HPLC/MS/MS-based SRM for biomarkers of rhGH administration

Phil Teale

S. Fenwick and R. Kay
Background

• Initial biomarker discovery project
  – LC-MS of proteolytic peptides
  – Simple extraction / enrichment (crash)
  – LRG as upregulated following GH admin
  – Extended to multiplexed semi-quant analysis (50 proteins)

• Multiplexed quant analysis of GH markers possible?
GH Biomarker targets

- IGF’s (IGF-I, IGF-II, etc)
- IGF binding proteins
- LRG (leucine-rich α-2-glycoprotein)
- PIIINP
MS approaches

• Selective
• Provide confirmatory quality data
• Ability to multiplex?
  – Minimal sample usage
• Eliminate biological reagents?
• Intact protein or proteotypic peptide?
Analytical requirements

• Efficient (and reproducible) extraction / isolation
• Reproducible digestion
• Effective internal standard (heavy label)

• Issues
  – Binding (both selective and non-selective)
  – Blank matrix (true blank or standard addition)
  – Reference materials
  – Incurred matrices (QC’s)
Generic approach

- Pretreatment / extraction
- Reduction, alkylation and digestion
- Quench / dilute and inject
- Integrate and quantitate

Heavy label internal standard
Extraction methods and the effect of binding

IGF-I T1 effect of IGFBP-III spike (1 % ammonia)
Extraction methods and the effect of binding

\[ y = -1.6014x + 112118 \]

\[ R^2 = 0.1635 \]
Bond Elut Plexa polymeric restricted access cartridge
Acidic loading for IGFs (2 % formic acid)
    Breaks IGF=IGFBP interaction
    Optimal for LRG
Basic loading for IGFBPs (0.5 % ammonia)

Wash 1mL 5% acetonitrile

Elute 2 x 250mL 0.1% formic acid, 75% acetonitrile
Incubation of sample (50µL) at appropriate pH

- RT, 10 min

SPE extraction on Bond Elut Plexa 96 well plate

Evaporation to dryness overnight

Addition of 50 µL dithiothreitol (10 mM) in ammonium bicarbonate

- 60 °C, 1 h

Addition of 10 µL iodoacetamide (100 mM) in ammonium bicarbonate

- RT, 30 min

Addition of 7.5 µL trypsin (100 µg/mL) in 50 mM acetic acid

- 37 °C, overnight

Addition of 15 µL formic acid (1 % v/v) in methanol

Injection (20 µL) onto LC-MS/MS system
Why uHPLC

• High separation power (appropriate gradient)
• Fast, well suited to quantification
• Robust
• Widely employed / available
• Sensitive?
Chromatographic conditions

- 100 x 2.1 mm 1.7 μm BEH C18 or
- Ascentis Express™ C$_{18}$ 100 x 2.1 mm, 2.7 μm fused core column

- Acetonitrile / formic acid gradient – column 50-60 °C, 700μl/min
- Sciex 5000 / 5500 Turbo V™ source
LLOQ in plasma

IGF-I T1

IGF-I T4
LLOQ in plasma

IGF-II T6

IGFBP-3 T3
IGF-II (T6) in buffer and plasma

IGF-II T6 (585.2/900.4)

\[ y = 9.29 \times 10^{-5} x + 0.0048 \]

\[ r^2 = 0.9979 \]

IGF-II T6 (585.2/900.4)

\[ y = 3.98 \times 10^{-5} x + 0.0015 \]

\[ r^2 = 0.9918 \]
IGFBP-II (T30) IGFBP-III (T3) in plasma

IGFBP-II T30 (484.9/614.4)

\[ y = 4.03 \times 10^{-6} x + 2.93 \times 10^{-5} \]

\[ r^2 = 0.9970 \]

IGFBP-III T3 (912.0/1208.6)

\[ y = 6.07 \times 10^{-6} x + 0.00105 \]

\[ r^2 = 0.9894 \]
IGF-I (T1) in plasma

\[ y = 8.69 \times 10^{-5} x - 0.000235 \]

\[ r^2 = 0.9944 \]

Incurred level corrected
Clinical serum samples

- Addenbrookes patient samples
- Monitored T1 and T4 peptide surrogates of IGF-I
- Good correlation between methodologies

<table>
<thead>
<tr>
<th>Reference matrix</th>
<th>Immulite™ (ng/mL)</th>
<th>LC-MS/MS (ng/mL)</th>
<th>RE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>119</td>
<td>117</td>
<td>1.7</td>
</tr>
<tr>
<td>Serum 1</td>
<td>171</td>
<td>172</td>
<td>0.6</td>
</tr>
<tr>
<td>Serum 2</td>
<td>346</td>
<td>336</td>
<td>2.9</td>
</tr>
</tbody>
</table>
Clinical serum samples

IGF-I concentrations in clinical samples

\[ y = 1.0787x - 4.1262 \]

\[ R^2 = 0.8516 \]

- **Normals**
- **Discordants**

IGF-I (ng/mL) Immulite™ vs. IGF-I (ng/mL) LC-MS/MS
PIIIINP

Possible target - endoproteinase Glu-C  G3 peptide  GGCSHLGQSYAD

No suitable tryptic fragment

42 kDa

Low levels in plasma (~ 3 ng/mL)
Summary

• LC-MS quantification of IGF’s and IGFBP’s √
• Generic extraction and uHPLC √
• Multiplex analysis?
• Confirmatory quality data √
• Powerful tool to validate immunoassays √
• Transferable?
• Worthy of further investigation
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