N-Glycan Profiling Analysis of a Monoclonal Antibody Using UHPLC/FLD/Q-TOF

Application Note

Authors

Xianming Liu, Wei Zhang, Yi Du, Sheng Yin, Hong Que, and Weichang Zhou

WuXi AppTec
Biopharmaceuticals Co., Ltd
Waigaoqiao Free Trade Zone
Shanghai 200131
China

Abstract

A high-sensitivity, high-resolution method for quantitative and qualitative glycan profiling of monoclonal antibodies has been developed. The method enables high confidence identification of even minor glycoforms and isoforms, using UHPLC/FLD/Q-TOF.

Agilent Technologies

Introduction

Most protein therapeutics developed are glycoproteins, including monoclonal antibodies (mAbs). The presence, absence, and profile of the complex oligosaccharide moieties on these mAbs can have significant impact on their therapeutic efficacy, pharmacokinetics, immunogenicity, folding, and stability[1,2,3,4,5]. For example, the N-Glycans located generally on amino acid Asn297 of mAbs are required for binding to the neonatal Fc receptor, which prevents lysosomal degradation of the mAb and extends its half-life in the plasma[6]. Certain glycan structures are also known to cause aggregation and decrease drug efficacy[1,5].

There is a strong understanding of which glycans contribute to both positive and negative drug performance. Hence, glycan profiles are determined early in the discovery and development of mAbs so they can be optimized. Once the best profile is determined, it must be monitored through bioprocess development to ensure that the mAb biotherapeutic is consistent and stable.

Analysis of the glycan moieties attached to a mAb is commonly conducted by enzymatic deglycosylation using N-Glycosidase F (PNGase F) to cleave asparagine-linked oligosaccharides from the antibody. The removed glycans are then derivatized, fluorescently labeled, and separated using ultra high performance liquid chromatography (UHPLC). A fluorescence detector (FLD) provides sensitive quantitation of the various glycan structures. However, HPLC/FLD methods do not always provide positive identification of all the glycan isoforms. Quadrupole time-of-flight mass spectrometry (Q-TOF MS) generates accurate mass information that can be used to definitively identify all glycan isoforms.

This Application Note describes a method that uses UHPLC and FLD detection online with Q-TOF MS to provide quantitative and qualitative profiling (positive identification) of N-glycans associated with mAbs, including minor glycoforms (Figure 1).

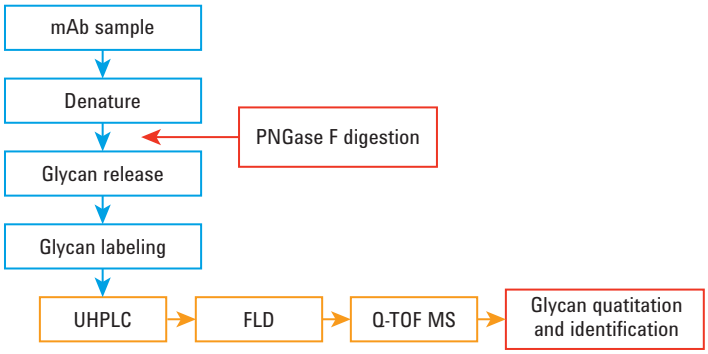

Figure 1. Workflow for glycan profiling of mAbs using UHPLC/FLD/Q-TOF MS.

Experimental

Reagents and standards

Sodium dodecyl sulfate (SDS), 2-Mercaptoethanol, 2-aminobenzamide (2-AB), dimethyl sulfoxide (DMSO), and sodium cyanoborohydride were obtained from Sigma-Aldrich. PNGase F was received from Prozyme.

Instruments

This study was conducted using an Agilent 1290 Infinity LC System coupled to an Agilent 1260 Infinity Fluorescence Detector, which in turn was coupled to an Agilent 6520 Q-TOF LC/MS system. The instrument conditions are shown in Table 1.

Sample preparation

A 250 µg mAb sample was denatured with 20 mg/mL SDS (final concentration 1 mg/mL) and reduced with 1 M 2-mercaptoethanol (final concentration 50 mM) for 5 minutes at room temperature. The sample was then incubated with 2.5 µL of PNGase F overnight at 37 °C to release the N-Glycans efficiently. After PNGase F digestion, ice-cold ethanol was added to the sample. The deglycosylated protein was removed by centrifugation. The supernatant containing the free N-glycans was then vacuum dried, and the dried glycan samples were labeled with the fluorophore 2-AB. The 2-AB labeling solution was composed of acetic acid/DMSO/2-AB/sodium cyanoborohydride (30 µL/70 µL/5 mg/6 mg), and the labeling reaction was carried out at 65 °C for 4 hours. The labeled sample was vacuum dried and reconstituted in 50 µL of ultrapure water for analysis.

Table 1. LC, FLD, and Q-TOF MS conditions.

Agilent 1290 Infinity LC run conditions				
Column	Waters, HILIC Glycan Amide column, 2.1 × 150 mm, 1.7 µm			
Column temperature	60 °C			
Injection volume	3 µL			
Mobile phase	A) 100 mM ammonium formate, pH 4.5 B) 100 % acetonitrile			
Gradient	Time (min)	Flow rate (mL/min)	% A	% B
	0	0.5	15	85
	5	0.5	25	75
	35	0.5	36	64
	40	0.5	50	50
	42	0.25	50	50
	43	0.25	80	20
	48	0.25	80	20
	52	0.5	15	85
	57	0.5	15	85
Agilent 1260 Infinity Fluorescence Detector	Excitation wavelength: 330 nm Emission wavelength: 420 nm			
Agilent 6520 Q-TOF LC/MS conditions				
Instrument mode	Auto MS/MS			
Ion mode	Dual ESI source, positive			
Drying gas	N_2, 10 L/min, 325 °C			
Fragmentor voltage	175 V			
Skimmer voltage	65 V			
OCT 1 RF Vpp	750 V			
Capillary voltage	3,500 V			
Acquisition rate	3 spectra/sec			
Mass range (MS)	500–3,200 *m/z*			
Mass range (MS/MS)	100–2,000 *m/z*			
Collision energy	Slope 3.6 and offset –4.8			

Results and Discussion

Glycan separation

The typical FLD chromatogram and Q-TOF total ions chromatogram (TIC) reveal well resolved glycan profiles (Figure 2). The FLD chromatogram provides quantitation, and reveals some of the minor glycan peaks. The Q-TOF MS data provide accurate mass information in addition to the chromatographic retention time, both of which can be used for glycan identification. The tandem mass spectrometry (MS/MS) capability of the Q-TOF also provides fragmentation information that aids in determining glycan identity with very high confidence.

Glycan identification

The identity of each peak was confirmed by accurate MS data, as shown in Table 2 and Figure 2. The sensitivity of the Agilent 6520 Q-TOF LC/MS system was sufficient to identify even the minor components. For example, Figure 3 shows the mass spectrum of the sialylated glycan G2FS1, which did not appear as a clear peak in the TIC (panel A), and gave a minor peak on the FLD chromatogram (panel B) at retention time (RT) 29.5 minutes. The extracted ion chromatogram (EIC, panel C) revealed an ion at this RT that matched the calculated mass for the $[M+H]^{2+}$ adduct of G2FS1 (panel D). Figure 4 shows similar results for the G0F glycan at RT 17 minutes, which appeared as a major peak on both chromatograms.

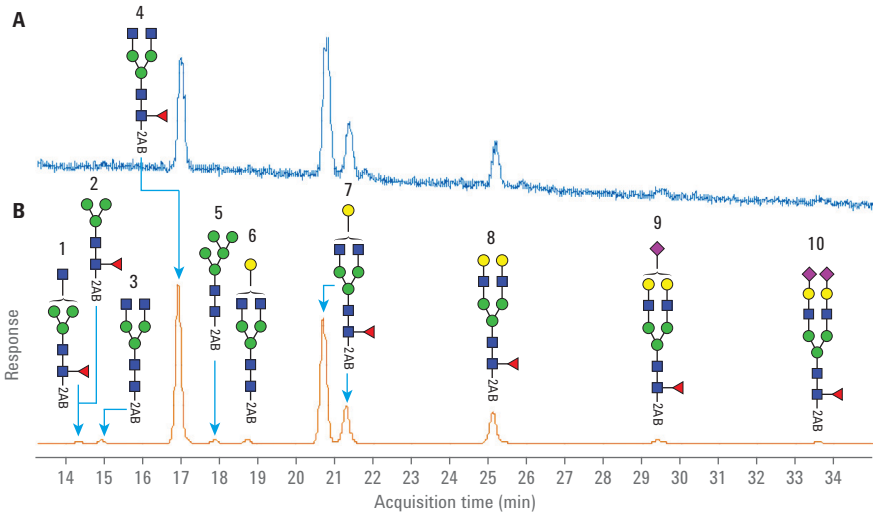

Figure 2. Typical Q-TOF total ions chromatogram (TIC) (A) and FLD chromatogram of N-glycans (B) from an mAb.

Table 2. Identification of N-glycans using accurate mass.

Peak no.	Glycan	Theoretical mass (Da)	Observed mass (Da)	Mass error (ppm)
1	2AB-G0F-GN	1,379.5338	1,379.5278	–4
2	2AB-Man3+1F	1,176.4544	1,176.4501	–4
3	2AB-G0	1,436.5552	1,436.5532	–1
4	2AB-G0F	1,582.6132	1,582.6075	–4
5	2AB-Man5	1,354.5022	1,354.5019	0
6	2AB-G1	1,598.6081	1,598.6008	–5
7	2AB-G1F	1,744.666	1,744.6575	–5
8	2AB-G2F	1,906.7188	1,906.7114	–4
9	2AB-G2FS1	2,197.8142	2,197.8034	–1
10	2AB-G2FS2	2,488.9096	2,488.9054	–2

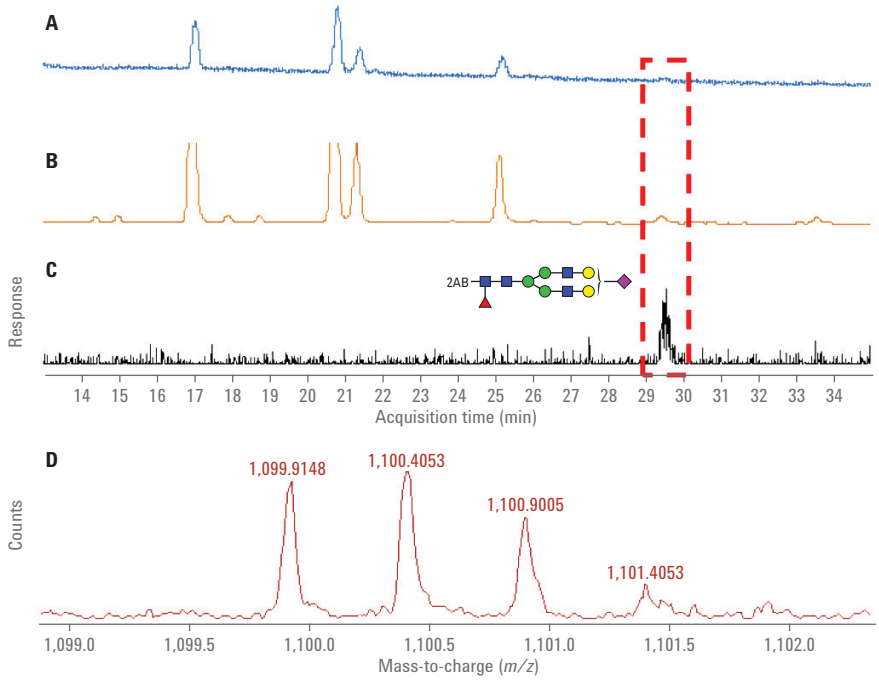

Figure 3. Identification of the G2FS1 glycan. A) expanded view of the N-Glycan TIC, B) expanded view of the FLD chromatogram, C) the extracted ion chromatogram (EIC) of the peak at 29.5 minutes, and D) the spectrum of $[M+H]^{2+}$ of the G2FS1 glycan, *m/z* 1,099.9144, which elutes at 29.5 minutes.

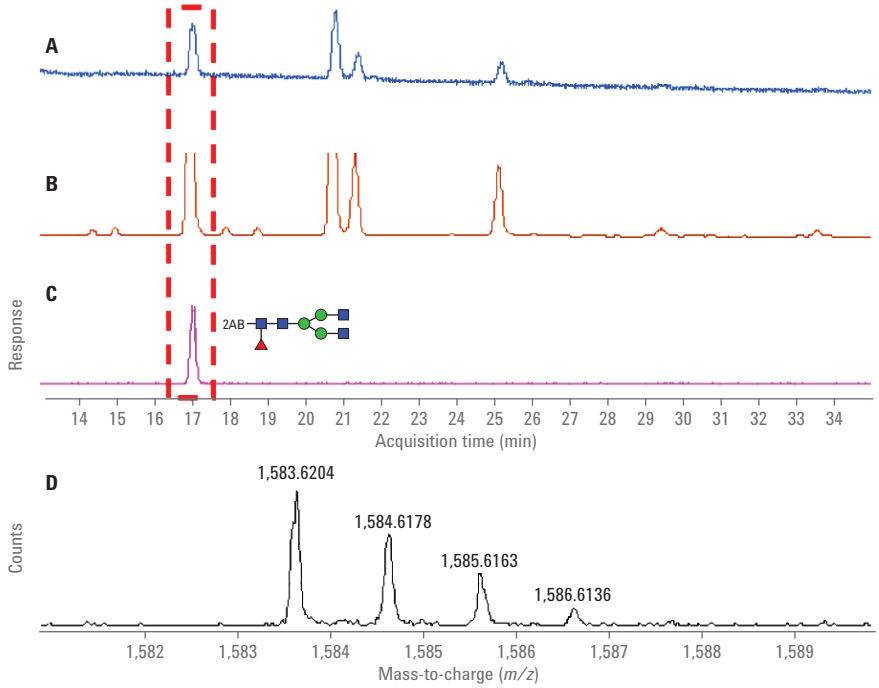

Figure 4. Identification of the G0F glycan. A) expanded view of the N-Glycan TIC, B) expanded view of the FLD chromatogram, C) the extracted ion chromatogram (EIC) of the major peak at 17 minutes, and D) the spectrum of $[M+H]^{1+}$ of the G0F glycan, *m/z* 1583.6205, which elutes at 29.5 minutes.

Given the importance of the glycosylation pattern for the efficacy and safety of mAbs, it is important to identify the variant and minor glycan structures. Tandem mass spectrometry (MS/MS) fragment ion data were collected to characterize and confirm the identities of the N-glycan structures (Figure 5). All of the fragment ions of the glycan chain were assigned to known glycan structures.

Conclusions

Glycan analysis using UHPLC/FLD/Q-TOF MS on the Agilent 1290 Infinity LC System and the Agilent 6520 Q-TOF LC/MS system is a powerful platform for quantitative and qualitative glycan profiling. Fluorescent detection provides sensitive and quantitative analysis of the labeled glycans. Mass spectrometry analysis is useful for the identification and structural characterization of glycans. The chromatographic resolution, reproducibility, sensitivity, and MS/MS capability enable good separation and analysis of minor glycoforms, which are otherwise challenging to assign.

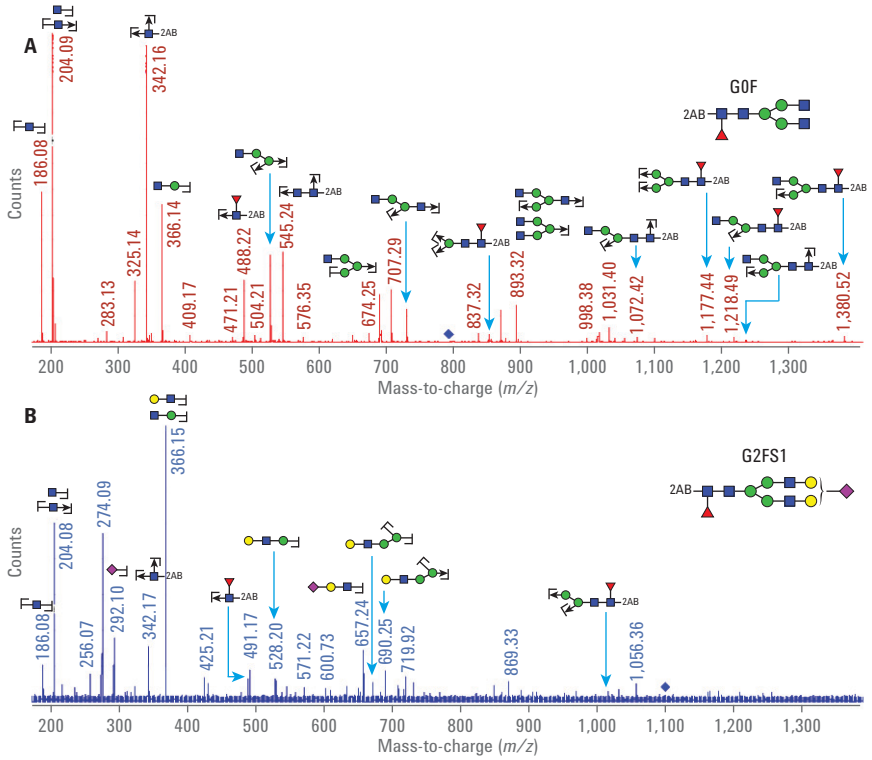

Figure 5. MS/MS spectra of (A) G0F and (B) G2FS1, with the major fragment ions assigned to b/y ions of G0F and G2FS1 respectively.

References

1. Jefferis, R. Glycosylation as a strategy to improve antibody-based therapeutics. *Nat. Rev. Drug Discov*. **2009**, *8*, pp 226-234.

2. Junttila, T. T; *et al.* Superior *in vivo* efficacy of afucosylated trastuzumab in the treatment of HER2-amplified breast cancer. *Cancer Res.* **2010**, *70*, pp 4481-4489.

3. Tao, M. H; Morrison, S.L. Studies of aglycosylated chimeric mouse-human IgG. Role of carbohydrate in the structure and effector functions mediated by the human IgG constant region. *J. Immunol*. **1989**, *143*, pp 2595-2601.

4. Sibéril, S; *et al.* The key to optimize therapeutic antibodies? *Crit. Rev. Oncol. Hematol*. **2007**, *62*, pp 26-33.

5. Petrescu, A. J; Wormald, M. R; Dwek, R. A. Structural aspects of glycomes with a focus on N-glycosylation and glycoprotein folding. *Curr. Opin. Struct. Biol*. **2006**, *16(5)*, pp 600-7.

6. Nelson, A. L; Reichert, J. M. Development trends for therapeutic antibody fragments. *Nat. Biotechnol.* **2009**, *27*, pp 331-337.

www.agilent.com/chem

This information is subject to change without notice.

© Agilent Technologies, Inc., 2014

Published in the USA, August 7, 2014
5991-5067EN

Agilent Technologies