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

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Agilent Technologies  
Santa Clara, CA  
USA

EBF Conference  
Barcelona  
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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

- 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.
SCAP System from Prolab Instruments

- Robotic Gripper
- ISTD Dilutor
- DBS CardRacks
- Camera
- Clamp Module
- Switching Valves
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

- Analyte transfer from Trap 1 to Trap 2
- Simultaneous dilution
- Start Analytical run
- Elute from Trap 2
- Analytical HPLC separation and MS detection

Clamped DBS Card

Extraction Gradient Pump

Dilution Pump

Analytical Gradient Pump

ISTD Loop

Trap 1

Trap 2

Analytical Column

Waste

6460 QQQ

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Dried Blood Spot Card

- Label Area for Customer Incl. Customer Barcode
- Card Barcode Number
- Redundant Card Barcode
- Substrate
- DBS Sample No. Field

Do not touch or contaminate sample carriers
Do not apply samples on this side
Software Control - Initial Implementation

Clamped DBS Card

10-Port Valve

ISTD Loop

ISTD

6460 QQQ

Trap 1

Analytical Column

6-Port Valve

Trap 2

Waste

Extraction Gradient Pump

Dilution Pump

Analytical Gradient Pump

A B

A

A C

SCAP

PC Controller

PC Controller

PC

MassHunter

Cycle Composer

Start Signal
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…

- 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

<table>
<thead>
<tr>
<th>Compound Name</th>
<th>Formula</th>
<th>Mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clozapine</td>
<td>C_{18}H_{19}ClN_{4}</td>
<td>326.8</td>
</tr>
<tr>
<td>Norclozapine</td>
<td>C_{17}H_{17}ClN_{4}</td>
<td>312.1</td>
</tr>
<tr>
<td>Clozapine-N-oxide</td>
<td>C_{18}H_{19}ClN_{4}O</td>
<td>342.1</td>
</tr>
</tbody>
</table>

- **N-Oxidation**
  - Clozapine-N-oxide

- **Hydroxylation**
  - 3-HydroxyClozapine
  - 3-Hydroxynorclozapine

- **Thiomethylation**
  - 3-Thiomethylclozapine
  - 3-Thiomethylnorclozapine

- **Demethylation**
  - Parent: Clozapine
  - Norclozapine
Clozapine DBS Sample Preparation

Clozapine Calibration Samples in Rat Whole Blood

<table>
<thead>
<tr>
<th>Level Name</th>
<th>Conc. (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clozapine</td>
<td>0.1 0.5 1 5 10 50 100 500 1000 5000 10000</td>
</tr>
<tr>
<td>Norclozapine</td>
<td>0.1 0.5 1 5 10 50 100 500 1000 5000 10000</td>
</tr>
<tr>
<td>Clozapine-NO</td>
<td>0.1 0.5 1 5 10 50 100 500 1000 5000 10000</td>
</tr>
</tbody>
</table>

Note 1: Rat whole blood in sodium heparin, pooled, mixed gender, was used for sample preparation.
Note 2: D₄-Clozapine was used as internal standard for quantitation of clozapine and metabolites.

- **Spot**: 15μL blood (diameter ~7.5 mm)
- **Dry**: Overnight at room temperature
- **Punch**: 6mm (Harris Uni Core)
- **Transfer**: into 2mL Eppendorf tube
- **Dry**: Speed-vac (~20min)
- **Add 200μL** ISTD solution (80% ACN [1%FA])
- **Mix**: Vortex (few sec) + ultra sonic (10-15min)
- **Spin**: 15,000rpm (15min)
- **Transfer Supernatant**: into AS-vial (~150μL)
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)

- ESI MRM Frag=150.0V CID@51.0 (327.0000 -> 192.1000) 02_Clozapine-Mix_Extraction-Test.d

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
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</thead>
<tbody>
<tr>
<td>Trap 1 Sample Wash (s)</td>
<td>70</td>
</tr>
<tr>
<td>from DBS to Trap 1 (s)</td>
<td>90</td>
</tr>
<tr>
<td>from Trap 1 to Trap 2 (s)</td>
<td>30</td>
</tr>
<tr>
<td>Clean and Cond Trap 1 (s)</td>
<td>240</td>
</tr>
<tr>
<td>ISTD_LoadVolume (µl)</td>
<td>50</td>
</tr>
<tr>
<td>GripperForce (mm)</td>
<td>19</td>
</tr>
<tr>
<td>SCAP_Auto_DBS_PosX (mm)</td>
<td>0</td>
</tr>
<tr>
<td>SCAP_Auto_DBS_PosY (mm)</td>
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</tr>
<tr>
<td>SCAP_Auto_DBS_PosZ (mm)</td>
<td>0</td>
</tr>
</tbody>
</table>

Counts vs. Acquisition Time (sec)
Elution of Clozapine and Metabolites on Analytical column

- **Clozapine-NO**
  - ESI MRM Frag=150.0V CID@51.0 (343.0000 -> 192.2000) 11_Cal10_5000ppb-r002.d
  - Elution time: 2.87 minutes

- **D4-Clozapine**
  - ESI MRM Frag=150.0V CID@51.0 (331.0000 -> 192.2000) 11_Cal10_5000ppb-r002.d
  - Elution time: 2.80 minutes

- **Clozapine**
  - ESI MRM Frag=150.0V CID@51.0 (327.0000 -> 192.1000) 11_Cal10_5000ppb-r002.d
  - Elution time: 2.81 minutes

- **Norclozapine**
  - ESI MRM Frag=150.0V CID@51.0 (313.0000 -> 192.2000) 11_Cal10_5000ppb-r002.d
  - Elution time: 2.75 minutes
Sensitivity LLOQ: 0.5 ng/mL (1.5 nM) in Blood

**Clozapine**

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

+ MR (327.0000 -> 192.1000) 03_Cal2_500ppt...
  Noise (RMS) = 12.24; SNR (2.81min) = 29.8

+ MR (327.0000 -> 192.1000) 03_Cal2_500ppt...
  Noise (RMS) = 12.34; SNR (2.81min) = 33.4

**Norclozapine**

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

+ MR (313.0000 -> 192.2000) 03_Cal2_500ppt...
  Noise (RMS) = 3.6; SNR (2.76min) = 32.3

**Clozapine-NO**

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

+ MR (343.0000 -> 192.2000) 03_Cal2_500ppt...
  Noise (RMS) = 2.34; SNR (2.87min) = 44.0

The Measure of Confidence

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

Accuracy, Reproducibility and Precision

<table>
<thead>
<tr>
<th>Concentration (ng/mL)</th>
<th>Calibration Standards</th>
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<tbody>
<tr>
<td></td>
<td>0.5</td>
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<td>%Accuracy</td>
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<tr>
<td>0.5</td>
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<tr>
<td>Reproducibility (%RSD, n = 3)</td>
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<tr>
<td>Response factor</td>
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<tr>
<td>Precision (%RSD, n = 10)</td>
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<tr>
<td></td>
<td>4.82</td>
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</tbody>
</table>
Calibration Curve: Norclozapine, 0.5 - 5000 ng/mL in blood

**Accuracy, Reproducibility and Precision**

<table>
<thead>
<tr>
<th>Concentration (ng/mL)</th>
<th>Calibration Standards</th>
</tr>
</thead>
<tbody>
<tr>
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<td>%Accuracy</td>
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<td>Reproducibility (%RSD, n = 3)</td>
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<td>Precision (%RSD, n = 9)</td>
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</table>
Calibration Curve: Clozapine NO, 0.5 - 5000 ng/mL in blood

Dynamic Range = 4 Orders

\[ R^2 = 0.9981 \]

### Accuracy, Reproducibility and Precision

<table>
<thead>
<tr>
<th>Concentration (ng/mL)</th>
<th>Calibration Standards</th>
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<td>%Accuracy</td>
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<td>Precision (%RSD, n = 9)</td>
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</table>
SCAP 6460 Reporting

Chain of custody

Correct spotting

Quality of sampling

Inspection
1. **Punch:** 6mm (Harris Uni Core)
2. **Transfer:** into 2mL Eppendorf tube
3. **Dry:** Speed-vac (~20min)
4. **Add 200μL** 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 (~150μL)
Sensitivity LLOQ: 0.5 ng/mL (1.5 nM) in Blood

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

- + MRM (327.0000 -> 192.1000) 03_Cal2_500ppt:
  - Noise (RMS) = 0.84; SNR (2.25min) = 16.4

- + MRM (327.0000 -> 192.1000) 03_Cal2_500ppt:
  - Noise (RMS) = 1.33; SNR (2.25min) = 9.8

- + MRM (327.0000 -> 192.1000) 03_Cal2_500ppt:
  - Noise (RMS) = 1.21; SNR (2.25min) = 10.0

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

- + MRM (313.0000 -> 192.2000) 03_Cal2_500ppt:
  - Noise (RMS) = 0.27; SNR (2.19min) = 32.2

- + MRM (313.0000 -> 192.2000) 03_Cal2_500ppt:
  - Noise (RMS) = 0.21; SNR (2.20min) = 53.4

- + MRM (313.0000 -> 192.2000) 03_Cal2_500ppt:
  - Noise (RMS) = 0.68; SNR (2.19min) = 14.9

**Clozapine N-Oxide**
LLOQ = 0.5 ng/mL (1.5 nM) in blood

- + MRM (343.0000 -> 192.2000) 03_Cal2_500ppt:
  - Noise (RMS) = 0.75; SNR (2.28min) = 13.2

- + MRM (343.0000 -> 192.2000) 03_Cal2_500ppt:
  - Noise (RMS) = 0.65; SNR (2.27min) = 6.9

- + MRM (343.0000 -> 192.2000) 03_Cal2_500ppt:
  - Noise (RMS) = 0.63; SNR (2.29min) = 5.9
Calibration Curve: Clozapine, 0.5 - 10000 ng/mL in blood

Accuracy, Reproducibility and Precision

<table>
<thead>
<tr>
<th>Concentration (ng/mL)</th>
<th>Calibration Standards</th>
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<tbody>
<tr>
<td></td>
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<td>Precision (%RSD, n = 10)</td>
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</table>

Response factor

Precision (%RSD, n = 10)

4.89
# Summary: DBS SCAP vs. Offline DBS

## Comparable Quantitation Performance Parameters

<table>
<thead>
<tr>
<th>Compound</th>
<th>DBS SCAP</th>
<th>Offline DBS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LOQ (ng/mL in blood)</td>
<td>Range (ng/mL)</td>
</tr>
<tr>
<td>Clozapine</td>
<td>0.5</td>
<td>0.5 – 10000</td>
</tr>
<tr>
<td>Norclozapine</td>
<td>0.5</td>
<td>0.5 – 5000</td>
</tr>
<tr>
<td>Clozapine N-oxide</td>
<td>0.5</td>
<td>0.5 – 5000</td>
</tr>
<tr>
<td>Sample Prep Time</td>
<td>~ 1 hr</td>
<td>~ 5 hrs</td>
</tr>
</tbody>
</table>

*The Measure of Confidence*
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
Acknowledgments

Agilent

- Doug McIntyre
- Na Parra

Prolab

- Werner Döbelin
- Bernhard Nemec
- Mirko Glinski