Presentation Overview

► A brief introduction to LC-MS/MS quantification of proteins and the aims of our work

► Presentation of two strategies for LC-MS/MS quantification of therapeutic antibodies

► Some (surprising) findings regarding ADA effects
Considerations for LC-MS/MS Quantification of Proteins in Biological Samples

Most bioanalytical strategies are based on tryptic digestion…
… but beyond that everyone does it differently!

What digestion approach:
- Classical guanidine/urea denaturation and overnight digestion?
- Detergent mediated denaturation and facilitated digestion?
- Microwave or methanol assisted digestion?
- Immobilised Trypsin?

Sample pre-treatment:
- Direct digestion of plasma?
- Protein precipitation or size exclusion?
- Protein A/G mediated extraction of antibodies?
- Bespoke ultra-selective immunoaffinity cleanup?

Post-digestion analysis:
- Additional cleanup after digestion?
- Conventional reversed phase LC-MS/MS
- Or more ‘fancy’ techniques (nanoflow, ion mobility)
- Which tryptic peptide do we choose?

To reduce or not to reduce?
Do we really need to reduce/alkylate?

Considerations
Introduction

► LC-MS/MS can provide complementary data to ligand binding assays as well as offering merits as a standalone bioanalytical approach

► We wanted an approach that was cost effective, sensitive, quick/easy to develop, no requirement for analyte-specific reagents, and high throughput for use with conventional UHPLC-MS/MS instrumentation

AB Sciex API5000™ and Waters Acquity™
Case Studies

► Herceptin® (Trastuzumab) used as a model therapeutic mAb

► SMART Digest™ (Thermo Fisher Scientific™)
  • Immobilised thermally stable trypsin
  • Supplied pre-aliquotted in strips of PCR tubes
  • Allows digestion at 70°C in up to two hours
  • No reduction/alkylation necessary*
    *(if signature peptide has no cysteine)

► Tests showed enhanced digestion efficiency compared to many traditional approaches and at much lower cost
Strategy One: Whole Serum/plasma Digestion

25 µL plasma + 25 µL of 50 µg/mL chicken lysozyme
(Generic protein internal standard)

Add 150 µL supplied digestion buffer and mix briefly

Load all into the SMART Digest™ tubes

Two hour digestion at 70°C

Perform SPE (Waters Oasis® HLB)

Eliminates salts and undigested proteins

Evaporative concentration and reconstitution

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

Total extraction time <5.5 hours per 96x sample run
Herceptin® Results

► Several peptides from variable region predicted

► After optimisation five of these gave desirable analytical performance

► Peptide IYPTNGYTR provided best signal/noise

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Light chain

DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLILYSASFLYSQVPSRFSGRSGTDFTLTISSLQPEDFATYYCQHQHTPTFQGGTKVEIKRTVAAPSFIFPPSDEQLKSGTASVVCLN FayENPPEAKVQWKVDNALQSGNSQESVTEQDKDSTYSLSSSTLKLADYEAEHHVYACEVTHQLSSPVTKSFNRGEC

Heavy Chain

EVQLVESGGGLVQPSGGLRLSCAASGFIKNDTYIHWRQAPGKGLEWVARQIYPTNGYTRYADSVKGFTISADTSKNTALQLMSLRAETDVAAYCSRWGDGSFYMDSWGWGQTLTVTSSATKPSVFPAPSSKSTSGTAALGCLVKDYTFPEPVTSWNSGALTSGVHTFPAVLQPSGLSYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDDKVEPDKSCDKTHTCPCPAPCELLGGPSVFLFPPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYGVEVHQNAKTPEEEQYNSTYRVSFLTVLHQDVLWNLGKEYVCKVSNKALPAPIEPITKSAKQGPQREMPPVYTLPAPSDSLTQNSLCLVKGIGYPSDIAYAWEQESNGQEPNYYKTKTPVLDGSDFSLYKSLTDVDSRWQGQNVSVCVMHEALHNHYTQKSLSLGK
Method Validation
(Whole plasma digestion, Herceptin, 0.25-250 µg/mL)

Three precision and accuracy runs with selectivity and stability tests

- Six individual lots of matrix at LoQC: 93-99% accuracy
- Haemolysed and lipidaemic LoQC and HiQC samples: 88-95% accuracy
- Stability samples acceptable:
  - 18 hour room temp
  - 18 hr refrigerated
  - 3x freeze-thaws
  - 3 days for extracts
Method Validation
(Whole plasma digestion, Herceptin®)

**LLOQ (0.25 µg/mL)**
- Retention time: 2.29 minutes

**Surrogate peptide of Lysozyme (ISTD)**
- Retention time: 2.56 minutes

**Matrix blank**
- (No peak)
Whole Plasma Digestion Applied to Clinical Assay for Other mAbs

► Whole plasma/serum digestion worked well for Herceptin®

► Applied to other therapeutic antibodies:
  • Humira® (Adalimumab)
  • Avastin® (Bevacizumab)

► Inadequate selectivity achieved for all the variable region surrogate peptides. (Lowest achievable LLOQ limited to 0.5-1 µg/mL due to interfering peaks)

Pre-digestion cleanup required!!!
Protein G-cleanup Introduced into Workflow

30 µL plasma + 30 µL of analogue mAb protein

GE Healthcare Protein G HP Multitrap 96-well plate

Eliminates all non-antibody proteins

Elute with acidic solution into neutralising buffer

Achieve pH 7.5 using Tris buffering

Load into SMART Digest™ tubes

Two hour digestion at 70°C

Perform SPE (Waters Oasis® HLB)

Eliminates salts and undigested proteins

LC-MS/MS (as before)

Total extraction time <6 hours per 96x samples
# Protein G/SMART Digest™ Method Validation
(Herceptin®, 0.1-100 µg/mL human serum, Avastin® ISTD)

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 5 µg/mL (HiQC)</th>
<th>QC 80 µg/mL (HiQC)</th>
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- **Six individual lots of matrix at LoQC: 98-109% accuracy**
- **Haemolysed and lipidaemic LoQC and HiQC samples: 89-112% accuracy**
- **Stability samples acceptable:**
  - 18 hour room temp
  - 18 hr refrigerated
  - 3x freeze-thaws
  - 3 days for extracts
Method Validation
(Protein G, SMART Digest™, Herceptin®)

LLOQ (0.1 µg/mL) Retention time 2.39 minutes

Avastin® surrogate peptide (ISTD) Retention time 4.30 minutes

Matrix blank
Testing for Other Antibodies

► Same methodology applied for Avastin® and Humira®

► Optimum surrogate peptides are:
  • Avastin®: FTFSLDTSK (Heavy chain 68-76)
  • Humira®: GLEWVSATWNSGHIDYADSVEGR (Heavy chain 44-67)

► Using Herceptin® as internal standard for both
Protein G/SMART Digest™ Method Validation
(Avastin®, 0.1-100 µg/mL human serum, Herceptin® ISTD)

Three precision and accuracy runs with selectivity and stability tests

<table>
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<th>QC 0.1 µg/mL (LLOQ QC)</th>
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Mean (µg/mL) 0.0995 0.299 5.12 79.1

Standard deviation (n=1) 0.0169 0.0011 0.220 4.98

RSD (%) 11.0 7.2 4.3 6.4

Accuracy (%) 99.5 99.7 102.4 97.6

Six individual lots of serum, haemolysis, lipidaemic and stability tests all acceptable

Avastin® LLOQ (0.1 µg/mL)

Retention time 4.82 minutes

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Protein G/SMART Digest™ Method Qualification
(Humira®, 0.2-100 µg/mL human serum, Herceptin® ISTD)

► One precision and accuracy run performed
► Sensitivity/selectivity at 0.1 µg/mL not acceptable
► Work ongoing to validate over range 0.2-100 µg/mL

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</table>

Humira® LLOQ (0.2 µg/mL)
Retention time 7.08 minutes
Conclusions

► Whole plasma/serum digestion can work very well if you have a very selective peptide or non-challenging LLOQ (≥0.25-1 µg/mL)

► Better selectivity/sensitivity achieved using the protein G cleanup prior to tryptic digestion (but at some additional cost per sample)

► Protein G approach has been successfully validated for Herceptin® and Avastin® (0.1-100 µg/mL in human serum, 4-6-15 criteria)

► Also qualified for Humira® in serum with higher LLOQ (0.2 µg/mL)

► Method can be easily adapted for other mAb therapeutics
What About Anti-Drug-Antibody Effects (ADA)?

► Common assumption that LC-MS/MS assays will always measure total (free, soluble target-bound and ADA-bound)

► Is this REALLY true for these methods?

► The impact of Anti-Herceptin® rabbit polyclonal antibody (pADA)
  Anti-Herceptin® human monoclonal antibody (mADA)

Was investigated for the following validated assays:

(1) Ligand binding assay
(2) Whole serum digestion LC-MS/MS method
What effect does these levels of ADA have on our LC-MS/MS assay?
Sample Preparation

The following samples were tested:

(Molar ratio Herceptin®: ADA)

- LoQC (0.3 µg/mL) control
- LoQC (0.3 µg/mL) with 160 µg/mL polyclonal ADA (1:533)
- LoQC (0.3 µg/mL) with 40 µg/mL monoclonal ADA (1:133)
- HiQC (80 µg/mL) control
- HiQC (80 µg/mL) with 160 µg/mL polyclonal ADA (1:2)
- HiQC (80 µg/mL) with 40 µg/mL monoclonal ADA (2:1)

Accuracy using **WHOLE SERUM DIGESTION LC-MS/MS** approach

- 107%
- 106%
- 35%
- 99%
- 90%
- 97%

**GREEN** = Accurately quantified by Ligand Binding Assay

**YELLOW** = Show 60-80% accuracy by Ligand Binding Assay

**RED** = Show no response by Ligand Binding Assay
Results Using LC-MS/MS Assays

Using **WHOLE SERUM DIGESTION** approach

- No impact of mADA on quantification

- Where the polyclonal antibody is present at high molar excess there is serious negative bias on the LC-MS/MS assay

- Hence: **LC-MS/MS assays can be affected by ADA effect!**
Clinical Relevance

► Polyclonal antibody used raised in rabbit to Fab fragment

► Therefore significant amount of antibody unique to variable region therefore could mimic a true ADA response in clinical samples

► What might be going on here??? (hypothesis…)

Immobilised Trypsin

ADA

Target mAb (variable region protected from trypsin)
Strategies investigated to dissociate Herceptin® ADA prior to digestion

► **Denaturation with DMSO – Solves the problem!**
- Impact of anti-Herceptin® polyclonal antibody (530x molar excess) eliminated
- DMSO has improved selectivity/sensitivity of whole plasma digestion (lower LLOQ)
- Work ongoing to assess ADA effects on protein G methodology

<table>
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<tr>
<th>Run</th>
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</table>
Conclusions

► We have shown that for LC-MS/MS, an anti-drug-antibody effect can result in an underestimation of the total drug even when digesting whole plasma/serum.

► However careful optimisation can overcome this to ensure total quantification
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