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Comparison of Released N-Glycans in Biosimilar mAb Drug Products Using the BioAccord™ LC-MS System

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Abstract

N-linked glycans are typically part of the set of critical quality attributes of therapeutic monoclonal antibodies (mAbs), as they play an important role in safety and efficacy. Therefore, they need to be well characterized for both an innovator product and subsequent biosimilar mAb candidates. This study demonstrates how a small footprint, compliance-ready LC-MS platform executing an analytical workflow for released N-linked glycan analysis can be used for biosimilar mAb product assessment. The compliance-ready waters_connect™ platform offers a streamlined informatics workflow for automatic assignment of labeled released glycans based on HILIC separation with fluorescence and mass detection, utilizing calibrated glycan unit retention times and accurate mass confirmation. This workflow was utilized for comparing the glycosylation profile of infliximab biosimilar drug products to the innovator drug product. Samples were prepared using an automated protocol with the Andrew+™ Pipetting Robot following the GlycoWorks™ RapiFluor-MS™ protocol and then analyzed on a BioAccord LC-MS System. A total of 36 N-glycans were detected across the four samples. Notable differences were observed in the glycans identified and the relative abundance of each glycan between samples. The results establish that this workflow can be readily deployed for profiling and monitoring released glycans in mAb drug products.

Benefits

- Automated, easy-to-deploy protocol for reproducible sample preparation

- Streamlined informatics workflow for automated assignment of labeled released N-glycans facilitated by glycan ladder calibrated chromatographic retention times and accurate mass confirmation

- Quantitative comparison of assigned N-glycans and their relative abundances between samples

Introduction

Glycosylation is an important modification that can impact the quality of a biotherapeutic drug product. The complex network of enzymes involved in the biosynthesis of N-glycans at various stages results in a diverse structures of N-glycans at each site that can vary based on upstream and downstream bioprocesses, as well as with the host cell line.[1] These post-translational modifications affect critical functions of the protein, including folding, conformation, stability, receptor interactions, and intra- and extracellular targeting.[2] The type and amount of glycosylation can impact drug safety, efficacy, and stability. Consequently, N-glycans are commonly selected as critical quality attributes (CQAs) to be monitored during the development and manufacturing of drug products. Even in the case of biosimilar drug products, characterization of glycoforms is critical, as glycosylation profiles can vary with differences in manufacturing processes, especially when the drug products originate from different cell lines. These variations in glycosylation can lead to differences in a drug's immunogenicity, biological function, and circulation half-life, making it critical to systematically assess any differences in glycosylation between biosimilar drug products compared to the originator.[1]

This work demonstrates how the workflow can be used to quickly assign N-glycans, compare profiles, and determine potential CQAs for later monitoring. Glycosylation profiles can be characterized at various levels, including analysis of the intact glycoprotein, glycoprotein subunits, glycopeptides, and released glycans. Full characterization of the set of released glycans provides knowledge of the monosaccharide composition, linkages, and relative abundance of N-glycans comprising the glycoprotein, but for most antibodies a set of known biantennary glycans would be expected. Released glycan analysis can be challenging, as glycans do not contain a chromophore and are typically present at low abundances in a diverse array of structures. To address this, released glycans can be labeled with a fluorescence tag to enable sensitive detection with a fluorescence detector coupled to liquid chromatography (LC) and mass spectrometry (MS). The GlycoWorks *Rapi*Fluor-MS tag offers an advantage of enabling effective glycoform resolution by HILIC chromatography, sensitive fluorescence detection, and high MS ionization efficiency.[3] Here, we demonstrate how the released glycan analysis workflow

can be implemented with a BioAccord LC-MS (ToF) System with waters_connect informatics to compare glycoprofiles of biosimilar infliximab drug products to the innovator product. Sample preparation was performed using an Andrew+ Pipetting Robot, and the resulting released glycan samples were analyzed with a BioAccord LC-MS System equipped with an inline ACQUITY™ Premier FLR Detector. The glycosylation profiles of each infliximab product were analyzed, and the relative abundances of N-glycans were compared between products.

Experimental

Sample Description

Infliximab released glycan samples, including Remicade™, Inflectra™, Avsola™, and Renflexis™, were prepared following the GlycoWorks *Rapi*Fluor-MS (RFMS) automation protocol using the Andrew+ Pipetting Robot (Figure 1).[4] For each sample, 5 μL (~2 μg mAb) was injected onto the column. The *Rapi*Fluor-MS Dextran Calibration Ladder (p/n: 186007982 <https://www.waters.com/nextgen/global/shop/standards--reagents/186007982-rapifluor-ms-dextran-calibration-ladder.html>) was reconstituted in 100 μL of water and 2 μL was injected onto the column. The *Rapi*Fluor-MS Glycan Performance Test Standard (p/n: 186007983 < https://www.waters.com/nextgen/global/shop/standards--reagents/186007983-rapifluor-ms-glycan-performance-test-standard.html>) was reconstituted in 50 μL of water and 2.5 μL was injected onto the column.

LC Conditions

LC system:	ACQUITY Premier BSM UPLC™
Detection:	ACQUITY Premier FLR Detector ($\lambda_{excitation}$=265 nm, $\lambda_{emission}$=425 nm, 2 *Hz*)
Vials:	QuanRecovery™ with MaxPeak™ HPS vials (p/n: 186009186)
Column(s):	ACQUITY Premier Glycan BEH™

Amide Column (1.7 µm, 130 Å, 2.1 ×
150 mm) (p/n: 186009524)

Column temperature: 60 $^{\circ}$C

Sample temperature: 8 $^{\circ}$C

Injection volume: 5 µL

Mobile phase A: 50 mM ammonium formate (pH 4.4)
                             prepared from Waters™ Ammonium
                             Formate Solution – Glycan Analysis
                             (p/n: 186007081)

Mobile phase B: Acetonitrile

Gradient Table

Time (min)	Flow (mL/min)	%A	%B	Curve
0.00	0.400	25	75	6
35.00	0.400	46	54	6
36.50	0.200	80	20	6
39.50	0.200	80	20	6
43.10	0.200	25	75	6
47.60	0.400	25	75	6
55.00	0.400	25	75	6

MS Conditions

MS system: BioAccord System

Ionization mode: ESI, positive

Acquisition range: 50–2,000 *m/z*

Capillary voltage: 1.5 kV

Low Energy cone voltage: 45 V

High Energy cone voltage: 70–90 V

Desolvation temperature: 350 °C

Intelligent data capture (IDC):   On

Data Management

Data were acquired and processed with the waters_connect informatics platform containing the integrated UNIFI™ App (version 3.1.0.16), utilizing both the Glycan FLR with MS confirmation and accurate mass screening workflows.

Results and Discussion

For biosimilar mAb manufacturers, characterizing and monitoring N-glycans can be challenging, as their levels can vary substantially in biosimilar drug products compared to the innovator product. Here, we present a robust and readily deployable automated workflow for assigning and comparing released glycans in infliximab drug products using the BioAccord LC-MS System with automated data processing for *Rapi*Fluor-MS labeled released N-linked glycans.

Sample Preparation Using the Andrew+ Pipetting Robot

Released glycan sample preparation workflows consisted of three steps prior to LC-MS analysis: (1) deglycosylation using PNGase F, (2) labeling using the *Rapi*Fluor-MS tag, and (3) SPE cleanup to remove excess

label. Automation of these steps is beneficial for ensuring reproducibility between samples, both for assesment of individual samples and comparing profiles across samples. The Andrew+ Pipetting Robot executed the sample preparation workflow for sixteen samples using the configuration shown in Figure 1.[4] This protocol can be used to prepare up to 48 samples in less than ninety minutes. The protocol was used to prepare four replicate samples of each of the four infliximab products, including the innovator and three biosimilars. As an added benefit, automating the protocol frees the user to perform other tasks while samples are being prepared. This automated glycan sample procedure is part of the pre-configured Andrew+ OneLab™ script that can be downloaded and applied by users.
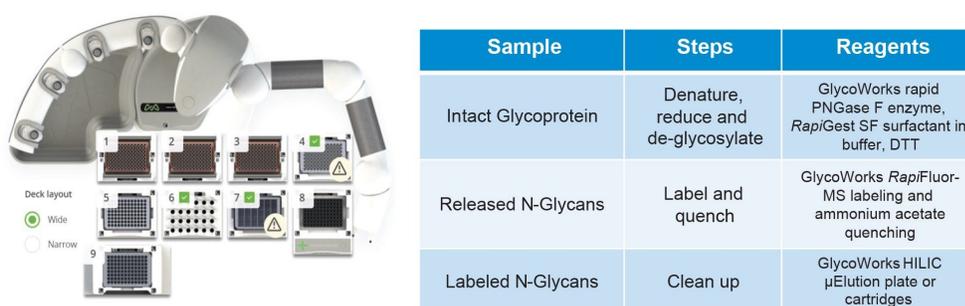

Sample	Steps	Reagents
Intact Glycoprotein	Denature, reduce and de-glycosylate	GlycoWorks rapid PNGase F enzyme, *Rapi*Gest SF surfactant in buffer, DTT
Released N-Glycans	Label and quench	GlycoWorks *Rapi*Fluor-MS labeling and ammonium acetate quenching
Labeled N-Glycans	Clean up	GlycoWorks HILIC µElution plate or cartridges

Figure 1. Automated protocol using the Andrew+ Pipetting Robot, including (left) the component setup and (right) the main protocol steps.

Retention Time Calibration and Performance Check

To ensure accurate assignment of glycans in a sample from a library-based search, chromatographic peak retentions are matched against entries of the Glucose Unit (GU) Scientific Library, which contains 177 N-glycan structures.[5] The library includes GU values (calibrated retention time in glucose unit values) and accurate masses of each glycan that can be used to confirm glycan peaks with increased confidence. Prior to running samples on the BioAccord System, the *Rapi*Fluor-MS Dextran Calibration Ladder (p/n: 186007982 < https://www.waters.com/nextgen/global/shop/standards--reagents/186007982-rapifluor-ms-dextran-calibration-ladder.html>) was used to calibrate the system with the ACQUITY Premier BEH Glycan Column and demonstrate system suitability for GU retention time-based assignment of the released N-glycans.[6] GU values from 4 to 12 were used in calibration to cover the elution range of N-glycans within the sample. The dextran ladder is shown in Figure 2 with GU values and retention times labeled. The accuracy of the calibration was

confirmed using a *Rapi*Fluor-MS Glycan Performance Test Standard (p/n: 186007983 <

https://www.waters.com/nextgen/global/shop/standards--reagents/186007983-rapifluor-ms-glycan-

performance-test-standard.html>), shown in the bottom of Figure 2. Several of the identified glycan structures

are shown in the chromatogram, along with the measured GU value and the difference between the expected

and observed GU values (Δ GU). The Δ GU values for identified glycans were less than 0.10, indicating excellent

retention time calibration.
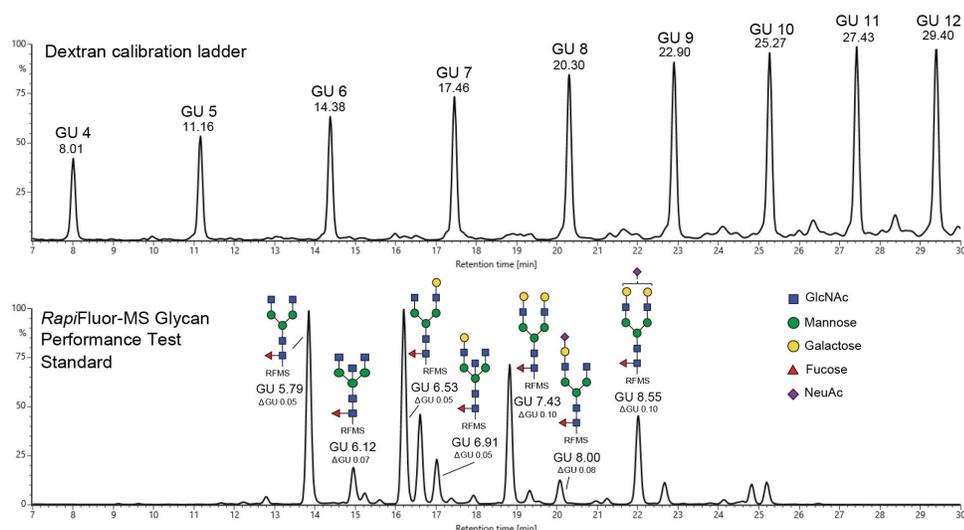
Figure 2. Retention time calibration and performance check, including (top) Glycan Unit (GU) calibration of retention times using a labeled dextran ladder and (bottom) establishment of system suitability of calibrated GU retention time assignments using a standard glycan sample.

Identification of N-Glycans in Infliximab Samples

After confirming suitable retention time calibration of the system, the four infliximab samples were run on the

system using the same separation conditions. The results were processed in the UNIFI App of waters_connect

using the Glycan Assay (FLR with MS Confirmation) workflow.[5] Using this workflow, glycan chromatographic

calibrated retention times and *m/z* values were considered for glycan structure assignment by matching the

glycan entries within the UNIFI scientific library (closest match in GU value and MW). The FLR peak areas were

used for relative quantitation measurements. Figure 3 shows an example of the results page for data processed

using this workflow. The component summary table lists the identified glycans and their pertinent information. The chromatogram window shows detected peaks and the name and structure of corresponding glycans. Clicking on a component in the table or a peak in the chromatogram pulls up a list of tabulated library search results, as shown in the bottom right. Here, all possible library matches are shown, along with relevant information, including Δ GU and Δ *m/z*, to facilitate rapid review of each peak assignment. In the example shown in Figure 3, there are two possible library matches for the peak eluting at 12.80 min, A2 and F(6)M4. However, only A2 matches based on expected *m/z*, and it also has a more closely matching GU value. Therefore, the automated A2 was confirmed as the glycan structure corresponding to this peak.
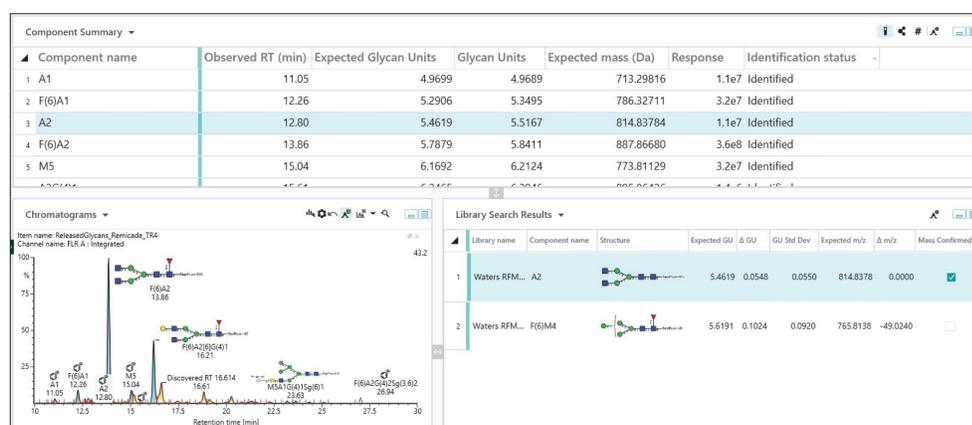

Figure 3. Glycan Unit based assignment of released N-glycans using the Glycan Assay (FLR with MS Confirmation) workflow in the UNIFI App within waters_connect.

Across all four infliximab samples, 36 unique N-glycan structures were assigned, but N-glycans assigned varied considerably between samples, with 18 to 21 glycans measured for each sample. Figure 4 shows a comparison of the identified N-glycans between samples. The Venn diagram on the left shows glycans common to each sample. While approximately a third of the identified glycans were consistently identified across all four samples, there were also glycans unique to each sample. Unsurprisingly, mAb samples that originated from the same cell line showed a greater profile similarity, *i.e.*, Remicade and Inflectra that both originate from a murine cell line, and Renflexis and Inflectra that both originate from a Chinese hamster ovary cell line. Figure 4 also contains an overlay of chromatograms from all four samples with the abundant glycans labeled.
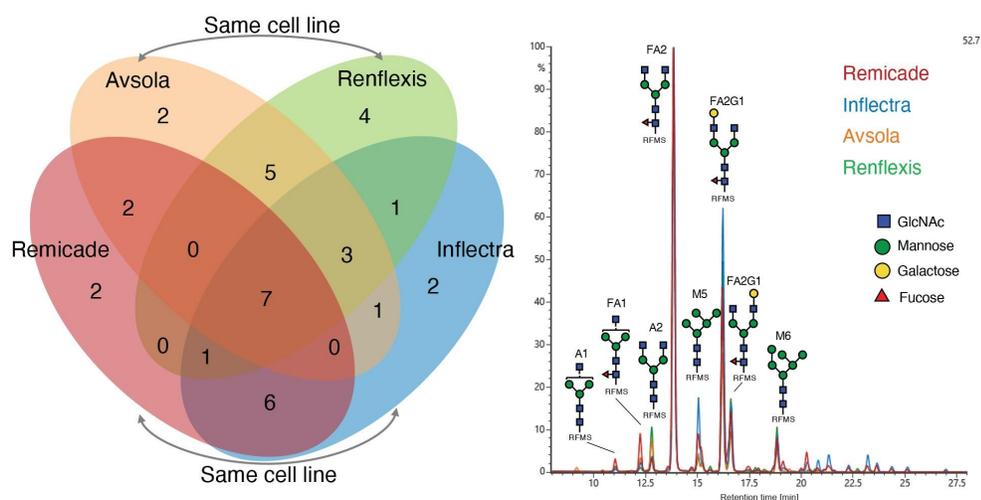
Figure 4. Comparison of identified N-glycans in the innovator and three biosimilar infliximab samples. A Venn diagram (left) was produced showing the number of glycans identified uniquely and in common between the samples. The overlaid chromatograms from the four infliximab samples was generated with the prominent glycans labeled, demonstrating differences in relative abundance of the glycoforms in each sample.

As is evident from the chromatogram, even glycans identified in all four samples showed variations in relative abundance. Figure 5 shows a more detailed examination of differences between the innovator and two of the biosimilar products, displayed as mirror plots. The biosimilar chromatogram is displayed on top and the innovator is shown in the bottom plot. The difference in peak intensity is displayed in the purple trace. Insets in each plot show a close-up of the low-abundance later-eluting N-glycans. These plots enable rapid visualization of differences between the two samples. Simplified Venn diagrams are also shown, comparing the glycans measured in each sample.
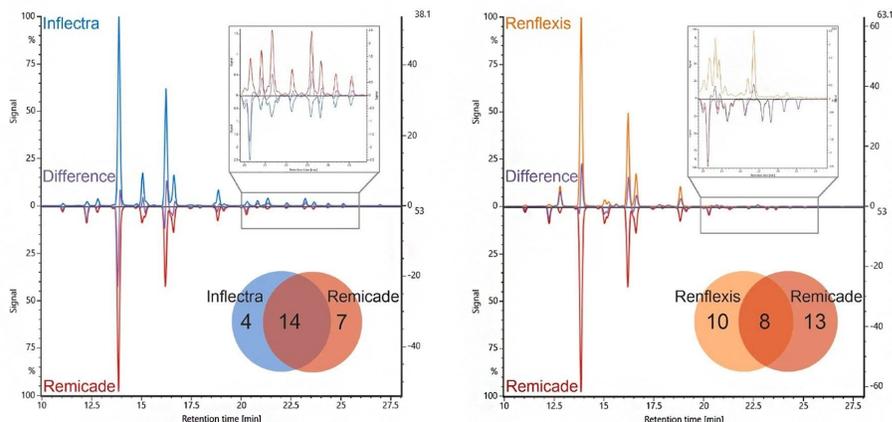
Figure 5. Mirror plots comparing the innovator infliximab sample (Remicade) to two biosimilar samples, (left) Inflectra and (right) Renflexis. The difference in peak intensity is shown as an overlay on each plot, and the Venn diagrams indicate the number of identified glycoforms unique to each sample and those present in both samples.

Quantification of N-Glycans in Infliximab Samples Using the Accurate Mass Screening Workflow

Once glycans have been identified, the accurate mass screening workflow can be implemented to monitor and quantify targeted glycans across samples. To quantify differences in the relative abundance of glycans in each sample, the relative percentage was calculated and compared across samples for a targeted list of identified glycans. As an example, Figure 6 shows the relative percentage area of seven N-glycans identified across all samples. FA2 and FA2G1 were the most abundant glycans in all four samples, with similar relative abundances in each sample. A2 (a bi-antennary glycan) tended to be more abundant in Avsola and Renflexis, while Man5 (a high-mannose glycan) was more abundant in Remicade and Inflectra. As expected, infliximab samples originating from the same cell line type (Remicade/Inflectra and Avsola/Renflexis) tended to have greater profile similarity. However, there were still notable differences between all samples, highlighting the importance of performing a systematic analysis of glycan profiles.
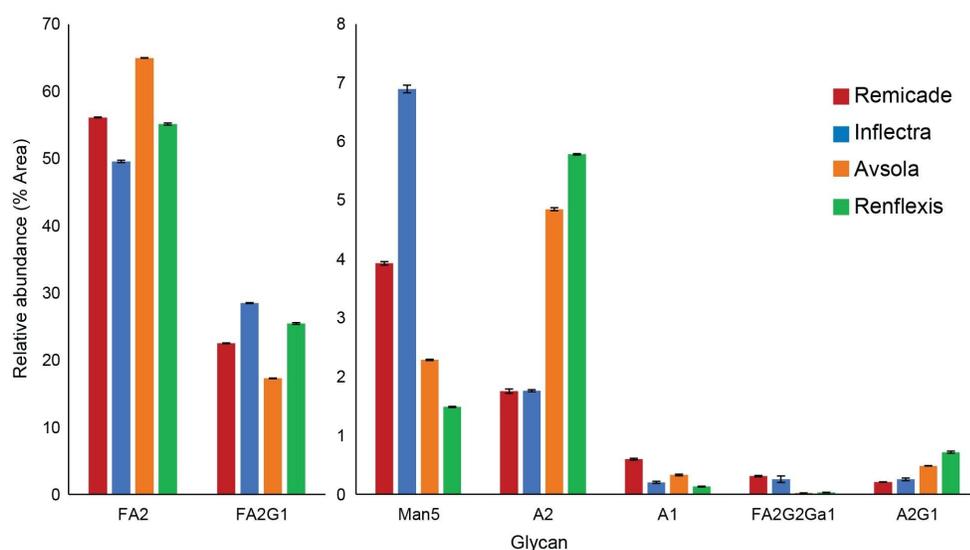
Figure 6. Comparison of the relative abundance (% area) of individual glycoforms across the four infliximab samples using the accurate mass screening workflow.

Conclusion

The structure and relative abundance of glycans can vary considerably between biosimilar mAb products, especially those that originate from different cell lines. As glycosylation impacts important functions of biotherapeutics, glycans are often considered as CQAs and require detailed characterization and close monitoring across samples and establish analytical comparability between innovator and biosimilar drugs. Here, a workflow was demonstrated for comparing the N-glycosylation profiles of biosimilar infliximab drug products to the innovator product using the BioAccord LC-MS System. The Andrew+ Pipetting Robot automated sample preparation with the GlycoWorks *Rapi*Fluor-MS Protocol, and the released N-glycan workflow in the waters_connect UNIFI App automated data acquisition, processing, and reporting, enabling rapid comparison of N-glycan profiles across samples. The workflow included calibration of retention times for high-confidence assignment of glycans using fluorescence and mass detection data and the UNIFI RFMS Glucose Unit (GU) Library. These tools enabled a comparison of glycans in each sample and monitoring of the relative abundance of key glycan structures in all four samples. These results show how the glycosylation profile of biosimilar mAb

drug products can be readily analyzed, for establishing process variability, biosimilar comparability, and facilitating the selection of CQAs for QC product release monitoring.

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