Peptide Mapping of Glycoprotein Erythropoietin by HILIC LC/MS and RP-LC/MS

Application Note

BioPharma

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Introduction

Peptide mapping is an important technique for the comprehensive characterization of protein biotherapeutics. Reversed-phase (RP) UHPLC/HPLC is routinely used, but if the digest contains hydrophilic peptides, valuable information can be missed. This application note demonstrates peptide mapping of digested glycoprotein erythropoietin (EPO) using HILIC chromatography as a complementary approach to RP peptide analysis. An Agilent ZORBAX Rapid Resolution High Definition 300-HILIC 1.8 µm LC column and an Agilent AdvanceBio Peptide Mapping RP column, in combination with time of flight (TOF) mass-spectrometry (MS), were used for mapping EPO protein. Taking advantage of the high organic solvent system of the mobile phase for HILIC (hydrophilic interaction chromatography), the digested peptides from these analyses were evaluated and compared for sequence coverage and peptide identification. This application note demonstrates the utility of HILIC as an orthogonal and complementary approach to RP LC/MS for peptide analysis.

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Experimental

Sample preparation

A sample of trypsin-digested EPO glycoprotein was purchased from Bio Creative, Shirley, NY; 100 µL of sample (2 mg/mL) was mixed with 100 µL of HILIC or RP eluent A solvent, as appropriate.

Operating conditions

Experiments were performed on a UHPLC/TOF system, consisting of an Agilent 1290 Infinity LC, accurate-mass 6224 TOF LC/MS, with dual ESI source in positive mode. Peptides from trypsin-digested EPO protein were separated using different HILIC and RP conditions.

HILIC conditions

Columns:	Agilent ZORBAX Rapid Resolution High Definition 300-HILIC, 2.1 × 100 mm, 1.8 µm (p/n 858750-901)	
Eluent:	A, 95% ACN + 5% water; B, 50 mM ammonium formate, pH 4.0	
Flow rate:	0.4 mL/min	
Gradient:	Time (min)	% B
	0	0
	15	100
	15.1	0
	20	0
Temperature:	55 °C	

Reversed-phase conditions

Column:	Agilent AdvanceBio Peptide Mapping, 2.1 × 250 mm, 2.7 µm (p/n 653750-902)	
Eluent:	A, 100% water, 0.1% formic acid; B, 100% ACN, 0.1% formic acid	
Flow rate:	0.4 mL/min	
Gradient:	Time (min)	% B
	0	3
	3	3
	33	45
	38	60
Temperature:	55 °C	

MS conditions

Gas temperature:	350 °C
Gas flow:	10 L/min
Nebulizer:	45 psi
Capillary voltage:	3,500 V
Fragmentor:	170 V
Scan rate:	2 spec/s
Mass range:	400 to 3,200 *m/z*

Results and Discussion

The elution order in reversed-phase and hydrophilic interaction chromatography is orthogonal. In reversed-phase separation, the digested peptides from EPO protein are eluted in order of increasing hydrophobicity. With hydrophilic interaction chromatography, the least hydrophobic peptides (hydrophilic) will be retained most strongly by the column, thus, the elution order is reversed. The use of HILIC columns for the analysis of the peptides obtained from an enzymatic digest of a protein would, compared with RP columns, be expected to provide increased retention and resolution of the hydrophilic peptides, including glycopeptides. Hence, digested peptides, that may not have been retained and resolved by RP, can be identified by HILIC.

The biotherapeutic glycoprotein, EPO, is a small protein and has a molecular weight of approximately 34,000 Da. It is known to be heavily glycosylated and, therefore, a tryptic digest would be expected to contain a range of peptides, including hydrophilic peptides and glycopeptides.

Figures 1A and 1B show a comparison of mass-spectrometry analysis of digested peptides from EPO glycoprotein using a ZORBAX RRHD 300-HILIC column and an AdvanceBio Pepping Mapping RP column.

The HILIC LC/MS results were extracted using the Agilent MassHunter molecular feature extractor and then matched to the digested EPO protein sequence, showing that the sequence coverage was 100% (Figure 1 A). Note that the separation took less than 15 minutes.

The same sample was then analyzed using the AdvanceBio Peptide Mapping RP column. Extracted compounds of matching EPO digested peptides again showed 100% sequence coverage (Figure 1 B).

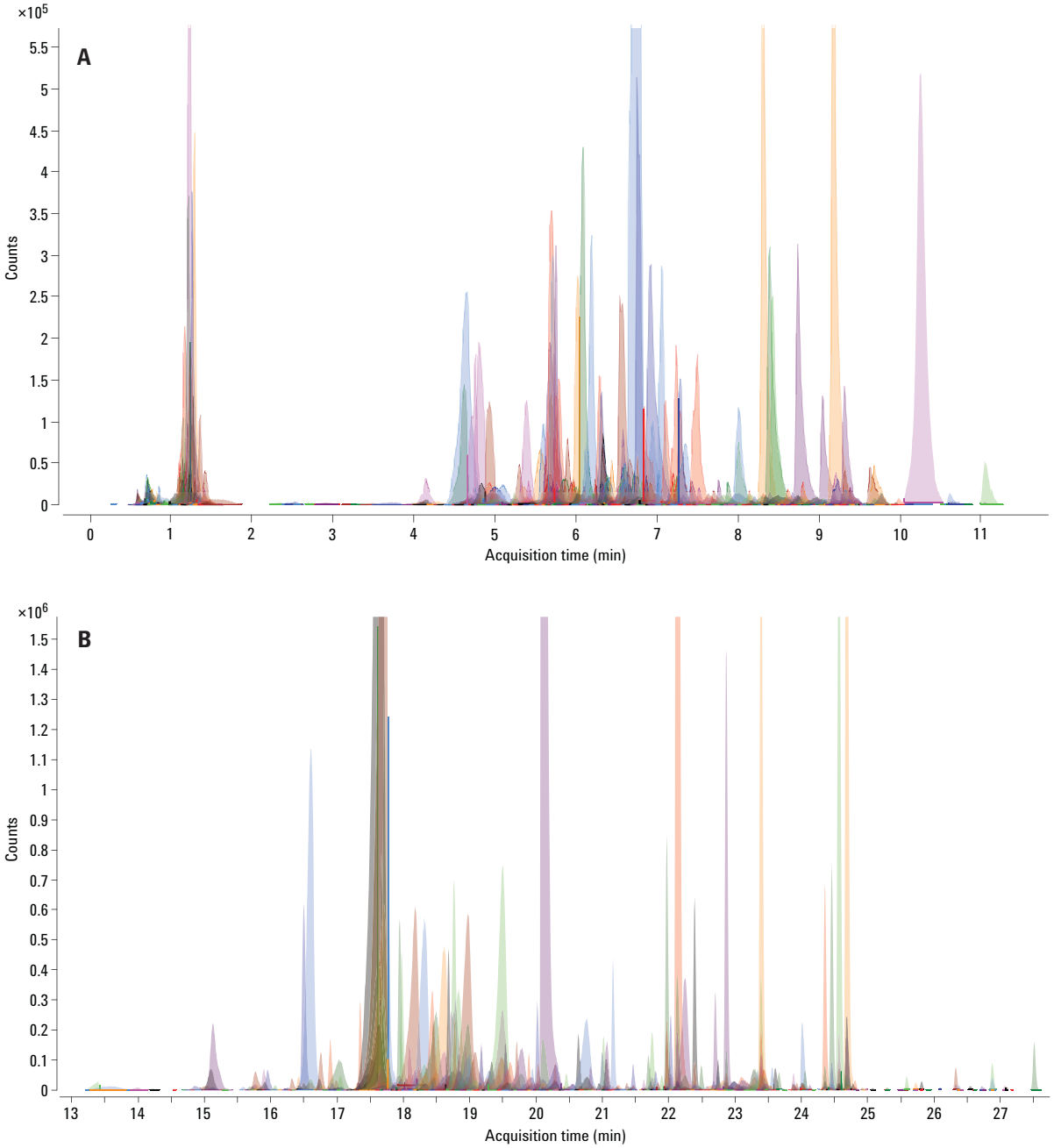

Figure 1. (A) Extracted compound chromatograms of matched EPO digested peptides from an Agilent ZORBAX RRHD 300-HILIC column and (B) an Agilent AdvanceBio Peptide Mapping RP column, both using the Agilent MassHunter molecular feature extractor.

Peptides common to HILIC and RP

Eight peptides were present from both columns when the data were compared. This indicated that these peptides had affinity for both modes of chromatography (Table 1). Generally, but not always, the elution order of the HILIC profile will be opposite that of the RP profile. Elution orders are dictated by hydrophobicity and charge (on the peptides). Therefore, the HILIC order does not necessarily go from 8 to 1 as shown in Figure 2.

Figure 2 shows that peptides P1 to P4 were resolved better with the ZORBAX RRHD 300-HILIC column. They eluted together on the reversed-phase column.

Table 1. Peptides common to both columns.

Peptide	Sequence	Hydrophobicity	RP retention time (min)	HILIC retention time (min)
P1	APPR	1.83	2.103	10.259
P2	GKLK	3.84	2.118	8.437
P3	ALGAQK	4.57	2.119	9.181
P4	AVSGLR	9.15	2.13	8.316
P5	YLLEAK	19.64	15.698	8.014
P6	VYSNFLRGK	23.14	18.742	6.583
P7	SLTTLLR	24.79	20.109	7.492
P8	VNFYAWKR	27.64	22.87	0.587

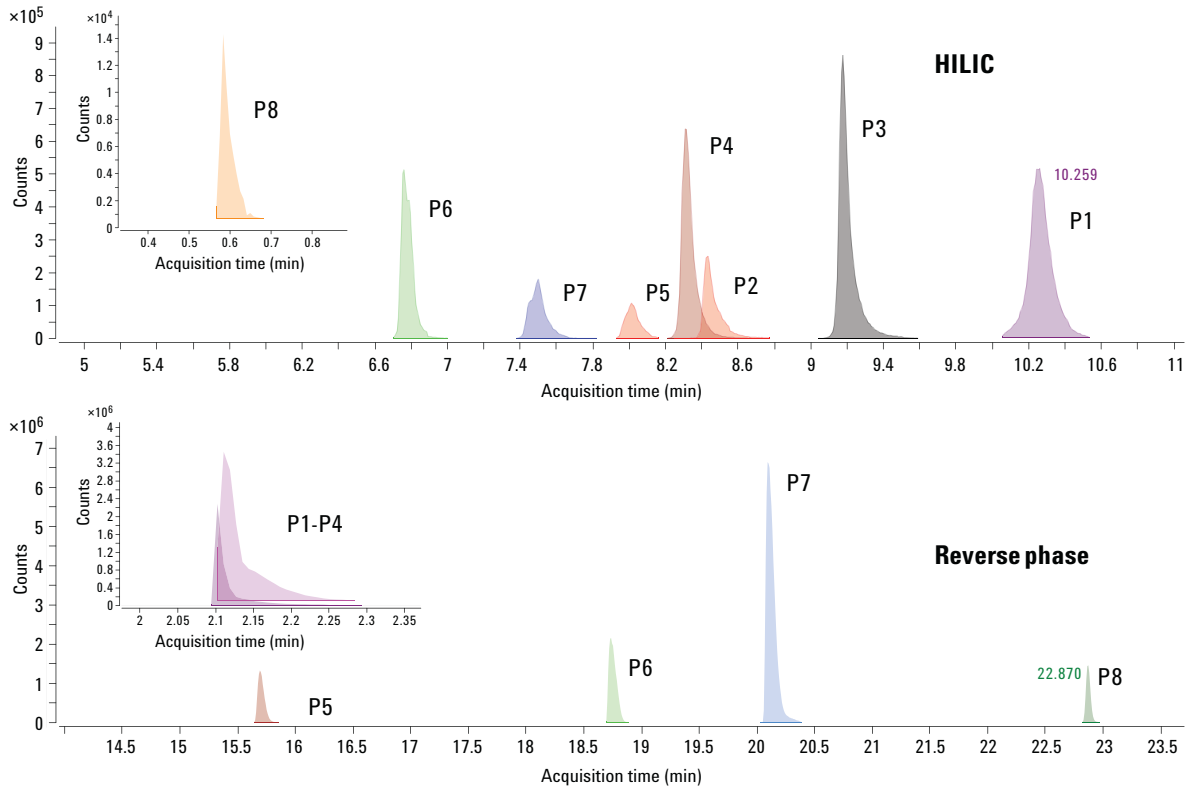

Figure 2. Comparing eight peptides from both columns for their retention times and resolutions.

Peptides found only from HILIC

Generally, under RP conditions, the least hydrophobic peptides (hydrophilic) will elute early making their quantitation by MS analysis more difficult. Some very hydrophobic peptides are difficult to dissolve in aqueous conditions, which are usually used as solvents for RP LC/MS analysis. This also leads to lower sensitivity. Therefore, with high organic solvent mobile phase, and the sample mixed with a high percentage organic solvent, some highly hydrophobic peptides will be dissolved and separated better with the HILIC column. Data from Table 2 provides an example showing hydrophilic peptides that are only be identified by HILIC LC/MS.

Table 2. Peptides identified only from the HILIC column.

No	Sequence	Hydrophobicity	Retention time (min)	Height
1	VLER	6.24	6.747	1396603
2	LKLYTGEACRTGDR	18.13	9.175	2192
3	ALGAQKEAISPPDAASAAPLRTITADTFR	37.09	11.059	5263
4	APPRLICDSRVLER	27.7	6.493	1485
5	GQALLVNSSQPWEPLQLHVDK	40.19	9.103	1995
6	KLFRVYSNFLR	36.51	4.629	3745
7	LFRVYSNFLR	35.24	4.945	1972

Glycopeptides from trypsin-digested EPO protein found from HILIC column

The data in Table 2 shows that peptide number 5 is the glycopeptide found only in the HILIC column. Its sequence location, retention, and glycosylation identification are indicated in Table 3. Using a ZORBAX RRHD 300-HILIC column, one additional glycopeptide with the sequence EAENITTGCAEHCSLNENITVPDTK was identified as having four different glycoforms (Table 3).

EPO glycoprotein has three N-glycosylation sites at sequence locations 24, 38, and 83. The glycopeptides identified by the HILIC column are listed in Table 3 with the glycosylation sites in red. Based on their retention times of the MS chromatogram (Fig 1A), these glycopeptides are very hydrophilic peptides. Similarly, four glycoforms of glycopeptide EAENITTGCAEHCSLNENITVPDTK were identified by the RP column. However, these glycoforms were eluted in the void volume of the RP column without being resolved.

Table 3. Glycopeptides from trypsin digested EPO glycoprotein found using an Agilent ZORBAX RRHD 300-HILIC column.

No	Sequence	Sequence Location	Retention time (min)	Glycosylation
1	EAENITTGCAEHCSLNENITVPDTK	21–45	9.425	1111 0A 1G (+1710.5977)
2	EAENITTGCAEHCSLNENITVPDTK	21–45	9.392	3022 2A 0G (+2407.8518)
3	EAENITTGCAEHCSLNENITVPDTK	21–45	9.425	3021 1A 0G (+2407.8518)
4	EAENITTGCAEHCSLNENITVPDTK	21–45	9.500	3020 0A 0G (+1825.6610)
5	GQALLVNSSQPWEPLQLHVDK	77–97	9.103	0100 0A 0G (+1038.3751)

Conclusions

The use of an AgilentZORBAX Rapid Resolution High Definition 300-HILIC could aid the mapping and identification of hydrophilic peptides that were not resolved by RP chromatography. Therefore, coupling this column with MS could be an orthogonal and complementary approach to RP LC/MS, to provide better retention for hydrophilic peptides including glycopeptides thus potentially providing better sequence coverage and protein characterization.

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