Little to LARGE

LC-MS/MS peptide method development for small molecule specialists

Science for a safer world
Peptide Quantitation

• Peptide biomarkers
  – Diagnosis (e.g. cancers, diabetes, Alzheimer's, cardiovascular)
  – Measure effectiveness of novel treatments

• Peptide drugs
  – For the treatment of a wide variety of diseases (pain, cancer, CVD)

The emergence of peptides in the pharmaceutical business: From exploration to exploitation - EuPA Open Proteomics Volume 4, September 2014, Pages 58–69

• Peptide quantitation in blood plasma samples - Needed to support the pharmacodynamic (PD) characterisation of biomarkers and pharmacokinetic (PK) characterisation of drugs
Peptide Quantitation

- Immunochemistry - e.g. RIAs, ELISAs
- However - antibodies may not be available, can overestimate concentrations (nonspecific antibody binding), can have long extractions, radioactivity precautions (RIA) or poor P&A

- LC-MS/MS - as an alternative
- Method development is similar to small molecules, but is **not** the same
Research: Physicochemical Properties

Ibuprofen, 206 Da

Glucagon, 3483 Da
# Sensitivity

## Small Molecules

<table>
<thead>
<tr>
<th>Plasma LLOQ (pg/mL)</th>
<th>Approx. Mass (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>340</td>
</tr>
<tr>
<td>0.25</td>
<td>390</td>
</tr>
<tr>
<td>1</td>
<td>420</td>
</tr>
<tr>
<td>1</td>
<td>500</td>
</tr>
<tr>
<td>5</td>
<td>470</td>
</tr>
</tbody>
</table>

**Ibuprofen**- 206 Da

1 pg = $2.92 \times 10^9$ molecules

\[
\div 17 \quad 1 \text{ pg} = 1.72 \times 10^8 \text{ molecules}
\]

## Peptides

<table>
<thead>
<tr>
<th>Plasma LLOQ (pg/mL)</th>
<th>Approx. Mass (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>3,500</td>
</tr>
<tr>
<td>15</td>
<td>4,100</td>
</tr>
<tr>
<td>25</td>
<td>3,300</td>
</tr>
<tr>
<td>30</td>
<td>4,400</td>
</tr>
<tr>
<td>40</td>
<td>4,100</td>
</tr>
</tbody>
</table>

**Glucagon**- 3,483 Da
**Sensitivity**

**Ibuprofen**
- [M-H]⁻¹
- [M+5H]⁺⁵
- [M+4H]⁺⁴
- [M+3H]⁺³
- [M+6H]⁺⁶

**Glucagon**
- [M+5H]⁺⁵
- [M+4H]⁺⁴
- [M+3H]⁺³

**m-NBA concentration**
- Maximum response (%)
- 100
- 80
- 60
- 40
- 20
- 0

**m-NBA concentration (%)**
- 0.00
- 0.03
- 0.05
- 0.08
- 0.10

**Intensity, cps**
- 0.00 0.03 0.05 0.08 0.10%
- 2.0e5 4.0e5 6.0e5 8.0e5 1.0e6
- 2.0e6 4.0e6 6.0e6 8.0e6 1.0e7

**m/z, Da**
- 0.0 20 40 60 80 100 200 300 400 500 600 700 800 900 1000 1100 1200 1300 1400 1500
- 587.8 697.5 871.6
- 625.2 705.0 877.3
- 513.3 685.0 856.7
- 466.4 637.5 808.2
- 411.2 580.5 751.8
- 365.7 536.9 708.1
- 319.8 490.2 661.5
- 274.0 442.1 613.4
- 227.2 394.4 565.7
- 180.3 356.6 527.9
- 133.4 285.6 457.9
- 86.5 237.8 409.0
- 39.6 191.1 362.4

**Max. % m-NBA**
- 0.00 0.03 0.05 0.08 0.10
- 0 5 10 15 20

**Maxim. sensitivity**
- 1.340 to 1.391 min from Sample 2 (0.1% FA 0.1% m-NBA (95:5)- 18-28 1000 ng/mL glucagon) of 27Apr14FS-008.wiff (Turbo Spray) Max. 5.1e6 cps.
Non-specific binding

• Blocking buffers- e.g. BSA
• Optimising pH
• Serial dilution in plasma
• Selection of vials
Plasma Extraction

**Ibuprofen**

- ACN
- 0.1% FA
- 75/25 (ACN/H2O)
- 0.1% FA
- 75/25 (ACN/H2O)
- 50/50 (ACN/H2O)
- 0.1% FA
- 50/50 (ACN/H2O)

**Glucagon**

- ACN
- 0.1% FA
- 75/25 (ACN/H2O)
- 0.1% FA
- 75/25 (ACN/H2O)
- 50/50 (ACN/H2O)
- 0.1% FA
- 50/50 (ACN/H2O)

**LC Pressure Plot**

ACN, 96 injections

75/25 ACN/H2O, 96 injections

400 µL plasma sample extracted
Plasma Extraction

2D Extraction: Protein Precipitation then SPE

548 injections

-1100 psi

LC Pressure Plot

Internal Standard Response

% Initial Response

0 50 100

Injection number

400 µL plasma sample extracted

Injection 021

Injection 494
Stability in plasma

Stabilisers
- Acid denaturartion
  Citric acid
- Enzyme inhibitors
  Aprotinin
  DPP-IV inhibitor
  P800 cocktail inhibitor
- Anticoagulant
  Lith Hep vs. EDTA
Established Glucagon Peptide Method

- Glass vials + BSA blocking buffer
- m-NBA and regular formic acid (FA) mobile phases gave equivalent sensitivity (SRM)
- 2D Extraction
- Performed on ice
- Surrogate matrix approach

15 pg/mL solution  Endogenous plasma  15 – 2000 pg/mL

<table>
<thead>
<tr>
<th>QC Level</th>
<th>Matrix</th>
<th>n</th>
<th>% Nominal</th>
<th>%CV</th>
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</thead>
<tbody>
<tr>
<td>15</td>
<td>Surrogate</td>
<td>6</td>
<td>110.3</td>
<td>11.9</td>
</tr>
<tr>
<td>25</td>
<td>Surrogate</td>
<td>6</td>
<td>114.0</td>
<td>10.0</td>
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<tr>
<td>45</td>
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<td>6</td>
<td>113.8</td>
<td>6.2</td>
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<tr>
<td>56.9</td>
<td>Plasma</td>
<td>6</td>
<td>92.6</td>
<td>7.0</td>
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<tr>
<td>106.9</td>
<td>Plasma</td>
<td>6</td>
<td>92.4</td>
<td>11.3</td>
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<tr>
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<td>Plasma</td>
<td>6</td>
<td>91.4</td>
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<td>1781.9</td>
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<td>98.0</td>
<td>10.7</td>
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<tr>
<td>10</td>
<td>Surrogate</td>
<td>6</td>
<td>87.7</td>
<td>25.3</td>
</tr>
<tr>
<td>7.5</td>
<td>Surrogate</td>
<td>5</td>
<td>68.6</td>
<td>40.4</td>
</tr>
</tbody>
</table>

Volunteers 1-9: Endogenous glucagon

Volunteer 10: Infused glucagon
Conclusion

- Small molecule and peptide method development is similar but not the same

- Need to consider
  - Binding: Plastic v.s glass, blocking buffers
  - MS Sensitivity: Multiple charge states, supercharging agents
  - Extraction: Diluted organic precipitation solvents, 2D extractions
  - Stability: Stabilisers, extraction temperature

- Can routinely develop precise, accurate, and robust peptide methods using LC-MS/MS. These can be used as an alternative to immunoassays
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Any Questions?

Science
for a safer world