Bioanalysis of Intact Biotherapeutics by Hybrid LBA/LCMS: Challenges & Solutions

Regulated Bioanalysis Interest Group (RBIG)
Tuesday June 5th 2018

Presiding:
Dian Su (Genentech), Jian Wang (Bristol-Myers Squibb) and Fabio Garofolo (Angelini Pharma)

Panelists:
Kevin Bateman (Merck), Matthew Szapacs (GlaxoSmithKline), John Mehl (Bristol-Myers Squibb) and Shawna Hengel (Seattle Genetics)
Estimate of attendance: ~150

Agenda: presentation followed by panel discussion (please see page 3)

Presentations (Please see pages 4-36, about 5min/presentation)

Pre-workshop survey questions for discussion (Please see pages 37-42)

Discussion was pretty live. Topics ranged from data process to sample preparation. Challenges were brought up in the aspects in sensitivity. Hot topics are summarized as below:

- **Subunits vs. Intact detection**: different observation by difference organization: increase vs. similar in MS signal at low level; increase in MS signal at higher level
- **Benefits** of intact quantification compared to surrogate peptide quantification: capture important biotransformation such as oxidation (*Matt Szapacs from GSK*)
- **General challenges** for intact quantification:
  1. Overall sensitivity is about 10x lower for intact LC-MS quantification vs ELISA assay, what can we do to improve the sensitivity: affinity capture step for enrichment;/subunits for quantification (*Kevin Bateman from Merck*) Some audience proposed to use HCD technique to measure product ion
  2. Not pretty chromatography of intact quantification relative to peptide quantification
- **Data processing**: Deconvolution vs. charge states
  1. Deconvolution: deconvolution should be used with caution-compound dependent (*Matt Szapacs from GSK*); some deconvolution software result in ghost peaks.
  2. Charge states: Should just a couple most intense m/z ions or more should be used for quantification (*Matt Szapacs from GSK*)
  3. Deconvolution vs. charge states for intact quantification: some didn’t observe significant difference between the two approaches however, there is a need for software improvements to enable more efficient and complete use of deconvolution for intact mass quantification. (*John T. Mehl from BMS*)
  4. Sample preparation is important to mitigating detection bias by intact quantification (*Shawna Hengel from Seattle Genetics*)
05:45pm-05:55pm – Introduction (Discussion Topics & Panelists)

05:55pm-06:00pm – Intact MS of Proteins for Quantitative Analysis at Merck
- Kevin Bateman (Merck)

06:00pm-06:05pm – Intact and Subunit-level Antibody Analysis from In-life Samples at GSK
- Matthew Szapacs (GlaxoSmithKline)

06:05pm-06:10pm – Intact mAb Quantification -Does Deconvolution Make a Difference?
- John Mehl (Bristol-Myers Squibb)

06:10pm- 06:15pm – Intact MS of Proteins for Quantitative Analysis at Seattle Genetics
- Shawna Hengel (Seattle Genetics)

06:15pm-07:00pm – Panel Discussion
Introduction

• Pros/Cons of using HRMS (QTOF & Orbitrap) for bioanalysis of intact therapeutic proteins and/or subunits

• "Bottom-up" (signature peptide) and "Top-down" strategies in Bioanalysis

• limitation of signature peptide approach to provide sufficient information on the biotherapeutics measured

• “Lost in digestion” - how to preserve the therapeutic protein for intact quantification
Introduction (Cont.)

• Identification and quantitation of **catabolites** for a better understanding of the various circulating biotherapeutic forms, biotransformation, glycoforms quantitation and post-translational modifications.

• How to overcome **sensitivity** issues in therapeutic intact protein quantification

• Advantages of **summing isotope signals** on charge state & isotope effects on S/N;

• Optimizing extraction window (**XIC**) for quantitation,

• Intact biotherapeutics bioanalysis by **Hybrid LBA/LCMS** in a fully **Regulated Environment**.
INTACT MS OF PROTEINS FOR QUANTITATIVE ANALYSIS AT MERCK

Lisa O’Callaghan, Daniel Spellman, Kevin Bateman
Pharmacokinetics, Pharmacodynamics and Drug Metabolism (PPDM)

June 5, 2018
ASMS Conference on Mass Spectrometry and Allied Topics
How can we Increase Sensitivity?

Increase amount of analyte
- Process more sample
- More specific capture (antigen)

Make analyte more detectable
- Deglycosylation
- Hinge digestion

[Diagram showing antibody structure and analysis graphs]
Sample Automation – Agilent AssayMap

Increase amount of analyte
- 20 → 30 μL sample volume
- 50 → 25 μL elution
- Target Capture

Make analyte more detectable
- On-cartridge deglycosylation (PNGase F)
- On-cartridge hinge digestion (IDES)
Increase in Sample Volume; Anti-human Fc vs Target Capture

10x increase in LLOQ by sample volume increase and automated AM IA
No improvement from anti-hFC to target capture
Detection Limit = 0.1 μg/mL

Reds = anti-FC
Greens = Target

Can we improve further with middle-down approach?

MERCK
INVENTING FOR LIFE
AssayMap On-Cartridge Reactions

**PNGase F Digestion**
- Cleaves N-linked glycans
- Collapse multiple glycoforms into one “naked” protein
- Potential to improve signal to noise

**IdeS Digestion**
- Cleaves IgG at hinge region
- Smaller fragment has the potential to improve sensitivity

Capture → React → Elute → PNGase F → Deglycosylation
Middle-Down Compared to Fully Intact

Highly concentrated samples showed strong signal increase for middle down approaches.

Low concentration samples showed no improvement.
Flow Through Evaluation

Previous hypothesis: No increase in sensitivity due to losses from on-cartridge reactions

Flow through captured and assessed

No intact or digested protein captured in flow through from either on-cartridge reaction
Middle-down Stability

Samples reassayed 24 hrs later and large signal decrease observed
• Deglycosylated species less stable than intact?
• Deglycosylation may result in increased aggregation and decreased stability

<table>
<thead>
<tr>
<th></th>
<th>% loss</th>
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<tr>
<td>Intact</td>
<td>67%</td>
</tr>
<tr>
<td>Hinge</td>
<td>88%</td>
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<tr>
<td>Deglycosylation</td>
<td>96%</td>
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Fusion Protein Optimization

Surrogate peptide approach requires changes in detection for each analyte
Intact detection does not change

<table>
<thead>
<tr>
<th>Sample Volume</th>
<th>Sample Prep Time</th>
<th>LLOQ</th>
<th>ULOQ</th>
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<td>30 μL</td>
<td>2.5 hrs</td>
<td>0.5 μg/mL</td>
<td>60 μg/mL</td>
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<td>5 μL</td>
<td>8 hrs</td>
<td>0.5  μg/mL</td>
<td>100 μg/mL</td>
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<table>
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<tr>
<th>STD</th>
<th>Intact HRMS (μg/mL)</th>
<th>Surrogate Peptide (μg/mL)</th>
<th>Accuracy</th>
<th>Avg. Calc. Conc. (μg/mL)</th>
<th>Accuracy</th>
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<td>25.1</td>
<td>100%</td>
<td>24.9</td>
<td>101%</td>
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</tbody>
</table>
Fusion Protein PK

Intact PK achieved for several protein variants without any method changes
Intact shows slightly different PK at later time points
- Possible catabolism
Backups
Optimization of Elution Conditions

Seven common IA elution buffers evaluated

Max Signal: 0.1% TFA, 5% Acetic and 2% FA

Least Interference: 5% Acetic and 2% FA

Most Stable: 0.1% TFA and 2% FA
PK Study mAb IgG1: LBA vs Surrogate vs Intact

Similar PK demonstrated by all analysis techniques
Less than 30% variability across all measurements

Linearity, Precision and Accuracy for Intact HRMS

<table>
<thead>
<tr>
<th>Conc. (µg/mL)</th>
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<th>Intraday</th>
<th>Interday</th>
<th>Intraday</th>
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<td>2.0</td>
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<td>12.9</td>
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### Current Sensitivity for Intact mAb PK Quantitation

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<tr>
<th>Sample</th>
<th>Capture</th>
<th>Volume (µL)</th>
<th>LLOQ (µg/mL)</th>
<th>Dynamic Range (µg/mL)</th>
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<td>Anti-Fc</td>
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<td>Top-Down</td>
<td>Anti-Fc</td>
<td>20</td>
<td>2.5</td>
<td>2.5 - 200</td>
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<td></td>
<td>Target</td>
<td>30</td>
<td>0.1</td>
<td>0.1 - 50</td>
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<tr>
<td>Deglycosylation</td>
<td>Target</td>
<td>30</td>
<td>0.1</td>
<td>0.1 - 100</td>
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<tr>
<td>Hinge Digestion</td>
<td>Target</td>
<td>30</td>
<td>0.1</td>
<td>0.1 - 100</td>
</tr>
</tbody>
</table>

No improvement with hinge digestion or deglycosylation

Increased sensitivity with larger sample volume and on-cartridge enrichment
• 2.5 → 0.1 µg/mL
Intact and Subunit-level Antibody Analysis from In-life Samples at GSK

Matt Szapacs
Outline

• Intact domain antibody example – method validation
• mAb subunit LC-MS review
• Measuring intact and subunit concentration by MS: 2 assays, 3 analytes
• Large & intact mass quantitation strategies
Intact Mass Quantitation of Domain Antibody (~15 kDa)

3 P&A runs: **Passed acceptance criteria**
(5 QC levels: LLQ/low/mid/high/HLQ), n=4
+/- 20% bias & Precision at each STD and QC level
+/- 25% bias & Precision at LLQ STD and QC level

Selectivity: **Passed acceptance criteria**
- Total blanks n=1 for 6 lots (no greater than 20% of the analyte response at the LLQ)
- Spike recovery at 3x LLQ in selectivity lots; n=3 per lot (+/- 20% bias and precision)

Stability: **Passed acceptance criteria (+/- 20% bias and precision)**
- Long term plasma stability @ -80°C: established 220 days
- 3 Freeze/Thaw cycles @ -80°C
- RT plasma stability for 24 hours
- Whole blood stability : Passed RT and wet ice.

- Evidence for robust assay performance at intact mass level
- Promising for application at larger masses
- Long-term stability an important consideration
LC-MS Quantitation of the Whole Protein

Demonstrate Selectivity and Linearity in Quantitation Down to 100 ng/mL

Advantages of mAb Subunit MS

- Easily monitor mass variants as low as +16 Da (oxidation)
- Localize mass variants to a particular subunit
- Subunits should agree in concentration measurement – if not then indication of unknown biotransformation on subunit(s)

Kellie et al. Bioanalysis, 2016, 8 (20), 2103-2114
2 Assays, 3 Analytes: Intact and Reduced LC-MS Assays

Bind Drug (1000 – 10,000 ng/mL in Rhesus Serum)

Disulfide reduction

Acid Elute

Intact LC-MS Analysis

Intact Mass

Pannullo, Wednesday Poster 060
Intact and Reduced Assay: Sample Results

**Mean concentrations (3 subjects)**
- **Intact Mass**
- **Heavy Chain**
- **Light Chain**

<table>
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<tr>
<th>Time (hr)</th>
<th>HC vs. LC % Difference</th>
<th>Intact vs. HC/LC mean % Difference</th>
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<td>96</td>
<td>-25.94</td>
<td>-41.05</td>
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- Reasonable agreement within assay tolerance
- Heavy Chain slightly lower than Light Chain
- Ongoing research to better characterize & understand these relationships

Pannullo, Wednesday Poster 060
LC-MS Data: Quantitation Strategies

- Try to use “simpler is better” approach - Use the fewest peaks / charge states possible for quantitation
- Similar assay performance for using few vs. many charge states
- Use of many peaks places greater burden on data review (e.g. is that peak real?)
- Deconvolution use is appropriate sometimes, but may be problematic in a regulated setting

Intact mAb Quantification
Does Deconvolution Make a Difference?

John T. Mehl, Ph.D.
Bioanalytical Research
Bristol-Myers Squibb
Princeton, NJ

ASMS RBIG Workshop, San Diego, CA, June-5 2018
Quantitative analysis of mAbs using Intact Mass Spectrum Deconvolution or Extracted-ion chromatogram

Jian, W., et al., *Bioanalysis* (2016) 8(16), 1679-1691


EIC

Rat plasma

Deglycosylated

Sum 6 charge states
Immunocapture sample preparation; Hybrid LC-MS

**Immobilized anti-human-Fc capture**

Capture

serum sample or tissue homogenate

Wash

mAb

Acid elution

**HRMS Full-scan**

**LC-MS**

Elution time (min)
Intact mAb Quantification

What level of sensitivity can be achieved?

How to process data; EIC or Deconvolution?
Example 1

Intact mAb Sample Prep and LC-MS conditions

Mouse plasma (100μL)
anti-Human Fc magnetic beads

**Immunocapture**

**Elution**

LC-MS analysis (Xevo G2-XS Q-TOF),

6.5 min LC method

Acquity UPLC Protein BEH C4 Column 300A, @ 80°C
Intact Mass Quantification of SiLu™ MAb immunocaptured from mouse plasma

TIC 3.75 µg/mL

EIC 3.75 µg/mL

EIC Blank plasma

Annoying background peak in blank

2577.5 + 2623.5 + 2671.0 + 2720.5 + 2771.8 + 2825.1 + 2880.5 m/z
Intact Spectrum of SiLu™ MAb
immunocaptured from mouse plasma

Combined spectra from 2.75 – 2.95 min

7.5 µg/mL

0.5 µg/mL

BLK mouse plasma
Peak in EIC is from Increased Baseline Signal

What is leading to the LC peak observed in window #2 of the blank mouse plasma?
Intact Mass mAb Quantification
EIC or Deconvolution?

**EIC**
- 0.5 µg/mL standard
- Blank plasma

**Deconvolution**
- 1.0 µg/mL
- Blank
- 0.5 µg/mL
Standard Curve of Intact SiLu™ MAb in mouse plasma
EIC and Deconvoluted Standard Curves

EIC Standard Curve
(1.0 - 15 µg/mL)

Deconvolution Standard Curve
(0.5 – 15 µg/mL)

<table>
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<tr>
<th>Conc. µg/mL</th>
<th>% Accuracy</th>
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<tr>
<td>15</td>
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</table>
1A. What are the **current industry strategies** for overcoming **sensitivity issues** in intact protein quantification?

1B. Which are the ones really working and which are not?

- Maximizing enrichment by IA by using best capturing antibody for improving mass spectrometry S/N?
- Deglycosylation?
- Subunits quantification?
- Summing isotope signals
- Charge state coalescence with DMSO
- Optimizing extraction window (XIC) for quantitation?
- Declustering potential & accumulation time?
- Optimization of chromatographic condition for intact proteins
- Others
2. **Why** intact protein quantification should be performed?

3. **How** intact protein quantification should be performed?

4. What are the **Pros/Cons** of using **HRMS** for quantitation of intact proteins and/or subunits?
5. “Lost in digestion” fact or myth?

6. Is the use of the “Bottom-up” (signature peptide) approach impacting the bioanalysis of biotherapeutics?

7. Are the risks of not providing sufficient information on the biotherapeutics bioanalysis by Hybrid LBA/LCMS signature peptide approach confirmed by actual data?
8. What are the **most common applications** for intact proteins quantification?

- Identification of intact biotherapeutics and their catabolites?
- Quantitation of intact biotherapeutics and their catabolites?
- Studies for better understanding the various circulating biotherapeutic forms?
- Biotransformation studies?
- Glycoforms quantitation?
- Post-translational modifications evaluations?
- Others…. 
9. What are the best stationary phases for intact proteins quantification?

10. Is the use of stable isotope-labeled internal standard version of the protein crucial?

11. Is there any concern raised by Regulatory Agencies when the intact quantification is used instead of the traditional bottom-up approach?
12. What are the current industry standards in “Top-down” protein Bioanalysis?