Meeting the LC-MS needs of a high-throughput clinical laboratory

April 18th
10:00 [New York] / 15:00 [London]
Meeting the LC-MS Needs of a High-Throughput Clinical Chemistry Laboratory

With examples from endocrinology and biochemical genetics unit disciplines within healthcare laboratories, this presentation will cover alternative sample extraction and liquid chromatography solutions developed to meet the challenges of a busy hospital laboratory.

Today’s Hosts: Isaac Bruce
Commissioning Editor

Today’s Presenter: Michael J.P. Wright
Cambridge University Hospital Trust
Our Speaker

Michael J.P. Wright
Lead Method Developer
Cambridge University Hospital Trust
Meeting the LC-MS Needs of a High-Throughput Clinical Chemistry Laboratory

Michael Wright

Biochemistry Department
Addenbrooke’s Hospital
Cambridge University Hospital Trust
What is Clinical Chemistry?

**The Question**
“What’s wrong with this person?”

What does it look/sound like?
“I think it might be disease X, send a sample to the lab”

**The Matrix** - The human body

- Urine
- Blood
- Faeces
- Saliva
- Bile
- Dried Blood Spot
- Cerebrospinal Fluid
- Amniotic Fluid

**The Target** - Biomarkers
Why LC-MS/MS?

- Seen as a sensitive and "specific"/selective way of measuring target analytes
- Replacement/Confirmation test for expensive, imprecise or inaccurate Immunoassays (IA)
- Replacement for traditional UV, flourescent, electrochemical detection for HPLC
- Replacement for lengthy GC-MS methods that have extensive sample clean-up
Topics to cover

1) Selecting the correct stationary phase
2) Is ultra high-pressure chromatography always the best option?
3) Sample preparation options for a high-throughput clinical laboratory
4) Where are we now with clinical chemistry LC-MS?
1: Choosing the appropriate chromatography

- Target analytes often differ greatly to those found in TDM or Toxicology
  - Endogenous,
  - Low concentration (pg/mL)
  - Often part of large, closely related families of endogenous compounds

- In many cases “dilute and shoot, trap & elute” methods are not suitable for accurate analysis

- Use of MS detection limits the buffers/ ion pair reagents that we have available – more reliant on the stationary phase
Isobaric interferences

**Urinary Free Cortisol**

Ascentis Express 100x2.1mm 2.7μm Phenyl Hexyl
Unusual patient samples

Serum testosterone

Serum 25OH Vitamin D3
25OH Vitamin D analysis in infants

Polar analytes

Ascentis Express 50x2.1mm 2.7µm HILIC
Urinary Xanthine, Hyperxanthine and Sulphocysteine

S-sulphocysteine

Ascentis Express 100x 2.1mm 2.7µm C18

Ascentis Express 100x 2.1mm 2.7µm HILIC
Chiral Columns for Non-Chiral Separations

Teicoplanin
(Chirobiotic T)

Key interaction sites; A, B, C and D are cavities
Urinary S-sulphocysteine, Xanthine & Hyperxanthine on Chirobiotic-T

Astec 100x2.1 5µm Chirobiotic T
2: Is ultra high-pressure chromatography always the best option?

\[ \Delta P = \frac{1000F \eta L}{\pi r^2 d_p^2} \]

Performance (Plates)

<table>
<thead>
<tr>
<th>Diameter (µm)</th>
<th>Performance (Plates)</th>
<th>Pressure (psi)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>12000</td>
<td>1100</td>
</tr>
<tr>
<td>3</td>
<td>22000</td>
<td>2900</td>
</tr>
<tr>
<td>1.7</td>
<td>32500</td>
<td>8600</td>
</tr>
</tbody>
</table>

Is ultra high-pressure chromatography always the best option?
Superficially porous particle columns

*Ascentis Express Particle*

*Totally Porous Particle*
Ascentis Express Fused Core columns

Sub-2μm efficiency achieved at near 3μm pressures.

*50x4.6 mm columns, 55/45 ACN/Water
Advantages – higher performance: pressure

Urine 5HIAA

Ascentis® Express C18
Dim: 50x4.6mm 2.7µm

BP = 2070 psi

1.5min run time
3: Sample preparation options for a high-throughput clinical laboratory

- Target analytes present in samples at low concentrations – require sample extraction to remove matrix effects & possibly concentrate sample

**Liquid-Liquid Extraction?**

1. Aliquot sample + Internal Standard + Solvent
2. Vortex
3. Centrifuge
4. Aspirate the solvent layer (freeze the aq. layer)
5. Dry down under N$_2$ or in rotary evaporator
6. Reconstitute in mobile phase
7. Centrifuge
8. Transfer to auto-sampler vials/plates

Time consuming Not always suitable Expensive to automate SLE?
Solid Phase Extraction?

- Time consuming
- Expensive to automate
- Consumable costs
Online Solid Phase Extraction
Protein Precipitation step

- 100µL Serum
- 25µL Internal Standard
- 25µL 0.2M ZnSO₄
- 200µL Methanol

- Process a plate of Calibrators, QC and 84 patient samples in 30min
Online Solid Phase Extraction

Autosampler

Eluting Pumps

Waste

Analytical Column

Strata 20 × 2.0mm 20μm C8
Meeting the needs of the department

- **Staff Time:**
  - LLE > Off-line SPE > **On-Line SPE**

- **Consumable Cost:**
  - Off-line SPE > **On-line SPE** > LLE

- **Automation Cost:**
  - LLE > Off-line SPE = **On-line SPE**

- **Sample Extraction Time**
  - LLE > Off-line SPE > **On-line SPE**
LC-MS Workload at Addenbrooke’s

- Newborn Screening MCADD/PKU
- TDM Tacrolimus, Cyclosporin, Sirolimus
- 25OH Vitamin D2/3
- Serum androgens
- Urine Metanephrines
- B/S Carnitines
- Urine, Serum and Salivary Cortisol
- Urine & Plasma Creatine/Guanidinoacetate/Creatinine
- Urine 5HIAA
- Homocysteines
- Total thyroxine

**Studies**
- DBS Testosterone
- Skin steroid panel
- Cell culture steroid panels
- 3-epi-25OH Vitamin D2/3

**Evaluation/validation**
- Plasma metanephrines
- Androstenedione
- Aldosterone
- 17OHP/DHEAS
- Insulin
- T3, rT3
Multiplexing

“What’s the point of running an overnight batch at 2min/sample when the system then sits unused for 10 hours throughout the rest of the night?”
Pump(s) 1 -> Autosampler -> Solvent selector -> 6 position selection valve -> Analytical column -> Column Oven -> Mass Spectrometer
Adding Online SPE

- Pump(s) 1
- Pump(s) 2
- Autosampler
- Column Oven
- Mass Spectrometer
- 6 position selection valve
- 2 position divert valve
- On-line SPE cartridge
- Analytical column
- Solvent selector
Example from Addenbrookes

1) Serum androgens – 48 samples by Online SPE (3½ hours)
2) Conditioning (40min)
3) Urine Cortisols – 36 samples by Online SPE (4 hours)
4) Conditioning (40min)
5) Serum 25OH Vit D3/2 – 96 samples by Online SPE (8 hours)

Run put on at 4pm has finished at 9am the following morning
Confirmation Analysis

**A**

![Graph A](image)

**B**

![Graph B](image)

### Calculating the standard ratio

<table>
<thead>
<tr>
<th>Std</th>
<th>Quantifier</th>
<th>Qualifier</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.68E+04</td>
<td>1.92E+04</td>
<td>0.72</td>
</tr>
<tr>
<td>2</td>
<td>1.18E+05</td>
<td>8.81E+04</td>
<td>0.75</td>
</tr>
<tr>
<td>3</td>
<td>2.02E+05</td>
<td>1.45E+05</td>
<td>0.72</td>
</tr>
<tr>
<td>4</td>
<td>4.24E+05</td>
<td>3.08E+05</td>
<td>0.73</td>
</tr>
</tbody>
</table>

**Mean** 0.73

**Range** ±15%

<table>
<thead>
<tr>
<th>Samples</th>
<th>Quantifier</th>
<th>Qualifier</th>
<th>Ratio</th>
<th>Accuracy</th>
</tr>
</thead>
<tbody>
<tr>
<td>UTAK QC_Low</td>
<td>6.68E+04</td>
<td>4.75E+04</td>
<td>0.71</td>
<td>98%</td>
</tr>
<tr>
<td>UTAK QC1</td>
<td>1.55E+05</td>
<td>1.19E+05</td>
<td>0.77</td>
<td>106%</td>
</tr>
<tr>
<td>UTAK QC2</td>
<td>4.34E+05</td>
<td>3.23E+05</td>
<td>0.74</td>
<td>102%</td>
</tr>
<tr>
<td>NIST Sera 1</td>
<td>1.58E+05</td>
<td>1.13E+05</td>
<td>0.72</td>
<td>98%</td>
</tr>
<tr>
<td>NIST Sera 2</td>
<td>7.67E+04</td>
<td>5.48E+04</td>
<td>0.71</td>
<td>98%</td>
</tr>
<tr>
<td>NIST Sera 3</td>
<td>1.15E+05</td>
<td>8.70E+04</td>
<td>0.76</td>
<td>104%</td>
</tr>
<tr>
<td>NIST Sera 4</td>
<td>4.74E+05</td>
<td>2.76E+05</td>
<td>0.58</td>
<td>80%</td>
</tr>
</tbody>
</table>
Multiplexing points to remember

- There is a larger amount of tubing - ensure that this is as small ID as possible to reduce extra column volumes and thus longitudinal diffusion (B-term)
  - Using 2.1mm ID columns we found 500µl/min to be the lowest flow rate we could use whilst not suffering from longitudinal diffusion

- Incompatible mobile phases require longer equilibration

- Keep Curtain Gas as high as you can on all methods
HybridSPE

Simplify the procedures of protein precipitation and phospholipid removal into one step.

- 5 µm PTFE Frit
- ZrO2-coated silica particles, 50 mg
- 0.2µm filter membrane
Phospholipid depletion filter plates

- 100µL Serum
- 25µL Internal Standard
- 25µL 0.2M ZnSO₄
- 200µL Methanol

Protein precipitation plate

Hybrid SPE plate

Collection plate
Figure 3. LC–MS/MS chromatograms of an injection of an in-house serum QC sample containing 25-hydroxyvitamin D₃, 3-epi-25-hydroxyvitamin D₃, 25-hydroxyvitamin D₂, and 3-epi-25-hydroxyvitamin D₂. Monitoring (A, C & E) m/z 383–257 for 25(OH)D₃ and
Figure 5. Comparison of epimer and non-epimer resolving methods in patient samples aged 1–91 years old. (A) Deming regression and (B) Bland Altman analysis comparing serum 25(OH)D₃ concentrations.

Figure 7. Comparison of two epimer-resolving methods in infant samples. (A) Deming regression and (B) Bland Altman analysis comparing serum 25(OH)D₃ concentrations.
Topic 4. Where are we now?

- Certified serum based reference standards

- Urinary Free Cortisol
  - Reference Range?

- Serum Testosterone
  - Clinically useful cross reactivity
  - Steroid Panels

<table>
<thead>
<tr>
<th>Sample</th>
<th>Immulite</th>
<th>LCMSMS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine blank</td>
<td>67</td>
<td>35</td>
</tr>
<tr>
<td>Urine + 2µg/ml THE</td>
<td>100</td>
<td>33</td>
</tr>
<tr>
<td>Urine + 2µg/ml THF 5α</td>
<td>593</td>
<td>38</td>
</tr>
<tr>
<td>Urine + 2µg/ml THF 5β</td>
<td>&gt;1300</td>
<td>33</td>
</tr>
<tr>
<td>Urine + 2µg/ml α-Cortolone</td>
<td>74</td>
<td>32</td>
</tr>
</tbody>
</table>
Acknowledgements

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Colin Stone
Clare Glicksman

Evelina Children’s Hospital
Neil Dalton
Charles Turner

Shimadzu
Earl McCoy

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Questions
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- Ascentis Express HPLC Columns
  - Sigma-aldrich.com/express
- HybridSPE-Phospholipid removal
  - sigma-aldrich.com/hybridspe-pl
- Chiral HPLC Columns
  - sigma-aldrich.com/chiral
Thank you

Today’s presentation has been recorded and a copy will shortly be available on

Bioanalysiszone

www.bioanalysis-zone.com/webinars/

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