Sensitive and Reproducible Glycan Analysis of Human Immunoglobulin G

The Agilent 1260 Infinity Bio-inert Quaternary LC System with an Agilent AdvanceBio 2.7 µm Glycan Mapping Column and Fluorescence Detection

Application Note

Biotherapeutics & Biosimilars

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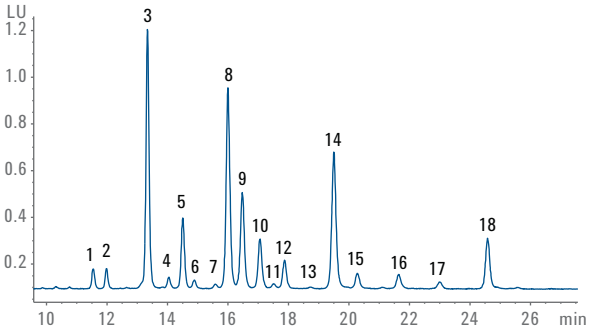
Abstract

This Application Note demonstrates the sensitive and reproducible analysis of N-linked glycans of human immunoglobulin G using the Agilent 1260 Infinity Bio-inert Quaternary LC System, with a novel HILIC column and fluorescence detection. Precision of retention time and area was determined for analysis of an N-linked human IgG glycan library. In addition, good linearity ranging from 0.016 to 1 pmol was determined for a mix of five glycan standards (M5, A2G2, A2G2S1, FA2G2S1, and A2G2S2) with low limits of detection and quantification.

The Agilent 1260 Infinity Bio-inert Quaternary LC System, with the Agilent AdvanceBio 2.7 µm Glycan Mapping Column and fluorescence detection is an optimal system for sensitive and reproducible analysis of 2-AB derivatized N-glycans released from human immunoglobulin G.



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Introduction

Antibodies represent the largest group of recombinantly-produced therapeutic proteins as a major drug class. The efficacy of these therapeutics is highly dependent on the correct glycosylation pattern. Currently, all licensed therapeutic monoclonal antibodies (mAbs) are immunoglobulines G (IgGs)[1]. The analysis of their glycosylation pattern is an important part of the QA/QC process. In addition, the glycosylation pattern of plasma-derived human IgGs can reflect the healthy or diseased status of a patient[2].

Human IgG has a single conserved N-linked glycosylation site located on the Fc region of each heavy chain at Asn-297[3]. This fact results in two sugar moieties per IgG, which are highly heterogeneous, and can result in a mixture of at least 30 glycoforms[1]. Plasma-derived human IgG N-linked glycans are predominantly biantennary complex-type structures, primarily core-fucosylated with zero to two galactose residues and one to two sialic acids. In addition, human IgGs carry a small amount of bisecting N-acetylglycosamine (GlcNAc) residues. In general, recombinantly-produced mAbs reveal a marginally less complex pattern without nonfucosylated or bisecting GlcNAc glycan structures[4].

Other more immature high-mannose structures such as mannose-5 are also found, primarily in small amounts[5].

The analysis of protein glycosylation pattern can be challenging, because the glycan analytes are often of low abundance in complex biological samples. The separation of AB-labeled glycan structures using Hydrophilic Interaction Chromatography (HILIC)-HPLC with fluorescence detection is a robust and sensitive method for glycan analysis[6]. This method was used in this Application Note for the determination of precision, linearity, and analytical sensitivity.

In particular, we demonstrate a sensitive and reproducible HPLC analysis of N-linked glycans of human immunoglobulin G using the Agilent 1260 Infinity Bio-inert Quaternary LC System with an Agilent AdvanceBio 2.7 µm Glycan Mapping column, and fluorescence detection.

The glycan symbol structures, according to the Consortium for Functional Glycomics (CFG), were used for glycan assignment (Figure 1). The Oxford glycan nomenclature, as well as the Biopharma mAb style, was used for the assigned glycans.

Experimental

The Agilent 1260 Infinity Bio-inert Quaternary LC System consisted of the following modules:

- Agilent 1260 Infinity Bio-inert Quaternary Pump (G5611A)
- Agilent 1260 Infinity Bio-inert High-Performance Autosampler (G5667A)
- Agilent 1290 Infinity Thermostat (G1330B)
- Agilent 1290 Infinity Thermostatted Column Compartment (G1316C) with bio-inert solvent heat exchangers (G5616-81000)
- Agilent 1260 Infinity Fluorescence Detector (G1321B), equipped with bio-inert standard FLD flow cell

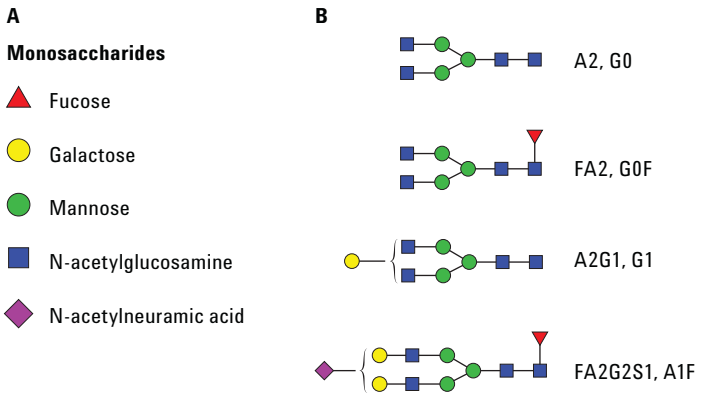


Figure 1. Glycan structure and isoforms. A) Monosaccharide description after the Consortium for Functional Glycomics (CFG). B) Predominant glycan structures of IgGs.

Column

Agilent AdvanceBio Glycan Mapping Column, 2.1 × 150 mm, 2.7 µm (p/n 683775-913)

Software

Agilent OpenLAB CDS, ChemStation Edition Systems Rev. C.01.05 [38]

Solvents and samples

Solvent A) Acetonitrile

Solvent B) 100 mM ammonium formate, pH 4.5

Glycan standards

- Agilent 2-AB labeled IgG N-linked glycan library 200 pmol (p/n 5190-6996)
- GLYKO 2-AB-(MAN-5) → M5, 100 pmol
- GLYKO 2-AB-(NA2) → A2G2, 100 pmol
- GLYKO 2-AB(A1) → A2G2S1, 100 pmol
- GLYKO 2-AB-(A1F) → FA2G2S1, 100 pmol
- GLYKO 2-AB-(A2) → A2G2S2, 100 pmol

All glycan standards were dissolved in 50 µL 100 mM ammonium formate, pH 4.5.

All solvents used were LC grade. Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22-µm membrane point-of-use cartridge (Millipak). Ammonium formate was purchased from Sigma-Aldrich, St. Louis, USA. All other individual 2AB-labeled glycan standards were purchased from Prozyme, Hayward, USA.

Chromatographic conditions

Parameter	Value		
Gradient	min	% B	Flow rate (mL/min)
	0	15	0.5
	5	25	
	35	36	
	40	50	
	42		0.5
	42.01		0.25
	43	100	
	48	100	
	50	15	0.25
	50.01		0.5
	51		
Stop time	51 minutes		
Post time	20 minutes		
Injection volume	2, 2.5 µL		
Thermostat	6 °C		
Column temperature	60 °C		
FLD	Ex. 260 nm, Em. 430 nm		
Peak width	0.025 minutes (0.5 seconds response time) (18.52 Hz)		

Results and Discussion

Reproducible analysis of N-glycans from AB-labeled human IgG standard

Figure 2 shows the separation of the AB-labeled human IgG N-linked glycan library. All major glycan structures found in human IgGs were efficiently resolved on a 2.1 × 150 mm, 2.7 µm Agilent AdvanceBio Glycan Mapping column. With respect to the assignments, the nomenclature employed in various publications [3,7,8] was used (Table 1). Most dominant glycans are core-fucosylated with zero, one, or two galactosylated structures. The last three peaks represent sialylated glycan structures, whereas the residual pre-eluting peaks are neutral glycans. Glycan structures were assigned after Haxo *et al*[9].

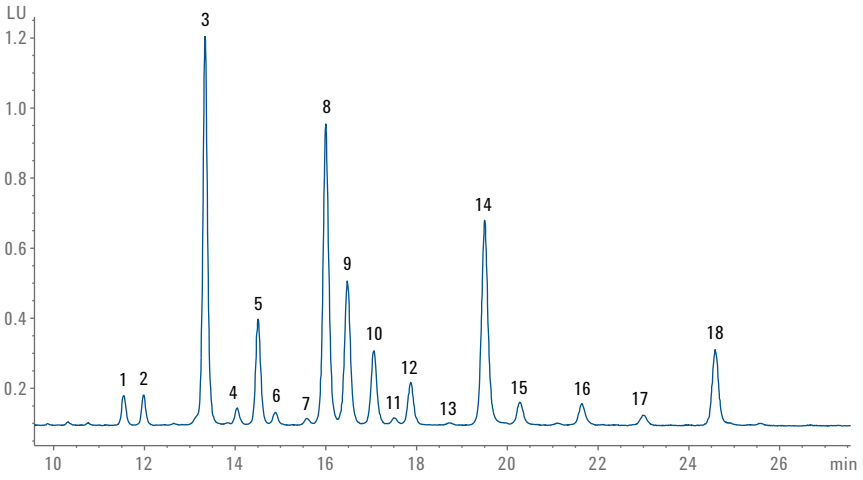


Figure 2. Separation of AB-labeled human IgG N-linked glycan library.

Precision of retention time and area was determined for six consecutive injections of the human IgG N-linked glycan library. For 15 dominant glycan structures, the retention time precision was below 0.27 %, and the area precision was below 3.5 % (Table 2). All measured RSD values for RT and area were within the commonly reported range[10].

Table 1. Nomenclature Oxford Style and mAb acronyms as well as structure of the assigned N-glycans in the human IgG glycan library.

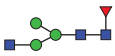
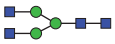
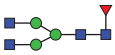
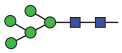
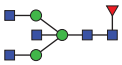
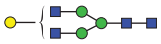
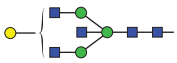
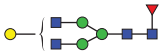
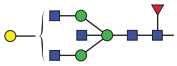
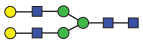
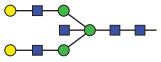
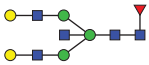
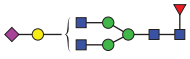
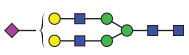
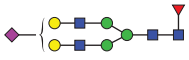
Peak	Oxford	Biopharma mAb style	Structure
1	FA1	G0F-GlcNAc	
2	A2	G0	
3	FA2	G0F	
4	M5	Man5	
5	FA2B	G0FB	
6	A2G1	G1	
7	A2BG1	G1B	
8, 9	FA2G1	G1F	
10, 11	FA2BG1	G1FB	
12	A2G2	G2	
13	A2BG2	G2B	
14	FA2G2	G2F	
15	FA2BG2	G2FB	
16	FA2G1S1	G1FS1	
17	A2G2S1	A1	
18	FA2G2S1	A1F	

Table 2. Precision of retention time and area for six consecutive injections of human N-linked IgG glycans.

Peak	Precision of RT (%)	Precision of area (%)
1	0.154	1.19
2	0.16	1.47
3	0.152	0.78
4	0.144	2.49
5	0.146	0.65
6	0.141	1.84
8	0.175	0.96
9	0.183	0.89
10	0.196	0.46
11	0.196	2.64
12	0.192	0.81
14	0.208	0.68
15	0.207	3.44
16	0.228	1.91
18	0.261	1.01

Linearity

Linearity was determined with a mix of five glycan standards (M5, A2G2, A2G2S1, FA2G2S1, and A2G2S2) diluted in series 1:2 ranging from 0.016 to 1 pmol, separated on a 2.1 × 150 mm, 2.7 µm Agilent AdvanceBio Glycan Mapping Column. Figure 3 shows a chromatogram of the five N-glycan mix at a sample amount of 1 pmol on column.

Limits of detection (LOD) were found between 9 and 12 fmol for signal-to-noise (S/N) = 3. Limits of quantification (LOQ) were found between 30 and 40 fmol for S/N = 10. All correlation curves showed excellent linearity, with correlation coefficients over 0.999, except for A2G2S1 with 0.997 (Table 3).

Using the option of the Agilent 1260 Series Fluorescence Detector for the analysis of up to four different excitation and emission wavelengths, optimal method development was possible, resulting in an excitation wavelength of 260 nm and an emission wavelength of 430 nm. This wavelength combination resulted in much higher intensity values compared to the wavelength normally used for glycan analysis (such as recommended by Anumula 2005[11]).

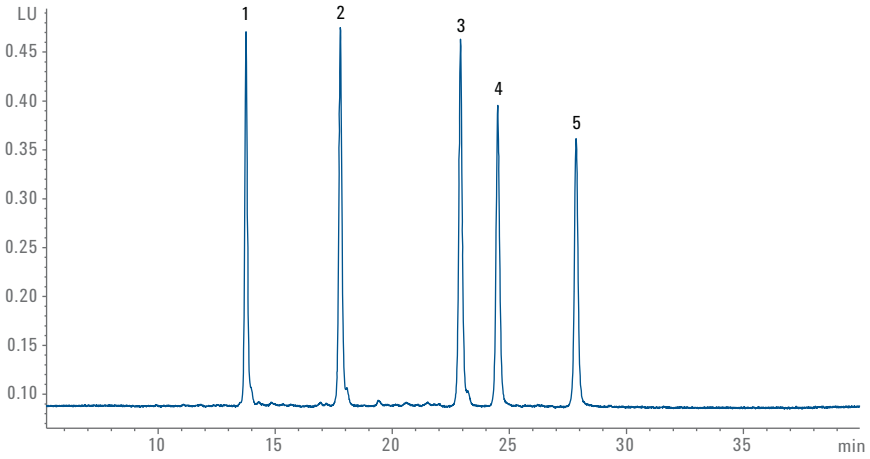


Figure 3. Mix of five AB-labeled N-linked glycan standards.

Table 3. Linearity, LOD, and LOQ for five AB-labeled N-linked glycan standards.

Peak	Oxford	Biopharma mAb style	Structure	LOD (fmol)	LOQ (fmol)	Correlation coefficient
1	M5	Man5		9	30	0.9996
2	A2G2	G2		9	30	0.9998
3	A2G2S1	A1		10	33	0.997
4	FA2G2S1	A1F		10	33	0.9997
5	A2G2S2	A2		12	40	0.9999

Conclusion

This Application Note demonstrates that the Agilent 1260 Infinity Bio-inert Quaternary LC System with the Agilent AdvanceBio Glycan Mapping Column and fluorescence detection is an ideal system for the sensitive and reproducible HPLC analysis of AB-labeled N-linked glycans derived from human IgG.

The analysis of a N-linked glycan library from human IgG showed good resolution for all major glycan structures. Precision of retention time was found to be below 0.27 % , and below 3.5 % for area precision. All measured RSD values for RT and area were within the commonly reported range[10]. With regard to structure assignment software, as presented by Campbell *et al*.[12], high precision of retention time is extremely important for correct assignment of the glycan structures.

Linearity was determined with a mix of five glycan standards (M5, A2G2, A2G2S1, FA2G2S1, and A2G2S2) diluted in series 1:2 ranging from 0.016 to 1 pmol. All correlation curves showed excellent linearity with correlation coefficients over 0.999, except for A2G2S1 with 0.997. LODs were found between 9 and 12 and LOQs were found between 30 and 40 fmol.

With fluorescence wavelengths optimized for our system, using the Agilent 1260 Infinity Series Fluorescence Detector, higher intensities and, therefore, better S/N ratios were achieved. Instead of an excitation wavelength of 330 nm (as recommended by Anumula 2005[11]), the lower wavelength 260 nm was used.

The Agilent 1260 Infinity Bio-inert Quaternary LC System and Glycan Mapping Column, with fluorescence detection provides an optimal system for sensitive and reproducible HPLC analysis of 2-AB derivatized N-linked glycans.

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