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Glycosimilarity assessment of biotherapeutics 1: Quantitative comparison of the N-glycosylation of the innovator and a biosimilar version of etanercept

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Highlights

- Glycosimilarity is introduced to quantitatively address N-glycosylation differences
- Practical examples of glycosimilarity assessment are given (innovator and biosimilar)
- Quantitative differences between the N-glycan profiles are discussed

Abstract

The carbohydrate moieties on the polypeptide chains in most glycoprotein based biotherapeutics and their biosimilars plays essential roles in such major mechanisms of actions as antibody-dependent cell-mediated cytotoxicity, complement-dependent cytotoxicity, anti-inflammatory functions and serum clearance. In addition, alteration in glycosylation may influence the safety and efficacy of the product. Glycosylation, therefore, is considered as one of the important critical quality attributes of glycoprotein biotherapeutics, and consequently for their biosimilar counterparts. Thus, the carbohydrate moieties of such biopharmaceuticals (both innovator and biosimilar products) should be closely scrutinized during all stages of the manufacturing process. In this paper we introduce a rapid, capillary gel electrophoresis based process to quantitatively assess the glycosylation aspect of biosimilarity (referred to as glycosimilarity) between the innovator and a biosimilar versions of etanercept (Enbrel® and Benepali®, respectively), based on their N-linked carbohydrate profiles. Differences in sialylated, core fucosylated, galactosylated and high mannose glycans were all quantified. Since the mechanism of action of etanercept is

TNFα binding, only mannosylation was deemed as critical quality attribute for glycosimilarity assessment due to its influence on serum half-life.

Abbreviations: APTS: 8-aminopyrene-1,3,6-trisulfonic acid; CGE-LIF: capillary electrophoresis – laser induced fluorescence; CQA: critical quality attributes; ADCC: antibody-dependent cell-mediated cytotoxicity; CDC: complement-dependent cytotoxicity; Fc: Fragment crystallizable; TNFα: tumor necrosis factor alpha; MOA: mode of action

Keywords: glycosylation, biosimilarity, biologics, capillary gel electrophoresis

1 Introduction

Patent expiration on numerous biotherapeutics has created new opportunities for the pharmaceutical industry to develop biosimilars [1], i.e., versions of the innovative biological products, which are similar but not identical to the innovator product [2]. Most recombinant therapeutic proteins, e.g., monoclonal antibodies, fusion proteins with the Fc fragment of IgG, erythropoietin, etc., and subsequently their biosimilars are possessing various levels of N-glycosylation. Even minor changes in their oligosaccharide structures (linkage, position, and site occupancy) can significantly influence their safety, efficacy, serum half-life and immunogenicity [3, 4]. Thus, information about the carbohydrate moieties of biosimilars is crucial to properly demonstrate similarity from the glycosylation point of view [5]. Regulatory agencies require comprehensive analysis of all critical quality attributes (CQA) to prove biosimilarity during the development and release of biosimilars, including their glycosylation [2, 6]. This special and important subset of biosimilarity is referred to as glycosimilarity [7].

Adequate determination of glycosimilarity requires proper evaluation of all important carbohydrate associated features like core fucosylation, galactosylation, sialylation and the presence of high mannose structures. The anticipated mechanism of action of a glycoprotein biopharmaceutical assumes appropriate glycosylation, which in turn represents carbohydrate related critical quality attributes [8]. For example, antibody-dependent cell-mediated cytotoxicity (ADCC) requires the absence of core fucosylation at the conserved Fc glycosylation of monoclonal antibodies or fusion proteins with Fc fragments. In the case of complement-dependent cytotoxicity (CDC), antennary galactosylation is an important CQA feature [9].

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Mannosylation at the Fc region of monoclonal antibody therapeutics and IgG fusion proteins enhances clearance, so should be closely monitored during production and release [10].

Etanercept (Enbrel®) was developed to treat Rheumatoid Arthritis and other autoimmune diseases like Psoriasis, Ankylosing Spondylitis, etc. [11]. It is a highly glycosylated IgG Fc fusion protein that binds tumor necrosis factor alpha (TNFα), a cytokine involved in systemic inflammation [12]. Etanercept possesses three N-glycosylation sites on the TNFα receptor part (Asn149, Asn171, and Asn317) and one on the conserved N-linked site of the IgG Fc portion (Asn297). The two parts are connected with a heavily O-glycosylated (13 sites) linker [13]. Since TNFα binding represents the mechanism of action of etanercept, Fc function-associated sugar residues like sialylation, core fucosylation or terminal galactosylation are not of high significance from glycosimilarity point of view. Information about the mannosylation level of the product, on the other hand, is important from the perspective of serum half-life [14].

Bioanalytical techniques such as nuclear magnetic resonance spectroscopy, mass spectrometry, slab gel electrophoresis and high-performance liquid chromatography, commonly used for the analysis of complex carbohydrates [15], are slow and/or offer less than adequate resolution. Capillary electrophoresis is an electric field driven high performance separation technique featuring high separation power and excellent detection limits for oligosaccharides [16]. In this paper we introduce this technique for comparative quantitative characterization of the N-glycosylation and associated glycosimilarity assessment for an innovator biologics fusion protein (Enbrel®) and one of its biosimilars (Benepali®) using capillary gel electrophoresis with laser induced fluorescent detection.

2 Materials and methods

2.1 Chemicals and reagents

The biopharmaceutical products of Enbrel® (innovator) and Benepali® (biosimilar) were kindly provided by the Medical School of University of Debrecen (Debrecen, Hungary). The Fast Glycan Sample Preparation and Analysis kit was from SCIEX (Brea, CA), the PNGase F enzyme from New England Biolabs (Ipswich, MA). The sodium cyanoborohydride (1 M, in THF) and all other chemicals were obtained from Sigma-Aldrich (St Louis, MO).

2.2 Sample preparation

The Fast Glycan kit (SCIEX) was used for sample preparation and analysis including fluorophore labeling with 8-aminopyrene-1,3,6-trisulfonic acid (APTS), magnetic bead based sample purification and capillary electrophoresis separation. Briefly, 100 μgs of both the innovator and the biosimilar products first underwent PNGase F digestion to liberate their N-glycans, followed by magnetic bead-mediated capture of the free oligosaccharides. This was followed by fluorescent labeling of the released carbohydrates with APTS, and another magnetic bead-mediated purification for excess labeling dye removal as reported before in detail [17]. The labeled glycans were then ready for CGE-LIF analysis, or were stored at -20ºC until further processing.

2.3 Separation and data analysis

The PA 800 Plus Pharmaceutical Analysis System (SCIEX) used in all separation experiments was equipped with a solid state laser-based fluorescent detector (λ_{ex}=488 nm/λ_{em}=520 nm). The separations were accomplished using either 20 cm (EZ-CE cartridge) or 50 cm effective length (50 μm I.D.) bare fused-silica capillary columns, filled with HR-NCHO gel buffer system (SCIEX). Reversed polarity separation mode was used in all analyses by applying 30 kV electric field strength either at 30°C or by the implementation of the temperature gradient of 15-55°C. The samples were injected by a three-stage injection protocol: 1) 3.0 psi for 5.0 sec water, 2) 2.0 kV for 2.0 sec sample and 3) 1.0 kV for 1.0 sec bracketing standard (DP2 and DP15). The 32 Karat software, version 10.1, (SCIEX) was employed for data acquisition and processing. Glycans were identified by their GU value from the built-in database of the software. Relative peak areas were used for quantitative comparison of the N-glycan profiles of the innovator product and its biosimilar counterpart.

3 Results and discussion

Capillary electrophoresis with laser-induced fluorescent detection (CGE-LIF) is a fast and efficient method to identify and quantitatively compare N-glycan profiles of biotherapeutics. In this work, we first optimized the separation temperature to obtain the highest resolution among the etanercept N-glycans, then quantitatively compared the released carbohydrates between the originator and the biosimilar products.

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3.1 Separation temperature optimization

As it has been previously reported, the electromigration properties of carbohydrates are activation energy dependent [18], thus first the separation temperature was optimized to obtain the highest resolution between the peaks of interest. One of the fastest way of separation temperature optimization in capillary electrophoresis is the application of a temperature gradient [19]. Figure 1 depicts the separation of the APTS labeled etanercept N-glycans using a temperature gradient from 15°C to 55°C. Based on the results, 30°C separation temperature seemed to be adequate to obtain the required resolution for all 18 carbohydrates of interest, especially between the A2 and Man5 glycans (peaks 7 and 8, respectively), which are important CQA features. Individual separation temperatures of 25°C, 30°C and 35°C were also evaluated using a 20 cm effective (30 cm total) length capillary tubing to validate the gradient findings of 30°C optimum (data not shown).

3.2 Comparative CGE-LIF analysis of the N-glycosylation of the innovator and biosimilar products

Next, N-glycan profiles of the the innovator biologics, Enbrel®, and the biosimilar product, Benepali®, were comparatively analyzed by CGE-LIF at 30°C using 20 cm separation distance. The resulting electropherograms are shown in Figure 2. The lower and upper traces depict the separations of the APTS labeled N-linked oligosaccharides of the two products, respectively. As one can observe, the same peaks were detected in both instances, but with different peak distribution, thus, the different relative amounts were quantitated due to micro-heterogeneity observed. Quantitative assessment was obtained from the relative peak areas and discussed in detail in the next paragraph.

3.3 Quantitative glycosimilarity assessment

Quantitative peak distribution changes are visualized in Figure 3, where the bars represent the individual glycan structures shown in the X axis corresponding to the originator (dark) and the biosimilar (gray) products. Each bar denotes the average corrected peak areas of 6 runs. Table 1 lists peak identities (numbers correspond to peaks in Figures 1), relative peak area percent values, similarity ratios and similarity percentages (tolerance %). The generally accepted ±20% tolerance window was applied for glycosimilarity assessment [7]. Only peaks with larger than

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1% peak area (peaks 1, 2, 5-11, 13, 15-18) were evaluated in this study in respect to glycosimilarity.

The peak areas of all di- and mono-sialo structures complying with the >1% relative peak area criteria (peaks 1, 2, 5, 6 and 9) were smaller in the biosimilar that of in the innovator product. While the di-sialo glycans (peaks 1 and 2) fall out of the 20% tolerance window (-30 and -35%, respectively), the significant mono-sialo products (peaks 6 and 9) were in the range (-16 and -14%, respectively). Since anti-inflammatory characteristics of these products were no MOA importance, even with the average of ~33% difference in their abundance did not represent a CQA issue. Similarly, the higher representation of the afucosylated neutral glycans (peaks 7 and 17) found in the biosimilar in comparison to the innovator drug, while well outside of the tolerance window (+63 and +130%, respectively), were not of CQA importance as the ADCC was not an MOA requirement for this product. The largest peak in both electropherograms was the core fucosylated biantennary glycan (FA2). This feature was represented in more than 36% in the innovator product than in the biosimilar, however, as CDC was not the MOA, it was not considered in the glycosimilarity assessment. Conversely, the abundance of highly galactosylated glycans (peaks 17 and 18), which were apparently much greater in the biosimilar than in the innovator (+130 and +124%, respectively), did not play a glycosimilarity role either. Interestingly, the peak area differences for the monogalactosylated glycans (peaks 15-16) were very similar and within the tolerance window (+7 and +10%, respectively). This was also true for the high mannose structures (peaks 8 and 11), where the difference between the innovator and biosimilar was below the tolerance window (-13 and +19%, respectively). Due to the fact that mannosylation on the Fc region of therapeutic IgG antibodies as well as Fc fragment containing fusion proteins, such as etanercept, affects the rate of serum clearance in humans, the presence (if any) and the extent of high mannose glycans were deemed as an important critical quality attribute of glycosimilarity prospective.

4 Conclusions

N-glycosylation plays an essential role in the mechanism of action for most glycoprotein biotherapeutics, thus represents an important CQA subset for biosimilarity. Comprehensive N-glycosylation characterization by capillary gel electrophoresis with laser-induced fluorescent detection (CGE-LIF) provided rapid, high-resolution separations with the option of quantitative

assessment of the carbohydrates of interest from glycosimilarity point of view. Identification and quantitative comparison of the relative peak areas between the N-glycan profiles of the innovator product (Enbrel®) and its biosimilar counterpart (Benepali®) were used as a model system to demonstrate glycosimilarity assessment analysis. Albeit, significant quantitative differences were found in sialylated, core-fucosylated and galactosylated structures between the innovator and the biosimilar, since ADCC and CDC functions were not critical to the mechanisms of action of these products, this subset of the data was not considered in glycosimilarity assessment. Mannosylation, on the other hand, plays an important role in serum clearance, so quantification based similarity evaluation of the high mannose structures represented an important CQA, with which, based on our results, this particular biosimilar version of etanercept (Benepali®) complied.

Acknowledgment

References

[1] G.B. Kresse, Biosimilars - Science, status, and strategic perspective, Eur. J. Pharm. Biopharm. 72 (2009) 479-486.

[2] FDA, Guidance for Industry: Quality Considerations in Demonstrating Biosimilarity of a Therapeutic Protein Product to a Reference Product. https://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM291134.pdf, 2015 (accessed April, 2015.).

[3] P. Azadi, Challenges in Comparability Studies of Glycoprotein Biosimilars. http://www.pharmoutsourcing.com/Featured-Articles/180537-Challenges-in-Comparability-Studies-of-Glycoprotein-Biosimilars/, 2015. 16(7)).

[4] N.P. Nikolov, M.A. Shapiro, An FDA perspective on the assessment of proposed biosimilar therapeutic proteins in rheumatology, Nat. Rev. Rheumatol. 13 (2017) 123-128.

[5] V. Azevedo, B. Hassett, J.E. Fonseca, T. Atsumi, J. Coindreau, I. Jacobs, E. Mahgoub, J. O'Brien, E. Singh, S. Vicik, B. Fitzpatrick, Differentiating biosimilarity and comparability in biotherapeutics, Clin. Rheumatol. 35 (2016) 2877-2886.

[6] EMA, Guideline on similar biological medicinal products. http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2014/10/WC500176768.pdf, 2014 (accessed October 23, 2014.).

[7] L. Hajba, B. Borza, A. Szekrenyes, A. Guttman, On the Glycosylation Aspects of Biosimilarity, Drug Discovery Today (2018) In Press.

[8] R. Jefferis, Glycosylation as a strategy to improve antibody-based therapeutics, Nature reviews. Drug Discovery 8 (2009) 226-234.

[9] F. Cymer, H. Beck, A. Rohde, D. Reusch, Therapeutic monoclonal antibody N-glycosylation - Structure, function and therapeutic potential, Biologicals : Journal of the International Association of Biological Standardization (2017) https://doi.org/10.1016/j.biologicals.2017.11.001.

[10] A.M. Goetze, Y.D. Liu, Z.Q. Zhang, B. Shah, E. Lee, P.V. Bondarenko, G.C. Flynn, High-mannose glycans on the Fc region of therapeutic IgG antibodies increase serum clearance in humans, Glycobiology 21 (2011) 949-959.

[11] M.M. Goldenberg, Etanercept, a novel drug for the treatment of patients with severe, active rheumatoid arthritis, Clinical Therapeutics 21 (1999) 75-87; discussion 1-2.

[12] K. Peppel, D. Crawford, B. Beutler, A tumor necrosis factor (TNF) receptor-IgG heavy chain chimeric protein as a bivalent antagonist of TNF activity, The Journal of Experimental Medicine 174 (1991) 1483-1489.

[13] S. Houel, M. Hilliard, Y.Q. Yu, N. McLoughlin, S.M. Martin, P.M. Rudd, J.P. Williams, W. Chen, N- and O-glycosylation analysis of etanercept using liquid chromatography and quadrupole time-of-flight mass spectrometry equipped with electron-transfer dissociation functionality, Analytical Chemistry 86 (2014) 576-584.

[14] H. Liu, C. Nowak, B. Andrien, M. Shao, G. Ponniah, A. Neill, Impact of IgG Fc-Oligosaccharides on Recombinant Monoclonal Antibody Structure, Stability, Safety, and Efficacy, Biotechnology Progress 33 (2017) 1173-1181.

[15] R.R. Townsend, A.T. Hotchkiss, Techniques in Glycobiolgy, Marcel Dekker, New York, NY, 1997.

[16] A. Guttman, High-resolution carbohydrate profiling by capillary gel electrophoresis, Nature, 380(6573) (1996) 461-462.

[17] M. Szigeti, A. Guttman, High-Throughput N-Glycan Analysis with Rapid Magnetic Bead-Based Sample Preparation, Methods in Molecular Biology (Clifton, N.J.) 1503 (2017) 265-272.

[18] A. Guttman, M. Kerekgyarto, G. Jarvas, Effect of Separation Temperature on Structure Specific Glycan Migration in Capillary Electrophoresis, Analytical Chemistry 87 (2015) 11630-4.

[19] M. Szigeti, A. Guttman, High-Resolution Glycan Analysis by Temperature Gradient Capillary Electrophoresis, Analytical Chemistry 89 (2017) 2201-2204.

Figures and tables

Figure 1. Temperature gradient capillary gel electrophoresis of released and APTS labeled etanercept N-glycans. Structures corresponding to the peak numbers are delineated in Table 1. DP2 (maltose), DP3 (maltotriose) and DP15 (maltopentadecaose) are bracketing and internal standards. Conditions: 50 cm effective (60 cm total, 50 μm ID) fused silica capillary column filled with HR-NCHO gel buffer. Applied electric field: 500 V/cm. Separation temperature (dashed line): 0-14 min: 15°C isotherm, 14-18 min: linear increase from 15 to 55°C, 18-22 min: 55°C, isotherm. Injection sequence: 3.0 psi/5.0 sec water; 2.0 kV/2.0 sec sample and 1.0 kV/1.0 sec bracketing standard.

Figure 2. CGE-LIF analysis of PNGase F-released and APTS labeled asparagine-linked oligosaccharides from Enbrel® (innovator, lower trace) and Benepali® (biosimilar, upper trace). Separation conditions were the same as in Figure 1 except: 20 cm effective (30 cm total, 50 μm ID) fused silica capillary column filled with HR-NCHO gel buffer. Applied electric field: 1000 V/cm; Separation temperature: 30°C (isotherm). The upper X-axis depicts the degree of polymerization values (DP) of the maltooligosaccharide ladder to help structural elucidation.

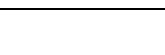Figure 3. Quantitative N-glycosylation comparison of Enbrel® and Benepali® at the individual carbohydrate level.

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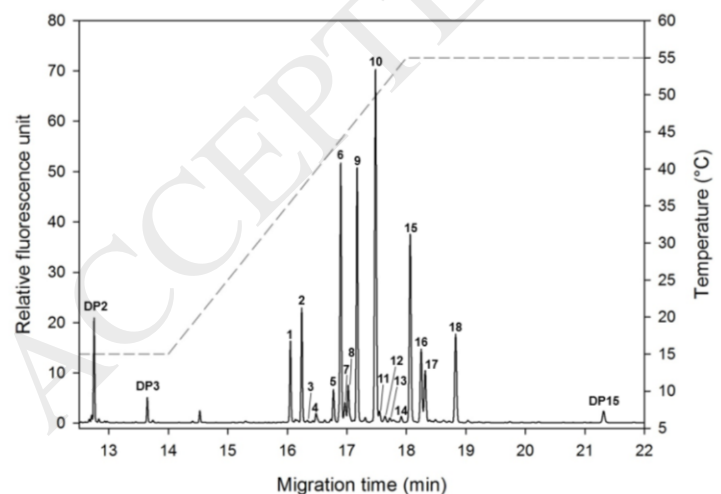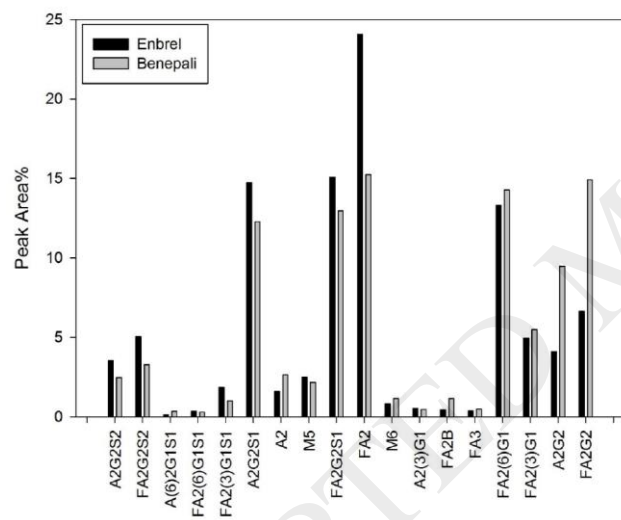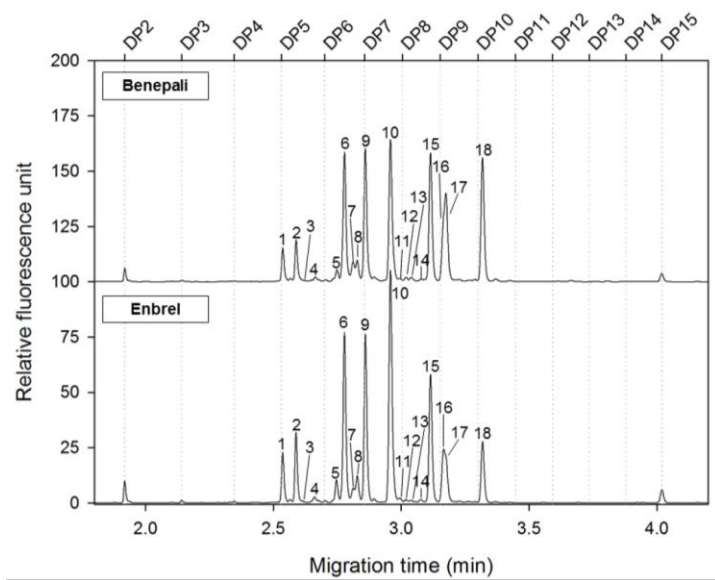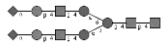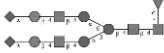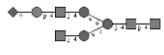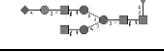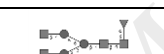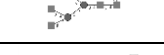
Table1. Quantitative N-glycosylation similarity analysis of the innovator (Enbrel®) and biosimilar (Benepali®) products. Features with less than 1% peak area (gray lines) were not evaluated in this study in respect to glycosimilarity.

Peak	Abbreviation	Glycan comp. letter	Stucture	Peak Area%		Similarity ratio	Tolarence (%)
				Enbrel	Benepali		
1	A2G2S2	H5N4A2		3.53	2.45	0.70	-30.44
2	FA2G2S2	H5N4A2F1		5.04	3.27	0.65	-35.02
3	A2G(6)1S1	H4N4A1		0.11	0.34	3.09	209.09
4	FA2(6)G1S1	H4N4A1F1		0.35	0.28	0.80	-20.00
5	FA2(3)G1S1	H4N4A1F1		1.86	1.01	0.54	-45.68
6	A2G2S1	H5N4A1		14.74	12.28	0.83	-16.65
7	A2	H3N4		1.61	2.64	1.64	63.81
8	M5	H5N2		2.49	2.16	0.87	-13.41
9	FA2G2S1	H5N4A1F1		15.09	12.96	0.86	-14.15
10	FA2	H3N4F1		24.06	15.24	0.63	-36.69
11	M6	H6N2		0.88	1.05	1.19	19.32
12	A2(3)G1	H4N4		0.51	0.46	0.90	-9.80
13	FA2B	H3N3F1		0.44	1.13	2.59	159.18
14	FA3	H3N5F1		0.35	0.49	1.40	40.00
15	FA2(6)G1	H4N4F1		13.33	14.29	1.07	7.20
16	FA2(3)G1	H4N4F1		4.95	5.49	1.11	10.85
17	A2G2	H5N4		4.11	9.47	2.31	130.69
18	FA2G2	H5N4F1		6.63	14.91	2.25	124.94

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