Separation of IgG Glycopeptides using HILIC-LC/MS in Comparison to RP-LC/MS

Application Note

Biotherapeutics and Biosimilars

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Introduction

Peptide mapping by reversed-phase (RP) HPLC is an important and routinely used technique for the comprehensive characterization of protein biotherapeutics. However, RP chromatography retains peptides based on hydrophobicity and does not adequately resolve the hydrophilic glycosylated peptides. Although Peptide mapping by RP chromatography does regularly result in maps containing both glycopeptides and non-glycosylated peptides, this mode of separation does not favor glycopeptides that elute at similar times. In particular, peptides with the same peptide sequence that have different glycosylation cannot be fully resolved by RP methods. However In recent years, HILIC chromatography has emerged as a powerful tool to analyze released glycans and glycopeptides, and would therefore be expected to provide increased retention and resolution of glycosylated peptides compared with RP separations.

In this work, HILIC chromatography has been applied as an orthogonal peptide mapping approach to RP methods for the enhanced separation, and identification, of glycopeptides in an IgG tryptic digest. Using a novel 2.7um superficially-porous HILIC column coupled to a time-of-flight mass spectrometer (TOF MS) we have evaluated the glycopeptides in comparison to the RP analysis. With the HILIC column, glycopeptides were more strongly retained while different glyco-forms of the same peptide were chromatographically separated, providing unique characterization data for the identity of the carrier IgG and the specific glycan attachment sites. Additionally this work will compare IgG reversed-phase and HILIC mass spectral data for sequence coverage and elution order of the same glycopeptides to demonstrate the utility of HILIC as an orthogonal and complementary approach to RP LC/MS for peptide analysis.

Materials and Methods

To achieve IgG1 tryptic digestion, 20 µL mAb IgG1 (30 mg/mL) was mixed with 20 µL 100 mM ammonium bicarbonate (pH 8). Trifluoroethanol (50 µL) and 5 µL 200 mM dithiothreitol (DTT) was added to the protein sample and heated at 65 °C for 30 minutes to denature and reduce the protein. After the protein had cooled to room temperature, 20 µL 200 mM iodoacetamide (IAM) was added to the sample. The sample was kept at room temperature in the dark for 1 hour. DTT (5 µL) was added to the sample to react with the excess IAM for 1 hour. The sample was diluted with 200 µL 100 mM ammonium bicarbonate and 600 µL water. Trypsin (40 µg) was added to the sample and incubated at 37 °C, with shaking at 300 rpm overnight. Undiluted formic acid (5 µL) was added to quench the digest. The final IgG concentration was 1 µg/µL.

Conditions - reversed-phase LC

Column:	Agilent AdvanceBio Peptide Mapping, 2.1 x 150 mm, 2.7 µm (p/n 653750-902)
Mobile phase:	A, water + 0.1% formic acid; B, ACN + 0.1% formic acid
Gradient:	3 to 45% B in 25 min, 45 to 90% B in 1 min, hold 2 min at 90% B, 90 to 3% B re-equilibration for 5 min
Flow rate:	0.5 mL/min
Column temp:	50 °C
Inj vol:	15 µL
Detection:	DAD, 215 nm
Instrument:	Agilent 1290 Infinity LC

Conditions - HILIC

Column:	AdvanceBio Glycan Mapping, 2.1 x 250 mm, 2.7 µm (p/n 651750-913)
Mobile phase:	A, 90 ACN:10 mM NH4 formate, pH 4.0; B, 10 mM NH_4 formate, pH 4.0
Gradient:	0 to 45% B in 60 min, 45% B hold 2 min, 45 to 100% B 62 to 64min, re-equilibration 0% B for 10 min
Flow rate:	0.5 mL/min
Column temp:	50 °C
Inj vol:	15 µL
Detection:	DAD, 280 nm
Instrument:	Agilent 1290 Infinity LC

Conditions - mass spectrometry, reversed-phase LC and HILIC

Instrument:	Agilent 6530 Accurate-Mass Q-TOF with Agilent Jet Stream Source
Source:	Dual ESI in positive mode
Drying gas temp:	250 °C
Drying gas flow:	10 L/min
Sheath gas temp:	250 °C
Sheath gas flow:	12 L/min
Nebulizer pressure:	25 psi
Capillary voltage:	3,500 V
Fragmentor voltage:	170 V
Skimmer voltage:	65 V
MS range:	250 to 2,500 m/z
MS scan rate:	8 spectra/s

Results and Discussion

HILIC versus RP – human IgG mapping

The panels in Figure 1 compare the performance of reversed-phase and HILIC for IgG mapping. In general, under reversed-phase chromatographic conditions, more hydrophilic peptides elute earlier than more hydrophobic peptides, whereas the order is reversed under HILIC conditions. Under reversed-phase conditions, very hydrophilic peptides usually have little retention and will elute close together, or co-elute. Panel A provides an example of this retention behavior for glycopeptides (hydrophilic) from human IgG. In this RP separation, four glycopeptides (Panel B) were identified by MS eluting at the beginning of the RP gradient, displaying poor resolution and overlap. In comparison, the HILIC separation in Panel C shows the same four glycopeptides. However, Panel D reveals greater resolution and demonstrates the resolving power of the HILIC column towards hydrophilic compounds by revealing a 5th glycoform, G1FB, not identified by the RP column. Tables 1 and 2 show he extracted compound chromatograms.

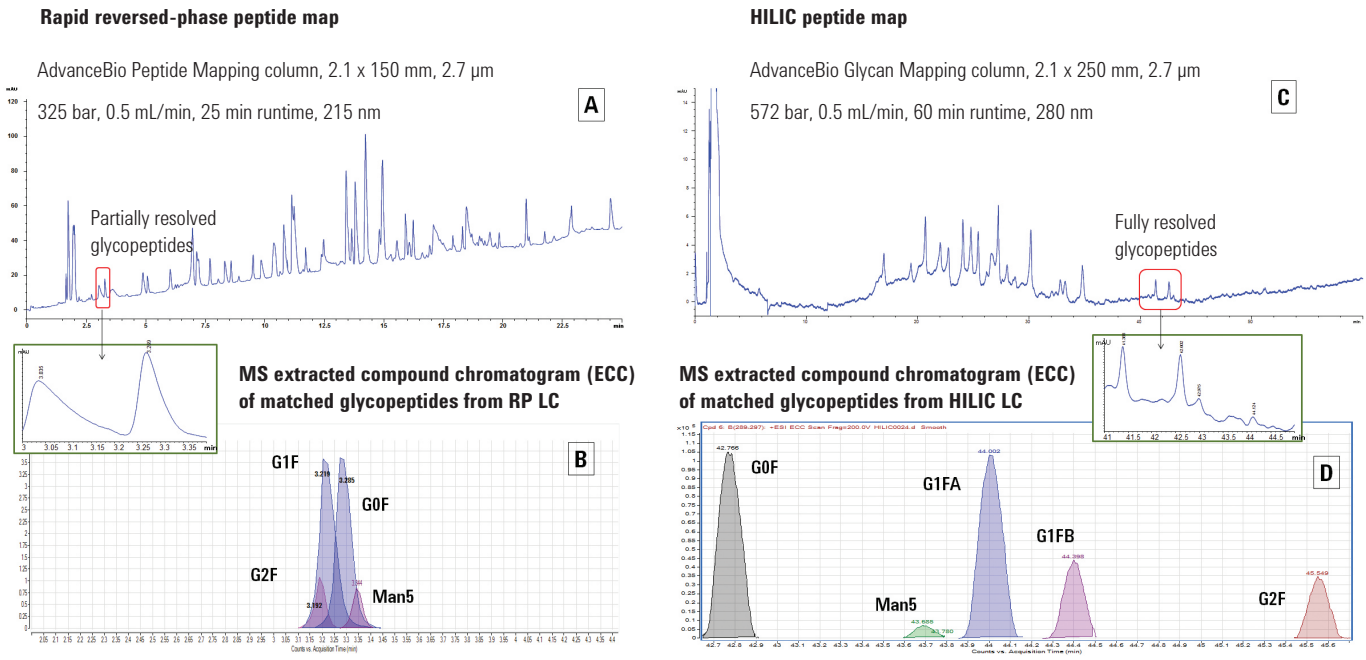

Figure 1. *Comparison of reversed-phase and HILIC for mapping human IgG. HILIC revealed the presence of a 5th glycoform that was not identified when using reversed-phase chromatography.*

Table 1. *Extracted compound chromatogram of matched glycopeptides from the RP LC separation in Panels A and B, Figure 1.*

Sequenece	Ret. Time	Volume	Mass	Modification	Structure
EEQYNSTYR	3.192	347440	2957.1443	G2F	
EEQYNSTYR	3.219	1765513	2795.0914	G1F	
EEQYNSTYR	3.285	1694571	2633.0386	G0F	
EEQYNSTYR	3.344	262780	2404.9276	Man5	

Table 2. *Extracted compound chromatogram of matched glycopeptides from the HILIC separation in Panels C and D, Figure 1.*

Sequenece	Ret. Time	Volume	Mass	Modification	Structure
EEQYNSTYR	42.766	789820	2633.0382	G0F	
EEQYNSTYR	43.685	48119	2404.9248	Man5	
EEQYNSTYR	44.002	791438	2795.0897	G1FA	
EEQYNSTYR	44.398	297427	2795.0896	G1FB	

HILIC versus RP – matched sequences

The RPLC and HILIC MS results shown in Figure 2 were extracted by Agilent MassHunter molecular feature extractor and then matched to the digested IgG protein sequences. Sequence coverage was 99.87% for the RP separation and 90.37% for the HILIC separation.

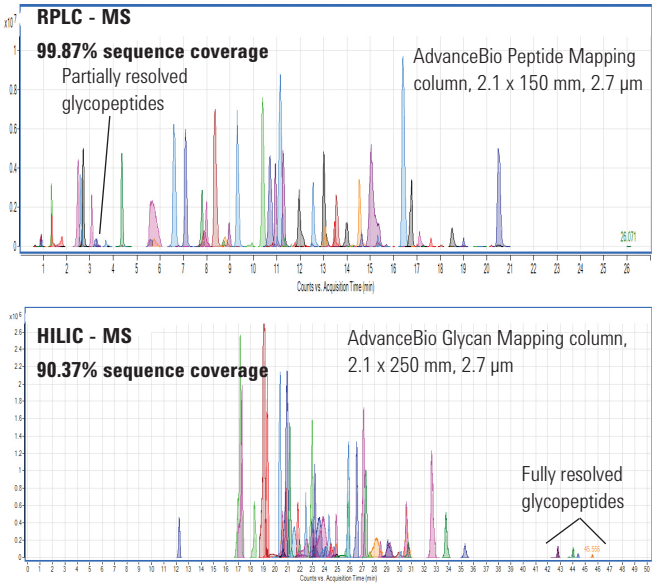

Figure 2. *Comparison of reversed-phase and HILIC for matched sequences.*

Glycopeptide resolution

Figure 3 shows stacked views of the separation of the glycopeptides in RP mode (left) and HILIC mode (right). The glycoforms of the peptide EEQYNSTYR were more resolved in the HILIC separation compared to the near co-eluting glycopeptides in the RP separation. In addition, HILIC enabled the identification of G1FB.

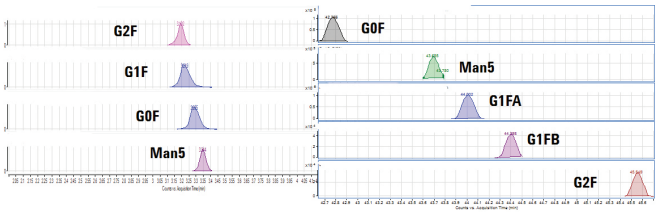

Figure 3. *Extracted compound chromatogram comparison of matched glycopeptides from the RP and HILIC separations shown in Panels B to D, Figure 1.*

Conclusions

HILIC mapping and RP peptide mapping columns, independently coupled to a quadrupole time-of-flight mass spectrometer, successfully identified IgG1 glycopeptides. The 2.7 µm superficially-porous HILIC column demonstrated strong retention and increased resolution of hydrophilic glycoforms of the same peptide, EEQYNSTYR, which were poorly retained and only partially resolved by reversed-phase chromatography. What's more, the HILIC column provided identification of an additional glycopeptide, G1FB, which was not readily identified by the reversed-phase separation. However, both HILIC and RP columns enabled high sequence coverage of IgG and demonstrated that HILIC/MS could be an orthogonal and complementary approach to RPLC/MS to provide better retention for hydrophilic peptides.

Acknowledgement

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