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ASSESSMENT OF THERMAL AGGREGATION BEHAVIOR OF BEVACIZUMAB BIOSIMILARS COMPARED WITH THE INNOVATOR: CORRELATION WITH N-GLYCAN PROFILE BY LC-MS AND SEC-HPLC

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ABSTRACT

This study primarily examined the thermal aggregation behaviour of bevacizumab biosimilars in comparison to the innovator. It focused on the variations in their N-glycan compositions. The percentages of aggregate variations were quantified using size exclusion chromatography. The study successfully measured and quantified the N-glycans of four bevacizumab biosimilars (AVZ, BRX, BVZ, and IVZ) and the innovator (AST) using LC-MS/Q-TOF. It was identified that all bevacizumab biosimilars contained different quantities of N-glycan compositions compared to the innovator. Compared to all biosimilars, innovators have a higher percentage of fucosylated N-glycans (86.50%), while Bryxta (BRX) has a lower percentage (65.40%). This study revealed variations in glycan composition, emphasising that fucose-related glycans play a substantial role in the stability and formation of aggregates in monoclonal antibodies. Research showed that higher levels of fucose-related glycans are linked to a lower percentage of aggregates observed during thermal aggregation studies. Conversely, lower levels of these glycans result in a higher percentage of aggregates.

Keywords: Bevacizumab, N-glycans, Avastin, SEC-HPLC, LC-MS

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INTRODUCTION

The biopharmaceutical industry is experiencing a surge in acquisitions, driven by the potential for more affordable and effective treatments compared to traditional small-molecule drugs.[1,2] Governments are also contributing to this trend by encouraging research and development in this sector. One of the most promising and rapidly growing areas is the development of therapeutic proteins, particularly monoclonal antibodies (mAbs).[3,4] These mAbs are used to cure a comprehensive selection of illnesses, such as cancer, arthritis, chronic conditions, and rare diseases.[5,6] Additionally, biosimilars, which are lower-cost versions of existing biopharmaceuticals, are increasing access to these therapies for more patients. Over the past decade, the US and European countries have approved fewer than five new mAbs per year.[7,8] Day by day, the mAbs market is growing, and currently, around 300 mAbs are queued in development status. Chinese hamster ovary (CHO) cells have become the dominant choice for producing mAbs due to several advantages.[9] Generally, Chinese hamster (CHO) cells are used for the expression of mAbs because they produce human-like translational changes and show less adverse properties in humans. Additionally, CHO cell lines boast a proven track record spanning over 30 years.[10,11] They consistently deliver high-quality and safe mAbs, even during cell engineering. This translates to a higher yield (titer value) and the ability to adapt to different culturing methods (adhesion and suspension). Most of the mAbs produced from CHO cell lines have asialylated or fucosylated N-glycans.[12] In monoclonal antibodies (mAbs), glycosylation is the most prevalent post-translational modification, occurring within the endoplasmic reticulum and Golgi complex.[13] Glycans are typically attached to the constant region (Fc) of the mAb's heavy chain through a covalent glycosidic bond. Specifically, N-glycans link to Asn (Asparagine), while O-glycans link to Ser (Serine) and Thr (Threonine).[14,15] These glycans are categorised into three main

types: oligomannose, complex, and hybrid, based on their structural modifications, which are influenced by the cell's expression system.[16-19] Glycosylation mostly affects the mAb's stability and activity, such as antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC).[20] Bevacizumab is a humanised immunoglobulin monoclonal antibody. The USFDA first approved it for the treatment of metastatic colorectal cancer in the year 2004 under the brand name Avastin.[21,22,23,24] Several biosimilars of bevacizumab were developed after the expiration of the relevant patents. Marketed under the brand names Avastin (AST), Avzivi (AVZ), Advamab (AVM), Bryxta (BRX), Bevazza (BVZ), Ivzumab (IVZ), and Krabeva (KRV), etc., these biosimilars are produced by various pharmaceutical laboratories. To our knowledge, no studies have compared aggregate profiles encompassing more than two bevacizumab biosimilars with the innovator product. This study primarily focused on quantifying mAb aggregates under thermal conditions using SEC chromatography. Analyzing the differences in aggregate formation between the innovator and its biosimilars concerning their N-glycan content using LC-MS-QTOF.

EXPERIMENTAL

Material and Methods

For this analysis, all analytical-grade consumable chemicals were used, including ammonium formate, acetonitrile, NaH_2PO_4, Na_2HPO_4, NaCl, NaN_3, HCOOH, and milli-Q water.

Size Exclusion Chromatography

The size exclusion chromatography (SEC) technique has been used to quantify the percentage of aggregate formation while exposing the samples to various temperatures. Thermo Scientific High-performance liquid chromatography (Ultimate-300, CA, USA) equipment was used for this study. This method uses phosphate buffer (pH 6.8) as the mobile phase and filters through 0.22µ pore size filters. A Superdex 200 (300 mm length and 10 mm width) column (GE Healthcare Biosciences, Pittsburgh, USA) was used to separate the aggregates. The sample in the developed SEC method was held at 5 °C, and the column temperature was held at 25 °C. Aggregates separation was done under isocratic elution conditions, and the total run time was 60 minutes. The aggregates were measured by determining the area under the chromatographic peak that was obtained during the analysis.

General Procedure

The glycans were separated with the Agilent InstantPC labelling kit according to the recommended procedure with minor changes. In a nutshell, monoclonal antibody (mAb) samples were initially subjected to buffer exchange using water, followed by concentration to a final concentration of 2 mg/mL. After that, 2.0 uL of denaturation solution was put into each sample to denature the protein structure, and the mixture was left in a water bath at 90 deg C to incubate the sample. The samples were left to cool to room temperature after the denaturation step was done. To release N-linked glycans, 2 uL of a pre-prepared digestion solution of digestion buffer and PNGase F enzyme (50:50, v/v) was added to each sample. The mixtures of the reactions were then incubated in a water bath at 50 °C for 5 minutes to allow the process of deglycosylation. After the release of glycans, the samples were once more cooled to room temperature and 5 uL of InstantPC labelling reagent was added to derivatise the released glycans. The reaction of labeling was conducted in a water bath at 50 °C for 2 minutes. Lastly, the labelled glycan samples were diluted using 150 uL of cleanup solution that consisted of formic acid and acetonitrile (2.5:95.5, v/v) before proceeding with the analysis. Glycans were extracted from the sample mixture by utilising the cleanup plate provided by the kit. Initially, the diluted sample mixture was loaded into the cleanup plate and washed three times with the cleanup solution. After completing the cleanup process, the glycans were extracted from clean plates using 100 µL of the eluent solution provided by the kit. Solutions were directly injected into the mass spectrometer to identify and quantify the released glycans.

Detection Method

LC-MS Conditions for Released Glycan's Analysis

The released glycans of all five mAbs were identified and quantified using the Agilent Advance Bio 6545XT LC-QTOF mass spectrometry (Santa Clara, CA, United States). The following mass parameters have been used to acquire the released glycans data. Source: Dual AJS ESI, Ion polarity: Positive mode,

Gas temperature: 150°C, Drying gas flow: 9 L/min, Nebulizer pressure: 35 psi, Sheath gas temperature: 300°C, the sheath gas flow was kept at 10 L/min and the capillary voltage (vCap) was adjusted to 3000 V and the nozzle voltage was adjusted to 500 V. Fragmentor voltage and skimmer voltage were set at 120 V and 65 V respectively. The data collection was done in a mass-to-charge (m/z) range of 600-3000 at a scan rate of 1 spectrum/s. The analysis was performed at high-resolution acquisition mode (4 GHz). N-glycans that were released were effectively separated by a HILIC-MS method with an Agilent Advance Bio Glycan Map column (2.1 x 100 mm, 2.7 um). Mobile phase A and mobile phase B were a buffer solution of 50 mM ammonium formate (pH 4.5 +- 0.2) and 100 per cent acetonitrile, respectively. The overall chromatographic run time was 40 minutes, and the separation was done by a programmed gradient of both the mobile phase composition and flow rate. The gradient conditions were as follows: MP-A 22% with 0.6 mL/min flow rate at 0 min; MP-A 22% with 0.6 mL/min flow rate at 1.0 min; MP-A 27.5% with 0.6 mL/min flow rate at 12 min; MP-A 50% with 0.5 mL/min flow rate at 27 min; MP-A 50% with 0.5 mL/min flow rate at 27. N-glycan chromatographic profiles obtained were then processed and interpreted with Bioconfirm software.

RESULTS AND DISCUSSION

N-Glycans profile identification and quantification N-linked glycans that are attached to the Fc region of monoclonal antibodies (mAbs) play a vital role in the stability of these antibodies. The glycans acted as a protective shield for the monoclonal antibodies (mAbs), preventing them from aggregating and degrading. In addition, fucosylated glycans have enhanced the stability of mAbs as compared with nonfucosylated glycans. Firstly, the N-glycans were extracted and quantified from all four biosimilars (AVZ, BRX, BVZ, and IVZ), including innovator Avastin (AST), as mentioned in the above sample preparation procedure and mass spectrometry. The results obtained are depicted in Table-1. The results clearly show the variations in glycan compositions among four biosimilars compared to the innovator. Here, the G0F is the major percentage of glycan presence in all biosimilars, including innovators (AST – 75.93 %, AVZ – 67.58 %, BRX – 48.65 %, BVZ – 61.35 % and IVZ – 59.13 %). Reaming glycans G0, G1, G1F, G2, and G2F were occupied with lower quantities from 1.76 to 16.65 %.

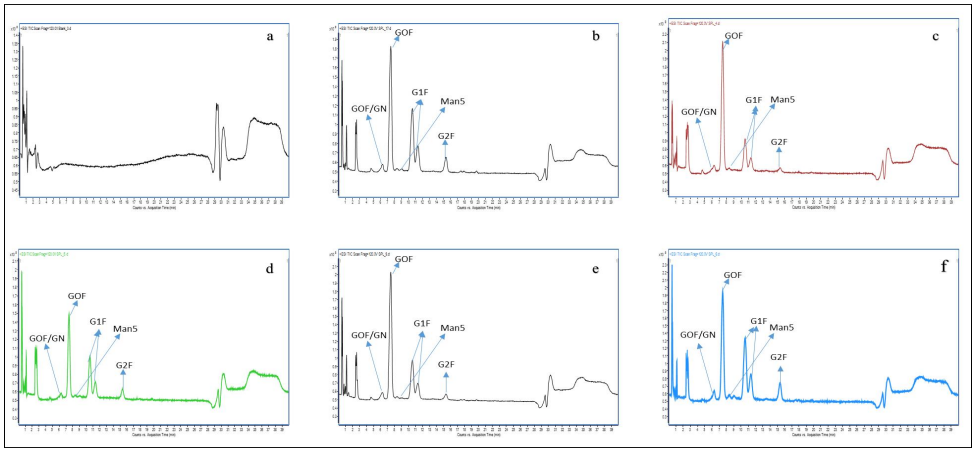

Fig.-1: Mass Spectrometry TIC Chromatograms for the N-glycan Profiles of Bevacizumab Innovator and its biosimilars. (a) Blank Chromatogram, (b) Glycan Profile of AST, (c) Glycan Profile of AVZ, (d) Glycan Profile of BRX, (e) Glycan Profile of BVZ and (f) Glycan Profile of IVZ

Table-1: N-glycans Composition Variation in Biosimilars Compared to the Innovator

Glycans	AST (%)	AVZ (%)	BRX (%)	BVZ (%)	IVZ (%)
G2F	2.21	2.82	4.52	7.32	1.76
G2	5.37	4.24	9.45	5.21	4.31
G1F	8.36	12.57	12.23	16.51	16.65
G1	2.55	2.82	9.41	2.33	4.23
G0F	75.93	67.58	48.65	61.35	59.13
G0	5.58	9.97	15.73	7.28	13.92
Total fucosylated N-glycans	86.5	82.97	65.4	85.18	77.54

Thermal Aggregation Study of Bevacizumab Biosimilars

Size exclusion chromatography is a versatile technique to separate the analytes based on their size variations. In this study, we focused on quantifying the aggregate formation of various biosimilars of bevacizumab while exposing them to multiple temperatures with different time durations. In this study, we mainly focused on the minute changes in aggregate formation concerning their glycan compositions. Samples were first exchanged in a 0.9% saline solution to study aggregation kinetics. They were then subjected to thermal stress (n=3) at temperatures ranging from 40°C to 70°C for different durations. After the completion of the respective time, the samples were cooled at room temperature and directly injected into the SEC-HPLC for the quantification of aggregates. The percentage of high molecular weight (HMW) aggregates was intended based on the area under the curve (AUC) of the aggregate peak relative to the total AUC of all peaks. Integration was performed using the base-to-base method to define the boundary between the aggregate and monomer peaks when they were not baseline resolved. No significant aggregation was observed at 40°C and 45°C, even after 48 hours of incubation. However, at 50°C, higher molecular weight aggregate (HMWA-1) formation began to occur after 24 hours. When the temperature was raised to 55°C, two distinct HMWA populations formed within 24 hours. At 60°C, HMWA formation was rapid, with significant increases noted within just one hour. After 8 hours, a specific HMWA-2 species showed substantial growth across all samples. At 65°C, extensive aggregation happened within 30 minutes, resulting in a dominant HMWA-2 species. Finally, at 70°C, rapid degradation occurred, along with the formation of a single type of HMWA-2 within just 10 minutes. The formation of quantity of aggregates in all biosimilars has been showing variations while exposing all biosimilars to the same temperatures and time durations. The obtained results are depicted in the Fig.-2.

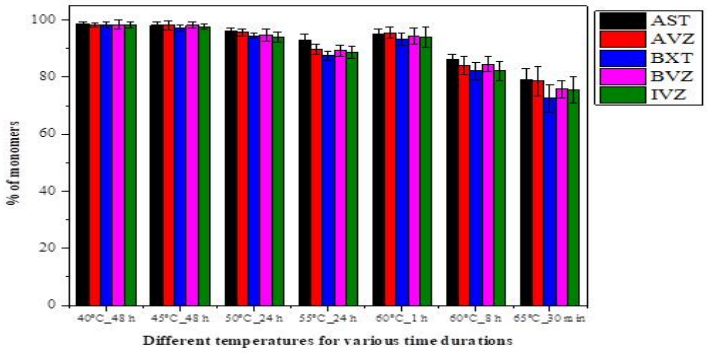

Fig.-2: Variation of the Aggregate Formation of all Biosimilars

Figure-2 clearly shows the difference in the quantity of aggregates in all biosimilars compared with the innovator. Numerous studies have shown that glycosylation is an essential post-translational modification (PTM) that influences protein properties.[25,26] Glycans enhance protein folding and stability, prevent abnormal aggregation, increase water solubility, and slow down thermal denaturation and degradation by forming hydrogen bonds and non-covalent interactions with amino acid residues.[27,28] Fucose-containing glycans are typically hydrophilic, meaning they attract water. This property enhances the overall hydrophilicity of the IgG protein. As a result, a hydration layer can form around the protein due to interactions with water molecules, which are made possible by this increased hydrophilicity. This hydration layer can act as a barrier, preventing protein-protein interactions that might lead to aggregation.[29,30] In this study, biosimilars and innovators have been exposed to various temperatures with different time durations as mentioned in Fig.-2. The results have revealed that biosimilars containing high percentages of fucose-related N-glycans have been formed in smaller aggregates than others. Aggregation studies comparing biosimilars and innovator products have demonstrated the effects of deglycosylation.

We used endoglycosidases such as PNGase-F to perform direct deglycosylation of monoclonal antibodies (MABs). Deglycosylated samples showed a 10% increase in aggregate formation at 45°C after 24 hours, compared to glycosylated samples. In contrast, glycosylated samples remained relatively stable under the same conditions. Observed that the form of the deglycosylated samples has been showing almost the same percentage of aggregates. This study found that in the case of deglycosylation, the samples exhibited

similar behaviour in thermal aggregation studies. However, when glycosylation was involved, the samples showed differences in their aggregation patterns during these thermal aggregation studies. The aggregation differentiations indicate the relative quantities of fucosylated glycans across all biosimilars. We quantified that the innovator (AST) exhibits a high percentage of fucosylated glycans, potentially contributing to its low percentage of aggregate formation, as illustrated in Fig.-2. In contrast, the biosimilar (BRX) demonstrates a reduced level of fucosylated glycans, which correlates with a higher percentage of aggregates compared to the other samples, as depicted in Fig.-3.

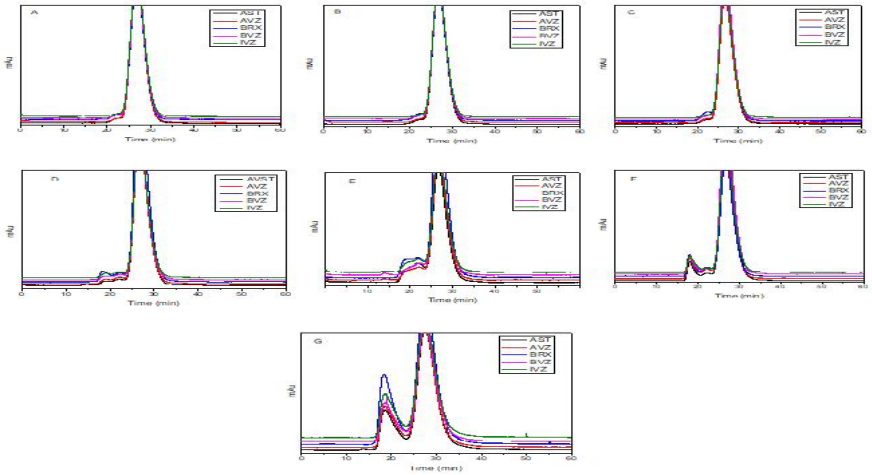

Fig.-3: SEC Aggregate Chromatograms of Bevacizumab Innovator and Biosimilars Under Thermal Stress Conditions. (A) 40°C for 48 hours, (B) 45°C for 48 hours, (C) 50°C for 24 hours, (D) 55°C for 24 hours, (E) 60°C for 1 hour, (F) 60°C for 8 hours, and (G) 65°C for 30 min

CONCLUSION

Biosimilars are exposed to various environmental conditions during the synthetic process, research and development, and formulation. This study mainly focused on the thermal aggregation behavior of different bevacizumab biosimilars by comparing the percentage variations of N-glycans. This study examined the thermal aggregation behaviour of the bevacizumab innovator and its available biosimilars. Before starting this thermal aggregation study, we quantified the percentage of released N-glycans from the innovator and four biosimilars. The released N-glycans varied among the different biosimilars and innovators. Fucosylated N-glycans are especially responsible for preventing the aggregation behaviour of proteins. In this study, all biosimilars have shown variations in formation aggregation quantities while exposed to different thermal temperatures. Monoclonal antibodies with higher levels of fucosylated N-glycans exhibit reduced aggregation compared to others. All mAbs in the deglycosylated form formed approximately the same amount of aggregates when exposed to thermal conditions. This study concluded that variations in fucosylated N-glycans also influence the quantity of aggregates formed when mAbs are exposed to higher thermal conditions. This research may be beneficial to the biopharmaceutical industry in the development of biosimilars.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

The present work is related to *SDG-3: Good Health and Well-being*. All the authors contributed significantly to this manuscript, participated in reviewing/editing and approved the final draft for publication. The research profile of the authors can be verified from their ORCID ids, given below:

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