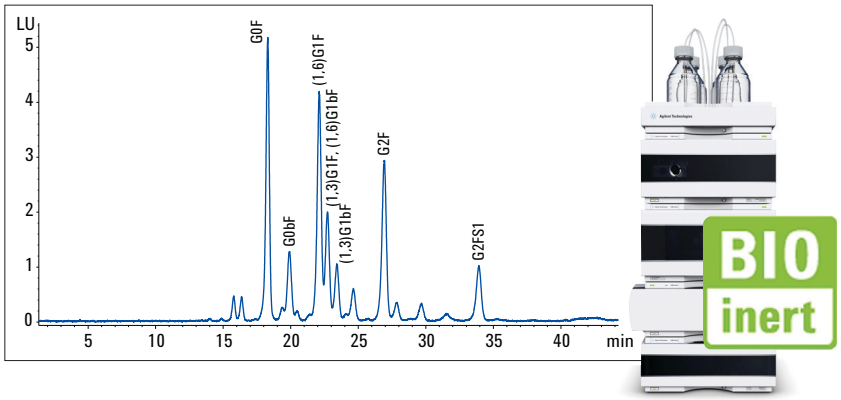
Sensitive and reproducible glycan analysis of human immunoglobulin G

Agilent 1260 Infinity Bio-inert Quaternary LC system with fluorescence detection

Application Note

Biopharmaceuticals



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Abstract

In this Application Note, sensitive and reproducible HPLC analysis of N-linked glycans of human immunoglobulin G is demonstrated using the Agilent 1260 Infinity Bio-inert Quaternary LC System together with fluorescence detection. Precision of retention times and areas was determined for analysis of a N-linked human IgG glycan library. In addition, good linearity in a range from 0.008 to 1 pmol was determined for a mix of five glycan standards (Man5, G2, G2S1, G2FS1, and G2S2) with low limits of detection and quantification.

The Agilent 1260 Infinity Bio-inert Quaternary LC System together with fluorescence detection is an optimal system for sensitive and reproducible analysis of 2-AB derivatized N-glycans released from human immunoglobulin G.

Agilent Technologies

Introduction

Antibodies represent the largest group of recombinantly produced therapeutic proteins as a major new class of drug. The efficacy of these therapeutics is highly dependent on the correct glycosylation pattern and, so far, all licensed therapeutic 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, mostly core-fucosylated with none 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 monoclonal antibodies (mAbs) reveal a marginally less complex pattern without nonfucosylated or bisecting GlcNAc glycan structures[4]. Other more immature structures are also found, for example high-mannose structures like mannose-5, although primarily in small amounts[5].

The analysis of protein glycosylation pattern can be challenging, especially due to the fact that 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] and was used in this Application Note for the determination of precision, linearity, and sensitivity.

Figure 1 shows the glycan structures, isoforms and nomenclature used in this Application Note, which have already been used in a previous publication[7]. The glycans linked to IgG are classified according to the number of terminal galactose residues attached, see Figure 1b. G0, G1, and G2 contain none, one and two terminal galactose residues.

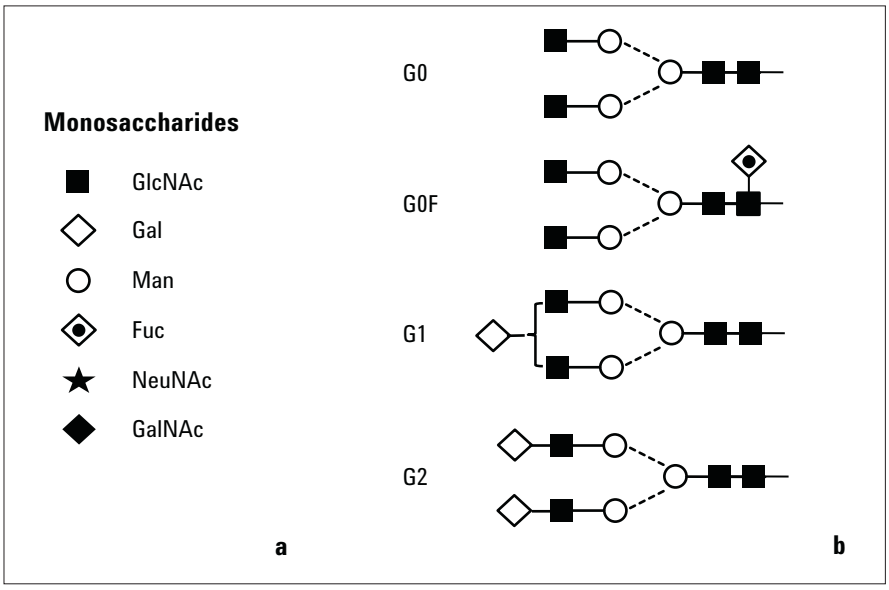


Figure 1
Glycan structure and isoforms. a) General nomenclature for glycans, b) Predominant glycan structures of IgGs. G = Galactose units, F = Fucose units. Modified after Arnold *et al.*[1].

Experimental

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

- Agilent 1260 Infinity Bio-inert Quaternary Pump (G5611A)
- Agilent 1260 Infinity High Performance Bio-inert Autosampler (G5667A)
- Agilent 1290 Infinity Thermostat (G1330B)
- Agilent 1290 Infinity Thermostatted Column Compartment (G1316C)
- Agilent 1260 Infinity Diode Array Detector VL (G1315D), equipped with bio-inert standard flow cell, 10 mm
- Agilent 1260 Infinity Fluorescence Detector (G1321B), equipped with bio-inert FLD flow cell

Column
TOSOH TSK-GEL Amide-80, 2 × 150 mm, 3 µm

Software
Agilent OpenLAB CDS, ChemStation Edition for LC & LC MS Systems, Rev. C.01.02 [14]

Solvents and samples
Buffer A: 100 mM ammonium formate, pH 4.5
Buffer B: acetonitrile

Glycan standards
- GLYKO 2-AB-(HUMAN IgG N-LINKED GLYCAN LIBRARY), 200 pmol
- GLYKO 2-AB-(MAN-5) → *Man5*, 100 pmol
- GLYKO 2-AB-(NA2) → *G2*, 100 pmol
- GLYKO 2-AB(A1) → *G2S1*, 100 pmol
- GLYKO 2-AB-(A1F) → *G2FS1*, 100 pmol
- GLYKO 2-AB-(A2) → *G2S2*, 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. AB-labeled glycan standards were purchased from Prozyme, Hayward, USA.

Chromatographic conditions

Gradient	Minutes	% B	Flow rate [mL/min]
	0	85	0.65
	5	75	
	10	72	
	30	67	
	40	64	
	40.01		0.65
	42	50	
	42.01		0.4
	45	100	
	50	100	
	55	85	
	55.01		0.4
	60		0.65
Stop time:	60.01		
Post time:	20 minutes		
Injection volume:	2.5 µL		
Column temperature:	50 °C		
FLD:	Ex. 260 nm Em. 430 nm		
Peak width:	Peak width: > 0.05 minutes (9.26 Hz)		
PMT:	PMT Gain: 14		

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 well resolved. For peak labeling, the nomenclature employed in various publications [3, 8, 9] was used. All dominant glycans are core fucosylated with none, one or two galactosylated structures, see Table 1. Except for the mono-sialylated G2FS1 (last peak), all assigned glycans have neutral structures.

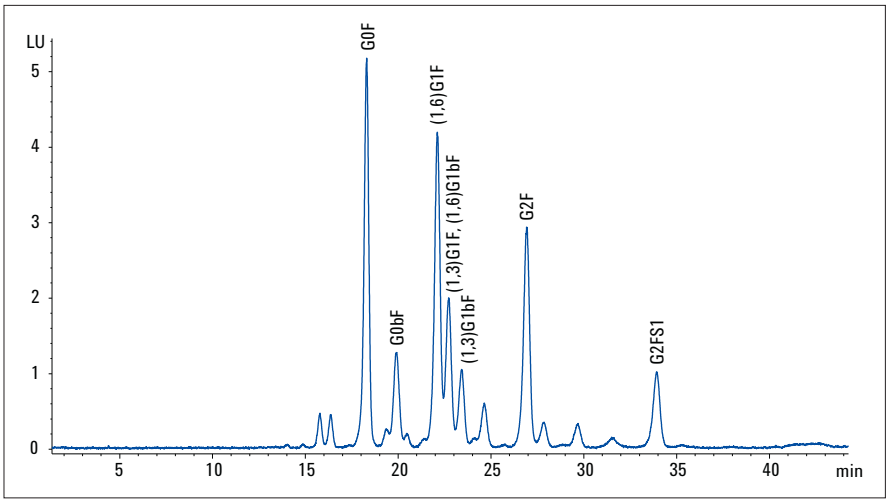


Figure 2
Separation of AB-labeled human IgG N-linked glycan library. Assignments were made based on Omtvedt *et al.* 2006.

Name	Monosaccharide composition	Structure
G0F	$GlcNAc_2Man_3GlcNAc_2Fuc$	
G0bF	$GlcNAc_3Man_3GlcNAc_2Fuc$	
(1,6)G1F	$GalGlcNAc_2Man_3GlcNAc_2Fuc$	
(1,3)G1F	$GalGlcNAc_2Man_3GlcNAc_2Fuc$	
(1,6)G1bF	$GalGlcNAc_3Man_3GlcNAc_2Fuc$	
(1,3)G1bF	$GalGlcNAc_3Man_3GlcNAc_2Fuc$	
G2F	$Gal_2GlcNAc_2Man_3GlcNAc_2Fuc$	
G2FS1	$NeuNAcGal_2GlcNAc_2Man_3GlcNAc_2Fuc$	

Table 1
Names, monosaccharide composition and structure of the assigned N-glycans in the human IgG glycan library.

Precision of retention time and area was determined for five consecutive injections of the human IgG N-linked glycan library. For all glycan structures, the retention time precision was below 0.16% and area precision between 0.66% and 4.11%, see Table 2. All measured RSD values for RT and area were within the commonly reported range[10].

Glycan	Precision of RT [%]	Precision of area [%]
G0F	0.135	0.74
G0bF	0.146	4.11
G1F	0.142	0.66
G1bF	0.153	1.03
G2	0.149	1.92
G2F	0.155	2.44
G2FS1	0.151	1.82

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

Linearity

Linearity was determined with a mix of five glycan standards (Man5, G2, G2S1, G2FS1, and G2S2) diluted in series 1:3 in a range from 0.008 to 1 pmol. Figure 3 shows a chromatogram of the five N-glycan mix at a sample amount of 1 pmol on column.

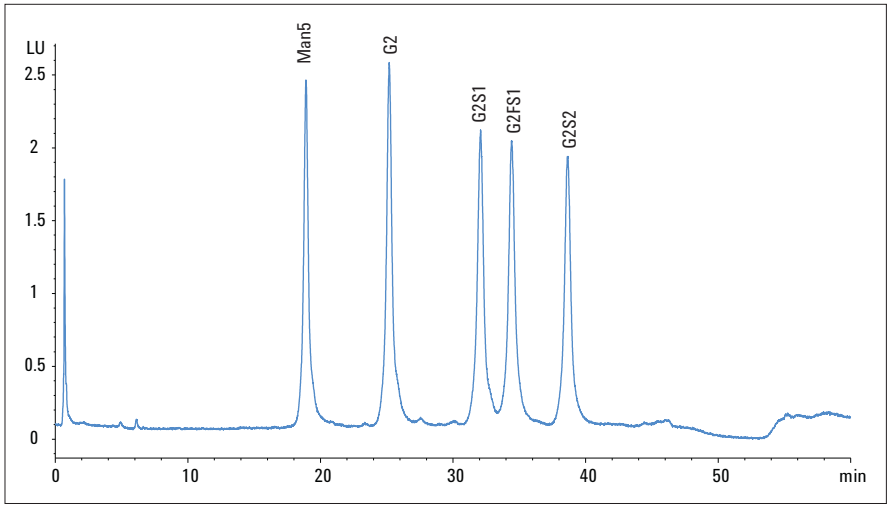


Figure 3
Mix of five AB-labeled N-linked glycan standards.

Limit of detections (LOD) were found between 10 and 15 fmol for S/N = 3. Limits of quantifications (LOQ) were found between 34 and 51 fmol for S/N = 10. All correlation curves showed good linearity with correlation coefficients over 0.999, see Table 3.

The PMT (photomultiplier tube) gain of the FLD detector was set to 14 for maximal sensitivity. In addition, using the Agilent 1260 Infinity Fluorescence Detector for the analysis of up to four different excitation and emission wavelengths, an optimal method development was possible, resulting in an excitation wavelength of 260 nm and an emission wavelength of 430 nm. This wavelength combination showed much higher intensity values compared to the wavelength normally used for glycan analysis (as, for example, recommended by Anumula 2005[11])

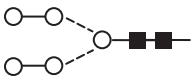
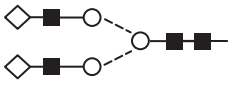
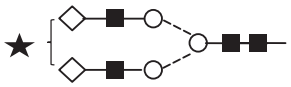
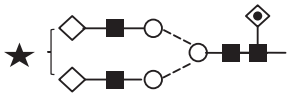
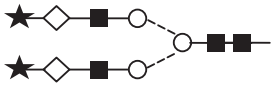
Glycan standard	Structure	LOD [fmol]	LOQ [fmol]	Correlation coefficient
Man5		10	34	0.9994
G2 (NA2)		11	37	0.9997
G2S1 (A1)		13	45	0.9998
G2FS1 (A1F)		14	47	0.9997
G2S2 (A2)		15	51	0.9993

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

Conclusions

This Application Note demonstrates that the Agilent 1260 Infinity Bio-inert Quaternary LC System together with 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 0.16% respectively between 0.66% and 4.11% 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 (Man5, G2, G2S1, G2FS1, and G2S2) diluted in series 1:3 in a range from 0.008 to 1 pmol. All correlation curves showed good linearity with correlation coefficients over 0.999. Limit of detections (LOD) were found between 10 and 15 and limits of quantifications (LOQ) were found between 34 and 51 fmol.

With optimized fluorescence wavelengths from the Agilent 1260 Infinity Fluorescence Detector, higher intensities and, therefore, better signal to noise ratios were achieved. Instead of an excitation wavelength of 330 nm (as recommended by Anumula 2005[12]) the lower wavelength 260 nm was used.

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

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Published in the USA, March 1, 2012
5991-0024EN

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