Fast and Efficient HILIC Methods for Improved Analysis of Complex Glycan Structures

Application Note

Biotherapeutics and Biosimilars

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Introduction

N-linked glycosylation is a critically important and very complex post-translational modification. It therefore needs to be controlled and monitored throughout development, processing, and manufacture of drug glycoproteins. Therapeutic protein characteristics, including safety, efficacy, and serum half-life, can be affected by differences in their glycosylation pattern, and so the analysis of these patterns is an important part of the characterization of therapeutic glycoproteins, particularly mAbs. Separation using HILIC with fluorescence detection is a robust method for glycan analysis, whereas HILIC/LC can also be coupled to mass spectrometry to obtain important mass and structure information.

One of the growing challenges in HILIC/LC, however, is achieving high-resolution separations with fast analysis times. With ever-increasing demands placed on biocharacterization for higher throughput, researchers are looking for improved separation (HILIC) methods, but not at the cost of lost separation performance. Since glycans include many closely related structures, it is critical to achieve the highest resolution possible, and preferably during a fast analysis time.

In this work, we used a sub-2 µm UHPLC HILIC column with amide chemistry for high-throughput glycosylation profiling. Specifically, we profiled 2-AB labeled human IgG and bovine fetuin N-linked glycans using a 1.8 µm, 2.1 x 150 mm column with fluorescence detection. Rapid, sensitive and selective separations were achieved to provide ultra-high resolution of these complex glycans in run times as short as 9 minutes. In a run time comparison to a currently available UHPLC glycan column, we observed a 40% reduction in analysis speed for human IgG N-linked glycans under identical conditions.

Materials and Methods

Conditions - recombinant human IgG1

Sample:	Agilent 2-AB labelled IgG N-linked glycan library, 200 pmol (p/n 5190-6996)
Column:	Agilent AdvanceBio Glycan Mapping, 2.1 x 150 mm, 1.8 µm (p/n 859700-913)
Mobile phase:	A, 100 mM NH_4 formate, pH 4.5; B, ACN
Inj vol:	2 µL in 70/30 ACN:water
Column temp:	55 °C
Sample thermostat:	10 °C
Detection:	Fluorescence, excitation 260 nm, emission 430 nm
Instrument:	Agilent 1290 Infinity LC with 1260 Fluorescence Detector

Conditions - bovine fetuin

Sample:	ProZyme Glyko 2-AB bovine fetuin N-linked library
Column:	Agilent AdvanceBio Glycan Mapping, 2.1 x 150 mm, 1.8 µm (p/n 859700-913)
Mobile phase:	A, 100 mM NH_4 formate, pH 4.5; B, ACN
Inj vol:	5 µL (20 pmol) in 70/30 ACN:water
Column temp:	55 °C

Other conditions as above

Conditions - dextran ladder

Sample:	Agilent 2-AB labelled dextran ladder standard (p/n 5190-6998)
Column:	Agilent AdvanceBio Glycan Mapping, 2.1 x 150 mm, 1.8 µm (p/n 859700-913)
Mobile phase:	A, 100 mM NH_4 formate, pH 4.5; B, ACN
Inj vol:	2 L 75:25 ACN:water (10 pmol total glycan)

Other conditions as above

Conditions - mass spectrometry, recombinant human IgG1

Instrument:	Agilent 6550 iFunnel Q-TOF LC/MS
Source:	Agilent Dual JetStream
Drying gas temp:	200 °C
Drying gas flow:	12 L/min
Sheath gas temp:	250 °C
Sheath gas flow:	12 L/min
Nebulizer pressure:	25 psi
Capillary voltage:	3,500 V
Nozzle voltage:	500 V
Fragmentor voltage:	250 V
Skimmer voltage:	45 V
Octupole RF Vpp:	550 V
MS range:	100 to 1,700 *m/z*
MS scan rate:	2 spectra/s

The workflow is shown in Figure 1.

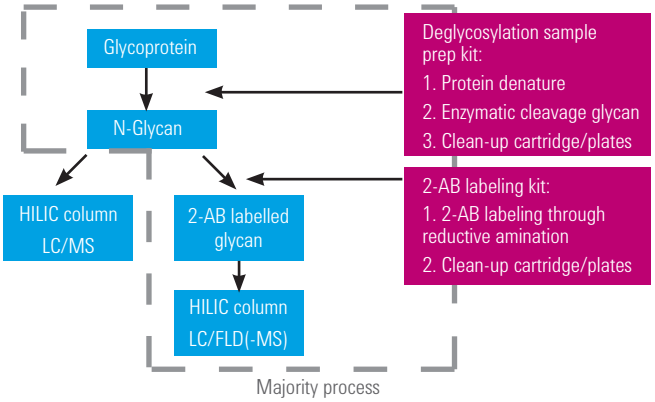


Figure 1. *Total workflow solution used in an investigation of 2-AB-labeled antennary glycans using an Agilent AdvanceBio Glycan Mapping HILIC column with fluorescence detection.*

Results and Discussion

Dextran ladder

Figure 2 shows the separation of a 2-AB labeled dextran ladder. In this separation, a homopolymeric series of 20 glucose oligomers were efficiently baseline resolved in less than 15 minutes.

Time	%A	%B	Flow rate (mL/min)
0	25	75	1.0
12	40	60	1.0
12.15	60	40	0.5
12.5	60	40	0.5
12.9	25	75	0.5
13.05	25	75	1.0
15	25	75	1.0

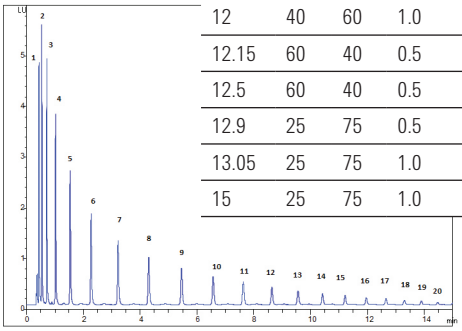
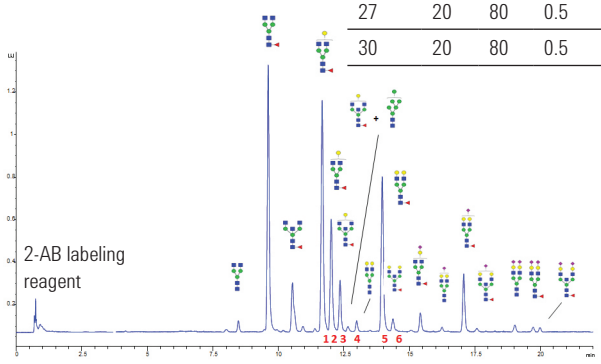


Figure 2. *The Agilent AdvanceBio Glycan Mapping column separates a 2-AB labeled dextran ladder to baseline in less than 15 minutes*

Human IgG glycans

Figure 3 demonstrates an ultra-high resolution separation of 2-AB labeled N-linked human IgG glycans.

Time	%A	%B	Flow rate (mL/min)
0	20	80	0.5
25	40	60	0.5
26	100	0	0.5
27	20	80	0.5
30	20	80	0.5

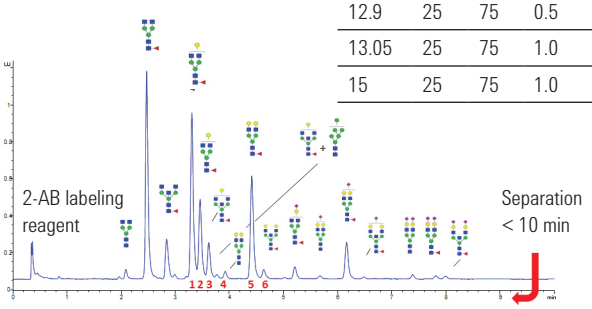


Avg RT (min)	RS 2,1	RS 3,2	RS 4,3	RS 6,5	Avg PW (min)	Bp (bar) at 30% B	Peak capacity
12.7	2.60	2.90	5.43	2.81	0.0741	298	221

Figure 3. *Very high resolution of 2-AB labeled N-linked human IgG glycans on the Agilent AdvanceBio Glycan Mapping column.*

Fast separation in less than 10 minutes was also achieved, as shown in Figure 4.

Time	%A	%B	Flow rate (mL/min)
0	25	75	1.0
12	40	60	1.0
12.15	60	40	0.5
12.5	60	40	0.5
12.9	25	75	0.5
13.05	25	75	1.0
15	25	75	1.0



Avg RT (min)	RS 2,1	RS 3,2	RS 4,3	RS 6,5	Avg PW (min)	Bp (bar) at 30% B	Peak capacity
3.93	1.63	1.70	3.05	2.00	0.059	298	135

Figure 4. *The Agilent AdvanceBio Glycan Mapping column separates 2-AB labeled N-linked human IgG glycans in less than 10 minutes.*

MS N-linked glycans

Figure 5 shows eight representative mass spectra from the ultra-high resolution separation of human IgG glycans. The spectra were generated by Q-TOF analysis (experimental). All spectra matched theoretical masses to within 6 ppm.

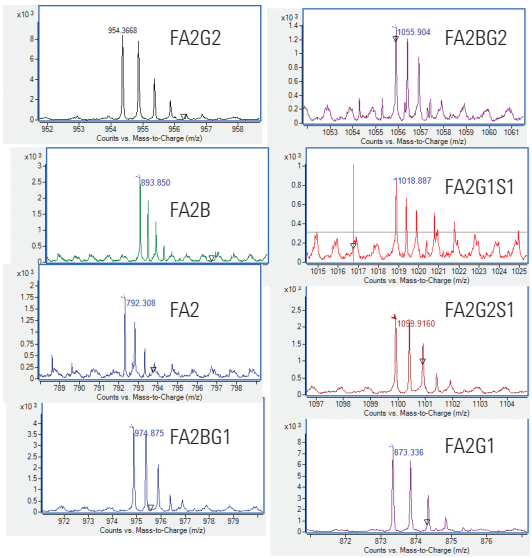
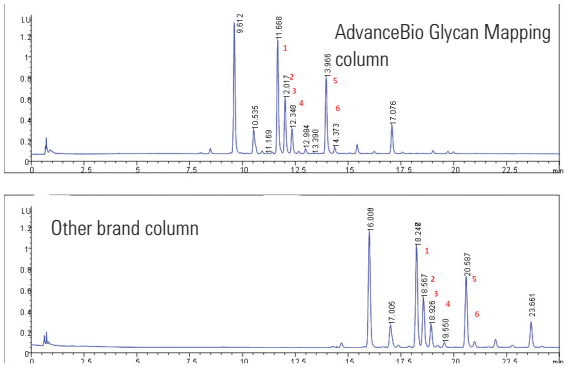


Figure 5. *Eight representative mass spectra from the ultra-high resolution separation of human IgG glycans (fluorescence detector separations are displayed on the panel to the left).*

Sub-2 µm HILIC comparison

Figure 6 shows the results of a comparison of glycan amide columns. Using the same chromatographic conditions, the AdvanceBio Glycan Mapping column delivered better resolution and narrower bands, with higher peak capacity, at a 40% faster separation time than another brand of sub-2 µm HILIC column in a 2.1 x 150 mm configuration.

Time	%A	%B	Flow rate (mL/min)
0	20	80	0.5
25	40	60	0.5
26	100	0	0.5
27	20	80	0.5
30	20	80	0.5



Column	Avg RT (min)	RS 2,1	RS 3,2	RS 4,3	RS 6,5	Avg PW (min)	Bp (bar) at 30% B	Peak capacity
Agilent AdvanceBio Glycan Mapping	12.7	2.60	2.90	5.43	2.81	0.0741	298	221
Other brand	20.2	1.77	1.94	3.39	2.10	0.1085	349	214

Figure 6. *The AdvanceBio Glycan Mapping column delivers better resolution and narrower bands, with higher peak capacity at a 40% faster separation time.*

Fetuin glycans

Finally, we revealed the fast and highly efficient performance of the AdvanceBio Glycan Mapping column in a separation of 2-AB labeled bovine fetuin N-linked glycans (Figure 7).

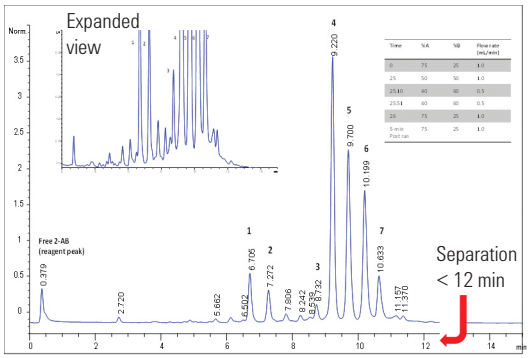


Figure 7. *Fast and highly efficient separation of 2-AB labeled bovine fetuin N-linked glycans, 2.1 x 150mm 1.8um AdvanceBio Glycan Mapping column.*

Table 1. *Glycan nomenclature and structure assignments for the optimized rapid separation of bovine fetuin 2-AB labeled N-linked glycans displayed in top chromatogram of Figure 7.*

Peak	Retention	GU value	Glycan structure	Structures
1	6.70	9.4	A2G2S2	
2	7.27	9.8	A2G2S2	
3	8.73	10.8	A3G3S3, A3G3S2 (trace)	
4	9.22	11.2	A3G3S3, A3G3S2 (trace)	
5	9.70	11.6	A3G3S3, A3G3S4 (trace)	
6	10.20	12	A3G3S4, A3G3S3	
7	10.63	12.4	A3G3S4	

Galactose
N-acetylglucosamine
Fucose
Mannose
N-acetylneuramic acid

Conclusions

The Agilent 1.8 µm HILIC amide AdvanceBio Glycan Mapping column provided separation of N-linked glycans with high speed, excellent resolution and increased efficiency. In a 2.1 x 150 mm configuration and under identical chromatographic conditions, the column enabled a well-resolved separation of 2-AB labeled IgG N-linked glycans, with a 40% reduction in elution time compared to another brand of sub-2 µm HILIC column. A separation of 2-AB labeled bovine fetuin N-linked glycans demonstrated the column's excellent selectivity and resolving power for separating these complex biantennary and triantennary glycans.

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