High Resolution Separation Media for High Throughput Monoclonal Antibody Analysis

Srinivasa Rao, Julia Baek, Ilze Birznieks, Yury Agroskin and Chris Pohl
Thermo Scientific, Sunnyvale, CA, USA
Overview

Purpose: Demonstrate advantages of high resolution media for high-throughput monoclonal antibody (MAb) analysis.

Methods: High throughput, high resolution separation of MAbs is achieved with the Thermo Scientific™ Dionex™ UltiMate™ 3000 Biocompatible Rapid Separation (BioRS) system using the Thermo Scientific™ Dionex™ Chromleon™ Chromatography Data System software.

Results: High pressure bio-inert column hardware was specifically used to achieve high flow rates without compromising the resolution of MAb analysis. By employing small particle resin packed into longer columns with higher flow rates are used to achieve fast, high resolution separation of MAbs.

Introduction

MAbs represent a major class of bio-therapeutic molecules that usually display complex micro-heterogeneity with several post-translational modifications including oxidation, isomerization, deamidation, glycation and others. Primary structure alterations such as lysine truncations are also known to occur in the C-terminus region of MAbs. Due to these possibilities, quality control and stability assessment of MAbs are very challenging tasks. The increasing utilization of MAbs in the pharmaceutical industry is also driving a growing demand for improved high resolution stationary phases for characterization of MAbs.

Previously introduced Thermo Scientific™ MAbPac™ strong cation-exchange phases are based on particle sizes of 10 μm, 5 μm and 3 μm resins for MAb charge variant separations. These small particle size phases were developed specifically to address the requirement of high resolution variant analysis of MAbs. However, there is a need in the industry to have analytical columns that combine uncompromised resolution power with high flow rate compatibility to achieve high-throughput separation of MAbs.

With the launch of a new, totally bio-inert high pressure UltiMate BioRS system with maximum pressure of 15000 psi, we have developed 5 μm polymeric particle size columns that are suitable for high throughput high resolution MAb analysis. Bio-inert column hardware is a critical component for any MAb separation to avoid metal interferences with analytes of interest. Here, we utilized a PEEK-lined stainless steel column bodies that are suitable for high pressure operations, providing a metal-free fluidic path.

This work describes the development and applications of 5 μm small particle columns in two different internal diameters (I.D; 4.6 and 2.1 mm) with various lengths for high-throughput, high-resolution MAb analysis. Isocratic and gradient analysis are performed to evaluate the asymmetry, efficiency, resolution and ruggedness of these different format columns.

Methods

Samples

Monoclonal antibody samples are received from local biotechnology companies. Cytochrome C (Equine) and other chemicals are obtained from Sigma/Aldrich®.

Columns

PEEK-lined Stainless Steel Columns

- MAbPac SCX-10 RS, 5 μm, 4.6 × 50 mm (P/N 082674)
- MAbPac SCX-10 RS, 5 μm, 4.6 × 150 mm (P/N 085209)
- MAbPac SCX-10 RS, 5 μm, 4.6 × 250 mm (P/N 082673)
- MAbPac SCX-10 RS, 5 μm, 2.1 × 50 mm (P/N 082675)
- MAbPac SCX-10 RS, 5 μm, 2.1 × 150 mm (P/N 088242)
- MAbPac SCX-10 RS, 5 μm, 2.1 × 250 mm (P/N 082515)

Methods

Salt Gradients are performed using MES buffers. pH gradients are performed using Thermo Scientific™ pH platform using CX-1 buffer kits (P/N: 083274; P/N: 085349)

Eluent and gradient details are given within the figures.
High Pressure Liquid Chromatography (HPLC)
HPLC experiments were carried out using a New UltiMate 3000 BioRS high pressure totally inert system equipped with:
Gradient Pump System; TCC-3000RS Thermostatted Column Compartment
WPS-3000 TRS Auto sampler;
VWD-3400RS UV Detector equipped with a Micro Flow Cell;

Chromatography was controlled by Chromeleon Chromatography Data System.

BioRS HPLC Instrument Specifications:
- Bio-inert materials
- Pressure of up to 1034 bar (~15000 Psi)
- Flow rates of up to 8 mL/min
- Short sampler cycle times
- High column temperatures
- Ultrafast data collection and processing

Separation media and mechanism of cation exchange column

- Substrate Monomers: Ethylvinylbenzene-divinylbenzene
- Substrate Pore Size: Non-porous
- Cross-linking: 55%
- Mode of Interaction: Cation Exchange
- Functional Group: Sulfonic Acid; SCX
- Separation Mechanism: Charge-Charge Interaction; By increasing ionic strength, or by pH

Results

Isocratic testing with Cytochrome C

Figure 1. Isocratic testing of MAbPac SCX-10 RS, 5 µm, 4.6 mm columns: Comparison of different lengths of columns.
(Please see Table 1 for chromatography data)

Table 1: Isocratic testing of MAbPac SCX-10 RS, 5 µm, 4.6 mm columns
Comparison of different lengths of columns (From Figure 1).

<table>
<thead>
<tr>
<th>Column</th>
<th>Flow Rate (mL/min)</th>
<th>Pressure (psi)</th>
<th>RT (minutes)</th>
<th>Asymmetry (AIA)</th>
<th>Efficiency (plates)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A MAbPac SCX-10, 5 µm, 4.6 × 50 mm</td>
<td>1.0</td>
<td>1,858</td>
<td>2.52</td>
<td>1.92</td>
<td>1,851</td>
</tr>
<tr>
<td>B MAbPac SCX-10 RS, 5 µm, 4.6 × 150 mm</td>
<td>1.0</td>
<td>3,216</td>
<td>8.43</td>
<td>1.69</td>
<td>6,285</td>
</tr>
<tr>
<td>C MAbPac SCX-10 RS, 5 µm, 4.6 × 250 mm</td>
<td>1.0</td>
<td>4,798</td>
<td>13.10</td>
<td>1.71</td>
<td>10,147</td>
</tr>
</tbody>
</table>
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Comparison of different lengths of columns.

Table 1: Isocratic testing of MAbPac SCX-10 RS, 5 μm, 2.1 mm columns:
Comparison of different lengths of columns. (From Figure 1).

<table>
<thead>
<tr>
<th>Column</th>
<th>Flow Rate (mL/min)</th>
<th>Pressure (psi)</th>
<th>RT (minutes)</th>
<th>Asymmetry (AIA)</th>
<th>Efficiency (plates)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.21</td>
<td>1.148</td>
<td>3.00</td>
<td>2.00</td>
<td>1,812</td>
</tr>
<tr>
<td>B</td>
<td>0.21</td>
<td>2.360</td>
<td>7.38</td>
<td>1.33</td>
<td>6,436</td>
</tr>
<tr>
<td>C</td>
<td>0.21</td>
<td>3.805</td>
<td>13.67</td>
<td>1.27</td>
<td>9,211</td>
</tr>
</tbody>
</table>

Isocratic testing with Cytochrome C

Figure 1. Isocratic testing of MAbPac SCX-10 RS, 5 μm, 2.1 mm columns:
Comparison of different lengths of columns. (Please see Table 2 for chromatography data)

Gradient testing with monoclonal antibodies

Figure 3. MAb separation on MAbPac SCX-10 RS, 5 μm 4.6 × 150 and 4.6 × 250 mm.
Resolution is maintained even at higher flow rates.

MAb Analysis on a 4.6 × 150 mm column at a flow rate of 2.0 mL/min (12 minute gradient; Figure 3B) resolution values are slightly diminished as compared to 1.3 mL/mL flow rate (20 minute gradient; Figure 3A). Highest resolution for MAb analysis is achieved with 4.6 × 250 mm column Figure 3C).
Methods

MAbPac SCX-10 RS, 5 µm, 2.1 × 250 mm (P/N 082515)

MAbPac SCX-10 RS, 5 µm, 2.1 × 150 mm (P/N 088242)

performed to evaluate the asymmetry, efficiency, resolution and ruggedness of these in two different internal diameters (I.D; 4.6 and 2.1 mm) with various lengths for high-fluidic path.

With the launch of a new, totally bio-inert high pressure UltiMate BioRS system with MAbPac SCX-10 RS, 5 µm, 4.6 × 50 mm (P/N 082674) PEAK-lined Stainless Steel Columns

Cytochrome C (Equine) and other chemicals are obtained from Sigma/Aldrich®.

Methods

High resolution separation media for high throughput monoclonal antibody analysis

Introduction

Chromatography was controlled by Chromeleon Chromatography Data System.

VWD-3400RS UV Detector equipped with a Micro Flow Cell;

BioRS HPLC Instrument Specifications:

• Pressure of up to 1034 bar (~15000 Psi)
• Short sampler cycle times
• Ultrafast data collection and processing
• High column temperatures

Previously introduced alterations such as lysine truncations are also known to occur in the C-terminus region of proteins. Oxidation, isomerization, deamidation, glycation and others. Primary structure

Table 1: Isocratic testing of MAbPac SCX-10 RS, 5 μm, 4.6 × 150 mm columns.

Comparison of different lengths of columns. (Please see Table 1 for chromatography data)

Table 2. Isocratic testing of MAbPac SCX-10 RS, 5 μm, 2.1 × 150 mm columns:

Table 3: Ruggedness testing of MAbPac SCX-10 RS, 5 μm, 4.6 × 150 mm column.

For pH gradient separation, CX-1 pH gradient buffers are used (P/N: 083274). pH gradient method offers ease of method development and excellent selectivity and separation of variants.

Figure 4. MAb separation using either salt, or pH gradients

A. Salt Gradient

Column: MAbPac SCX-10 RS, 5 μm, 4.6 × 150 mm
Eluent A: 20 mM MES (pH 5.5) + 300 mM NaCl
Eluent B: 20 mM MES (pH 5.5) + 300 mM NaCl
Gradient: 20-90% B in 10 minutes
Sample: MAb, 5 mg/mL
Injection Volume: 12 μL
Flow Rate: 2.0 mL/min

B. pH Gradient

Column: MAbPac SCX-10 RS, 5 μm, 4.6 × 150 mm
Eluent A: 1X CX-1, pH Gradient Buffer A (pH 5.6)
Eluent B: 1X CX-1, pH Gradient Buffer B (pH 10.2)
Gradient: 20-90% B in 10 minutes
Sample: MAb, 5 mg/mL
Injection Volume: 12 μL
Flow Rate: 2.0 mL/min

Figure 5. MAb separation on MAbPac SCX-10 RS, 5 μm, 2.1 × 150 mm column.

Column: MAbPac SCX-10 RS, 5 μm
Dimension: 2.1 × 150 mm
Sample: MAb 5mg/mL
Injection Volume: 2 μL

Eluents:
Eluent A: 20 mM MES (pH 5.5)
Eluent B: 20 mM MES (pH 5.5) + 300 mM NaCl

A. Flow Rate: 0.21 mL/min
Gradient: 30-60% B in 24 min

B. Flow Rate: 0.42 mL/min
Gradient: 30-60% B in 12 min

Resolution values are shown for lysine truncation variants.

Two different flow rates and gradient conditions are used. Even at 0.42 mL/min flow rate (Panel B), resolution values are comparable to 0.21 mL/min flow rate (Panel A). At high flow rates analysis is faster and improves throughput.

Figure 6. Ruggedness testing of MAbPac SCX-10 RS, 5 μm, 4.6 × 150 mm column.

MAb sample is injected intermittently. Peak width at half height (Minutes) is shown in Table 3 for lysine truncation peaks 1,2 and 3. More than 300 runs are performed indicating that the column is quite rugged.

For property rights of others.

Acknowledgements

Srinivasa Rao, Julia Baek, Ilze Birznieks, Yury Agroskin and Chris Pohl, Thermo Scientific, Sunnyvale, CA, USA

In insightful discussions.

Conclusion

High throughput, high resolution separation of MAb is achieved with the totally inert system equipped with:

- Bio-inert materials
- Pressure of up to 1034 bar (~15000 Psi)
- Short sampler cycle times
- Ultrafast data collection and processing
- High column temperatures

The method offers ease of method development and excellent selectivity and separation of variants.

For pH gradient separation, CX-1 pH gradient buffers are used (P/N: 083274). pH gradient method offers ease of method development and excellent selectivity and separation of variants.
Methods

MAbPac SCX-10 RS, 5 µm, 2.1 × 150 mm (P/N 088242)
MAbPac SCX-10 RS, 5 µm, 2.1 × 50 mm (P/N 082675)
MAbPac SCX-10 RS, 5 µm, 4.6 × 250 mm (P/N 082673)
MAbPac SCX-10 RS, 5 µm, 4.6 × 150 mm (P/N 085209)

In different formats columns.

performed to evaluate the asymmetry, efficiency, resolution and ruggedness of these
throughput, high-resolution MAb analysis. Isocratic and gradient analysis are

fluidic path.

column bodies that are suitable for high pressure operations, providing a metal-free

PEEK-lined Stainless Steel Columns

Cytochrome C (Equine) and other chemicals are obtained from Sigma/Aldrich®.

separations. These small particle size phases were developed specifically to address
are based on particle sizes of 10 μm, 5 μm and 3 μm resins for MAb charge

Previously introduced phases for characterization of MAbs.

industry is also driving a growing demand for improved high resolution stationary

oxidation, isomerization, deamidation, glycation and others. Primary structure
complex micro-heterogeneity with several post-translational modifications including

MAbs represent a major class of bio-therapeutic molecules that usually display

Table 1: Isocratic testing of MAbPac SCX-10 RS, 5 μm, 2.1 mm columns:

<table>
<thead>
<tr>
<th>Sample No</th>
<th>Peak 1</th>
<th>Peak 2</th>
<th>Peak 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>28</td>
<td>0.099</td>
<td>0.099</td>
<td>0.104</td>
</tr>
<tr>
<td>51</td>
<td>0.098</td>
<td>0.097</td>
<td>0.104</td>
</tr>
<tr>
<td>74</td>
<td>0.100</td>
<td>0.098</td>
<td>0.105</td>
</tr>
<tr>
<td>97</td>
<td>0.098</td>
<td>0.096</td>
<td>0.103</td>
</tr>
<tr>
<td>120</td>
<td>0.098</td>
<td>0.098</td>
<td>0.103</td>
</tr>
<tr>
<td>143</td>
<td>0.100</td>
<td>0.096</td>
<td>0.104</td>
</tr>
<tr>
<td>188</td>
<td>0.107</td>
<td>0.105</td>
<td>0.106</td>
</tr>
<tr>
<td>231</td>
<td>0.103</td>
<td>0.100</td>
<td>0.105</td>
</tr>
<tr>
<td>274</td>
<td>0.115</td>
<td>0.111</td>
<td>0.111</td>
</tr>
<tr>
<td>317</td>
<td>0.107</td>
<td>0.103</td>
<td>0.112</td>
</tr>
<tr>
<td>Average</td>
<td>0.103</td>
<td>0.100</td>
<td>0.106</td>
</tr>
<tr>
<td>RSD (%)</td>
<td>5.47</td>
<td>4.75</td>
<td>3.03</td>
</tr>
</tbody>
</table>

Peak width at half height (Minutes) is shown for MAb lysine truncation variants
(Data is derived from Figure 6).

Summary

• A New UltiMate 3000 BioRS high pressure totally inert system was used. PEEK-lined
stainless steel columns that are suitable for high pressure operations were used to
avoid any metal related interferences with MAb/protein chromatography.

• MAbPac SCX-10 RS, 5 µm columns are developed in 2.1 mm and 4.6 mm I.D
formats. Three different length columns (50 mm, 150 mm and 250 mm) are made
available to offer various method development requirements. While both formats offer
similar resolution and throughput, the larger I.D 4.6 mm columns are specifically
useful for high sample loadability and the smaller I.D 2.1 mm columns are for
conserving sample and eluent usage.

• A comparison of Isocratic separation of Cytochrome C on MAbPac SCX-10, 5 μm,
4.6 mm and 2.1 mm I.D columns with different lengths is shown in Figure 1 and
Table 1 and Figure 2 and Table 2 respectively. As expected, the highest plate number
for Cytochrome C separation is achieved with the longest column as compared to
other shorter columns.

• Higher pressure compatibility of the column hardware allows the use of high flow
rates 2 mL/min for 4.6 × 150 mm (Figure 3; Panel B); 0.42 mL/min for 2.1 × 150 mm
(Figure 5; Panel B), while maintaining decent resolution. This results in faster analysis
and improves throughput.

• MAb analysis is routinely performed by using either salt gradient, or pH gradient.
PH gradient offers ease in method development process as well as better selectivity
than salt gradients for a majority of MAbs (Figure 4; Panel B).

• Ruggedness of MAbPac SCX, 5 μm, 4.6 × 150 mm column for over 300 runs
without any major changes in peak width at half height clearly supports the view that
the column is quite rugged (Figure 6 and Table 3).

Conclusion

This study demonstrates successful development of MAbPac SCX-10 RS, 5 µm columns
in different formats for high throughput and high resolution MAb analysis. This study also,
shows successful usage of UltiMate 3000 BioRS high pressure inert system along with
the PEEK lined stainless steel column hardware for high pressure separation
applications.

Acknowledgements

We thank Yuanxue Hou, Hongmin Zhang for providing us the small particle resin and
Doug Jamieson for helping us with the column hardware acquisition, packing and other
insightful discussions.