LC-MS/MS based strategies for quantification of therapeutic antibody drug conjugates

By Ranbir Singh Mannu
Discovery Bioanalysis Manager
Requested Bioanalysis in ADC programs

LC-MS/MS assays for quantification of ADCs requested:

1. Quantification of free toxin – key to patient safety
   - Consideration of toxin-linker and toxin-metabolite
   - Traditional small molecule LC-MS/MS bioanalysis

2. Quantification of “total antibody” (i.e. including DAR0)
   - Whole serum digestion or affinity extraction of antibody prior to proteolytic digestion and LC-MS/MS

3. Quantification of drug-conjugated antibody (i.e. excluding DAR0)
   - Immunoaffinity extraction via anti-toxin prior to proteolytic digestion and LC-MS/MS
   - Highlight a number of considerations and technical challenges of MSIA

4. Evaluation of DAR (drug/antibody ratio) in vivo, or for in vitro serum/plasma stability studies
   - Link to BioCMC characterisation studies
Numerous analytical challenges come from any, or all, of the three components of the given ADC: the antibody, the linker, and/or the drug.

Initial demonstrative studies using Her vcE
- (Herceptin® Maleimidocaproyl valine-citruline monomethyl Auristatin E)

- We had Her vcE in the freezer!
- Conjugation to cysteine residues – no expected blocking of tryptic digestion
- Total antibody assays considered unlikely to be affected by toxin/linker stability
- Previous experience with this model ADC (incl. BioCMC characterisation studies) and with Herceptin
- Anti-toxin antibodies commercially available for immunoaffinity extraction methods

Average DAR 3.5 by HIC-UV, RP-HPLC-UV
Strategy One: Whole Serum/Plasma SMART Digest™

Total ADC

25 µL serum + 25 µL 50 µg/mL Lysozyme (ISTD)

Add 150 µL supplied digestion buffer, mix

Load into SMART Digest™ tubes; 70°C for 2 h with mixing (1200 rpm)

SPE with Waters Oasis® HLB 10 mg

Evaporative concentration & reconstitution

LC-MS/MS

(Sciex API 5000™, Waters Acquity®)
Phenomenex Kinetex® XB-C18 1.7 µm column
Water/acetonitrile phases + formic acid

Sample Processing Time: 5.5 h

Isotopically labelled internal standard not required

SPE step serves to remove salts and undigested protein prior to sample concentration and LC-MS/MS
Strategy One: Whole Human Serum SMART Digest™
Her vcE, Validation 0.1 - 50 µg/mL

LLOQ (0.1 µg/mL) Retention time 2.13 minutes

Surrogate peptide of Lysozyme (ISTD) Retention time 2.40 minutes

Matrix blank (No peak)
THREE PRECISION AND ACCURACY RUNS WITH SELECTIVITY AND STABILITY TESTS

<table>
<thead>
<tr>
<th>Run</th>
<th>Replicate</th>
<th>QC 0.1 µg/mL (LLOQ QC)</th>
<th>QC 0.3 µg/mL (LoQC)</th>
<th>QC 3.5 µg/mL (MeQC)</th>
<th>QC 40 µg/mL (HiQC)</th>
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Mean (µg/mL): 0.0998, 0.295, 3.55, 41.3
Standard deviation (n-1): 0.0120, 0.0229, 0.124, 1.94
RSD (%): 12.0, 7.8, 3.5, 4.7
Accuracy (%): 99.8, 98.3, 101.4, 103.3

- Six individual lots of matrix at LoQC: 97.3-109.1% accuracy
- Haemolysed and lipidaemic LoQC and HiQC samples: 98.3-110.3% accuracy
- Stability samples acceptable:
  - 24 hour room temp
  - 3x freeze-thaws
  - 3 days for extracts
Strategy Two: Protein G/ SMART Digest™
Total ADC

30 µL serum + 25 µL 50 µg/mL analogue mAb (ISTD)

↓

GE Healthcare Protein G HP MultiTrap™ 96-well plate

↓

Elute with acidic solution into neutralising buffer

↓

Load into SMART Digest™ tubes; 70°C for 2 h with mixing (1200 rpm)

↓

SPE with Waters Oasis® HLB µElution

↓

LC-MS/MS

(Sciex API 5000™, Waters Acquity®)
Phenomenex Kinetex® XB-C18 1.7 µm column
Water/acetonitrile phases + formic acid

Analogue mAb internal standard required for co-extraction with analyte mAb using Protein G

Neutralisation of acidic eluate is critical prior to tryptic digestion

Sample Processing Time: 6.5 h
Strategy Two: Protein G/ SMART Digest™
Her vcE, Human Serum Method Validation, 0.1-50 µg/mL

LLOQ (0.1 µg/mL) Retention time 2.28 minutes

Avastin® surrogate peptide (ISTD) Retention time 4.20 minutes

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Strategy Two: Protein G/ SMART Digest™
Her vcE, Human Serum Method Validation, 0.1 - 50 µg/mL

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Mean (µg/mL) | 0.0950 | 0.293 | 3.37 | 40.6 |
Standard deviation (n-1) | 0.0142 | 0.0220 | 0.279 | 2.47 |
RSD (%) | 14.9 | 7.5 | 8.3 | 6.1 |
Accuracy (%) | 95.0 | 97.7 | 96.3 | 101.5 |

- Six individual lots of matrix at LoQC: 87.1-97.6% accuracy
- Haemolysed and lipidaemic LoQC and HiQC samples: 89.3-98.8% accuracy
- Stability samples acceptable:
  - 24 hour room temp
  - 3x freeze-thaws

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Strategy Three: Immunoaffinity Extraction
Method Development Challenges

**Cost**
Cost of streptavidin MSIA tips/ magnetic beads. 1mg of anti-MMAE $8000

**Adsorption**
Evaluated adding BSA, other proteins and 2% serum to load and elution steps.

**Linear Range**
How much anti-MMAE to use?

**DAR Ratio**
Need at least 1µg to Assess DAR ratio?
Strategy Three: Immunoaffinity Extraction
Quantification of Drug-Conjugated Antibody (Excl. DAR0)

- Load into SMART Digest™ tubes; 70°C for 2 h with mixing (1200 rpm)
- Elute with acidic solution into neutralising buffer
- Anti-toxin Immunoaffinity extraction:
  - MSIA™ D.A.R.T'S™ Streptavidin & Versette™, Thermo Scientific™
  - Or Dynabeads® M-280 Streptavidin magnetic beads, Invitrogen
- Elute with acidic solution into neutralising buffer
- SPE with Waters Oasis® HLB µElution
- 50 µL serum

Sample Processing Time: 12 h

LC-MS/MS
(Sciex API 5000™, Waters Acquity®)
Phenomenex Kinetex® XB-C18 1.7 µm column
Water/acetonitrile phases + formic acid

- Availability of internal standard
- Analogue + toxin
- Analogue – toxin
Strategy Three: Anti-vcE/ SMART Digest™
Her vcE LLOQ from Human Serum using anti-MMAE

LLOQ (0.1 µg/mL) Retention time 2.21 minutes

Avastin® surrogate peptide (ISTD) Retention time 4.16 minutes

Herceptin® (10 µg/mL)

LC-MS/MS strategies for quantification of therapeutic antibodies and ADCs
Strategy Three: Anti-vcE/ SMART Digest™
Her vcE Calibration curve from Human Serum using anti-MMAE

Calibration range 0.1 to 5 µg/mL

Saturation could be resolved by increasing Anti-MMAE antibody from 1 µg to 2.5 µg (extra cost)
Strategy Three: Current Optimum Conditions

1) Binding anti-MMAE to streptavidin MSIA tips/ magnetic bead
   - Dilute anti-MMAE in BSA before binding to streptavidin MSIA tips/magnetic beads

2) Loading ADC and wash steps
   - MOPS buffer at pH 6.4 provided best sensitivity suggesting potential instability of ADC at pH 7.4

On-going work

1) Assess the amount of free MMAE in stock solution
   - Buffer exchange stock ADC solution

2) Move the analysis onto a API6500
   - Assess the lowest limit of quantification

3) Validate methodology
Middle-up LC-MS following IdeS digestion and DTT reduction

- A rapid approach to Characterisation of Thiol-conjugated ADCs and Calculation of DAR by LC-MS

- Injection of 0.1-2 µg
- Co-elution of Fc/2, Fd and LC subunits into ESI-QToF MS

- Adapt for serum extracts?
- Investigate any bias in anti-MMAE binding?
- Evaluation of DAR for serum stability studies/in vivo catabolism studies?
- 50 µL serum, 20 µg/mL Her vcE = 1 µg
Conclusions

- Whole plasma/ serum digestion can work very well if you have a suitably unique peptide, better selectivity/ sensitivity achieved using the protein G cleanup prior to tryptic digestion
- Immunoaffinity extraction of drug-conjugated antibody more costly due to cost of MSIA tips or magnetic beads, plus the cost of anti-toxin antibody
- Adsorption is a major issue at various points in the methodology
- Sub 0.1 µg/mL LLOQ achievable with ~ 100 fold calibration range
- Concerns of DAR bias and the ability to assess this at the lower end of the calibration range
- Significant amount of method development time required, long extraction time and the need for automation
Acknowledgements

- Mr James Duffy
- Mr Gregory Bogle
- Dr William Eborall
- Dr Matt Ewles
- Dr Johannes Stanta
- Dr David Firth
- Dr Lee Goodwin
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