Challenges and Solutions to the Bioanalysis of Large Molecules by LC-MS/MS: Development, Validation and Production

Shane Needham, Ph.D.
Co-Founder and Chief Scientific Officer
Alturas Analytics, Inc.
LARGE MOLECULE ANALYSIS:
LBA or LC-MS/MS

Selectivity/Sensitivity?
Signal/Noise!!!
GAINS IN SELECTIVITY AND SENSITIVITY

- Off-line sample preparation
- High resolution mass spectrometer
- MS/MS/MS
- Ion mobility applications
- Derivitization
- Chromatographic improvements
  - Microflow LC-MS – optimized source
  - More resolution
  - Different stationary phase or orthogonal chromatography
GAINS IN SELECTIVITY AND SENSITIVITY

• Sequence Selection
• Sample Preparation
  ▪ Mass Spectrometric Immunoassay (MSIA™)
  ▪ Microelution
• Microflow LC-MS
  ▪ Optimized source design
  ▪ Optimized MFLC
Conventional

With OptiFlow™

With OptiFlow™ and Column Heater
ADC MIMIC:

SigmaMAb antibody drug conjugate (ADC) mimic consists of the human universal monoclonal antibody standard (catalog number MSQC4, an IgG1 monoclonal antibody) conjugated to dansyl fluorophores via an LC-SMCC crosslinker.

IS

SI\textsuperscript{TM}LuMAb Infliximab is a recombinant, stable isotope-labeled, monoclonal antibody which incorporates [13C6, 15N4]-Arginine and [13C6, 15N2]-Lysine.
METHODS
Sample Preparation

- Sample volume: 50 μL
- Add 25 μL Infliximab internal standard
- Precipitate with acetonitrile 0.1% formic acid, remove supernatant
- Denature pellet with 100 μL of RapiGest™ and incubate
- Reduce with 10 μL of dithiothreitol and incubate
- Derivatize with 10 μL of iodoacetamide and incubate
- Digest with 25 μL of porcine trypsin at 0.200 mg/mL
- Incubate at 37°C for 2.5 hours
- Dilute 1:1 with 10% TCA solution
SEQUENCE SELECTION:
Skyline

FASTA records begin with '>', and have the protein name followed by the optional protein description.

>SGMASTABLELABEL_LIGHTCHAIN
DLLTQSPAILSYSPSGRSVPSSFGRTQGGPHWYQQRNGSPRRKLYASESMSGIPSRFSGSQGTDFTSLNTYVESEDIAYYCQSHSWFTRFGSTTNLEVKFTVAAPSVFIPFSDEQLKSATAVCLNNFYPREAKIVKVDNALQSGNVSQGESVTEGSKOSTYSLSTLKSADVIEKHRVYACEVTHQGLSSPVTFSNREGEC
SKYLINE PREDICTED PEPTIDE SEQUENCES: Q1 and Product Ion
METHODS
MFLC-MS/MS

- Waters Acquity M-Class Binary LC Systems
- Gradient using acetonitrile and water with 0.1% formic acid
- Flow rate: 50 μL/min
- Column: Phenomenex Biphenyl (50 X 1.0 mm, 3 μm)
- Column temperature: 50°C
- Sciex 5500/6500+ QTRAP operating in MRM mode
- ESI
- Positive ion mode
EXAMPLE MRM OF PREDICTIONS FROM SKYLINE

Improving Selectivity!
ADC MIMIC

OptiFlow™ vs. Conventional Source

**Electrodes:** OptiFlow™ 10-50 µL/min, Conventional 50 µm

**Columns:**
- 10-20 µL/min Halo Biphenyl 0.3 X 50 mm, 2.7 µm
- 50 µL/min Phenomenex Kinetex® Biphenyl 1.0 X 50 mm, 1.7 µm

**Peptide:** Digested ADC Mimic (Sigma)

**Concentration:** 100 ng/mL

**LC System:** Waters M Class
(MP A: Water 0.1% formic acid, B: Acetonitrile 0.1% formic acid)

**MS System:** API 6500

**Gradient:** 5%-25% Organic over 3 minutes
## ADC MIMIC

**OptiFlow™ vs. Conventional Source**

<table>
<thead>
<tr>
<th>Flowrate (µL/min)</th>
<th>Source</th>
<th>AVG Area</th>
<th>Area CV</th>
<th>AVG Height</th>
<th>Height CV</th>
<th>Area % Diff from Conv. and OptiFlow™</th>
<th>Height % Diff from Conv. and OptiFlow™</th>
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</thead>
<tbody>
<tr>
<td>10</td>
<td>OptiFlow™</td>
<td>3.44E+04</td>
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<td>1.02E+04</td>
<td>4.5</td>
<td>6.72E+03</td>
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<td>10</td>
<td><em>OptiFlow™</em></td>
<td>2.99E+04</td>
<td>3.5</td>
<td>8.53E+03</td>
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<td>59.7</td>
<td>39.4</td>
</tr>
</tbody>
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*1-10 µL electrode*
Selectivity or Sensitivity???

Signal/Noise!!
SMALL MOLECULE – PROPRANOLOL
OptiFlow™ vs. Conventional Source

**Electrode:** OptiFlow™ 10-50 μL/min, Conventional 50 μm
**Analyte:** Propranolol
**Concentration:** 1 ng/mL
**LC System:** Waters M Class
(MP A: Water 0.1% formic acid, B: Acetonitrile 0.1% formic acid)
**MS System:** AB Sciex QTRAP® 6500+
# SMALL MOLECULE – PROPRANOLOL

**OptiFlow™ vs. Conventional Source**

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<td>10</td>
<td>OptiFlow™</td>
<td>3.05E+04</td>
<td>2.46E+04</td>
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<td>Conventional</td>
<td>1.72E+04</td>
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<td>2.33E+04</td>
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<tr>
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<td>2.53E+04</td>
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<tr>
<td>50</td>
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<td>2.33E+04</td>
<td>1.87E+04</td>
<td>8.1</td>
<td>29.7</td>
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</table>
MICROFLOW SOURCE:
OptiFlow™ vs Conventional Source

Selectivity or Sensitivity???

Signal/Noise!!
MSIA™
Mass Spectrometric Immunoassay

Preparation Procedure:
1. Pre-rinse MSIA™ tips
2. Rinse MSIA™ tips with 2% formic acid
3. Capture biotinylated anti-IgG
4. Rinse
5. Capture ADC
6. Elute

Photos: thermofisher.com
OFF-LINE SAMPLE PREPARATION:

Imunoaffinity

Sigma ADC Mimic 100 ng/mL Pellet Digestion

Sigma ADC Mimic 10.0 ng/mL MSIA™

Selectivity or Sensitivity???

Signal/Noise!!
GENERIC METHODS FOR SCREENING OF PEPTIDE THERAPEUTICS BY MFLC-MS/MS
NORMAL FLOW
conventional source, (0.7 mL/min) 100 ng/mL, non-extracted
OPTIMIZED MFLC-MS
OptiFlow™ source, (50 µL/min) 100 ng/mL, extracted from 50 µL rat plasma

Selectivity or Sensitivity???

Signal/Noise!!
OFF-LINE SAMPLE PREPARATION:
Microelution SPE
Signal/Noise!!

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Recovery Microelution WCX (%)</th>
<th>Recovery WCX (%) Blowdown and Reconstitute</th>
<th>MW</th>
<th>Q1 (m/z)</th>
<th>Product Ion (m/z)</th>
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<tr>
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<td>2</td>
<td>83.6</td>
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<td>3</td>
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<td>3.0</td>
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<td>97.4</td>
<td>3.0</td>
<td>4090</td>
<td>818.7</td>
<td>954.2</td>
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</table>
LARGE MOLECULE AND MFLC BIOANALYSIS

Many options to improve selectivity/sensitivity

- SIGNAL/NOISE DRIVES ANALYTICAL CHEMISTRY – LLOQ

- Analyzed more than 2500 samples to support two clinical large molecule studies by MFLC-MS/MS with a >91% pass rate (GLP acceptance criteria)

- Additionally supporting two preclinical large molecule programs

- We are in the process of validating three small molecule assays using MFLC-MS/MS in support of multiple clinical and preclinical studies
ACKNOWLEDGEMENTS

• Team Alturas
  • Chad Christianson and Jennifer Zimmer
• SCIEX-LC-MS, OptiFlow™
• Thermo-MSIA™
• Waters-M Class MFLC
Alturas Analytics, Inc.
The LC/MS Experts™

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