Norclozapine	0.5	0.5 – 5000	0.9988	85.2 – 109.9	0.84 – 6.05	8.94
Clozapine N-oxide	0.5	0.5 - 5000	0.9981	93.2 – 104.1	0.68 – 6.15	3.82
Sample Prep Time	~ 1 hr					

Offline DBS

Compound	LOQ		Range (ng/mL)	Linearity (R ²)	Accuracy (%)	Reproducibility (%)	Precision (%)
	ng/mL in blood	fg on- column					
Clozapine	0.5	23	0.5 – 10000	0.9997	88.0 – 103.2	0.61 – 8.92	4.89
Norclozapine	0.5	23	0.5 – 10000	0.9991	89.3 – 107.1	0.08 – 8.98	8.36
Clozapine N-oxide	0.5	23	0.5 - 10000	0.9991	91.8 – 108.4	0.25 – 8.66	6.64
Sample Prep Time	~ 5 hrs						

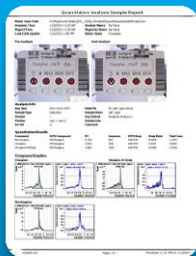
Summary

Integrated SCAP LC/MS



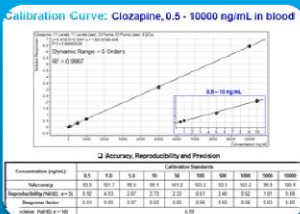
- Method Development
- SCAP software driven
- MassHunter LC/MS
- Data processing

Reporting



- Pre- and post- sampling card images
- Processed quantitative data
- Chain of custody
- Sampling integrity

Clozapine study



- Comparable results with hole punching
- 0.5 ng/mL LOQ
- Linear assay over 4 orders of magnitude
- Excellent assay precision and accuracy

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