Speeding Metabolic Stability Assays Using Automated High Throughput LC/MS Techniques

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Introduction

• In drug discovery, metabolic stability is often a key factor in whether or not a compound continues on in the development process
• Metabolic stability can be assessed in vitro using pooled liver microsomes obtained from humans or other species of interest
• Automation plays a key role to increase sample throughput
• LC/MS provides the required selectivity, sensitivity, and speed to produce quality data

Metabolic Stability Screening

• Major determinant of in vivo drug concentration is clearance in the major organ of metabolism - the liver
• Cytochromes P450 (CYP) are the principal enzymes involved in metabolizing drugs and are thoroughly investigated in drug discovery and development
• Determine in vitro metabolic stability of CYP isoform-selective inhibitors in the presence of human liver microsomes

Automated Assay Overview

• LC/MS analysis of 96 well plate
  – Detection of % parent ion (SIR) remaining at each time point
  – Data processing

Verification of Packard MultiPROBE® II Automated Assay

Propranolol, 5 µM, LC/MS analysis

<table>
<thead>
<tr>
<th>Time</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>Group 4</th>
<th>Group 5</th>
<th>Group 6</th>
<th>Group 7</th>
<th>Group 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
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<tr>
<td>60</td>
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<td>69</td>
<td>60</td>
<td>56</td>
<td>66</td>
<td>60</td>
</tr>
</tbody>
</table>

Common LC/MS Method Parameters

• Sample:
  – 4 component known inhibitor sample to test LC/MS conditions
• LC/MS Conditions:
  – Waters Alliance HT into a Waters single quadrupole MS (APCI+)
    • First 0.7 min diverted to waste before directed to MS
    • Mobile phase A: 10% ACN w/ 0.1% formic acid
    • Mobile phase B: 100% ACN w/ 0.1% formic acid
      – 0.00 - 2.00 min 10% B - 100% B
      – 2.25 min 100% B
      – 2.35 - 3.50 min 10% B

Packard MultiPROBE® II

Automated Assay Overview

• Lead: 5 µM, Pooled HLM: 0.5 mg/mL
• Times: 0, 10, 30 & 60 minutes
• Acetonitrile to stop reaction
• If desired, concurrent incubations with known CYP isoform-selective inhibitors:
  – 1 mM ketoconazole (CYP3A4)
  – 1 mM quinidine (CYP2D6)
• Centrifuge; transfer supernatant to new 96 well plate
Experiment 1
- Reflects Traditional Operation
- Elevated Column Temp Reduces Solvent Viscosity

<table>
<thead>
<tr>
<th>Column</th>
<th>Flow rate</th>
<th>Injection mode</th>
<th>Pre-column volume</th>
<th>Rapid equilibration</th>
<th>Column re-eq</th>
<th>Column temp</th>
<th>Run time</th>
<th>Cycle time</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.6 x 50 Symmetry® C18</td>
<td>1.5 mL/min</td>
<td>Sequential</td>
<td>0</td>
<td>OFF</td>
<td>0</td>
<td>40</td>
<td>3.5</td>
<td>5.0</td>
</tr>
</tbody>
</table>

Experiment 2
- Column re-equilibration is part of next sample – can hide it’s time behind sample draw and data system reset

<table>
<thead>
<tr>
<th>Column</th>
<th>Flow rate</th>
<th>Injection mode</th>
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<td>OFF</td>
<td>0</td>
<td>40</td>
<td>2.5</td>
<td>4.2</td>
</tr>
</tbody>
</table>

**Switching Valves**
- Built-in valves for the 2790
- Software controlled
- 3-Column Selection
- 6-Column Selection
- Column Regeneration

**Alternating Column Regeneration**
- With both an internal column selection valve and an external column regeneration valve plumbed inline, the flexibility of column choice and the increase in productivity of offline column regeneration can be achieved.

**Additional Approaches For Increasing Throughput**
- Cassette analysis
  - Several metabolic stability samples combined, analyzed simultaneously
- Two time points
  - Zero and 60 minutes
- Column Regeneration
  - 2790 Valve Option

**Alternating Column Regeneration**
- 2 Analytical Columns
  - No additional reequilibration time
  - Continuous data from the mass spectrometer
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**Alternating Column Regeneration**

Incorporate High Throughput Routines

2 columns with Alliance HT and Regeneration Valve

<table>
<thead>
<tr>
<th>Column 1 Cycle Time</th>
<th>Column 2 Cycle Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Run Time</td>
<td>Regenerate Column 2</td>
</tr>
<tr>
<td>Draw Sample</td>
<td>X</td>
</tr>
<tr>
<td>Needle Wash</td>
<td>Run Time</td>
</tr>
<tr>
<td>Load Sample</td>
<td>X</td>
</tr>
<tr>
<td>Regenerate Column 1</td>
<td></td>
</tr>
</tbody>
</table>

Incorporate Parallel Sampling

<table>
<thead>
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<th>Column 1 Cycle Time</th>
<th>Column 2 Cycle Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Run Time</td>
<td>Regenerate Column 2</td>
</tr>
<tr>
<td>Draw Sample</td>
<td>X</td>
</tr>
<tr>
<td>Needle Wash</td>
<td>Run Time</td>
</tr>
<tr>
<td>Load Sample</td>
<td>X</td>
</tr>
<tr>
<td>Regenerate Column 1</td>
<td></td>
</tr>
</tbody>
</table>

X= injection prep time

**Experiment 3**

- Alternating Column Regeneration
- 4.6 x 50 Symmetry® C18
- 1.5 mL/min
- Sequential
- Pre-column volume: 0
- Rapid equilibration: OFF
- Column re-equil: 40
- Column temp: 2.5
- Run time: 3.4

**Experiment 4**

- Alternating Column Regeneration
- Next sample is drawn right after needle wash and purge
- 4.6 x 50 Symmetry® C18
- 1.5 mL/min
- Parallel
- Pre-column volume: 0
- Rapid equilibration: OFF
- Column re-equil: 40
- Column temp: 2.5
- Run time: 2.8

**Metabolic Stability**

**LC/MS Method Development**

**Reducing Cycle Times**

- Original Method
  - 3.5 min run time
  - 3.0 min cycle time
- Column Re-equilibration
  - 2.5 min run time
  - 4.2 min cycle time
- Alternating Column Regeneration
  - 2.5 min run time
  - 3.4 min cycle time
- Alternating Column Regeneration and Parallel Sampling
  - 2.5 min run time
  - 2.8 min cycle time

**Increasing Metabolic Stability Assay Throughput**

Per 16 hour overnight run:
- Normal analysis allows 192 samples
- With column re-equilibration, 228 samples
- With offline column regeneration, 282 samples
- With offline column regeneration and parallel sampling, 342 samples

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