

Assessing Glycosimilarity of Biotherapeutics

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Introduction

Patent expiration on numerous protein therapeutics has created opportunities for development of biosimilars. However, many recombinant therapeutic proteins, and consequently their biosimilar counterparts, are heavily glycosylated. Glycosylation can significantly impact biological activity, effector function, serum half-life, and immunogenicity of the products. Even minor changes to linkages, position, and site occupancy in the carbohydrate structures of protein therapeutics can have significant effects on safety and efficacy. As such, comprehensive

information about glycosylation of biotherapeutics is critical to demonstrating similarity. Regulatory agencies require comprehensive analysis of glycosylation-related critical quality attributes (CQA) during the development, manufacturing, and release of biosimilars.¹

Many technologies commonly used for analysis of complex carbohydrates (e.g. high-performance liquid chromatography, mass spectrometry, and nuclear magnetic resonance) are slow, difficult, expensive, or offer less-than-ideal resolution. Capillary electrophoresis with laser-induced fluorescence

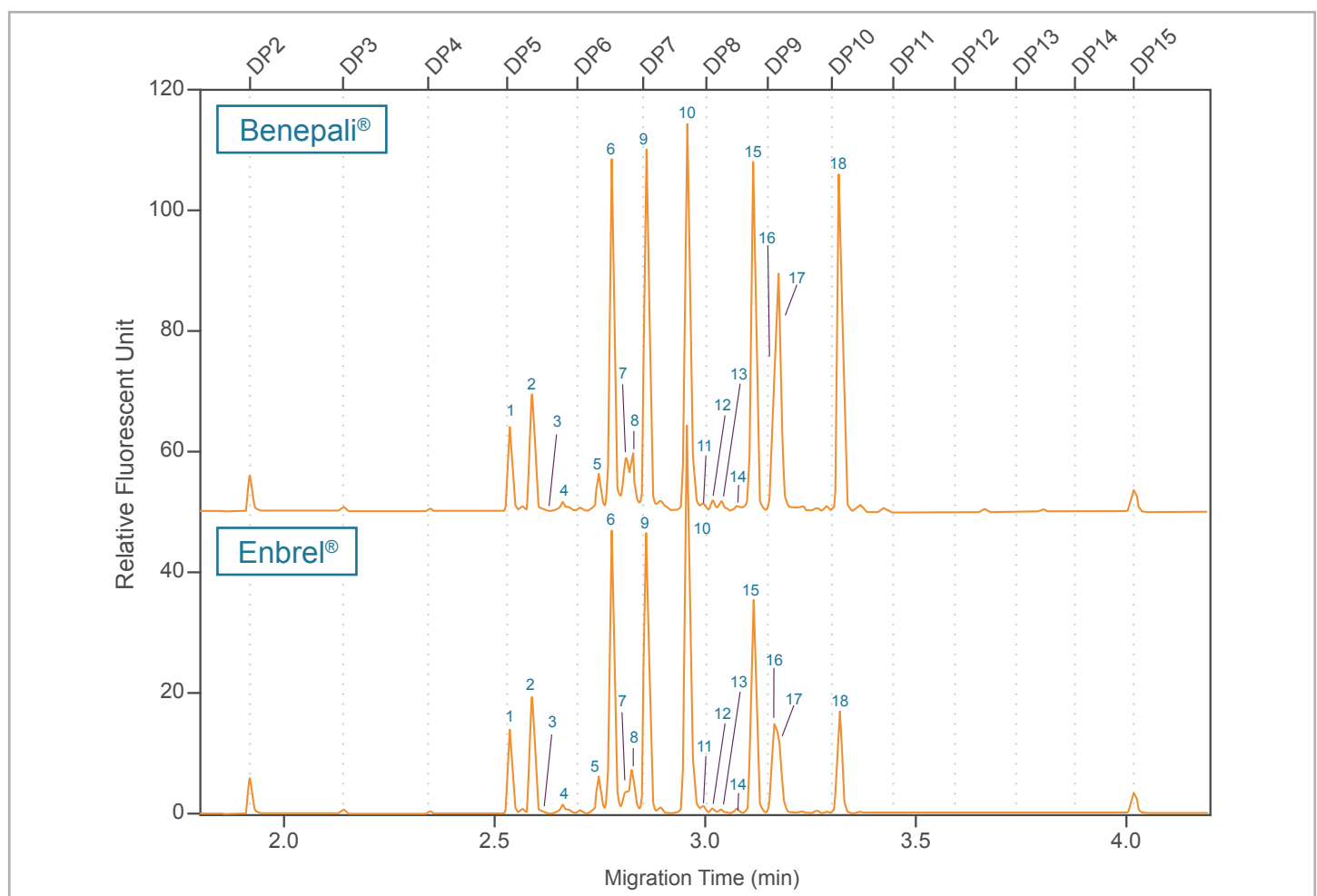


Figure 1. CE-LIF analysis of the PNGase F-released and aminopyrenyltrisulfonate (APTS)-labeled asparagine-linked oligosaccharides from Enbrel® (innovator, lower trace) and Benepali® (biosimilar, upper trace).

detection (CE-LIF) provides a fast, easy way to identify and quantitatively compare the N-glycan profiles of innovator products and their biosimilars. In this work, the CE-based SCIEX PA 800 Plus Pharmaceutical Analysis System was used to identify and quantitatively compare the N-glycan profiles of two biotherapeutics: the innovator product, Enbrel[®], and its biosimilar counterpart, Benepali[®]. The PA 800 Plus system, in conjunction with the SCIEX Fast Glycan Labeling and Analysis Kit, quickly and easily identified quantitative differences in fucosylation, galactosylation, and mannosylation between the two products (Figure 1). Given the mechanism of action these products, only the mannosylation was deemed as critical quality attribute for the assessment of biosimilarity.

Key Features:

- Simplified sample preparation workflow and analysis
- Rapid separations, less than five minutes for these analyses
- High-resolution separations
- Automated glycan identification based on GU values and carbohydrate sequencing
- Quantitative comparison of N-glycan profiles based on relative peak areas

Overview

To properly address glycosimilarity between an innovator product and its biosimilar, N-glycosylation related attributes such as total fucosylation, galactosylation, and mannosylation, as well as their criticality, have to be determined (this Technical Note focuses on Asn-linked sugars). The mechanism of action of a particular biotherapeutic determines which form(s) of glycosylation qualify as critical quality attributes. For example, when the mechanism of action of a therapeutic monoclonal antibody is antibody-dependent cell-mediated cytotoxicity (ADCC), information about the presence or absence of core fucosylation is of high importance. If complement-dependent cytotoxicity (CDC) is the mechanism of action, antennary galactosylation represents a CQA.² Mannosylation of the conserved glycosylation site on the Fc region of therapeutic IgG antibodies increases the rate of serum clearance in humans,³ so its presence and extent should be closely monitored.

Etanercept (trade name Enbrel[®], biosimilar Benepali[®]) was developed to treat autoimmune diseases like rheumatoid arthritis and psoriasis. It is a highly glycosylated Fc-fusion protein that binds tumor necrosis factor alpha (TNF α), a cytokine involved

in systemic inflammation. The two major parts of etanercept are the TNF α receptor (upper part) and the Fc portion (lower part) of an IgG1 antibody, connected with a heavily O-glycosylated (13 sites) linker (Figure 2). Enbrel has three N-linked glycosylation sites (Asn149, Asn171, and Asn317) that have recently been characterized in detail.⁴ The mechanism of action of etanercept is manifested via the TNF α receptor part, not through the Fc portion of the molecule. This suggests that some regular Fc function-related glycans such as core fucosylation for ADCC function or terminal galactosylation for CDC function, which might be critical quality attributes in other biotherapeutics, are likely not important from the glycosimilarity point of view of this particular product.

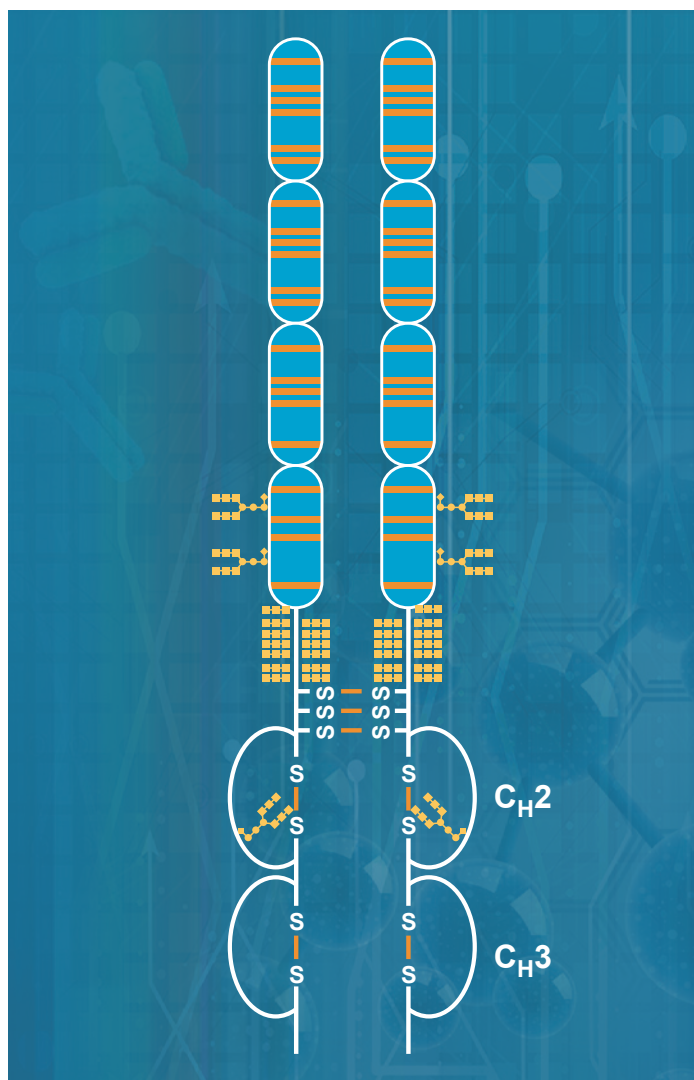


Figure 2. The Fc fusion protein etanercept and its glycosylation sites. The yellow structures depict the one and two N-glycosylation sites at the Fc and TNF α part, respectively, as well as the 13 O-glycosylation sites in the hinge region.

Figure 3. PA 800 Plus Pharmaceutical Analysis System



Experimental

Chemicals

Enbrel® and Benepali® were provided by the Medical School of the University of Debrecen (Hungary). All other chemicals not provided in SCIEX's Fast Glycan Sample Preparation and Analysis kit were from Sigma-Aldrich (St Louis, MO, USA).

Sample Preparation

The Fast Glycan Sample Preparation and Analysis Kit (SCIEX, Brea, CA) was used for sample preparation and analysis.

Samples of Enbrel® and Benepali® underwent:

1. Enzymatic release of the N-glycans from glycoproteins
2. Magnetic bead-mediated capture of the released glycans
3. Labeling of the released glycans with a charged fluorophore
4. Magnetic bead-mediated capture of the fluorophore-labeled glycans and dye removal, followed by elution of the labeled glycans

Separation and Analysis

A PA 800 Plus Pharmaceutical Analysis System (SCIEX, Figure 3) equipped with a solid state laser-based fluorescence detector ($\lambda_{\text{ex}}=488 \text{ nm}/\lambda_{\text{em}}=520 \text{ nm}$) was used for separation and detection. The separations were accomplished using a 20 cm (EZ-CE cartridge, SCIEX) ($50 \mu\text{m}$ I.D.) bare fused-silica capillary column.

32 Karat software, version 10.1, (SCIEX) was employed for data acquisition and processing, along with GU Value software for peak identification. Relative peak areas were used to quantitatively compare the N-glycan profiles of the innovator product and biosimilar.

Results and Discussion

The innovator product, Enbrel®, and the biosimilar product, Benepali®, were both analyzed using the previously outlined method.

Glycan structures were identified by their GU values and by carbohydrate sequencing.⁵ Peak areas of the quantitative N-glycosylation profile data were compared. Quantitative peak distribution changes are visualized in the bar diagram of Figure 4, where the bars represent individual glycan structures. Each bar denotes the average of 6 runs. The corresponding Table 1 lists peak identities, peak area values, similarity ratios, and percentages of similarity. An arbitrary 20% threshold was used to determine glycosimilarity. Red numbers indicate glycans whose abundance in the biosimilar differed more than 20% from the abundance in the innovator. Green numbers indicate glycans that were within the 20% threshold.

The abundance of the core fucosylated FA2 glycan was almost twice as high in the innovator as in the biosimilar. Conversely, the abundance of highly galactosylated A2G2 and FA2G2 glycans was much higher in the biosimilar than in the innovator. As noted previously, however, given the mechanism of action of etanercept, these were not considered to be critical quality attributes.

Because mannosylation on the Fc region of therapeutic antibodies and Fc-fusion proteins (such as etanercept) affects the rate of serum clearance in humans, the presence (if any) and the extent of high mannose structures were deemed as CQA. While the peak area of Man5 glycan was within the 20% arbitrary threshold between the originator and the biosimilar, the Man6 glycan differed by as much as 40%, although its absolute concentration was below 1%.

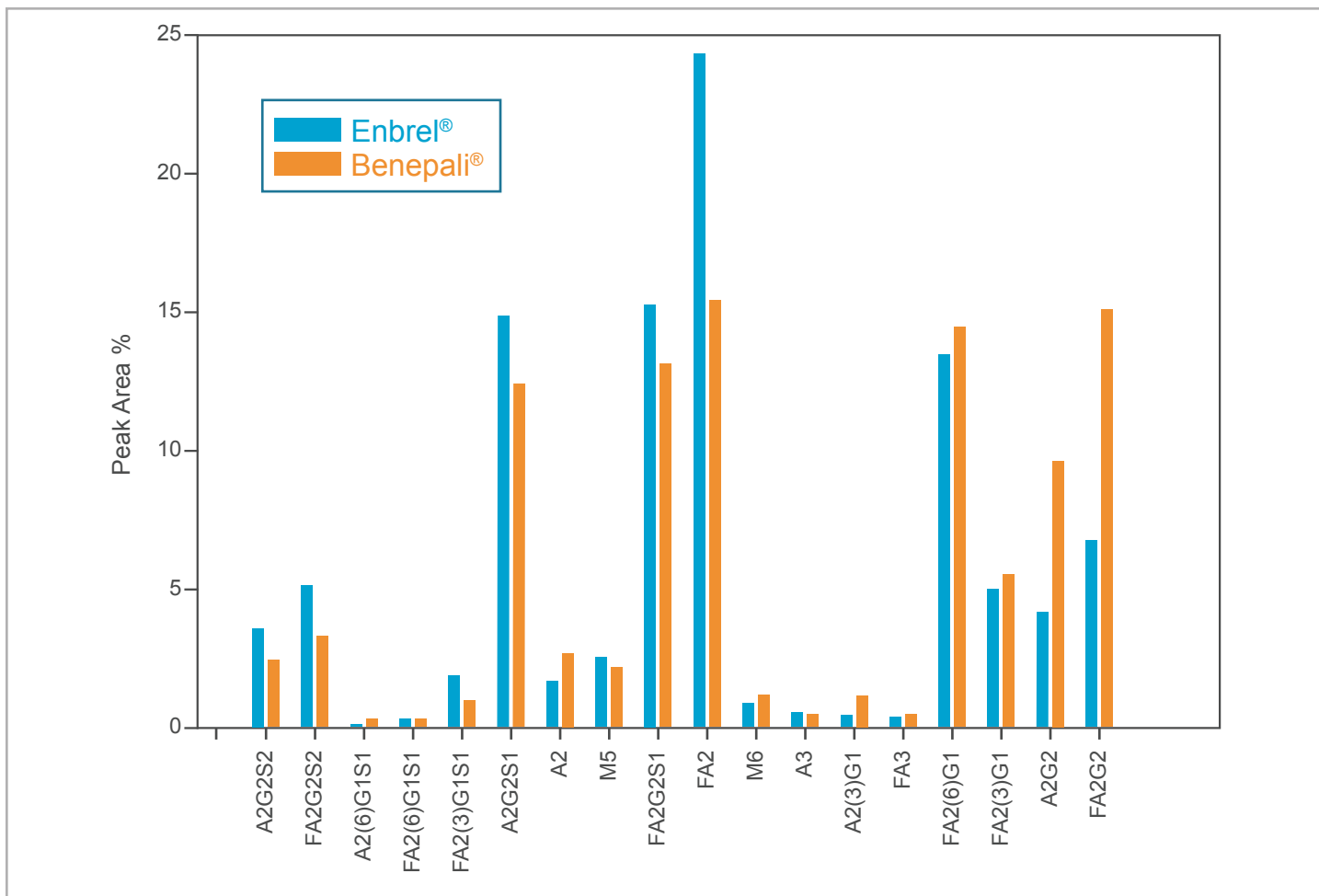


Figure 4. Quantitative N-glycosylation profile analysis of Enbrel® and Benepali® at the individual carbohydrate level for glycosimilarity assessment. Each bar denotes the average of 6 runs.

Table 1. Quantitative N-glycosylation profile analysis of Enbrel® and Benepali® at the individual carbohydrate level using an arbitrary 20% threshold for glycosimilarity. Red = abundance differences > 20%. Green = abundance differences < 20%.

| Peak | Peak ID | Peak Area % | | Similarity Ratio | Similarity % |
|------|------------|-------------|-----------|------------------|--------------|
| | | Enbrel® | Benepali® | | |
| 1 | A2G2S2 | 3.53 | 2.45 | 1.44 | 69.56 |
| 2 | FA2G2S2 | 5.04 | 3.27 | 1.54 | 65.98 |
| 3 | A2(6)G1S1 | 0.11 | 0.34 | 0.32 | 317.43 |
| 4 | FA2(6)G1S1 | 0.35 | 0.28 | 1.24 | 80.55 |
| 5 | FA2(3)G1S1 | 1.86 | 1.01 | 1.84 | 54.32 |
| 6 | A2G2S1 | 14.74 | 12.26 | 1.20 | 83.35 |
| 7 | A2 | 1.61 | 2.64 | 0.61 | 85.85 |
| 8 | M5 | 2.49 | 2.16 | 1.15 | 163.81 |
| 9 | FA2G2S1 | 15.09 | 12.96 | 1.16 | 86.59 |
| 10 | FA2 | 24.06 | 15.24 | 1.58 | 63.31 |
| 11 | M6 | 0.81 | 1.13 | 0.71 | 140.31 |
| 12 | A3 | 0.51 | 0.46 | 1.12 | 89.56 |
| 13 | A2(3)G1 | 0.44 | 1.13 | 0.39 | 259.18 |
| 14 | FA3 | 0.35 | 0.49 | 0.73 | 137.48 |
| 15 | FA2(6)G1 | 13.33 | 14.29 | 0.93 | 107.20 |
| 16 | FA2(3)G1 | 4.95 | 5.49 | 0.90 | 110.85 |
| 17 | A2G2 | 4.11 | 9.47 | 0.43 | 230.69 |
| 18 | FA2G2 | 6.63 | 14.91 | 0.44 | 224.94 |

Conclusions

N-glycosylation plays an essential role in the mechanism of action for most biotherapeutics and thus represents a critical quality attribute for biosimilarity. This work demonstrated the ability of capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) to provide rapid, high-resolution separations and comprehensive N-glycosylation characterization of these carbohydrate structures.

Identification and quantitative comparison of the relative peak areas between the N-glycan profiles of an innovator product, Enbrel®, and its biosimilar counterpart, Benepali®, were the first steps in defining glycosimilarity as an important subset of biosimilarity.⁶ Significant quantitative differences were found in major fucosylated and galactosylated structures (FA2, A2G2 and FA2G2) between the innovator and biosimilar.⁷ However, because ADCC and CDC functions are not critical to the mechanism of action of this product, these differences were not considered significant for glycosimilarity assessment. Mannosylation, on the other hand, plays an important role in the serum clearance of the product, so the quantitative differences found in mannose structures represented a glycosimilarity CQA.

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