SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Glycomic analysis of innovator and biosimilar bioterapeutics

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Glycomic Analysis of Innovator and Biosimilar Biotherapeutics

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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, 17 May 2024, 13:00.

1. Introduction

Biological drugs, also known as biopharmaceuticals or biologic therapeutics, are gaining increasing prominence in the pharmaceutical market. These products encompass a wide range of recombinant proteins, such as various hormones, blood products, growth factors, antibody-based products, fusion proteins, or recombinant vaccines. It is well illustrated by the fact that the market for biological drugs has doubled in the past 10 years.

The first recombinant protein-based drug was insulin developed by Genentech and introduced to the market by Eli Lilly in the 1980s. Following this, dozens of biopharmaceutical products developed for various diseases were introduced in subsequent years. However, there are currently several recombinant protein-based drugs on the market whose patents have expired, opening the opportunity for competing companies to develop their own versions known as biosimilars. According to the widely accepted nomenclature, biosimilars refer to those biopharmaceutical products developed afterwards that are highly similar to the innovator product but not identical. These differences arise from the use of different cell lines and manufacturing and purification technologies. During their development, the following considerations should be taken into account: (1) demonstration of similarity; (2) interchangeability with the original-innovator product; (3) unique nomenclature for distinctiveness; (4) regulatory requirements; (5) regulatory guidelines that aid the manufacturer in product development; (6) intellectual property rights; and finally, (7) safety.

In the following pages, I would like to summarize the characteristics of biosimilarity in relation to regulatory guidelines. Subsequently, I will discuss one particularly important quality attribute, N-glycosylation, with a specific focus on its regulation and examination.

2. Objectives

Since the detailed examination of critical quality attributes using orthogonal methods is a delicate question of biological drug manufacturing, the following objectives have been set for my doctoral work:

(1) Glycomic analysis of a traditional biologic therapy (adalimumab) and method development for precise evaluation.

(2) Quantitative comparison of the innovator and biosimilar versions of a fusion protein (etanercept) in terms of glycosimilarity.

(3) Application of an orthogonal method for the analyses instead of the commonly used UPLC in the pharmaceutical industry.

(4) Adaptation of the results obtained from CGE-LIF measurements to a high-throughput analysis using a multicapillary electrophoresis instrument, and establishment of a new glycan database for this purpose.

3. Results and discussion

3.1 Quantitative evaluation of adalimumab

I analyzed the N-linked glycans of adalimumab labeled with APTS using CGE-LIF. Among these glycans, a biantennary oligosaccharide with core fucosylation (FA2) was present in 72% proportion, which does not have a terminal galactose. Monogalactosylated forms at positions 6 (12%) and 3 (4.5%) of the same carbohydrate backbone were also present in high proportions. Additionally, the high-mannose structure Man5 was present at 2.47%. Since the complement-dependent cytotoxicity (CDC) and antibody-dependent cell-mediated cytotoxicity (ADCC) effector functions do not play a role in the mechanism of action for rheumatoid arthritis (RA) treatment, the structures affecting these functions (galactosylation and fucosylation) are not included in the analysis. However, the Man5 structure significantly affects serum clearance, so its quantitative analysis is of paramount importance (CQA). Therefore, I spiked with increasing amounts of Man5 standard to the adalimumab samples prior to APTS labeling using PNGase F to cleave the sugars. To ensure comparability of the increased peaks with each other, I used internal and bracketing standards (DP2, DP3, and DP15) to calculate the GU values. By using this approach, the GU values had %RSD values below 0.05%, indicating high accuracy was achieved. In order to proper resolution between Mannose-5 and A2 complex glycans, I used a capillary with an effective length of 50 cm (total length: 60 cm). The RFU values were normalized to the largest peak, which is the FA2 peak in the electropherogram. These normalization steps (on both axes) allow for easy comparison between runs and support accurate peak area calculations for quantitative CQA evaluation. To establish a precise and reproducible correlation between Man5 concentrations and corresponding peak areas, I examined the electrokinetic injection parameters as well as the effects of sample concentration and volume. First, I varied the injection voltage and time between 1.0 and 3.0 kV and 1.0 and 3.0 s, respectively, using sample volumes of 50 and 100 μL (concentration: 0.5 ng/μL). I introduced a combined injection parameter determined by the product of injection voltage and time, referred to as the injection coefficient during concentration calculations. Plotting the peak areas under different injection parameters showed a precise linear correlation. To evaluate the peak areas associated with the Man5 peak in both low and high concentration ranges, I selected an injection parameter that resulted in moderate-sized peak areas based on the above linear relationship. Thus, I performed the analysis of the standards with the concentration dependency study using the no. 4 injection factor (2.0 kV, 2.0 s). Additionally, for precise and reproducible quantitative determination, I investigated the effect of concentration on the peak areas under

the Man5 peak. The concentration of the stock solution made from Man5 standard was 0.5 ng/μL. I prepared serial dilutions using HPLC-grade water, achieving a 50-fold dilution, and then measured the samples using the 2.0 kV, 2.0 s injection parameters. It is important to note that in this concentration range, I obtained very good signal intensities at lower concentrations, and the peaks did not saturate even at higher concentrations. Comparing the sample quantities and peak areas, I obtained an exponential correlation.

Based on the results obtained with different injection parameters, Man5 concentrations, and variable sample volumes, I precisely calculated the concentration of Man5 in the adalimumab sample (2.94 ng).

3.2 Comparison of innovator and biosimilar etanercept based on their N-glycan profiles

When evaluating glycosimilarity, I applied the commonly accepted ±20% tolerance limit. In my work, I only evaluated peaks with a relative peak area greater than 1% for glycosimilarity, taking the average of six measurements for each structure into account.

All di- and monosialic acid structures that met the criterion of having a relative peak area greater than 1% had smaller peak areas in the biosimilar product compared to the innovator product. The disialic acid glycans fell outside the 20% tolerance window (-30% and -35%), whereas significant monosialic acid products fell within the tolerance range (-16% to -14%). Since the anti-inflammatory properties of the mentioned products were not MOA (mode of action) relevant, their average difference of approximately 33% did not pose a CQA (critical quality attribute) problem. Similarly, afucosylated neutral glycans were present in higher quantities in the biosimilar, exceeding the tolerance limit (+63% and +130%). However, since they have an impact on ADCC (antibody-dependent cell-mediated cytotoxicity) effector function and this is not relevant for adalimumab, as it is not part of the mechanism of action, their presence is not considered a criterion for glycosimilarity. In both electropherograms, the largest peak corresponds to a core-fucosylated biantennary glycan (FA2), which is present in the innovator product more than 36% higher than in the biosimilar. This particular structure has a modulatory role in CDC (complement-dependent cytotoxicity), which, similar to the previous cases, is not considered a criterion for glycosimilarity. In the case of the biosimilar, a prominent presence of highly galactosylated sugar structures can be observed (+130% and +124%). However, these structures do not have significance in terms of glycosimilarity. Interestingly, in contrast, monogalactosylated glycans are present in very similar proportions compared to the

innovator product (+7% and +10%). The same applies to high-mannose structures, where the difference also falls within the tolerance range (−13% and +19%). Considering that mannose glycosylation in the Fc region of humans influences serum clearance rate, the presence (if any) and extent of highly mannose-containing glycans can be considered an important critical quality attribute from the perspective of glycosimilarity.

Based on all of this, it can be stated that the bioequivalence of the two variants of etanercept is also supported by comparing the N-glycosylation profiles measured using the orthogonal technique of CGE-LIF (capillary gel electrophoresis with laser-induced fluorescence).

3.3 Establishing an N-glycan database for high-throughput analysis

I analyzed different N-glycan libraries using a multicapillary gel electrophoresis device suitable for high-throughput analysis. The aim was to establish a new database of glucose units by simultaneously analyzing the N-glycan libraries with biotherapeutics (etanercept and adalimumab).

During the analysis, I examined three neutral N-glycan libraries labeled with APTS (fucosylated biantennary, afucosylated biantennary, and oligomannose), as well as six sialylated libraries (bi-, tri-, and tetra-antennary sialylated libraries with α(2-3) and α(2-6) linked sialic acids). In addition, three positions were reserved for maltooligosaccharides to establish the new GU database, and a fusion protein (etanercept, Enbrel®), and a monoclonal antibody (adalimumab, Humira®) were included. It is worth noting that all 12 samples were measured simultaneously with the multicapillary gel electrophoresis device in less than 10 minutes, including the two biotherapeutics. For the fucosylated, afucosylated, and mannose-rich libraries, I identified 15, 15, and 9 structures, respectively. For the bi-, tri-, and tetra-antennary libraries with α(2-3) linked sialic acids, I determined 17, 6, and 6 N-glycan structures. In the case of α(2-6) linked sialic acids associated with bi-, tri-, and tetra-antennary structures, I identified 18, 6, and 6 structures, respectively.

Each glycan follows the Offord nomenclature, which can be found on the website www.GlycoStore.org. The corresponding calculated GU values at 25°C form the basis of the new GU database for separations using multicapillary gel electrophoresis. For the calculations, I used software downloaded for free from the website www.GUcal.hu.

After creating the GU database, I performed the analysis and evaluation of N-glycans from etanercept and adalimumab, which were PNGase F-treated and labeled with APTS. I identified nine sugar structures from adalimumab and 17 from etanercept samples. The latter had one less peak identified compared to the CGE-LIF measurements, which can be considered a satisfactory initial result considering the length of the capillaries and the speed of the method.

4. Summary

In the course of my work, I first developed a quantitative evaluation method based on an orthogonal analytical technique (CGE-LIF), which can be used to easily determine the exact amounts of CQA-relevant sugar structures in the N-glycan profile of biotherapeutics. To accomplish this part of the task, I used adalimumab, to which I added different amounts of Man5 sugar standard. I found a highly accurate (R2=0.9990) linear relationship between peak areas and the injection coefficient defined by the combination of electrokinetic sample introduction parameters. I also examined the effect of concentration on peak areas, and the result was also a very accurate (R2=0.9995) exponential relationship. The amount of original Man5 in the sample (2.94 ng) was calculated based on my results.

In the next step, instead of a monoclonal antibody a fusion protein (etanercept) was analyzed. I performed a quantitative comparison of the innovative and biosimilar version using CGE-LIF, for which I used the relative peak areas. Initially, I identified the N-glycans associated with etanercept by exoglycosidase matrix digestion, then determined the temperature optimum of the separation. I found no profile difference between the two N-glycan pools, but the individual structures were present in different amounts. Based on the theoretical considerations of the glycosimilarity index, I compared the relative peak areas for quantitative evaluation. When evaluating glycosimilarity, I used the generally accepted tolerance limit of ±20%. A core-fucosylated biantennary glycan (FA2) was expressed to the greatest extent in both versions, however, this structure has a CDC modulatory role, which is not part of the mode of action, i.e., it cannot be considered as glycosimilarity criterion. The biosimilar profile showed the abundance of highly galactosylated sugar structures (+130 and +124%), but these were not relevant from glycosimilarity point of view. Monogalactosylated and high-mannose glycans wre present in very similar proportions compared to the innovator (+7 and +10%) (−13 and +19%).

Finally, in order to ensure that the analytical methods and evaluations I developed for capillary electrophoresis can be applied to high throughput screening, fucosylated biantennary, afucosylated biantennary, high-mannose, in addition bi-, tri- and tertaantennary α(2-3) and α(2-6) sialylated glycan libraries were analyzed with a multicapillary electrophoresis device. With the help of the maltooligosaccharide ladder and the GUcal software, I generated a new glucose unit database, creating the opportunity for a more accurate evaluation when using a multicapillary electrophoresis device. After setting up the database, I identified the N-glycan

pools of therapeutic antibodies (etanercept and adalimumab) analyzed parallel with glycan libraries and the maltooligosaccharide ladder by multicapillary electrophoresis device.

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Registry number: DEENK/165/2023.PL
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Candidate: Beáta Borza
Doctoral School: Doctoral School of Molecular Medicine

List of publications related to the dissertation

1. Filep, C. B., **Borza, B.**, Járvás, G., Guttman, A.: N-glycosylation analysis of biopharmaceuticals by multicapillary gel electrophoresis: generation and application of a new glucose unit database. *J. Pharm. Biomed. Anal. 178*, 1-5, 2020.
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2. **Borza, B.**, Szigeti, M., Szekrényes, Á., Hajba, L., Guttman, A.: Glycosimilarity assessment of biotherapeutics 1: quantitative comparison of the N-glycosylation of the innovator and a biosimilar version of etanercept.
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   Electrophoresis. 39 (18), 2340-2343, 2018.
   DOI: http://dx.doi.org/10.1002/elps.201800076
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List of other publications

4. Sárközy, D., **Borza, B.**, Domokos, A., Váradi, E., Szigeti, M., Mészáros-Matwiejuk, Á., Molnár-Gábor, D., Guttman, A.: Ultrafast high-resolution analysis of human milk oligosaccharides by multicapillary gel electrophoresis.
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5. **Borza, B.**, Hajba, L., Guttman, A.: N-glycan Analysis in Molecular Medicine: innovator and Biosimilar Protein Therapeutics.
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