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

Kelly Johnson, John Erve*, Andre Dandeneau*, and Beverly Kenney

Waters Corporation, Milford, MA
*GENTEST Corporation, Woburn, MA

Alliance® HT LC/MS System

40 cm   59 cm   39 cm

Packard MultiPROBE® II
Robotic Liquid Handling System
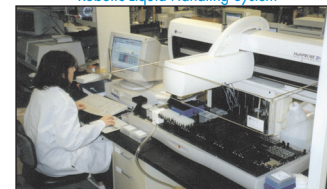
Introduction

- 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

Automated Assay Overview
High Throughput LC/MS

Waters Alliance® HT LC/MS System with MassLynx™ software

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

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

Verification of Packard MultiPROBE® II Automated Assay
Propranolol, 5 µM, LC/MS analysis

Time	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	Group 8
0	100	100	100	100	100	100	100	100
10	93	92	93	93	97	96	93	91
30	88	86	86	90	85	84	86	81
60	63	66	64	69	60	56	66	60

Time	Average	Stdev	CV%
0	100.0	0.00	0.0%
10	93.5	1.80	1.9%
30	85.9	2.72	3.2%
60	63.0	3.99	6.3%

Automated Assay Overview
Robotic Sample Prep

Packard Multi-PROBE® II

- 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

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

1

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Incorporate 2790 High Throughput Functionality

|	Experiment 1	Experiment 2
	• Reflects Traditional Operation • Elevated Column Temp Reduces Solvent Viscosity	• Column re-equilibration is part of next sample – can hide it's time behind sample draw and data system reset
Column:	4.6 x 50 Symmetry® C18	4.6 x 50 Symmetry® C18
Flow rate:	1.5 mL/min	1.5 mL/min
Injection mode:	Sequential	Sequential
Pre-column volume:	0	0
Rapid equilibration:	OFF	OFF
Column re-equil:	0	*1.0*
Column temp:	40	40
Run time:	3.5	2.5
Cycle time:	5.0	4.2

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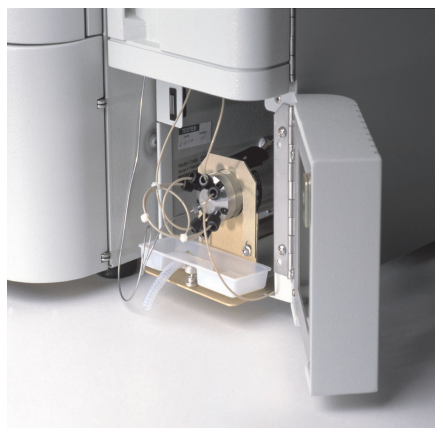
- Built-in valves for the 2790
- Software controlled
- 3-Column Selection
- 6-Column Selection
- Column Regeneration

Metabolic Stability LC/MS Method Development

Experiment 1

Experiment 2
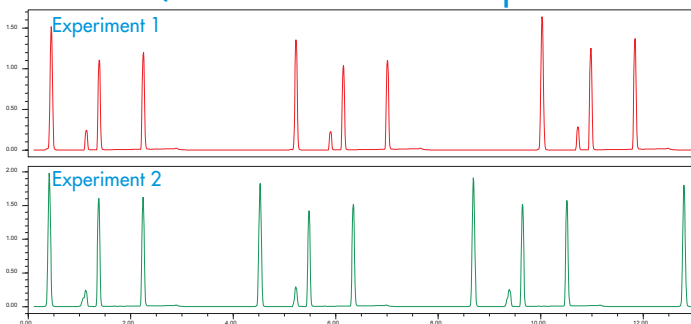
— Original Method 1.5 mL/min on 4.6 x 50 mm Symmetry® C_{18}

— Sequential w/Column Re-equilibration Method 1.5 mL/min on 4.6 x 50 mm Symmetry® C_{18}

Alternating Column Regeneration
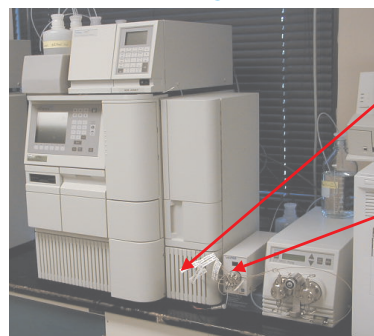
Built-in 2790 3-column select valve

Rheodyne Lab Pro™ Two-Position, Ten-Port Fluid Processor

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
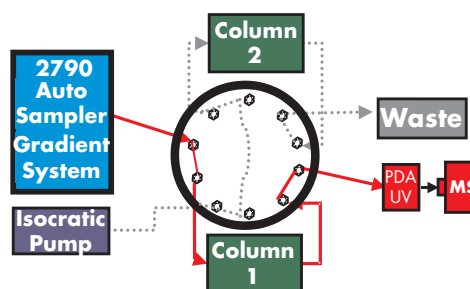
ADVANTAGES
- No additional reequilibration time
- Continuous data from the mass spectrometer

Speeding Metabolic Stability Assays Using Automated High Throughput LC/MS Techniques
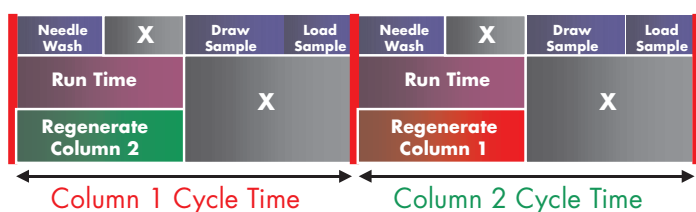
Kelly Johnson, John Erve*, Andre Dandeneau*, and Beverly Kenney
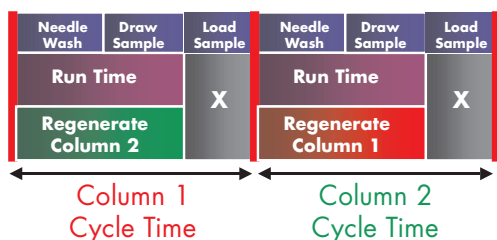
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Alternating Column Regeneration
Incorporate High Throughput Routines
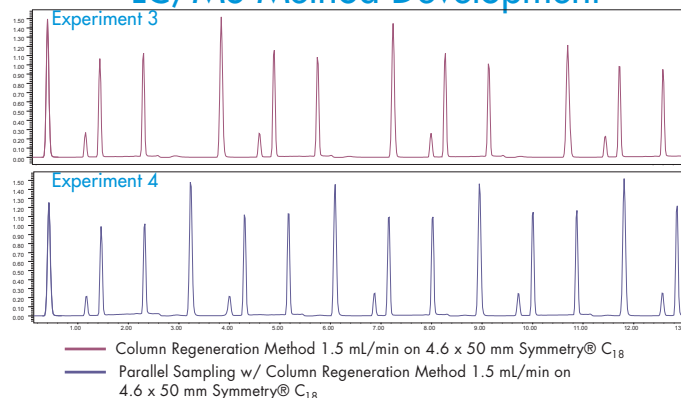
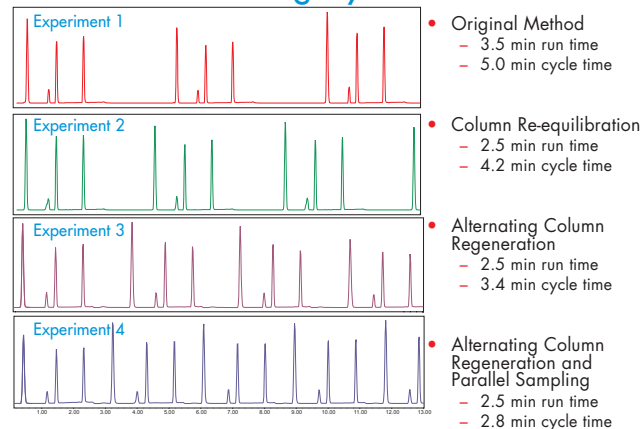2 columns with Alliance HT and Regeneration Valve

Column 1 Cycle Time | Column 2 Cycle Time

Incorporate Parallel Sampling

Column 1 Cycle Time | Column 2 Cycle Time

X= injection prep time

Metabolic Stability LC/MS Method Development

Experiment 3

Experiment 4

— Column Regeneration Method 1.5 mL/min on 4.6 x 50 mm Symmetry® C$_{18}$
— Parallel Sampling w/ Column Regeneration Method 1.5 mL/min on 4.6 x 50 mm Symmetry® C$_{18}$

Reducing Cycle Times

Experiment 1
Experiment 2
Experiment 3
Experiment 4

- Original Method
  - 3.5 min run time
  - 5.0 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

Incorporate Two Column Regeneration Hardware and 2790 High Throughput Routines	Experiment 3 • Alternating Column Regeneration	Experiment 4 • Alternating Column Regeneration • Next sample is drawn right after needle wash and purge
• Columns (2):	4.6 x 50 Symmetry® C18	4.6 x 50 Symmetry® C18
• Flow Rate:	1.5 mL/min	1.5 mL/min
• Injection Mode:	Sequential	Parallel
• Pre-column volume:	0	0
• Rapid equilibration:	OFF	OFF
• Column re-equil:	0	0
• Column temp:	40	40
• Run time:	2.5	2.5
• Cycle time:	3.4	2.8

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

3