A Novel HILIC Column for High Speed N-linked Glycan Analysis

Application Note

Biotherapeutics and Biosimilars

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Introduction

Recombinant monoclonal antibody therapeutics (mAbs) represents the largest group of therapeutic proteins. The efficacy of these therapeutics is highly dependent on the correct glycosylation patterns of the mAbs and, so far, all licensed therapeutic mAbs are immunoglobulins G (IgG) [1]. Human IgG has a single conserved N-linked glycosylation site located on each heavy chain [2]. N-linked glycosylation is a critically important and an elaborately complex post-translational modification that requires control, monitoring, and understanding during all phases of glycoprotein drug development, processing and manufacture [3]. Properties such as safety, efficacy, and the serum half-life of therapeutic proteins can be affected by differences in their glycosylation pattern. Therefore, analysis of the glycan pattern is an important part of characterization of therapeutic glycoproteins, especially mAbs.

Different strategies have been applied for the analysis of glycans. However, the majority of methods are based on enzymatic protein release of glycans from the mAb with subsequent derivatization with a labeling agent, such as 2-aminobenzamide (2-AB) [4]. Due to the lack of chromophores on the glycan, labeling with a 2-AB (neutral) tag enables fluorescence detection. Labeled glycans are very hydrophilic structures and the preferred separation technique is by hydrophilic interaction chromatography, commonly referred to as HILIC. Separation using HILIC with fluorescence detection is a robust method for glycan analysis, while HILIC/LC can also be coupled to mass spectrometry for obtaining important mass and structure information.

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In this application note, we introduce the AdvanceBio Glycan Mapping Column, a sub-2 µm HPLC column with a novel HILIC amide chemistry for high-throughput glycosylation analysis. The column and methods provide high resolution of glycans with a 40% reduction in elution time compared to currently available HPLC column technologies. To illustrate the utility of the AdvanceBio Glycan Mapping column, we studied a 2-AB labeled human IgG N-linked glycan sample.

Materials and Methods

Conditions, ultra high resolution

Column:	Agilent AdvanceBio Glycan Mapping, 2.1 × 150 mm, 1.8 µm (p/n 859700-913)
Glycan library:	Agilent 2-AB labeled human IgG N-linked glycans, 200 pmol (p/n 5190-6996)
Eluent:	A) 100 mM NH_4 formate, pH 4.5 B) ACN
Injection volume:	2 µL in 70:30 ACN: water

The same conditions were used for assessing fast separations, but with a different gradient.

The workflow is shown in Figure 1.

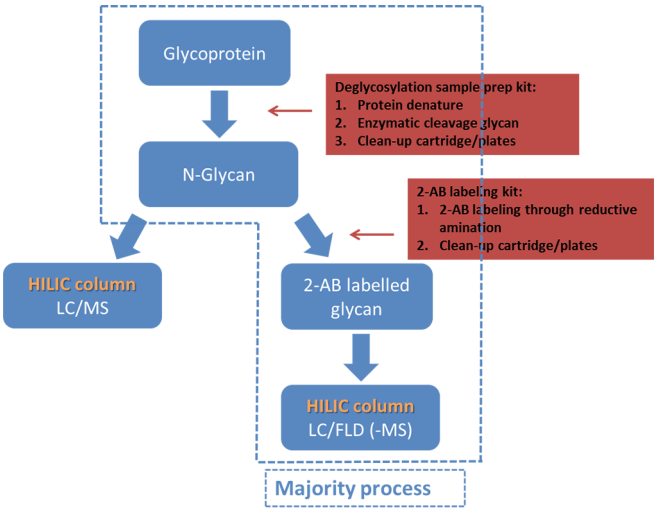


Figure 1. Total workflow solution used in an investigation of 2-AB-labeled human IgG glycan using an Agilent AdvanceBio Glycan Mapping Column HILIC column with fluorescence detection.

Results and Discussion

Fast separation

The AdvanceBio Glycan Mapping Column HILIC column with 1.8 µm particles delivers extremely fast glycan analysis, in less than 10 minutes (Figure 2 and Table 1). Table 2 shows the peak identities and predominant glycan structures of IgG.

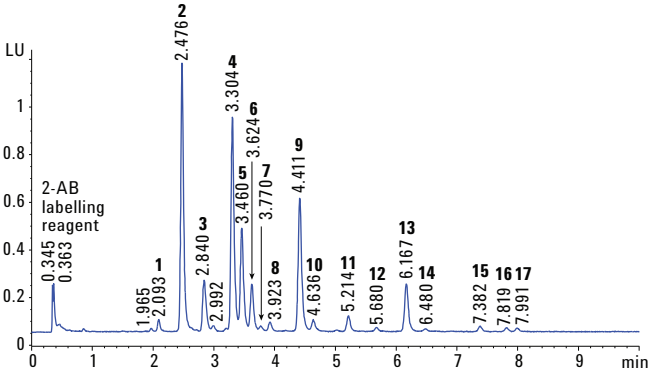


Figure 2 conditions

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

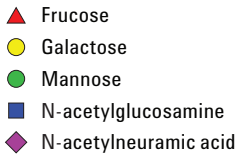
Figure 2. The fast analytical capability of the Agilent AdvanceBio Glycan Mapping column separates 2-AB labeled human IgG N-linked glycans in less than 10 minutes.

Table 1. Resolution results obtained during a fast separation of 2-AB labeled human IgG N-linked glycans.

Column	Average RT (min)	RS 2,1	RS 3,2	RS 4,3	RS 6,5	Average PW (min)	Bp (bar), 20% B	Peak capacity
Glycan Mapping column	3.93	1.63	1.70	3.05	2.09	0.059	298	135

Table 2. Peak identities and predominant glycan structures of IgG.

Peak	Glycan	Structure	Peak	Glycan	Structure
1	G0		10	G2FB	
2	G0F		11	G1FS1	
3	G0FB		12	A1	
4	G1F		13	A1F	
5	G1F′		14	A1FB	
6	G1FB		15	A2	
7	G1FB Man6		16	A2F	
8	G2		17	A2FB	
9	G2F				



Ultra high resolution separation

The AdvanceBio Glycan Mapping Column HILIC column with 1.8 µm particles can alternatively deliver ultra high resolution performance during a slightly increased run time (Figure 3 and Table 3). In this separation, the 2-AB labeled human IgG N-linked glycan are highly resolved, delivering excellent sensitivity.

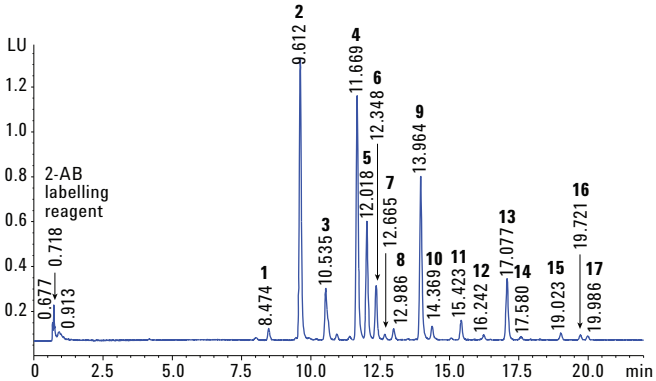


Figure 3 conditions

Column temperature:	55 °C		
Gradient:	Time (min)	% B	Flow rate (mL/min)
	0	80	0.5
	25	60	0.5
	26	0	0.5
	27	80	0.5
	30	80	0.5
Detection:	Fluorescence, excitation 260 nm, emission 430 nm		
Instrumentation:	Agilent 1290 Infinity LC System with an Agilent 1260 Infinity Fluorescence Detector		

Figure 3. A separation of 2-AB labeled human IgG N-linked glycans with ultra high resolution obtained using the Agilent 1.8 µm AdvanceBio Glycan Mapping Column HILIC column.

Table 3. Results of a high resolution separation of 2-AB labeled human IgG N-linked glycans.

Column	Average RT (min)	RS 2,1	RS 3,2	RS 4,3	RS 6,5	Average PW (min)	Bp (bar), 20% B	Peak capacity
Glycan Mapping column	12.7	2.60	2.90	5.43	2.81	0.0741	298	221

Performance comparison

Using the same chromatographic conditions, the AdvanceBio Glycan Mapping Column delivers better resolution and narrower bands with higher peak capacity at a 40% faster separation time than another supplier's column in a 2.1 × 150 mm configuration (Figure 4 and Table 4).

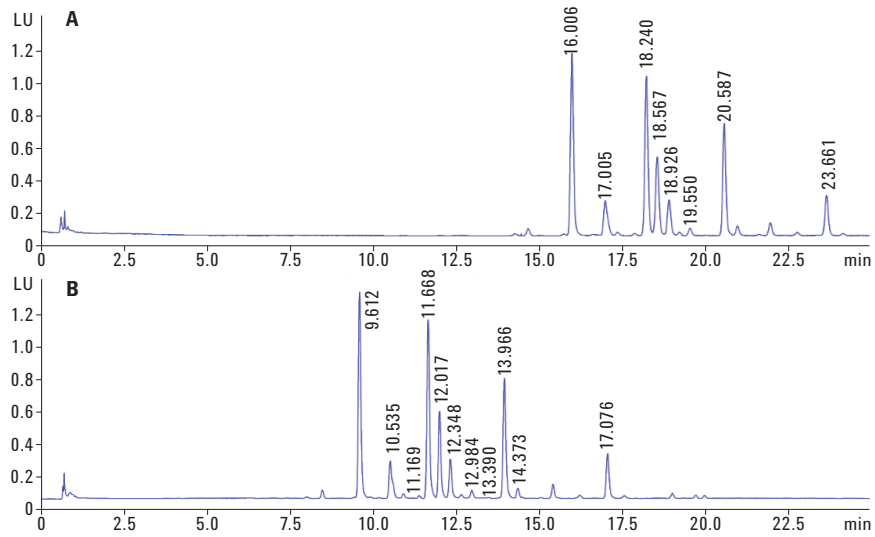


Figure 4. The Agilent AdvanceBio Glycan Mapping column (B) delivers better resolution, narrower peak widths and higher peak capacity, with a 40% faster separation, when compared to another supplier's column of the same dimensions and using the same conditions.

Table 4. Comparison of results delivered by the Agilent AdvanceBio Glycan Mapping column and another supplier's column showing speed advantage.

Column	Average RT (min)	RS 2,1	RS 3,2	RS 4,3	RS 6,5	Average PW (min)	Bp (bar), 20% B	Peak capacity
Other supplier's glycan (A)	20.2	1.77	1.94	3.39	2.10	0.1085	349	214
Agilent AdvanceBio Glycan Mapping (B)	12.7	2.60	2.90	5.43	2.81	0.0741	298	221

In another comparison, an ultra fast separation was applied to both columns using similar conditions (gradient offset adjusted by 5% B) for both columns. The AdvanceBio Glycan Mapping column produced better resolution and narrower bands with higher peak capacity than the other supplier's column, again in a 2.1 × 150 mm configuration (Figure 5 and Table 5).

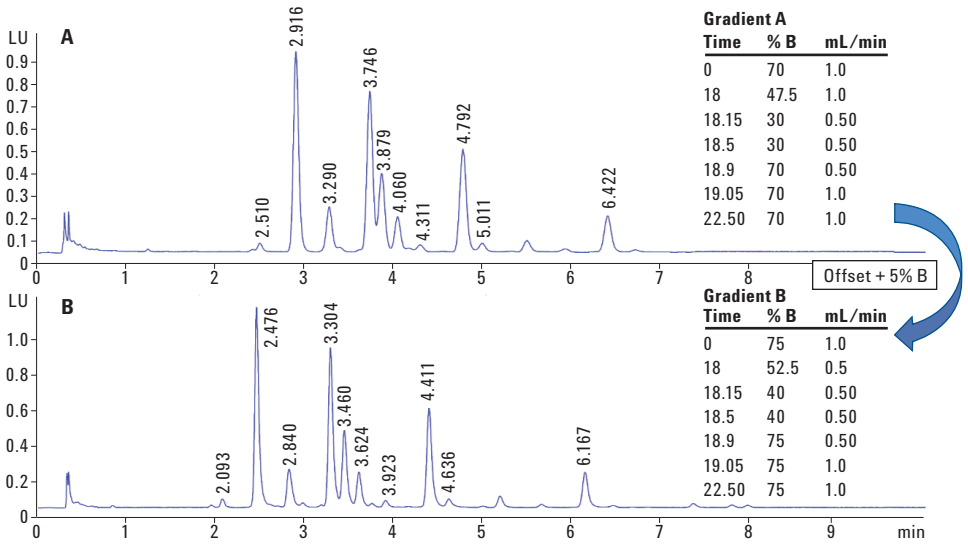


Figure 5. Ultra fast separation comparison: the AdvanceBio Glycan Mapping column (B) provides better resolution and narrower bands with higher peak capacity at equal separation time than another supplier's column of the same configuration.

Table 5. Comparison of results delivered by the Agilent AdvanceBio Glycan Mapping column and another supplier's column showing resolution advantage.

Column	Average RT (min)	RS 2,1	RS 3,2	RS 4,3	RS 6,5	Average PW (min)	Bp (bar), 20% B	Peak capacity
Other supplier's glycan (A)	4.32	1.02	1.39	1.92	1.59	0.078	375	101
Agilent AdvanceBio Glycan Mapping (B)	3.93	1.63	1.70	3.05	2.09	0.059	298	135

Chemical stability

The AdvanceBio Glycan Mapping column also demonstrated excellent lifetime stability at elevated temperature. Figure 6 details stable separation performance after 5,000 injections at 60 °C. In this comparison, peak shape, retention and sensitivity are maintained after repeated injections.

Conclusions

The Agilent HILIC-based AdvanceBio Glycan Mapping column provides separation of glycans with high resolution and efficiency, with a 40% reduction in elution time compared to other HPLC column technologies. In addition, the column showed excellent chemical stability for at least 5,000 column volumes.

Acknowledgement

This work was presented in a poster session at HPLC 2014, the 41st International Symposium on High performance Liquid Phase Separations and Related Techniques. 11–15 May, 2014, New Orleans.

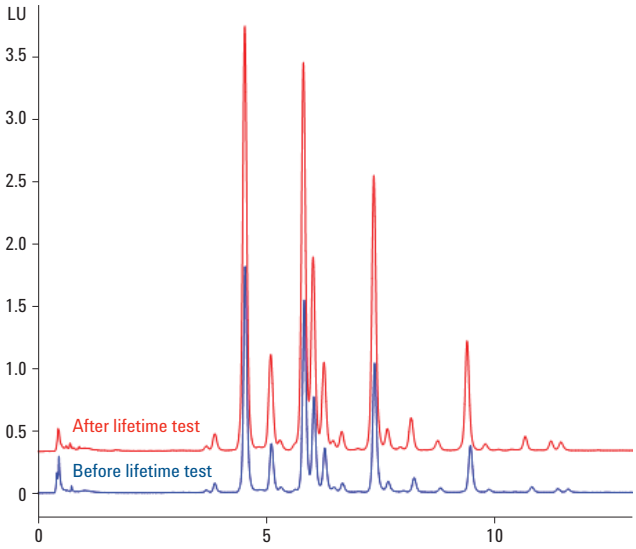


Figure 6. Glycan separation overlay before and after a lifetime test on the Agilent AdvanceBio Glycan Mapping column (HILIC QC conditions: 10:90 100 mM pH 4.4 ammonium formate:ACN, 0.2 mL/min, 4 minutes, 60 °C, cytosine).

References

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