An Overview of Current Tools for Quantitative LC-MS in the Clinical Laboratory

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Disclosure

- Employment: Waters Corporation
- Stock Ownership: Waters Corporation

During the course of this presentation I will speak about both Waters and non-Waters products. Any representation of any product is designed to be illustrative of a general principle of liquid chromatography or mass spectrometry and does not represent a product endorsement. I encourage you to speak with the respective vendor representatives for additional information about specific products.

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Outline

- Sample pretreatment
  - Single-use SPE, Multi-use SPE, SPE-MS/MS, Immuno-affinity purification
- HPLC vs. UHPLC
- Ionization
  - Differential Mobility Separation
- Mass Analyzers
  - Nominal mass vs. High Res./Exact Mass
  - Ion ratios and product ion spectra for confirmation
- Data reduction software
- What’s available and what’s needed
  - Audience participation: Your input is desired for this ever-growing list...

Strategies for sample pretreatment

- Dilute-and-shoot
- Protein precipitation (PPT)
- Liquid/liquid extraction (LLE)
- Solid phase extraction (SPE)
  - Manual, e.g. vacuum manifold
  - Integrated, e.g. on-line
    - Reusable extraction columns
    - Single-use extraction cartridges
- Supercritical fluid extraction (SFE)
  - extraction using supercritical carbon dioxide instead of an organic solvent

Less steps using an integrated pretreatment approach
Online SPE using reusable cartridge columns

![Diagram of SPE process]

Multiplexing Increases Throughput

System diagram above is representative of a Thermo Scientific Transcend™ LX-4 system.

Rapid determination of steroids for newborn screening for congenital adrenal hyperplasia (CAH) by turbulent flow liquid chromatography-tandem mass spectrometry (TFLC-MS/MS)

Jean M. Lacey1, Mark J. Magrane1, Joseph M. Di Bussolo2, Silvia Tortorelli1, SiHoun Hahn1, Piero Rinaldo1, Dietrich Matern1

1. Mayo Clinic, Rochester, MN; 2. Cohesive Technologies, Franklin, MA

54th ASMS Conference on Mass Spectrometry
On-line SPE using single-use extraction cartridges

- Gripper Places Cartridge In Right Clamp — Specified in sample list
- HPD Applies Conditioning And Equilibration Solvents To Cartridge

Sample Extraction Mode: Sample Loading

- Sample Manager Draws Sample During Conditioning And Equilibration Steps
- Loop Brought In Line With Loading Solvent From HPD
- Sample Pushed Through Cartridge, Analyte Trapped And Stream Diverted To Waste

Sample Extraction Mode: Sample Wash

- HPD Supplies Wash Solvents Across The Cartridges
- Up To 23 SPE Solvents Can Be Used — Mix ratios of 2 solvents can be used in steps of 10% for method development
Sample Extraction Mode: Sample Elution

- Gripper Moves Cartridge To Left Clamp For Elution Step
- Elution Time Is Defined In The Inlet Editor
  - Selectivity of extraction
- HDP Supplies Clamp Flush Solvent To Left Clamp Tubing
  - Numerous wash steps can be applied to minimize carryover

Improved Sensitivity: Online SPE vs Protein Crash

Sirolimus 1ng/mL

1 ng/mL tacrolimus from protein precipitated whole blood

Improved Sample Clean-up: Removal Of Phospholipids

Whole Blood without SPE
Whole Blood with Online SPE
Immuno-affinity purification

- Should I Be Embarrassed That My Mass Spec Assay Uses an Antibody?
  - NACBBlog, 06 March 2012 by Fred Strathmann
  - Short answer is “no”
  - Reduction in final sample complexity
  - Immunoassays possess sensitivity, but lack specificity. MS detection overcomes this

- Applied primarily to low abundance analytes, e.g. 1,25(OH)2-vitamin D and peptides/proteins, e.g. SISCAPA®

Clinical Chemistry 57:9;1279–1285 (2011)

Chromatography
HPLC vs. UHPLC
Particle Technology

60 μm Human Hair
(very fine hair)

5 μm
Analytical Particles
(can fit 12 across hair)

2 μm
UHPLC Particles
(can fit 33 across hair)

Images are on the same scale (Bar = 10 μm)

Particle Size and Mechanical Separating Power

Columns contain the same packing material chemistry, are the same length with the same mobile phase. One column has particles which are a third the size.

50mm
5 micron

50mm
1.7 micron

Smaller particle sizes provide for better separation with the same run time.

This is also called “Efficiency”

Analytical Challenges - Sensitivity and Throughput

Set to same scale

1970’s
10 μm – 250 mm
Rs (2,3) = 1.54
35.00 min.

1980’s
5 μm – 150 mm
Rs (2,3) = 2.69
9.50

1990’s
3.0 μm – 100 mm
Rs (2,3) = 2.29
4.50

2012
1.7 μm – 50 mm
Rs (2,3) = 2.25
1.10 min.
UHPLC Column on HPLC System:
What are the benefits?

**XTerra® MS C18**
2.1 x 50 mm, 2.5 µm
F = 0.5 mL/min
PSIMAX = 3,950
T(4) = 1.30
N (4) = 4,100
Rs (2,3) = 1.10

**ACQUITY UPLC® BEH C18**
2.1 x 50 mm, 1.7 µm
F = 0.3 mL/min
PSIMAX = 4,200
T(4) = 1.63
N (4) = 5,400
Rs (2,3) = 0.97

Fully optimized HPLC instrument:
Band spreading contribution reduced as much as possible, but still relatively high.

Fully optimized HPLC instrument:
Minimal benefits realized with UHPLC column
A) Flow rate too slow (pressure)
B) Still more instrument band spreading than UHPLC instrument

Reset AU Scale

HPLC Non-Optimal Linear Velocity
Higher Band Spreading
**UHPLC Column on UHPLC System:**

What are the benefits?

The results look much better!!

ACQUITY UPLC® BEH C18

2.1 x 50 mm, 1.7 µm
F = 0.3 mL/min
PSIMAX = 4,200
T (4) = 1.63
N (4) = 5,400
Rs (2,3) = 0.97

Reset AU Scale

ACQUITY UPLC® BEH C18

2.1 x 50 mm, 1.7 µm
F = 0.6 mL/min
PSIMAX = 8,400
T (4) = 1.02
N (4) = 10,100
Rs (2,3) = 2.25

~2 x Plate Count
~2 x Rs
~2 x Pressure
~8 x Sensitivity

**Differential Mobility Separation**

**Ion Mobility Technology**

Differential ion mobility device
Quantitation using tandem quadrupole and hi-res instruments

Specificity is achieved by: 1) chromatography, 2) pre-cursor ion selection (0.7 – 1.0 amu), and 3) product ion selection (0.7 – 1.5 amu).
Tacrolimus:
Single column method, SIR vs MRM

SIR m/z 821
3µg / L
30µg / L

MRM m/z 821>768

2 Cases – Pseudo-Cushing
Syndrome by HPLC-UV

<table>
<thead>
<tr>
<th></th>
<th>Case 1</th>
<th>Case 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>UFC (HPLC)</td>
<td>166</td>
<td>126</td>
</tr>
<tr>
<td></td>
<td>135</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td>463</td>
<td>156</td>
</tr>
<tr>
<td></td>
<td>180</td>
<td></td>
</tr>
<tr>
<td>UFC (GC-MS/MS)*</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>UFC (RIA)</td>
<td>35</td>
<td>n/a</td>
</tr>
<tr>
<td>PSH</td>
<td>0.9</td>
<td>1.1</td>
</tr>
<tr>
<td>Ratios</td>
<td>&lt; 0.4 - 1.1</td>
<td>&lt; 0.4 - 3.2</td>
</tr>
</tbody>
</table>

*Using qualifier ion transition

Ion ratios and product ion confirmation
Acquire Qualitative & Quantification Data at the Same Time

- Acquire qualitative (spectral) data & quantitative (MRM) data in a single injection

- MS spectral data at the same time as MRM data
  - Detect compounds not defined as MRMs
  - Valuable aid to method development
  - See all of the background interferences while developing your targeted MRM method
  - Tentatively identify compounds during routine quantitative analysis

- Use MRM data to trigger the acquisition of product ion spectra
  - Spectral data provides useful extra information about a suspect MRM quantification result

Multiple identification criteria improves confidence in results...

- MS2 Scan
- Peak @ 8.04 min
- Spectrum of Peak @ 8.04 min
- Product Ion Spectra
- Forward Match Scores
- Reverse Match Scores
- Cyclobenzaprine FW = 275.2
Now I understand – it’s so easy!

- Carbon has a mass of 12
- Hydrogen has a mass of 1
- Oxygen has a mass of 16
- Nitrogen has a mass of 14

**Exact mass**

<table>
<thead>
<tr>
<th>Element</th>
<th>Mass (u)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>12.0000</td>
</tr>
<tr>
<td>H</td>
<td>1.0078</td>
</tr>
<tr>
<td>O</td>
<td>15.9949</td>
</tr>
<tr>
<td>N</td>
<td>14.0031</td>
</tr>
</tbody>
</table>

If such compounds can be mass measured with sufficient accuracy it is possible to determine elemental composition.

A nominal mass instrument cannot distinguish these... requires "Hi-Res"

- CO: 27.9949
- N₂: 28.0062
- C₂H₄: 28.0312

The Basics of Exact Mass

**ToF basics:**

What is ‘Time-of-Flight’ analysis?

- ToF detectors
  - ToF analysers are VERY accurate clocks
  - Time taken to travel a specified path i.e. from A to B (flight tube)
  - The Time-of-Flight of a given ion depends directly upon on its mass-to-charge ratio.
  - If the charge-state of an ion is known then the mass can be determined from the time taken to complete the flight tube.

**Benzoylcyconine (Precursor Ion)**

Elemental Composition C₁₆H₁₉NO₄

- Carbon: 16 x 12.00000 = 192.00000
- Hydrogen: 19 x 1.007825 = 19.148675
- Nitrogen: 1 x 14.003074 = 14.003074
- Oxygen: 4 x 15.994915 = 63.97966

Exact mass [M+H]+ = m/z 290.1392

Both instruments are accurately calibrated for mass but resolution is significantly different.
**Hair Cocaine**

**UPLC-TOF/MS**

Sample Preparation
20 mg washed hair, analyze specific deuterated IS
pulverization with metal balls 4 min
1 hr extract at 60 °C in 1 mL methanol, microfilter
dried and reconstituted in 100 µL starting mobile phase

Instrumentation
UPLC: Waters Acquity UPLC HSS C18 150 x 2.1 mm, 1.8 µ, 50°C, 400 µL/min, 13-95% gradient 5.0mM NH4 formate pH3.0/acetonitrielle 0.1% formate, 5 µL, 15 min run time
Mass Spec: Waters Xevo G2 QToF, ESI+, cone 20V, source 120°C, capillary 1.5kV,
cone N2, 10 Lhr, desolvation 500°C, desolvation N2 800 Lhr, argon CE, high resolution 50-1000 m/z, lock mass mode (leucin enkephlin 20 µL/min, capillary 2 kV, CE 6kV), NaFomate cal.

**Ion Preparation**

**MS Function 1** for precursor data with low collision energy 6 eV
**MS* Function 2** for fragment ion data with MS* collision energy ramp (10-40 eV)

**Quantitation**
Precursor ion analysis by isotope dilution technique

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**Calibration Curves**

10 ng/mL = 500 pg/mg hair

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**Orbitrap – Principle of Operation**

Makarov A. Anal. Chem. 2000, 72, 1156-1162.
Benefit of resolution for co-eluting compound

Resolution 100,000  
Mass accuracy Bisoprolol 3 ppm

Resolution 10,000  
Mass accuracy Bisoprolol 9 ppm

Bisoprolol 12C
Quinine 13C

Sample with both drugs - confirmation compromised @10,000

Where does the specificity come from?

Tandem Quadrupole  
- Sample pretreatment protocols
- Chromatographic retention time
- Precursor ion selection
- Specific molecular fragments
- Product ion selection
- Ion ratio calculation

High Resolution/Exact Mass  
- Sample pretreatment protocols
- Chromatographic retention time
- Precursor ion selection
- Specific molecular fragments
- Product ion selection
- Ion ratio calculation

IGF-1 Quantitation using Hi-Res Mass Spectrometry

*Quantifier ion  
*Qualifier ions

Anal Chem. 2011, 83, 9005–9010
Things to look for in data reduction software:

- Analyte confirmatory ion ratios (at least one ratio, > 1 desirable)
- Concentrations above maximum reporting level (MRL)
- Signal-to-noise ratio
- Analyte or Internal Standard RT or RRT
- Analyte concentration below LOD or LOQ
- Standard Deviation of QCs too high
- $r^2$ of calibration too low
- Internal Standard area
- Peak Quality (width, skewness, kurtosis)

<table>
<thead>
<tr>
<th>What’s Needed</th>
<th>What’s Available</th>
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<tbody>
<tr>
<td>Efficient sample pretreatment strategies</td>
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<tr>
<td>Integrated sample clean up / concentration</td>
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<tr>
<td>Reliable chromatography systems and columns</td>
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<tr>
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Thank You – Questions?