Development of an Immunoprecipitation and LC-MS/MS based Method for Quantifying the 105 kDa Recombinant Protein SXN101959 in Plasma.

Richard Kay
Principal Scientist, Bioanalytical Sciences, Quotient Bioreserarch

EBF 2012

Answers Through Innovation
Movember
• Syntaxin and TSI technology (SXN101959)

• LC-MS/MS method development

• Comparison of ELISA and LC-MS/MS
UK based Life Sciences company

Develop biological drugs - Targeted Secretion Inhibitors (TSI)

TSI are based on botulinum toxins designed to inhibit secretion of disease mediators from target disease cells.

Synaptobrevin (V-SNARE)

Syntaxin TSI

SNAP-25 (N+C)

Syntaxin-1 (T-SNARE)

Botulinum light chain enzyme
SXN101959

- A TSI targeted to inhibit GH release from cells
- Designed to control Acromegaly
  - Normalise GH and IGF-1 in patients
Why LC-MS/MS?

• Syntaxin already had an ELISA developed
• Experienced relatively low LLOQ (5 ng/mL in plasma)

• Wanted to explore the use of LC-MS/MS
  – Can LC-MS be more sensitive than ELISA?

• Challenge: Develop LC-MS/MS method with sub ng/mL LLOQ in plasma
  – 1 ng/mL = 10 picomolar

• Stephanie Fischmann presented at EBF in 2011
  – Achieved an LLOQ of ~1 ng/mL for MAb = ~7 pM
Plasma protein dynamic range

- 30 miles (MAb 5 μg/mL)
- 0.3 miles (IGF-I ~ 100 ng/mL)
- 15 feet (Therapeutic protein ~ 1 ng/mL)
- 300,000 miles (Albumin 50 mg/mL)
Plasma protein dynamic range

Taken from plasma proteome institute website
SXN101959 LC-MS method development

- Tryptically digested protein
- Performed IDA based analysis to identify suitable peptides

Mascot matching

Peptide coverage
- Matched ~66% of entire protein sequence
- Selected 37 peptides from SXN101959
  - Mixture of light and heavy chain peptides
- Optimised LC-MS parameters on all 37 peptides.
  - Scheduled SRM definitely required!
Immunoprecipitation approach

- Uses specific antibodies attached to paramagnetic beads
- Syntaxin supplied us with both Monoclonal and polyclonal sera
  - Assessed both antibodies for IP method development.

- Challenges with developing IP methodology:

  - Binding of antibodies to paramagnetic beads
    - Is the antibody bound?

  - Are the beads functional?
    - Can the beads extract SXN101959 from buffer.

  - Can the beads extract SXN101959 from plasma?
    - Sensitivity, precision and accuracy,
    - Internal standardisation?
Antibody binding?

- Is the antibody bound to the bead after the coupling process?

Or...
Antibody binding?

- Tryptically digested both monoclonal and polyclonal Ab
- Identified a number of antibody derived tryptic peptides
- Generated SRM transitions to peptides from light and heavy chains:
  - Useful for monitoring Ab-bead conjugation
Can we extract SXN101959 from buffer?

• Prepared calibration line of SXN101959 in buffer
  – 0.5 to 1000 ng/mL

• Extracted using both MAb and PAb beads:

• Extraction methodology:
  – Overnight roll at 37 °C
  – Pellet beads and wash with buffer (repeat)
  – Reduce, alkylate and tryptically digest
  – Analyse by LC-MS/MS
Monoclonal beads (SXN101959 Light chain)

Calibration range 0.5 to 1000 ng/mL in buffer
Polyclonal beads (SXN101959 Light chain)

Calibration range 0.5 to 1000 ng/mL in buffer
Can we extract SXN101959 from plasma?

- Generated a calibration line in human and dog plasma
- Same range, 200 µL of plasma extracted
Rat plasma sample analysis

- Samples were available from a pre-clinical trial
  - Rats dosed with SXN101959
- SXN101959 concentration assigned by ELISA

- LC-MS/MS approach:
  - Analysed in a 96 well plate format
  - Also assessed precision and accuracy

- Issue - loss of beads during extractions
  - How to correct for bead loss?
  - No internal standard..
- Use the monoclonal antibody peptide to correct for bead losses..
Calibration line statistics

- Comparison of raw and adjusted data
- Data for heavy peptide only (n=2)

### Raw data

<table>
<thead>
<tr>
<th>STD</th>
<th>Conc. (ng/mL)</th>
<th>Mean (ng/mL)</th>
<th>%CV</th>
<th>Accuracy %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.00</td>
<td>5.26</td>
<td>9.9</td>
<td>105.3</td>
</tr>
<tr>
<td>2</td>
<td>10.0</td>
<td>9.07</td>
<td>9.7</td>
<td>90.7</td>
</tr>
<tr>
<td>3</td>
<td>50.0</td>
<td>47.9</td>
<td>13.8</td>
<td>95.8</td>
</tr>
<tr>
<td>4</td>
<td>100</td>
<td>93.1</td>
<td>0.2</td>
<td>93.1</td>
</tr>
<tr>
<td>5</td>
<td>500</td>
<td>528</td>
<td>2.2</td>
<td>105.6</td>
</tr>
<tr>
<td>6</td>
<td>1000</td>
<td>1090</td>
<td>6.4</td>
<td>109.5</td>
</tr>
</tbody>
</table>

### Adjusted data

<table>
<thead>
<tr>
<th>STD</th>
<th>Conc. (ng/mL)</th>
<th>Mean (ng/mL)</th>
<th>%CV</th>
<th>Accuracy %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.00</td>
<td>5.23</td>
<td>21.7</td>
<td>104.7</td>
</tr>
<tr>
<td>2</td>
<td>10.0</td>
<td>9.08</td>
<td>6.0</td>
<td>90.8</td>
</tr>
<tr>
<td>3</td>
<td>50.0</td>
<td>50.7</td>
<td>16.1</td>
<td>101.5</td>
</tr>
<tr>
<td>4</td>
<td>100</td>
<td>93.5</td>
<td>14.3</td>
<td>93.5</td>
</tr>
<tr>
<td>5</td>
<td>500</td>
<td>535</td>
<td>7.0</td>
<td>107.1</td>
</tr>
<tr>
<td>6</td>
<td>1000</td>
<td>1020</td>
<td>2.1</td>
<td>102.4</td>
</tr>
</tbody>
</table>
Quality control sample statistics

- Comparison of raw and adjusted data
- Data for heavy peptide only (n=6)

<table>
<thead>
<tr>
<th>QC</th>
<th>Conc. (ng/mL)</th>
<th>Mean (ng/mL)</th>
<th>%CV</th>
<th>Accuracy %</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOW</td>
<td>50.0</td>
<td>50.8</td>
<td>7.3</td>
<td>101.6</td>
</tr>
<tr>
<td>MED</td>
<td>250</td>
<td>260</td>
<td>8.2</td>
<td>104.1</td>
</tr>
<tr>
<td>HIGH</td>
<td>500</td>
<td>575</td>
<td>10.2</td>
<td>114.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>QC</th>
<th>Conc. (ng/mL)</th>
<th>Mean (ng/mL)</th>
<th>%CV</th>
<th>Accuracy %</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOW</td>
<td>50.0</td>
<td>47.5</td>
<td>6.6</td>
<td>94.9</td>
</tr>
<tr>
<td>MED</td>
<td>250</td>
<td>247</td>
<td>6.7</td>
<td>98.7</td>
</tr>
<tr>
<td>HIGH</td>
<td>500</td>
<td>557</td>
<td>12.1</td>
<td>111.3</td>
</tr>
</tbody>
</table>
LC-MS/MS vs ELISA (corrected for bead volume)

- Light chain peptide: $y = 0.8278x + 29.699$, $R^2 = 0.9357$
- Heavy chain peptide: $y = 0.8623x + 38.773$, $R^2 = 0.9113$
Inter peptide comparison

- SXN101959 concentration (heavy and light chain peptides)

\[ y = 0.9448x - 3.8637 \]

\[ R^2 = 0.9946 \]
Conclusions (and future work?)

• IP in combination with high flow rate LC-MS/MS can be sensitive
  – 0.5 ng/mL in plasma (10x more sensitive than ELISA)

• Robust approach

• Internal standardisation in absence of SIL protein
  – Monitor Ab specific peptide – to adjust for loss of bead
  – Add SIL peptide as soon as possible?

• Are LC-MS/MS methods subject to same issues with ADA’s?
  – Do ADA’s interfere with the capture of the analyte?
Acknowledgements

Syntaxin

• Alberto Martinez
• Andrew Splevins
• Aimee Cossins
• Helen Ludlow
Any questions?

Answers Through Innovation