Biotherapeutic Antibody Subunit LC-MS for Quantitation & Biotransformation Monitoring from In-Life Studies

Delaware Valley Drug Metabolism Discussion Group
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John F. Kellie
Associate Fellow & Investigator
GlaxoSmithKline
MS of Intact Proteins in a Bioanalysis Setting

- Small molecules (typically) have few charge states, with few naturally occurring isotopes
- Intact proteins have many charge states, with many isotopes under each charge state
- Protein digestion “levels the playing field” – while the mixture is more complex, molecules are smaller & as a result...
  - They can be better purified based on size or other properties
  - It’s easier to perform LC separation (and MS analysis)
  - For MS: Few charge states and few isotopes

- Best Practice for Mass Spectrometry:
  - **Small molecules** - quantify the whole molecule & metabolites
  - **Large molecules** - quantify a small, surrogate peptide to infer whole molecule concentration

Information about the whole molecule is lost in digestion

**CQA** - Chemical or other attribute that can be clearly defined and monitored to help ensure product quality

**Biotransformation** – Modification of a chemical or biological compound (Drug) occurring *in vivo* (during in-life study)
Immunocapture method from plasma for ex-vivo, pre-clinical, clinical samples

Carried out in ELISA plate
- Coat with capture Ab
- Use 1-10 µL plasma diluted to 100 µL
- Detergent-free washing steps
- Ides Digestion, DTT reduction (neutral pH, in H₂O (no salts))
- Waters iKey (C4) coupled to Synapt G2-Si MS system
- Chromatographically separate mAb subunits
- Long run time (20 min)

Kellie et al. Bioanalysis 8 (20), 2103-2114
Whole-mAb LC-MS Assay “Menu”

1. Coat with capture antibody
   - Block
   - Wash

2. Bind biotherapeutic
   - Acid Elute
   - Intact LC-MS Analysis
   - Wash
   - Intact Mass

3. Disulfide reduction
   - Acidify

Variants:
- IdeS digestion
- 25 kDa Subunits (IdeS Digestion)
- Reduced Subunits

- LC-MS analysis
- Fc/2 (x2)
- Fd (x2)
- Lc (x2)
- Hc (x2)
- Lc (x2)
Introduction

- Ligand binding assay (LBA) or peptide-based LC-MS assays are traditional methods for monitoring drug concentration from pre-clinical or clinical samples.
- Intact or “top-down” LC-MS methods are less proven for quantitative bioanalysis (and less sensitive than LBA)
- Assays offer unique advantages: detection of the whole molecule, reduced dependence on target binding, drug metabolism monitoring.
- Here we present data for a bi-specific antibody with a focus on subunit (heavy and light chain) masses and comparison to intact protein LC-MS & functional LBA.
- Specific Biotransformation & Quality Attributes were also monitored: Oxidation, Amino Acid Clipping, and Subunit Clipping.
Method Summary

● For intact and reduced subunit mass monitoring, an **anti-human IgG capture** antibody is coated on each well of a **96-well immunoassay plate**.

● Monkey serum samples containing human IgG drug are incubated on plate, and following detergent-free washing, samples are either subjected to **acid elution for intact mass LC-MS** analysis or **reduced with DTT** and then acidified for **subunit mass LC-MS** analysis.

● Samples are analyzed using a **C4 iKey microflow column** coupled to a Waters’ Synapt G2-Si system.

● For functional LBA concentration monitoring, target is coated onto an MSD plate, followed by human IgG detection
2 Assays, 3 Analytes: Intact and Reduced LC-MS Assays

- Coat with capture antibody
- Bind Drug (1000 – 10,000 ng/mL in Rhesus Serum)
- Block
- Acid Elute
- Wash

Disulfide reduction

- Acidify

Reduced Subunits

Heavy (black) & Light Chain (red) MS

Intact LC-MS Analysis

Intact Mass

MS

LC-MS Chromatograms

Light Chain

Heavy Chain

m/z 1150 1200 1250 1300 1350 1400 1450

% 0 100
**Assay I: Reduced Subunits**

- Can readily quantify Light & Heavy Chains

### Precision and Accuracy Results (n=4)

<table>
<thead>
<tr>
<th></th>
<th>Light Chain</th>
<th></th>
<th></th>
<th>Heavy Chain</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>QC Conc. (ng/mL)</td>
<td>Average</td>
<td>RSD %</td>
<td>Bias %</td>
<td>Average</td>
<td>RSD %</td>
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<tr>
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<td>10776.03</td>
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<td>15.28</td>
<td>5624.85</td>
<td>10.15</td>
<td>12.50</td>
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<td>1500</td>
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<td>1769.28</td>
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<td>500</td>
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<td>19.27</td>
<td>-5.89</td>
<td>NQ</td>
<td>NQ</td>
<td>NQ</td>
</tr>
</tbody>
</table>

Light Chain Range: 10,000 – 500 ng/mL  
Heavy Chain Range: 10,000 – 1000 ng/mL

![Graph](image1.png)  

$R^2=0.985$  

![Graph](image2.png)  

$R^2=0.971$
Assay II: Intact Mass

- Can quantitate intact mass down to 500 ng/mL
- Can differentiate large mass differences (e.g. 5-10 a.a. or loss of subunit)
- Most useful for quantification of intact mass only

**Intact Mass**

**LC Peak**

**Intact Mass – Std. curve range:**

10,000 – 500 ng/mL

R²=0.975

174,632 Da  
HLOQ – 10,000 ng/ml

LLOQ – 500 ng/ml
Concepts, Background & Goals

- Intact mass or subunit mass measurements can provide additional information about the **bispecific antibody** complementary to ligand binding assays.

- Utilize anti-human IgG for capture, thus measurement is not dependent on molecule function (unlike LBA).

- Provide subunit and intact mass measurements from serum samples of PK/PD rhesus macaque study dosing.

- Specifically:
  - Compare subunit and intact mass PK measurements to previous LBA results.
  - Evaluate if the C-terminal a.a. residue and linker remains present in serum or is there any evidence of clipping.
  - Look for methionine oxidation risk.
  - Determine whether other modifications to the Heavy Chain or Light Chain are observed over time in serum.
Quick Tutorial on Raw MS Data

- Data is collected at $m/z$, peaks have charge state ($z$) which corresponds to mass ($M$)
- Distribution of numbered charge states correspond to a particular mass and form a unique, identifying pattern
- Observe discordance in the pattern and identify missing or unknown peaks / masses
- Look in smaller regions of the spectrum free of interfering peaks for protein mass speaks of interest
- Quantify based on LC peak area corresponding to a few protein charge states
- Raw $m/z$ data is most useful for assessment of specific attributes / biotransformation

Heavy Chain Mass Spectrum

![Retention time (min)]

64,704 Da (Avg. Mass)
Oxidation on Heavy Chain

- M570 is located on dAb CDR and therefore may impact target engagement

- Can monitor charge state peak width for standard/QC samples, 5 min samples, and later stage post-dose

- The peak does not broaden or indicate shift toward oxidation

- Total of 9 Met Ox sites on heavy chain

- The apparent lack of increase in oxidation with 9 potential sites suggests the risk is minimal

+1 Ox on Heavy Chain not observed >50% relative to intact heavy chain
Oxidation on Light Chain

- Single Met site on light chain
- Light chain has smaller mass – the +16 Da mass shift is better resolved
- Increases confidence for detection
- Light chain oxidation does not appear to increase over time post-dose

Reference Standard 10,000 ng/mL

+1 Ox peak center

+1 Ox peak on light chain not observed >20 % relative to intact light chain

30 mg/kg Day 22
5 min

30 mg/kg Day 22
672 hr
Monitoring for HC C-terminal A.A. Clipping

- Delta (minus) A.A. material provided
- A.A. added on C-term for ADA fix; clipping would increase ADA risk

- Delta heavy chain material observed by immunocapture LC-MS method in similar ratio as spiked into serum

- Loss of Ala not detected in any samples

- A few examples from 30 mg/kg are shown

No Ala loss observed >20% relative to intact heavy chain
Monitoring for Cleavage of Linker (Heavy Chain)

- mAb material provided
- Spiked into serum and detected by immunocapture LC-MS

- Clearly observed linker cleavage in some (but not all) 72 hr & later post-dose
- Not observed in 1 or 10 mg/kg, nor in early dose 30 mg/kg
- A few examples from 30 mg/kg are shown on next slide
Monitoring for Cleavage of Linker (Heavy Chain)

- Observe constant increase in clipped product compared to original
- Eventually the most abundant form of heavy chain is clipped form
- Captured Biotransformation event!

Cleavage of amino acid linker readily observed when >20% raw instrument response relative to intact heavy chain.
Monitoring for Cleavage of Linker (Intact Mass)

- While mAb vs. Bi-spec mAb can be readily distinguished, monitoring cleavage by intact mass is less straightforward.
- Cleavage events on intact Ab mean a mass loss of 14 kDa (one Ab Hc) OR 28 kDa (both Ab Hc).
- The Intact mass assay is most useful for confirming the presence or absence of intact mass (+/- 30 Da).

Most prevalent clipped form
Most abundant Ab form
Clipped species calculations: Single Heavy Chain

Clipped species (mAb) has 1.6 fold higher instrument response than unclipped species (mAbdAb) for single and mixed species below 7000 ng/mL in assay:

“Clipped (corrected response)”

Final single heavy chain calculations are determined by

Unclipped (raw response) + Clipped (corrected response) = Total response

Clipped (corrected response) / Total response = % clipped (single heavy chain)

Intact LC-MS methods (150-170 kDa) are not optimized to adequately quantify clipped species

Possible to analyzed for clipped species in more controlled manner (e.g. mAb and mAbdAb reference standards utilized more thoroughly)
Mathematical Calculation for Ordered Pairs – Broad implications for comparisons of any reduced/peptide LC-MS method as it relates to LBAs

\[ x = \text{clipped fraction of all heavy chains} \]

\[ x^2 = \text{doubly clipped mAbdAb} \]

\[ (1 - x)^2 = \text{zero clipped mAbdAb} \]

\[ 1 - [x^2 + (1 - x)^2] = \text{singly clipped mAbdAb} \]
# Heavy Chain / Light Chain Assay Performance

## Standards

### Heavy Chain

<table>
<thead>
<tr>
<th></th>
<th>STD 1000 1000 ng/mL</th>
<th>STD 2000 2000 ng/mL</th>
<th>STD 4000 4000 ng/mL</th>
<th>STD 5000 5000 ng/mL</th>
<th>STD 8000 8000 ng/mL</th>
<th>STD 9000 9000 ng/mL</th>
<th>STD 10000 10000 ng/mL</th>
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</thead>
<tbody>
<tr>
<td>Mean</td>
<td>1012</td>
<td>1598</td>
<td>4013</td>
<td>4856</td>
<td>8046</td>
<td>9047</td>
<td>10237</td>
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<tr>
<td>Std Dev</td>
<td>129</td>
<td>282</td>
<td>449</td>
<td>458</td>
<td>1004</td>
<td>645</td>
<td>1152</td>
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<tr>
<td>Precision (%CV)</td>
<td>12.8</td>
<td>14.4</td>
<td>11.2</td>
<td>9.0</td>
<td>12.5</td>
<td>7.1</td>
<td>11.1</td>
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<tr>
<td>Accuracy (%)</td>
<td>101.2</td>
<td>97.9</td>
<td>100.3</td>
<td>97.1</td>
<td>100.6</td>
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### Light Chain

<table>
<thead>
<tr>
<th></th>
<th>STD 1000 1000 ng/mL</th>
<th>STD 2000 2000 ng/mL</th>
<th>STD 4000 4000 ng/mL</th>
<th>STD 5000 5000 ng/mL</th>
<th>STD 8000 8000 ng/mL</th>
<th>STD 9000 9000 ng/mL</th>
<th>STD 10000 10000 ng/mL</th>
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<tbody>
<tr>
<td>Mean</td>
<td>1001</td>
<td>1993</td>
<td>4032</td>
<td>4958</td>
<td>8129</td>
<td>8840</td>
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<tr>
<td>Std Dev</td>
<td>85</td>
<td>247</td>
<td>453</td>
<td>252</td>
<td>889</td>
<td>868</td>
<td>915</td>
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<tr>
<td>Precision (%CV)</td>
<td>8.5</td>
<td>12.4</td>
<td>11.3</td>
<td>5.7</td>
<td>10.9</td>
<td>9.8</td>
<td>9.5</td>
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<tr>
<td>Accuracy (%)</td>
<td>101.1</td>
<td>95.7</td>
<td>100.8</td>
<td>99.2</td>
<td>101.6</td>
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</table>

## Quality Control Samples

### QC 3000

<table>
<thead>
<tr>
<th>QC 3000 3000 ng/mL</th>
<th>QC 5000 5000 ng/mL</th>
<th>QC 8000 8000 ng/mL</th>
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</thead>
<tbody>
<tr>
<td>Mean</td>
<td>2542</td>
<td>4408</td>
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<tr>
<td>Std Dev</td>
<td>516</td>
<td>510</td>
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<tr>
<td>Precision (%CV)</td>
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<td>12.5</td>
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<tr>
<td>Bias (%)</td>
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<td>11.8</td>
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<tr>
<td>n</td>
<td>16</td>
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### QC 5000

<table>
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<th>QC 8000 8000 ng/mL</th>
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<tbody>
<tr>
<td>Mean</td>
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<tr>
<td>Std Dev</td>
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<tr>
<td>Precision (%CV)</td>
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<tr>
<td>Bias (%)</td>
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<tr>
<td>n</td>
<td>16</td>
<td>16</td>
</tr>
</tbody>
</table>

- Run Passed Criteria: 75% of all Standards and 66% of all QCs (50% at a given level) within ±25% (30% at LLOQ)
- Light Chain is the best performing analyte
Intact and Reduced Assay: Sample Results (1 mg/kg)

**Mean concentrations (3 subjects)**

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Intact Mass</th>
<th>Heavy Chain</th>
<th>Light Chain</th>
<th>Ligand Binding Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
<td>100</td>
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<tr>
<td>24</td>
<td>90.3</td>
<td>90.3</td>
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<tr>
<td>48</td>
<td>81.8</td>
<td>81.8</td>
<td>81.8</td>
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<tr>
<td>72</td>
<td>74.3</td>
<td>74.3</td>
<td>74.3</td>
<td>74.3</td>
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<tr>
<td>96</td>
<td>67.8</td>
<td>67.8</td>
<td>67.8</td>
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</tbody>
</table>

**% Difference**

<table>
<thead>
<tr>
<th></th>
<th>Lc vs. Hc</th>
<th>Lc vs. Intact</th>
<th>Lc vs. LBA</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.1</td>
<td>-26.3</td>
<td>-3.4</td>
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<tr>
<td>13.7</td>
<td>9.6</td>
<td>-35.6</td>
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<td>16.9</td>
<td>-6.8</td>
<td>-43.6</td>
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<tr>
<td>14.0</td>
<td>7.4</td>
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<tr>
<td>10.3</td>
<td>24.2</td>
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<tr>
<td>12.5</td>
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<td>-5.0</td>
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<tr>
<td>23.8</td>
<td>35.7</td>
<td>-3.5</td>
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</table>
Conclusions

- **Intact and subunit mass LC-MS assays** can provide **complimentary** data to **LBA** for in-life study sample analysis, particularly if immunocapture is not dependent on antigen binding function.

- Good agreement between LBA and LC-MS data.

- Ability to monitor for **in vivo oxidation** at subunit level.

- Provided qualitative evidence **single amino acid amino acid clipping** was not present in C-terminus of the heavy chain subunit.

- Observed **partial subunit clipping** of the heavy chain.
Acknowledgements & Statements

- Thanks to Kristen Pannullo for sample analysis
- Additional acknowledgements: Matt Szapacs, Chris Evans, Eric Yang, Molly Karlinsey, Scott Summerfield, Shari Gordon, Claire Ashman, Kristy Fraley, and Andrew Mayer.

- All studies were conducted in accordance with the GSK Policy on the Care, Welfare and Treatment of Laboratory Animals and were reviewed by the Institutional Animal Care and Use Committee either at GSK or by the ethical review process at the institution where the work was performed.
- All animal studies were ethically reviewed and carried out in accordance with Animals (Scientific Procedures) Act 1986 and the GSK Policy on the Care, Welfare and Treatment of Animals.