High Resolution Glycopeptide Mapping of EPO Using an Agilent AdvanceBio Peptide Mapping Column

Application Note

BioPharma

Authors

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Abstract

The 2.7 µm Agilent AdvanceBio Peptide Mapping column is specifically designed to improve separations of peptide and peptide mapping applications. The column is based on superficially porous technology and was developed for fast and efficient separation of complex peptide mapping mixtures. This application note demonstrates peptide mapping performance with the AdvanceBio Peptide Mapping column for profiling a tryptic digest of recombinant human erythropoietin (rhEPO) protein as a model digest for glycopeptide profiling. Like many protein therapeutics, rhEPO exhibits a great deal of heterogeneity due to modifications that occur during manufacturing. The AdvanceBio Peptide Mapping column, with an optimized C18 coating technology, provides excellent peptide selectivity and resolution across a broad elution range to enhance separation of the glycopeptide fragments for fast, efficient, and sensitive mass spectrometry analysis.
Introduction

Peptide mapping using reversed-phase HPLC of proteolytic peptides, combined with electrospray ionization mass spectrometry (ESI/MS), has become the method of choice for establishing the identity and efficacy of biotherapeutic products [1]. Peptide fragment analysis by liquid chromatography/mass spectrometry (LC/MS) is a crucial requirement for the release of these products and plays an important role to complete the protein characterization strategy. For example, the stability of a recombinant protein therapeutic requires long term monitoring throughout the shelflife of the product for important modifications, such as glycosylation. Although glycosylation can be analyzed on the intact protein, glycopeptide mapping is necessary to provide additional information of critical importance, such as sequence information, mass analyses, and identification of the glycosylation sites.

This application note used an rhEPO protein as a model glycoprotein to demonstrate high resolution peptide mapping and glycopeptide analysis by LC/MS using a 2.7 µm AdvanceBio Peptide Mapping column.

The column was specifically designed to improve separations for peptide mapping applications and provided increased resolution, higher sensitivity, and greater selectivity needed for high efficiency peptide profiling, such as that required for rhEPO mapping and enhanced glycopeptide mining. Furthermore, the 2.7 µm superficially porous particles enabled the use of a longer column (2.1×250 mm) at a higher flow rate delivering sub-2 µm type resolution within HPLC column pressures.

Materials and Methods

Recombinant human EPO was purchased from Creative Biolab, Shirley, NY. Trifluoroacetic acid was purchased from Sigma-Aldrich Corp., St. Louis, MO, and iso-propanol and acetonitrile were supplied from Honeywell-Burdick & Jackson, Muskegon, MI. High quality sequence-grade trypsin was obtained from the Stratagene division of Agilent Technologies.

Protease digestion was accomplished by adding trypsin protease to a solution including approximately 4.2 mg (2.1 mg/mL, 2 mL) EPO. The ratio of substrate and enzyme was 50:1 (w:w). The mixed solution was incubated at 37 °C for 12 hours. The digestion was quenched by storing the sample at -70 °C. After validation, 3.78 mg (2.1 mg/mL, 1.8 mL) of digested EPO was obtained.

An Agilent 1200 Infinity LC System was used, with auto injector (HiP-ALS), binary pump, thermostatted column compartment (TCC) and diode array detector (DAD), coupled to an Agilent 6224 TOF LC/MS. Data was processed using an Agilent MassHunter Qualitative Analysis software and an Agilent MassHunter BioConfirm.

TOF MS parameters

Spectra were recorded in positive ion and in centroid mode.

Gas temperature: 350 °C
Drying gas: 10 L/min
Nebulizer: 45 psi
V_{cap}': 3,500 V
Fragmentor: 170 V
Skimmer: 65 V
Octapole 1 RF: 750 V
MS: 4 Hz
Mass range: 100 to 7,000 m/z
Reference mass: 149.02332
922.009798
Acq. mode: Extended dynamic range mode (2 GHz)

Chromatographic conditions

Column: Agilent AdvanceBio Peptide Mapping, 2.1×250 mm, 2.7 µm (p/n 651750-902)
Eluent: A, H_{2}O + 0.1% formic acid (v/v);
B, acetonitrile + 0.1% formic acid (v/v)
Injection volume: 5 µL (2 µg/µL)
Flow rate: 0.4 mL/min
Gradient:

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>% B</th>
</tr>
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<tr>
<td>0</td>
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<tr>
<td>28</td>
<td>45</td>
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<td>33</td>
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<td>34</td>
<td>95</td>
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</tbody>
</table>
Temperature: 55 °C
Results and Discussion

Optimized rhEPO HPLC peptide mapping with an AdvanceBio Peptide Mapping column

Systematic method development was performed for mapping the rhEPO digest to determine the best resolved separation under water/ACN conditions. During these evaluations, 2.1 × 150 mm and 2.1 × 250 mm AdvanceBio Peptide Mapping columns were evaluated under various flow, temperature, and gradient profiles. Additionally, since peptide mapping separations work well under conditions that are favorable for ESI/MS, separations were evaluated with trifluoroacetic acid (TFA) and formic acid (FA) ion-pair additives to determine effects on retention and peak shape. The AdvanceBio Peptide Mapping column delivered excellent separation performance and compatibility with both TFA and FA ion-pair additives. The optimized separation displayed in Figure 1 provided the best separation profile for rhEPO mapping. The 2.1 × 250 mm AdvanceBio Peptide Mapping column and optimized chromatographic conditions with formic acid as the ion-pair additive enabled a high resolution separation of the rhEPO tryptic digest, providing very narrow peak widths, increased sensitivity, and unique selectivity across the gradient profile, making this separation highly amenable to ESI/MS analysis.

Figure 1. Reversed-phase rhEPO peptide map using a 2.1 × 250 mm, 2.7 μm Agilent AdvanceBio Peptide Mapping column (See Chromatographic conditions section).
Rapid rhEPO tryptic digest mapping by ESI-MS

Peptide maps often take 2 hours or longer using a 2.1 × 250 mm column to produce the needed resolution. Additionally, re-equilibration and run-to-run cycle times can add extended time to the completed analysis, significantly affecting laboratory production. While faster maps are desirable, it is critically important that resolution is not compromised. Figure 2 shows the total ion chromatogram (TIC) during a fast LC/MS analysis on a 2.1 × 250 mm column completed in less than 35 minutes. Excellent peak retention, peak shapes, and resolution across the gradient are all indicators of a robust method for LC/MS validation of the peptide mapping analysis.

Figure 2. Total ion chromatogram of an LC/MS analysis on a 2.1 × 250 mm Agilent AdvancedBio Peptide Mapping column accomplished in under 35 minutes.
The faster analysis gained from the AdvancedBio Peptide Mapping column did not compromise the chromatographic map of rhEPO separation. Figure 3 shows the results from the Agilent MassHunter Molecular Feature Extractor (MFE). MFE is an algorithm that finds compounds from complex data and creates averaged MS spectra for each compound. These MFE compound results are then matched back to the rhEPO protein sequence. This 34 minute LC/MS method resulted in 100% sequence coverage of the rhEPO protein.

Figure 3. 100% rhEPO sequence coverage achieved using the 2.1 × 250 mm Agilent AdvanceBio Peptide Mapping column. Data generated using the MFE in Agilent MassHunter qualitative analysis software.
The rhEPO has a predicted molecular mass of 24,000 Dalton and apparent glycosylated molecular mass of 30,400 Dalton, and, thus, serves as an effective glycoprotein model to demonstrate glycopeptide profiling using the AdvanceBio Peptide Mapping column. Figure 4 is the extracted peptide glycosylation profile from the total LC/MS analysis shown in Figure 3, while Table 1 shows the mass and glycosylated sequence information. The AdvanceBio Peptide Mapping column enabled the identity of 42 unique glycopeptides and demonstrated the performance advantage and utility of this column for highly complex peptide mapping, where post-translational modification information, such as glycosylation, is critical to the complete protein characterization.

![Figure 4. MFE extracted peptide glycosylation profile using the 2.1 x 250 mm Agilent AdvanceBio Peptide Mapping column. Data generated using Agilent MassHunter Molecular Feature Extractor (MFE) on an Agilent 6224 TOF LC/MS.](image)
<table>
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<th>RT</th>
<th>Glyco-Peptide Accurate Mass</th>
<th>Pred glycan modification [seq. location]</th>
<th>Sequence</th>
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Table 1: Predicted N-linked glycan modifications and sequence for recombinant human EPO (continued).

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<th>Glyco-Peptide Accurate Mass</th>
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</table>

Conclusions

The Agilent 2.7 µm AdvanceBio Peptide Mapping column demonstrated excellent utility for fast and efficient LC/MS peptide mapping using a recombinant human erythropoietin protein digest. A 2.1 × 250 mm column dimension, and chromatographic conditions optimized for mass spectrometry analysis, provided high resolution separation of the tryptic rhEPO digest across the entire gradient profile demonstrating unique selectivity and retention features critical to generating a well-defined and resolved peptide map. The AdvanceBio Peptide Mapping column enabled 100% rhEPO sequence coverage by ESI/MS and provided 42 glycopeptide matches, making this column an excellent choice for highly complex peptide mapping applications.

Reference


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