LC-MS/MS for the quantification of Peptide biomarker and mixture of closely related Protein in formulation

Luc-Alain SAVOY
Part I: SGS overview

Part II: Peptides in biological matrices

Peptides analysis by mass spectrometry
  - Biomarker peptides (Angiotensins)
  - Therapeutic peptides

Part III: Protein in formulations

Chorionic gonadotropin (CG) proteins
WHAT WE DO

DELiver
- Analytical Development
- Biologics
  - Characterization
  - Potency, Efficacy, Biosafety
- Quality Control
- Clinical Research for Phase I to IV Trials

ENSURE
- Quality, Safety and Effectiveness of Bio/pharmaceutical
- « High value for money » services
- Reduction time-to-market
LIFE SCIENCE SERVICES OVERVIEW

- Over 35 years experience - 1,500 full time employees with 28 facilities in 15 countries

- Global drug development partner from Molecule to Market with unique international analytical laboratory network
  - across America, Europe, Asia with Centers of Excellence matching Biopharmaceutical and Small molecules needs

- Expert biopharma analytical services
  - Research, QC, regulatory
    - Scientific consultancy
    - Biosimilars
    - Comparability
    - Bioanalysis & bioassays
    - Proteomics, glycomics
    - Extracables & leachables
    - Virus detection and identification
    - Molecular biology assays – q-PCR
  - Product analysis
    - Glycoproteins, proteins & peptides
    - Antibodies & vaccines
    - Gene & cell therapies
    - Oligonucleotides & polysaccharides
    - Small molecules & antibiotics

- Strong commitment to clinical and laboratory Quality and Operational Excellence in many areas
  - Harmonized QMS and Validation & Transfer methods, LIMS, Lean
ADVANTAGES OF LC-MS/MS

- Better selectivity between structurally or chemically similar peptides
- Better precision and accuracy
- Antibodies not required
- Low sensitivity (pg/mL)
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Stable isotope labeled peptides are used as Internal Standards (IS) to correct variabilities during the entire bioanalytical process (extraction, dilution, adsorption, evaporation, degradation...).

### Amino Acid Sequences of Angiotensin Peptides

<table>
<thead>
<tr>
<th></th>
<th>Sequences</th>
<th>MW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angiotensin I</td>
<td>Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu</td>
<td>1296.5</td>
</tr>
<tr>
<td>Angiotensin II</td>
<td>Asp-Arg-Val-Tyr-Ile-His-Pro-Phe</td>
<td>1046.2</td>
</tr>
<tr>
<td>Angiotensin III</td>
<td>Arg-Val-Tyr-Ile-His-Pro-Phe</td>
<td>931.1</td>
</tr>
<tr>
<td>Angiotensin IV</td>
<td>Val-Tyr-Ile-His-Pro-Phe</td>
<td>774.9</td>
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</table>

<table>
<thead>
<tr>
<th>Internal Standard</th>
<th>Sequences</th>
<th>MW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angiotensin I</td>
<td>Asp-Arg-Val-Tyr-Ile*-His-Pro-Phe-His-Leu</td>
<td>1303.5</td>
</tr>
<tr>
<td>Angiotensin II</td>
<td>Asp-Arg-Val-Tyr-Ile*-His-Pro-Phe</td>
<td>1053.2</td>
</tr>
<tr>
<td>Angiotensin III</td>
<td>Arg-Val-Tyr-Ile*-His-Pro-Phe</td>
<td>938.1</td>
</tr>
<tr>
<td>Angiotensin IV</td>
<td>Val-Tyr-Ile*-His-Pro-Phe</td>
<td>781.9</td>
</tr>
</tbody>
</table>

[Ile* = I(13C6,15N)]
POSITIVE ESI MASS SPECTRUM OF ANGIOTENSIN II (Precusor ion)

Sol 2 μg/mL débit 5 μL/min avec 0.4 mL/min MeOH/H2O (40/60) + 0.5 % HCOOH
ANGIOTENSIN II MS:001 1 (0.367) 523.39

[M+2H]^{2+}

MH^+
POSITIVE ESI MASS SPECTRUM ANGIOTENSIN II (PRODUCT IONS)

MRM transition 524 → 263

[M+2H]^{2+}
MASS SPECTROMETER SETTINGS FOR ANGIOTENSIN MRM TRANSITIONS

<table>
<thead>
<tr>
<th>Angiotensin</th>
<th>Pecursor ion (m/z)</th>
<th>Product ion (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angiotensin I</td>
<td>433.2 (3+)</td>
<td>110,1</td>
</tr>
<tr>
<td>Angiotensin I (IS)</td>
<td>435.4 (3+)</td>
<td>110,1</td>
</tr>
<tr>
<td>Angiotensin II</td>
<td>523.8 (2+)</td>
<td>263,1</td>
</tr>
<tr>
<td>Angiotensin II (IS)</td>
<td>527.4 (2+)</td>
<td>263,1</td>
</tr>
<tr>
<td>Angiotensin III</td>
<td>466.4 (2+)</td>
<td>263,1</td>
</tr>
<tr>
<td>Angiotensin III (IS)</td>
<td>469.9 (2+)</td>
<td>263,1</td>
</tr>
<tr>
<td>Angiotensin IV</td>
<td>388.4 (2+)</td>
<td>235,1</td>
</tr>
<tr>
<td>Angiotensin IV (IS)</td>
<td>391.8 (2+)</td>
<td>235,1</td>
</tr>
</tbody>
</table>

The selection of specific MRM transitions results in a highly sensitive and selective detection of the peptides.

MRM: Multiple Reaction Monitoring
UPLC-MS/MS CONDITIONS

- Column: Acquity (Waters)
  - Length: 100 mm
  - Internal diameter: 2.1 mm
  - Particle size: 1.7 µm
- Flow rate: 0.5 mL/min
- Mobile phase
  - A: H₂O (+ formic acid)
  - B: MeOH (+ formic acid)
- Gradient table:

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>%A</th>
<th>%B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>2,9</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>3,4</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>3,5</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>
LC-MS/MS CHROMATOGRAM FOR ANGIOTENSINS IN SOLUTION

Sol 250 pg/mL angiotensin I, II, III, IV et 1-7
Essai 310111-019

Angiotensin II
Angiotensin I
Angiotensin III
Angiotensin IV
Angiotensin 1-7

MRM of 5 Channels ES+
TIC
1.57e6
LC-MS/MS CHROMATOGRAM FOR ANGIOTENSINS IN HUMAN PLASMA

Plasma humain

Essai 310111-027 Sm (Mn, 2x3)

MRM of 5 Channels ES+
433.15 > 110.1 (Angiotensin I)
2.95e5
Area

Angiotensin I

Essai 310111-027 Sm (Mn, 2x3)

MRM of 5 Channels ES+
524 > 263.2 (Angiotensin II)
1.41e5
Area

Angiotensin II

Essai 310111-027

MRM of 5 Channels ES+
466.37 > 263.15 (Angiotensin III)
2.21e5

Angiotensin III

Essai 310111-027

MRM of 5 Channels ES+
388.43 > 235.15 (Angiotensin IV)
1.45e4

Angiotensin IV
**Calibration Curves for Angiotensins I & II**

**Angiotensin I**
2 to 2000 pg/mL

**Angiotensin II**
2 to 2000 pg/mL

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**Compound name: Angiotensin II**
Correlation coefficient: $r = 0.997474$, $r^2 = 0.994955$
Calibration curve: $0.0130392 \times x + 0.00329752$
Response type: Internal Std (Ref 3), Area * (IS Conc. / IS Area)
Curve type: Linear, Origin: Exclude, Weighting: 1/x^2, Axis trans: None

**Compound name: Angiotensin I**
Correlation coefficient: $r = 0.998615$, $r^2 = 0.997232$
Calibration curve: $0.0127264 \times x + 0.017328$
Response type: Internal Std (Ref 1), Area * (IS Conc. / IS Area)
Curve type: Linear, Origin: Exclude, Weighting: 1/x^2, Axis trans: None
**INTRA-RUN ASSAY PERFORMANCE AT THE LLOQ (2 pg/mL)**

<table>
<thead>
<tr>
<th>Replicate analysis of QC samples spiked at the LLOQ</th>
<th>Angiotensin I 2pg/mL</th>
<th>Angiotensin II 2pg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.03</td>
<td>2.27</td>
</tr>
<tr>
<td></td>
<td>1.79</td>
<td>1.95</td>
</tr>
<tr>
<td></td>
<td>2.55</td>
<td>1.57</td>
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<tr>
<td></td>
<td>1.61</td>
<td>2.10</td>
</tr>
<tr>
<td></td>
<td>2.32</td>
<td>2.10</td>
</tr>
<tr>
<td></td>
<td>1.81</td>
<td>2.19</td>
</tr>
<tr>
<td>Mean Concentration (pg/mL)</td>
<td>2.02</td>
<td>2.03</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>0.36</td>
<td>0.25</td>
</tr>
<tr>
<td>Precision (%CV)</td>
<td>17.7</td>
<td>12.3</td>
</tr>
<tr>
<td>Accuracy (Bias %)</td>
<td>0.9</td>
<td>1.5</td>
</tr>
<tr>
<td>n</td>
<td>6</td>
<td>6</td>
</tr>
</tbody>
</table>

Precision and accuracy acceptance criteria are within ±20 % at LLOQ
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**TRIPTORELIN**

- Triptorelin is a decapeptide (MW 1311.5 Da).
- Triptorelin is an agonist of gonadotropin releasing hormone.
- Treatment of prostate or breast cancer.
- New clinical phase III study with LLOQ 10pg/mL.
- Sequence:
  
  PyroGlu-His-Trp-Ser-Tyr-(D)Trp-Leu-Arg-Pro-Gly-NH₂
A stable isotope labelled internal standard was used

<table>
<thead>
<tr>
<th></th>
<th>Sequence</th>
<th>MW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triptorelin</td>
<td>PGlu-His-Trp-Ser-Tyr-D-Trp-Leu-Arg-Pro-Gly-NH₂</td>
<td>1311.5</td>
</tr>
<tr>
<td>Triptorelin (U-^{13}C⁵,^{15}N)</td>
<td>PGlu-His-Trp-Ser-Tyr-D-Trp-Leu-Arg-*Pro-Gly-NH₂</td>
<td>1317.5</td>
</tr>
</tbody>
</table>

[*Pro= I(^{13}C⁵,^{15}N)]

Assay volume: 100 µL of human serum

Runtime: 4 minutes.
LC-MS/MS CHROMATOGRAM OF A BLANK HUMAN SERUM AND OF A 50.0 pg/mL QC

50.0 pg/mL (100 µL of serum)  
IS labeled Pro ($^{13}$C$_5$, $^{15}$N)
<table>
<thead>
<tr>
<th></th>
<th>Precision (%CV)</th>
<th>50.0 pg/mL</th>
<th>150 pg/mL</th>
<th>1500 pg/mL</th>
<th>3500 pg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intra-run 1</td>
<td>Precision (%CV)</td>
<td>5.53</td>
<td>2.98</td>
<td>1.88</td>
<td>0.53</td>
</tr>
<tr>
<td></td>
<td>Accuracy (Bias %)</td>
<td>2.80</td>
<td>-4.67</td>
<td>2.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Intra-run 2</td>
<td>Precision (%CV)</td>
<td>4.29</td>
<td>2.31</td>
<td>0.54</td>
<td>1.88</td>
</tr>
<tr>
<td></td>
<td>Accuracy (Bias %)</td>
<td>-4.00</td>
<td>-7.33</td>
<td>-6.67</td>
<td>-8.86</td>
</tr>
<tr>
<td>Intra-run 3</td>
<td>Precision (%CV)</td>
<td>2.13</td>
<td>1.60</td>
<td>0.66</td>
<td>0.71</td>
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<tr>
<td></td>
<td>Accuracy (Bias %)</td>
<td>5.40</td>
<td>0.67</td>
<td>0.00</td>
<td>-2.29</td>
</tr>
<tr>
<td>Inter-run</td>
<td>Precision (%CV)</td>
<td>5.62</td>
<td>4.15</td>
<td>3.89</td>
<td>4.18</td>
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<tr>
<td></td>
<td>Accuracy (Bias %)</td>
<td>1.40</td>
<td>-3.33</td>
<td>-1.33</td>
<td>3.71</td>
</tr>
</tbody>
</table>

Precision and accuracy acceptance criteria are within ±15 %
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DOSAGE OF PROTEINS IN FORMULATIONS – CASE STUDY

- Sample: A mixture of closely related proteins (human CG, equine CG)
- Aim: Each variant needs to be quantified individually
- Preparation of experimental procedures: In silico search for specific signature peptide(s) produced by defined proteolysis (enzyme/chemical)
DOSAGE OF PROTEINS IN FORMULATIONS – CASE STUDY

- Sample process: Test of the selected proteolytic procedure on the complex sample
- Synthesis of cold labeled signature peptides
- Establishment of calibration curves
DOSAGE OF PROTEINS IN FORMULATIONS – CASE STUDY

- Signature peptides
  - Horse CG: ......RFASIRLPGPC......
  - Human CG: ......RFESIRLPGPC......

- Signature peptides and IS
  - Peptide 1: Mw 592.3, Peptide 1 IS: Mw 598.3 (Phe$^{13}$C$_6$)
  - Peptide 2: Mw 650.3, Peptide 2 IS: Mw 656.3 (Phe$^{13}$C$_6$)

- Transitions:
  - Peptide 1: 592.3 > 278.2
  - Peptide 1 IS: 598.3 > 284.2
DOSAGE OF PROTEINS IN FORMULATIONS – CASE STUDY

- UPLC-MS analysis of processed standard sample spiked with IS

  Peptide 1 IS

  Peptide 1

  Peptide 2 IS

  Peptide 2
DOSAGE OF PROTEINS IN FORMULATIONS – CASE STUDY

- Representative results

<table>
<thead>
<tr>
<th>BATCH No.:</th>
<th>Ratio eCG : hCG</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.0 : 1</td>
</tr>
<tr>
<td>2</td>
<td>8.3 : 1</td>
</tr>
<tr>
<td>3</td>
<td>8.2 : 1</td>
</tr>
<tr>
<td>4</td>
<td>5.3 : 1</td>
</tr>
<tr>
<td>5</td>
<td>7.9 : 1</td>
</tr>
</tbody>
</table>
CONCLUSION

- Detailed characterisation of your protein is essential as you will have to make sure that your quantitation assay is targeting:
  - A common feature of all variants if you want total amount (e.g. MAB PK studies).
  - A unique feature of each variant if you want relative amount.
THANK YOU FOR YOUR ATTENTION
QUESTIONS ?