High throughput LC-MS/MS of Pyrimethamine and Sulfadoxine in limited volume plasma samples:
combining selectivity from chromatography and fast ion separation by MS

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**Introduction**

- **Pyrimethamine + Sulfadoxine:**
  - Prevention and treatment of *Malaria P. falciparum*
  - Chloroquine in Eastern Africa
  - Antifolate-sulfonamide combination: *new first-line drug*

![Chemical Structures]

PYR

SUL
Drug resistance:

- Due to exposure to *sublethal concentrations*
  - Suboptimal dosage
  - Use of substandard drug formulations

Therefore:

**Tablet formulations on African market:**

Acceptable quality?  
Constant quality (shelf time under tropical conditions)?

- **In vitro availability:**  
  - Dissolution tests  
  - ≠ between formulations

- **In vivo bio-availability:**  
  - Clinical trial  
  - Same ≠ ?
**Aim**

From an **analytical viewpoint**:

Sound **simultaneous quantitation** of both drugs in human plasma samples

**Challenge:**

- Huge sample load
- Repetitive samples / volunteer
- Largely different concentrations

Fansidar®

Tablet: 25 mg PYR + 500 mg SUL
LC-MS/MS method: development

Focus

- Limited volume samples
- Fast analysis
  - Rapid sample clean-up
  - Fast chromatography
- « Strip and shoot »
- Short column

Selectivity
- Sensitivity
  - Tandem MS
Sample pretreatment:

- 250 µL crude plasma
- Deproteinization: 100 µL ZnSO₄ 0.1 M + 100 µL AcCN
- Removal of lipids: 300 µL CHCl₃

Chromatography:

- 10 µL supernatant injected
- XTerra MS C₁₈ column (3.5 µm p. s., 50*1 mm)
- Elution gradient: H₂O/ AcCN + 0.5% formic acid ammoniumformate (20 mM)
- IS: Sulfamerazine
MS/MS conditions:

- Triple quadrupole (Q. Ultima, Micromass)
- ESI +
- MRM
### MS/MS conditions:

<table>
<thead>
<tr>
<th>Compound</th>
<th>Precursor ion</th>
<th>Product ion</th>
<th>Cone voltage (V)</th>
<th>Collision energy (eV)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ion</td>
<td>m/z</td>
<td>m/z</td>
<td></td>
</tr>
<tr>
<td>PYR</td>
<td>[M+H]^+</td>
<td>249.1</td>
<td>233.1</td>
<td>30</td>
</tr>
<tr>
<td>SUL</td>
<td>[M+H]^+</td>
<td>311.1</td>
<td>156.0</td>
<td>70*</td>
</tr>
<tr>
<td>IS</td>
<td>[M+H]^+</td>
<td>265.2</td>
<td>110.0</td>
<td>35</td>
</tr>
</tbody>
</table>

Detuned to extend linear dynamic range
LC-MS/MS method: results & discussion

Fortified plasma sample

40 ng/mL PYR
4 µg/mL SUL

0.00 0.50 1.00 1.50 2.00 2.50 3.00 3.50 4.00 4.50 5.00 5.50 6.00 6.50
Time

PYR

1.00 1.50 2.00 2.50 3.00 3.50 4.00 4.50 5.00 5.50 6.00 6.50
Time

SUL

1.00 1.50 2.00 2.50 3.00 3.50 4.00 4.50 5.00 5.50 6.00 6.50
Time

IS

1.00 1.50 2.00 2.50 3.00 3.50 4.00 4.50 5.00 5.50 6.00 6.50
Time

BSMS 7th Annual Meeting
Sample clean-up

« Single step - one tube »

✓ Fast, efficient, simple
✓ No unduly dilution

Clear supernatant

Chromatography

✓ Fast
✓ Partial separation
✓ 1 mm-column → sensitivity ↑

MS/MS

✓ Selectivity → MRM
✓ Sensitivity

Sensitivity in bio-analysis ?
- **Low** [PYR] ↔ **High** [SUL]

Problems: carry-over saturation (ionisation and detector level)

⇒ Reconciliation in one-acquisition method?

1. Quantitative interval
2. Detuning for SUL: cone voltage
**LC-MS/MS method: validation**

- **Linearity:**
  - ✓ Range: [SUL] 0.1 - 50 µg/mL
    - [PYR] 5 - 1000 ng/mL
  - ✓ Weighed linear regression: 1/x
  - ✓ SUL $R = 0.9978$
    - PYR $R = 0.9984$

- **LOD:**
  - SUL 0.01 µg/mL
  - PYR 1 ng/mL

- **LOQ:** Lowest calibration point

- **Selectivity:** common antimalarial drugs + sulfonamides

**MRM**
### Other parameters:

<table>
<thead>
<tr>
<th>Compound</th>
<th>SUL (µg/mL)</th>
<th>PYR (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conc. level</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>Total reproducibility (CV, %) (n=5)</td>
<td>9.8</td>
<td>9.7</td>
</tr>
<tr>
<td>Within-day reproducibility (CV, %) (n=7)</td>
<td>7.0</td>
<td>3.8</td>
</tr>
<tr>
<td>Accuracy (Recovery ± SD, %) (n=5)</td>
<td>103.7 ± 8.7</td>
<td>103.9 ± 6.9</td>
</tr>
</tbody>
</table>
Conclusion

- Sensitivity:
  - « No longer an issue » for majority of applications
  - Shift towards reproducibility and carry-over

- Trend: « fast analysis »
  - Tempting response to ever increasing demands
  - However:
    - Rudimentary sample clean-up (+ fast chromatography) ≠ « Quick & dirty »