Development of Analysis Methods for Therapeutic Monoclonal Antibodies Using Innovative Superficially Porous Particle Biocolumns

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Agilent BioHPLC Columns
Outline of Talk

• Introduction – mAbs, Characterization
• Workflow Solutions
• Reversed-Phase Challenges
• RP Method Development/Optimization
• mAb Intact and Fragment Solutions
• Summary
Critical Quality Attributes and Analytical Methods

- Pyro- Glutamate Deamidation/Oxidation
- Fragmentation (Hinge)
- Glycosylation (G0, G1, G2)
- Truncation (Lys 0, 1, 2)
- Disulfide Shuffling

Analytical Methods:
- HILIC
- SEC
- RP
- IEX

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Comprehensive Portfolio of Analytical Instrumentation & Solutions for Biopharma & Biosimilars

Discovery > Development > QA/QC

Glycans
- CE/LIF/MS, CE/MS
- LC/FLD, LC/MS

Aggregation
- LC/UV Size Exclusion
- CE/UV Field-flow fractionation

Molecular Weight Determination
- CE/SDS-PAGE, CE/MS
- Microfluidic SDS-PAGE
- LC/UV or LC/MS

Charge Variants
- IEF analyzer (iCE 280)
- CE (cIEF), CE/MS
- Bio-LC/UV Ion Exchange

Peptide Mapping
- LC/UV LC/MS
- CE/UV, CE/MS

Oxidation
- CE/MS
- LC/UV LC/MS
- HIC and RP

Amino Acids
- LC/UV LC/MS
- CE, CE/MS

Protein PEGylation
- LC/UV SEC / Cation Exchange
- Microfluidic SDS-PAGE

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Characterization of Monoclonal Antibodies

- Titer determination and purification
  - Affinity Chromatography

- Protein identification and impurity profiling
  - Reversed-phase chromatography (RP)

- Glycan analysis
  - Hydrophilic interaction chromatography (HILIC)

- Charge variant analysis
  - Ion exchange chromatography (IEX)

- Aggregation analysis
  - Size exclusion chromatography (SEC)

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Agilent Bio-LC Column Portfolio

Reversed-Phase Columns: mAb Characterization

AdvanceBio RP mAb
Primary Structure Characterization Workflows

- **Intact mAb**
  - LC/MS

- **Reduction / alkylation**
  - Heavy / Light Chains
    - LC/MS, LC/UV

- **Enzymatic digestion**
  - Fab / Fc Regions
    - LC/MS, LC/UV

- **Enzymatic digestion**
  - Peptides
    - LC/UV

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The Challenges

<table>
<thead>
<tr>
<th>Challenge</th>
<th>Disadvantage</th>
<th>Consequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insufficient Resolution</td>
<td>Poor accuracy and precision for results</td>
<td>Lack of confidence in analysis results</td>
</tr>
<tr>
<td>Long analysis times</td>
<td>Low number of samples run per day</td>
<td>Low throughput</td>
</tr>
<tr>
<td>Short column lifetimes</td>
<td>Must purchase new columns often</td>
<td>Poor use of resources</td>
</tr>
<tr>
<td>Method transfer to multiple columns</td>
<td>Different columns for UV and MS</td>
<td>Time consuming</td>
</tr>
</tbody>
</table>
Particle
- 3.5 μm SP particle
- 0.25 μm porous layer depth
- 450Å pore diameter

Bonded Phases
- C4
- SB-C8
- Diphenyl

The optimum large molecule resolution for use with both HPLC and UHPLC systems

The most popular phases for proteins, plus a unique selectivity
Method Development: Factors Affecting the Protein Separation

AdvanceBio RP-mAb

- Column packing (silica type and bonded phase)
- Column dimension
- Mobile phase
- Column temperature
- Linear velocity
- Gradient profile
- Sample
Choose The Initial Bonded Phase: C18, C8, C4

Buy Them All and Try Them All.....

Not really feasible, right?
How do you narrow it down?
Selection of Bonded Phase:

<table>
<thead>
<tr>
<th>Application</th>
<th>RP column</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptides and proteins, &lt; 5000 MW</td>
<td>C18</td>
</tr>
<tr>
<td>Hydrophobic polypeptides and proteins, Antibodies, &gt; 5000 MW</td>
<td>C8</td>
</tr>
<tr>
<td>Larger, hydrophobic peptides and proteins, Antibodies, &gt; 5000 MW</td>
<td>C4</td>
</tr>
<tr>
<td>Proteins with aromatic sidechains, Antibodies, Fusion proteins, &gt; 5000 MW</td>
<td>Diphenyl</td>
</tr>
</tbody>
</table>

Increasing protein size and hydrophobicity

C18  C8  C4  Diphenyl

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Pore size

- Generally speaking, the “go to” pore size for most protein applications using RP is > 300Å.

≤ 4000 MW → Use smaller pore size columns to maximize loading capacity and retention

4000 – 500,000 MW → Use > 300Å pore columns to maintain high efficiency. Increase column diameter to increase loading capacity
Mobile phase

- In order of increasing eluotropic force and decreasing polarity: Water, Methanol, Acetonitrile, isopropanol, THF
  - Acetonitrile: Low UV Cutoff, 190 nm
  - Volatile
  - Good solubilization, denaturant

- Selectivity differences and sample retention will vary significantly between mobile phases

- pH and ionic strength of the aqueous portion of mobile phases are important in developing rugged methods

- It is very important to control pH to stabilize retention and selectivity

- % of organic solvent – there is a pressure maximum and minimum for organic:aqueous mobile phases and it differs depending on the organic

- Use solvents that are compatible with the shipping solvent
Ion-pairing agent

TFA

- Most common ion pairing agent for reversed-phase analysis
- Low UV absorbance
- Volatile
- Good solubilization, denaturant
- Acidic - improves peak shape
- Mild anionic ion pair - more retention of Lys, other free amines

TFA / Water : TFA / ACN mobile phase

- Low pH (0.1% TFA, pH ≈ 1.9) suppresses silanol interactions
- Relatively low viscosity - high efficiency, low pressure

If LC/MS is used, can substitute Formic or Acetic acid
Column Temperature

- Higher column temperature can dramatically improve resolution and recovery
- Column temperature can be used to fine tune a separation by affecting both the retention $k$ and selectivity $\alpha$
- Check manufacturer specs for compatibility
- Agilent AdvanceBio RP-mAb columns are rated to 90 °C

- About a 1% increase in T leads to a 1 to 2% decrease in $k$.
- Increase in T leads to decrease in pressure due to decrease in mobile phase viscosity.
- Increase in T also leads to decrease in peak widths.
- Also, use of a thermostat column compartment improves retention time precision.
Impact of Column Temperature on Intact mAb Analysis

AdvanceBio RP-mAb

80 °C
70 °C
60 °C
50 °C
40 °C

• Temperature has a significant impact on run time, peak shape and recovery.

• Higher temperatures better with optimum at ~ 80 °C for this example

AdvanceBio RP-mAb SB-C4, 2.1 x 100 mm

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Impact of Temperature on mAb Fragment Analysis

AdvanceBio RP-mAb

- High resolution separation of different variant peaks at higher temperature

AdvanceBio RP-mAb C4, 4.6 x 50 mm
The smaller the plate height, the higher the plate number and the greater the chromatographic resolution
Linear Velocity/Flow Rate

- Key Chromatographic Parameters Affected by Flow Rate
  - Resolution
  - Efficiency
  - Peak shape
  - Pressure

- Shorten analysis time by:
  - Reduced column length
  - Increase flow rate
Impact of Linear Velocity on Intact mAb Analysis

AdvanceBio RP-mAb

- Linear velocity has an impact on peak shape and resolution
- Higher velocities better with optimum at ~ 1mL/min for 2.1 mm id for this example

AdvanceBio RP-mAb SB-C4, 2.1 x 100 mm
Impact of Linear Velocity on mAb Fragment Analysis

AdvanceBio RP-mAb

- Linear velocity has strong impact on resolution.
- Higher velocities better with optimum at ~ 1 mL/min for 2.1 mm id for this example

AdvanceBio RP-mAb SB-C8, 2.1 x 100 mm
Gradient Slope

- Gradient steepness affects retention ($k^*$) and resolution.

- Adjust gradient slope to optimize resolution - accomplished by changing:
  - gradient time $t_G$ (most common way to change gradient steepness)
  - % change in organic modifier over time
Impact of Gradient Profile on mAb Fragment Analysis

AdvanceBio RP-mAb

• Gradient profile has impact on selectivity and resolution

• Steeper gradient better with optimum 5 – 50% for this example

AdvanceBio RP-mAb SB-C8, 2.1 x 100 mm
Column Robustness

- High Reproducibility
- Low Carry-over
- Long Column Lifetime
Reproducibility
AdvanceBio RP-mAb

- Injection to injection repeatability supports consistent column performance

<table>
<thead>
<tr>
<th>Analysis of intact mAb</th>
<th>Average</th>
<th>%RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RT</td>
<td>4.5404</td>
<td>0.09</td>
</tr>
<tr>
<td>Area</td>
<td>4521.294</td>
<td>0.09</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RT</td>
<td>4.8494</td>
<td>0.05</td>
</tr>
<tr>
<td>Area</td>
<td>158.43</td>
<td>0.80</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Analysis of reduced mAb</th>
<th>Average</th>
<th>%RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RT</td>
<td>3.234</td>
<td>0.06</td>
</tr>
<tr>
<td>Area</td>
<td>496.162</td>
<td>0.91</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RT</td>
<td>4.6574</td>
<td>0.04</td>
</tr>
<tr>
<td>Area</td>
<td>909.85</td>
<td>0.69</td>
</tr>
<tr>
<td>C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RT</td>
<td>5.0118</td>
<td>0.02</td>
</tr>
<tr>
<td>Area</td>
<td>127.028</td>
<td>1.10</td>
</tr>
</tbody>
</table>

AdvanceBio RP-mAb SB-C4, 4.6 x 50 mm
No Carry-Over

AdvanceBio RP-mAb

- No protein carry-over between runs

- Injection of 3 production batches of intact mAb each followed by 1 blank injection, shows no carry-over

- Injection of 3 production batches of reduced mAb each followed by 1 blank injection shows no carry-over

AdvanceBio RP-mAb SB-C4, 4.6 x 50 mm
Long Column Lifetime
AdvanceBio RP-mAb

- Stable over 1,000 injections = 40,000 column volumes

<table>
<thead>
<tr>
<th>Peak</th>
<th>Compound</th>
<th>RT_1st Injection</th>
<th>RT_1003 Injection</th>
<th>RT change (min)</th>
<th>RT change%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ribonuclease A</td>
<td>0.957</td>
<td>0.942</td>
<td>0.015</td>
<td>1.57%</td>
</tr>
<tr>
<td>2</td>
<td>Lysozyme</td>
<td>1.872</td>
<td>1.86</td>
<td>0.012</td>
<td>0.64%</td>
</tr>
<tr>
<td>3</td>
<td>Cytochrome C</td>
<td>2.111</td>
<td>2.097</td>
<td>0.014</td>
<td>0.66%</td>
</tr>
<tr>
<td>4</td>
<td>α-Lactalbumin</td>
<td>2.909</td>
<td>2.899</td>
<td>0.014</td>
<td>0.34%</td>
</tr>
<tr>
<td>5</td>
<td>Catalase</td>
<td>3.994</td>
<td>3.981</td>
<td>0.013</td>
<td>0.33%</td>
</tr>
<tr>
<td>6</td>
<td>Carbonic Anhydrase</td>
<td>3.994</td>
<td>3.981</td>
<td>0.013</td>
<td>0.33%</td>
</tr>
</tbody>
</table>

AdvanceBio RP-mAb SB-C8

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Excellent Batch-to-Batch Reproducibility
AdvanceBio RP-mAb (different column batches)

• Reproducible retention time and peak shapes over 6 different column batches

Samples
1. Ribonuclease A 13.7 kDa
2. Cytochrome C 12 kDa
3. Holo-Transferrin 80 kDa
4. α-Lactalbumin 14.2 kDa
5. Catalase 240 kDa
6. Carbonic Anhydrase 29 kDa

AdvanceBio RP-mAb SB-C4, 2.1 x 100 mm
Fast Intact mAb Analysis

AdvanceBio RP-mAb

- AdvanceBio RP-mAb C4 provides a sharp peak and resolves fine detail in less than two minutes
- AdvanceBio RP-mAb Diphenyl resolves additional fine detail - the Diphenyl phase is unique to Agilent

All phases provides good separation of intact mAb with AdvanceBio RP-mAb Diphenyl resolves additional fine detail.

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Fast Intact mAb Analysis

AdvanceBio RP-mAb

- AdvanceBio RP-mAb C4 provides better resolution than protein columns from competitors.
High Resolution Separation of Intact mAb

AdvanceBio RP-mAb

- AdvanceBio RP-mAb C4 provides superior peak shape at a lower pressure than a UHPLC protein column from a competitor.
Fast & High Efficiency Separation of Intact mAb

AdvanceBio RP-mAb

- AdvanceBio RP-mAb provides superior peak shape

Width = 0.219
Width = 0.187
Width = 0.227

AdvanceBio RP-mAb C4, 450Å, 3.5 μm
AdvanceBio RP-mAb Diphenyl, 450Å, 3.5 μm
Brand A, C4, 300Å, 3.5 μm

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High Resolution mAb Fragments: LC/UV

AdvanceBio RP-mAb

• AdvanceBio RP-mAb provides superior peak shape and high resolution than competitor protein column

Reduced mAb

\[ \text{AdvanceBio RP-mAb C4, 450Å, 3.5 μm} \]
\[ \text{Brand A , C4, 300Å, 3.5 μm} \]

Rs = 2.0
Rs = 0.6
Rs = 3.4
Rs = 0.8

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High Resolution ADC Fragments: LC/UV

AdvanceBio RP-mAb

- Separation of different drug conjugated species

Reduced ADC

Papain digested ADC

AdvanceBio RP-mAb C4, 450Å, 3.5 μm

Brand A, C4, 300Å, 3.5 μm

ADC: Lys conjugated

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High Resolution ADC Fragments: LC/UV

AdvanceBio RP-mAb

- Separation of different drug conjugated species

**Reduced ADC**

- Lys-conjugated ADC
- Cys-conjugated ADC

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High Resolution mAb Fragments: LC/UV

AdvanceBio RP-mAb

- Separation of stressed-induced antibody variants

Stressed variants

- mAb
- pH stressed mAb
- Oxidized mAb

AdvanceBio RP-mAb C4, 4.6 x 50 mm
Fast, High Resolution mAb Fragment Analysis

AdvanceBio RP-mAb

- AdvanceBio RP-mAb provides superior peak shape and resolution than other columns designed for protein separations

Papain digestion

Intact

2 * Fab

Fc

AdvanceBio RP-mAb C4, 450Å, 3.5 μm

Brand A       C4, 400Å, 3.4 μm

Brand B      C4, 200Å, 3.6 μm

Brand C      C4-30, 300Å, 2.6 μm

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Fast, High Resolution mAb Fragment Analysis

AdvanceBio RP-mAb

- Separation of mAb subunits with superior peak shape and resolution
## Benefits of AdvanceBio RP-mAb Columns

<table>
<thead>
<tr>
<th>Pain</th>
<th>Features and Advantages</th>
<th>Benefit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insufficient resolution</td>
<td>Superficially porous particles of smaller particles (3.5 µm) with wide pore (450Å) increase resolution for mAb but maintain compatibility with all LC instruments</td>
<td>Improved confidence in analysis results (accuracy)</td>
</tr>
<tr>
<td>Long analysis times</td>
<td>Due to Poroshell technology, analysis time of mAb characterization has been shown to decrease significantly over fully porous particles of the same size</td>
<td>Improved throughput - reduced costs</td>
</tr>
<tr>
<td>Short column lifetime</td>
<td>Column with robust Poroshell packed bed and with 2 µm frit decreases chances of bed-collapse or inlet blockage</td>
<td>Improved resource use - reduced costs</td>
</tr>
</tbody>
</table>
Summary
AdvanceBio RP-mAb

- AdvanceBio RP-mAb columns - analyzing monoclonal antibodies for biopharma discovery, development, and QA/QC applications

- **Improved accuracy**: Superficially porous particles (3.5 μm) with wide pores (450Å) increase mAb resolution while maintaining compatibility with all LC instruments

- **Speed**: Shorter analysis times compared to columns packed with fully porous particles of the same size

- **Lower costs**: The robust Poroshell packed bed and 2 μm inlet frit extend column lifetime by helping prevent inlet blockage

- **Flexible method development**: Range of chemistries – SB-C8, C4, and Diphenyl
Q & A