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WHITE PAPER

Analysis of the N-Glycan Profile of a Therapeutic Monoclonal Antibody

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ABSTRACT

The objective of this study was to determine the N-glycosylation profile in an antibody reference standard and product formulations.

The N-glycan profile of the antibody was analyzed by releasing the N-glycans from the molecule using PNGase F digestion. The released N-glycans were extracted and enriched by solid phase extraction, followed by 2-AB labeling. The labeled N-glycans were then resolved by HILIC chromatography and detected by fluorescence spectroscopy. N-glycan standards were used to identify the N-linked oligosaccharides present in the antibody formulations.

The fucosylated N-glycan with one terminal galactose was the major N-glycan species in both the reference standard and the product formulations at about 50 to 55% of the total N-glycans observed. The fucosylated N-glycan with two terminal galactose units was found at about 14 to 18% and an N-glycan without galactosylation was also observed at about 27 to 31% in both the reference standard and product formulations. Low levels of sialylated glycans were detected but no significant amounts of Man5 and N-linked oligosaccharides without a fucose moiety were observed in the test materials.

The N-glycan profile of the antibody product formulation was comparable with the reference standard. All the N-glycans observed in both antibody formulations were fucosylated N-linked oligosaccharides, with one terminal galactose as the major component. The N-glycan with two terminal galactose units and an N-glycan without galactosylation were also observed in these antibody formulations.

BACKGROUND

The current study describes the procedure for determining the glycan profile of a therapeutic monoclonal antibody and comparing a reference standard lot from a new drug formulation. The method involves labeling with 2-Aminobenzamide (2-AB) and analyzed on UPLC-HILIC with fluorescence detection. The intended use of the method is to provide analytical support for clinical drug development.

OBJECTIVE

The objective of this study was to determine the N-glycosylation profile in an antibody reference standard and product formulations.

METHODS

Materials

The main materials used in this project were a genetically-expressed recombinant monoclonal antibody therapeutic. The antibody is in highly purified form in a drug substance and product formulations.

Equipment

The primary equipment used in this project was a Waters Acquity Binary UPLC equipped with a Fluorescence detector.

Sample preparation

N-glycan release from the antibody was carried out by denaturing the molecule at 65°C for 10 minutes followed by incubation with PNGase F in 50 mM Tris-HCl, pH 8.0 for at least 16 h at 37°C.

Extraction and enrichment of the released N-glycan

The N-glycan released sample was loaded onto a pre-conditioned SPE cartridge and washed repeatedly with acetonitrile using a vacuum manifold. The bound N-glycan was eluted off the cartridge with aqueous solvent and dried on a vacuum evaporator (Speed VAC concentrator).

Derivatization with 2-AB

The 2-AB labeling solution was added to the sample, mixed, and incubated for 3 h at 65°C. Ammonium acetate and acetonitrile solutions were added to the resulting sample and centrifuged. The supernatant was transferred to a HPLC vial for analysis.

Preparation of standards

To the vial of the Performance Standard was added 100 µL of 100 mM Ammonium Formate, pH 4.5 and 100 µL Acetonitrile. The standard is aliquoted in small volumes and frozen at – 20 C until analysis.

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UPLC conditions:
Instrument Parameters:
Acquity Instrument Method

Excitation Wavelength	330 nm
Transmission Wavelength	420 nm
Gain	1
Auto Zero on Inject Start	Enabled
Data Rate	1 pts/sec
Time Constant	Fast

Waters Acquity UPLC system

Injection Volume	2µL
Sample Loop Option	Partial loop with needle overfilled
Weak Wash Solvent	95% Acetonitrile/5% Water
Weak Wash Volume	600 µL
Strong Wash Solvent	95% Water/5% Acetonitrile
Strong Wash Volume	200 µL
Column Temperature	60°C
Sample Temperature	Room temperature
Syringe Draw Rate (Advanced Settings)	30 µL/min
Needle Placement (from bottom) (Advanced Settings)	4.0mm Note: This setting is recommend for the Waters Total Recovery HPLC vials. Other sample vials may require a different setting.
Needle Overfill Flush	3.0 µL

(Advanced Settings)				
Run Time: 58.5 minutes				
Gradient Table				
Time (minutes)	Flow Rate (mL/min)	%Mobile Phase A	%Mobile Phase B	Curve
Initial	0.5	22.0	78.0	6
10.0	0.5	22.0	78.0	6
48.5	0.5	44.1	55.9	6
49.5	0.25	100	0	6
54.5	0.25	100	0	6
58.5	0.5	22.0	78.0	6

Mobile Phase A (100 mM Ammonium Formate, pH 4.5)
Mobile Phase B (100% Acetonitrile)

CALCULATIONS

1.1 Interference of Control Sample

$$\%\ \text{Peak Control} = \frac{\text{Peak Area}_{\text{Control}}}{\text{Peak Area}_{\text{1st Injection of Sample}}} \times 100\%$$

Note: Calculate the %Peak Control for G0F, G1Fa, GlFb, G2F and GlF + SA, and G2F + SA, respectively.

1.2 Ratio of G0F/G1Fa

$$\text{Ratio}_{\text{G0F/G1Fa}} = \frac{\text{Area}_{\text{G0F}}}{\text{Area}_{\text{G1Fa}}}$$

1.3 %RD of two (2) replicates of Sample injections for ratio of G0F/G1Fa

$$\%\ \text{RD} = \frac{\text{Ratio}_{\text{Sample1}} - \text{Ratio}_{\text{Sample2}}}{\text{Ratio}_{\text{Sample1}} + \text{Ratio}_{\text{Sample2}}} \times 2 \times 100\%$$

1.4 Glycan profiling calculation (Round to two (2) decimal points)

1.4.1
$$\%\ \text{G0F} = \frac{\text{Area}_{\text{G0F}}}{\text{Area}_{\text{(G0F + G1Fa + G1Fb + G2F)}}} \times 100\%$$

1.4.2
$$\%\ \text{G1Fa} = \frac{\text{Area}_{\text{G1Fa}}}{\text{Area}_{\text{(G0F + G1Fa + G1Fb + G2F)}}} \times 100\%$$

1.4.3
$$\%\ \text{G1Fb} = \frac{\text{Area}_{\text{G1Fb}}}{\text{Area}_{\text{(G0F + G1Fa + G1Fb + G2F)}}} \times 100\%$$

1.4.4
$$\%\ \text{Galactosylation} = \frac{2 \times \text{Area}_{\text{G2F}} + \text{Area}_{\text{G1Fa + G1Fb}}}{2 \times \text{Area}_{\text{(G2F + G1Fa + G1Fb + G0F)}}} \times 100\%$$

1.4.5
$$\%\ \text{Monosialyl Glycans} = \frac{\text{Area}_{\text{G1F + SA}} + \text{Area}_{\text{G2F + SA}}}{\text{Area}_{\text{(G0F + G1Fa + G1Fb + G2F + G1F - SA + G2F - SA)}}} \times 100\%$$

RESULTS

Analysis of the N-glycan profile of a therapeutic monoclonal antibody formulations was obtained from N-glycans cleaved from the antibody following N-glycanase digestion. The digest fraction was loaded onto a solid phase extraction cartridge to bind the hydrophilic oligosaccharides and washed off the hydrophobic proteinaceous fraction with high organic solvent. The bound glucosamine sugars were eluted off the cartridge with aqueous solvent and subsequently labeled with fluorescent 2-AB.

The various forms of N-glycans typically found in immunoglobulins were resolved by HILIC chromatography and detected by Fluorescence spectroscopy (Fig. 1). Immunoglobulin N-glycan standards were concurrently labeled and analyzed with the samples to identify the N-linked oligosaccharides present in the

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antibody formulations. The HILIC-fluorescence method showed good linearity and precision response for N-glycan determination in monoclonal antibodies (Fig. 2). Likewise, injection repeatability was also demonstrated (Fig. 3) in this method. Similar chromatographic profiles for N-glycans were observed between the reference standard lot and the new drug formulation (Fig. 4).

As shown in Table I, similar N-glycan compositions were obtained with the current HILIC method as compared with the previous method employing ion-exchange chromatography from another testing laboratory. In Table II, the N-glycan with one terminal galactosylation and fucosylation (G1Fa, b) was the major N-glycan species in both the reference standard and the product formulations at about 50 to 55% of the total N-glycans observed. The fucosylated N-glycan with both terminal galactosylation (G2F) was observed at about 14 to 18% and an N-glycan without galactosylation (G0F) was present at about 27 to 31% in both the antibody reference standard and product formulations. Other forms of N-glycans such as monosialylated glucosamines were detected at low levels at less than 2%. Man5 and unfucosylated N-linked oligosaccharides were not detected in the test materials.

Figure 1. Representative chromatogram of the expected (A) and observed (B) N-glycan standard profile by HILIC-fluorescence detection.

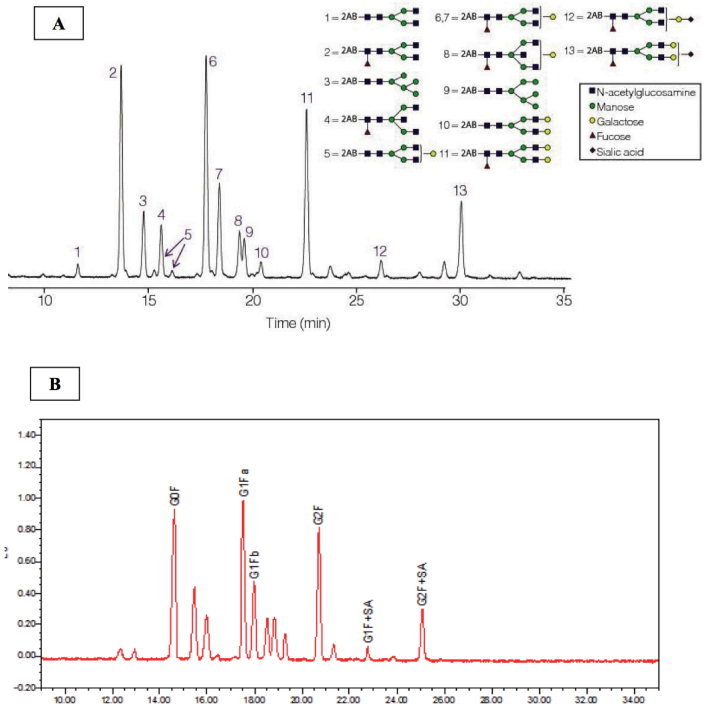
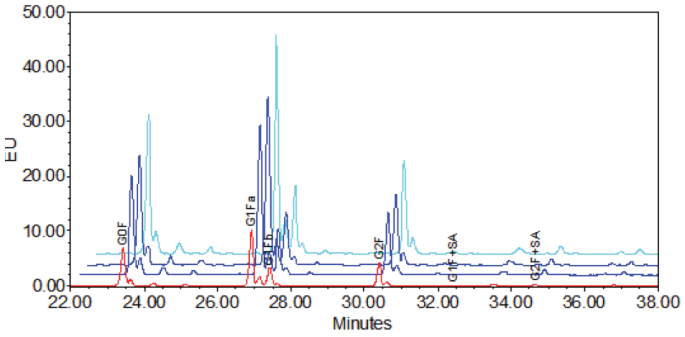


Figure 2. Linearity and precision assessment of the N-glycan profiling by HILIC-fluorescence spectroscopy.



Precision	G0F	G1F	G2F	Sialylation
%RSD	3.46	1.58	8.60	18.65

Figure 3. Injection repeatability of the N-glycan profile of a therapeutic monoclonal antibody.

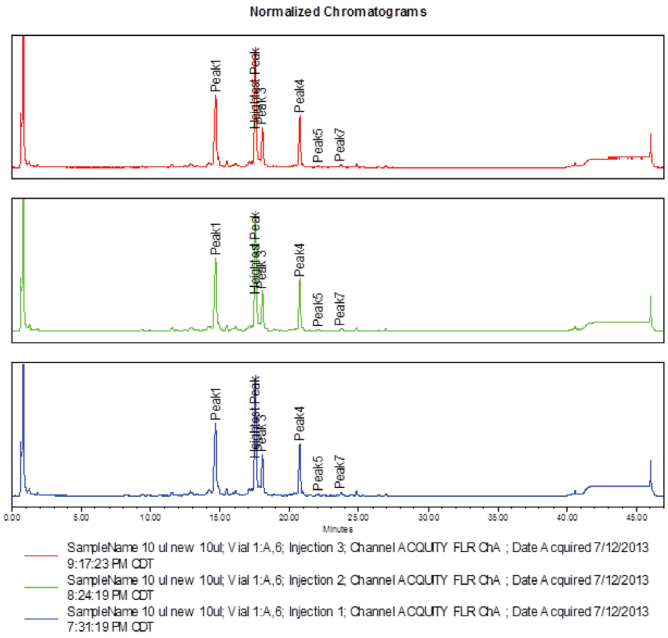


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Figure 4. HILIC chromatograms of N-glycoforms in the reference standard and antibody drug formulation.

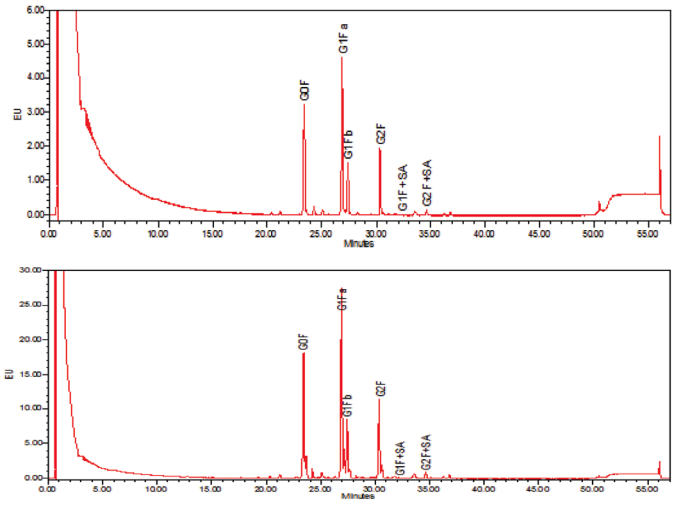

Table I. Comparison of Previous and Current Method of N-glycan profiling.

N-Glycoform	Previous Method	Current Method
(G0F)	17.4%	14.0%
(G1F)	51.3%	56.2%
(G2F)	31.3%	29.8%
% Biantennary Galactosylation	43.0%	42.1%
Monosialysation	1.6%	1.9%

Table II. Comparison of Reference Standard and Drug Formulation.

N-Glycoform	Reference Standard	Drug Formuation
(G2F)	17.7%	17.4%
(G1Fa)	42.4%	41.6%
(G1Fb)	12.6%	13.0%
(G0F)	27.3%	28.0%
% Biantennary Galactosylation	45.2%	44.7%
Monosialysation	1.3%	1.5%

CONCLUSIONS

Antibody N-glycan profiling was determined by PNGase F digestion. 2-AB labeling, HILIC chromatography and Fluorescence detection. Overall, the N-glycan profile of the antibody product formulation was comparable with the reference standard. All the N-glycans observed in both antibody formulations were fucosylated N-linked oligosaccharides with single galactosylation as the major glycoform species. The N-glycan with both terminal galactosylation and an N-glycan without galactosylation were also observed in these therapeutic antibody formulations.

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