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Comparative Analysis of Glycoform Profiles Between Biosimilar and Originator Monoclonal Antibodies by Liquid Chromatography–Mass Spectrometry

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Abstract: Glycosylation is considered as a critical quality attribute for monoclonal antibodies (mAbs) and needs routine monitoring during production. This study aims to compare the glycoform profiles of biosimilar and four originator mAbs using ultra-performance liquid chromatography (UPLC) coupled to electrospray ionization-quadrupole time of flight-mass spectrometry (ESI/Q-TOF MS). The resultant mass spectrum showed that seven different glycoform pairs, including G0F–GN/G0, G0F–GN/G0F, G0F/G0F, G0F/G1F, G1F/G1F, G1F/G2F, and G2F/G2F were identified via intact mass analysis for all tested mAb samples. The correct identification of each glycoform pair was achieved by comparing the observed mass with its theoretical mass using high-resolution mass spectrometry data (with mass accuracies of less than 100 ppm). The most abundant paired glycoforms detected at the intact protein level are G0F/G0F and G0F/G1F, with relative abundance ranges of 38.45 – 43.43% and 19.32 – 22.20%, respectively. The obtained data demonstrated that biosimilar and originators have the same types of glycoform pairs, and the relative abundances of each pair were comparable among biosimilar and four originator mAb samples. Additionally, the reduced mass analysis revealed that five different glycans (G0F–GN, G0, G0F, G1F, and G2F) were attached to the heavy chain of the mAb, and the relative abundance of G0F ranged from 75.21 to 77.90%. The detected mass accuracies for reduced mass analysis were below 25 ppm. The results of the intact and reduced mass analyses showed that the biosimilar is similar to its originator in terms of glycoform percentages and molecular masses.

Keywords: Biosimilar, monoclonal antibody, glycoform, liquid chromatography-mass spectrometry.

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1. INTRODUCTION

Immunoglobulin G (IgG)-based monoclonal antibodies (mAbs) are one of the most critical therapeutic biological products and have rapidly expanded in the biopharmaceutical field since the approval of the first mAb by the Food and Drug Administration (FDA) in 1986 (1). mAbs have been utilized for numerous therapeutic applications, such as treating inflammatory, autoimmune disorders, cardiovascular, and oncologic diseases due to their targeted selectivity, high functionality, and low adverse effects (2-3). Biosimilars are supposed to provide patients with more economical treatment alternatives by lowering overall healthcare costs. The patent protection for most of the top-selling therapeutic mAbs has expired or will soon expire; therefore, biosimilar versions have been developed by several biopharmaceutical companies. A biosimilar drug can be defined as a therapeutic protein that is highly similar to its originator product by showing no clinically meaningful differences in terms of quality, safety, and efficacy. To demonstrate the similarity, a biosimilar candidate has to be analyzed side-by-side with the originator regarding physicochemical and functional properties to confirm the quality of the product.

mAbs are recombinant glycoproteins with a total molecular mass of around 150 kDa. They have a Y-shaped homodimeric structure consisting of two identical light chains (LC, ~25 kDa each) and two heavy chains (HC, ~50 kDa each) linked together via disulfide bonds. The structure is inherently heterogeneous due to having various chemical and enzymatic post-translational modifications (PTMs) such as N-linked glycosylation, N-terminal pyroglutamic acid formation, C-terminal lysine truncation, deamidation of asparagine, and oxidation of methionine (4-5). Among them, N-linked glycosylation, a covalent attachment of carbohydrates (oligosaccharides) to the protein, is one of the most prominent PTM in mAbs, which adds heterogeneity to the structure. Most of the mAbs are glycosylated at the asparagine (Asn, N) residue in the fragment crystallizable (Fc) region of the constant heavy chain domain 2 (CH2). This attribute should be monitored throughout mAb development and production stages due to its potential impact on safety, stability, and efficacy profiles (6-8). Therefore, state-of-the-art analytical methodologies should be employed to characterize biosimilars comprehensively.

Today, mass spectrometry (MS)-based analytical methods have become a gold standard for characterizing mAbs at intact, reduced, subunit, and peptide levels (9-15). The preliminary assay in biosimilar characterization is intact and reduced mass analyses via high-resolution mass spectrometry, *e.g.*, ESI-Q-IM-TOF-MS and ESI-TOF-MS systems (16-18). Intact mass analysis is used to determine the molecular weight of therapeutic proteins and the relative quantification of major glycoform pairs with a minimum or no sample preparation step (19-20). Additionally, a reduced mass analysis provides a more accurate mass measurement for subunits (LC and HC) and allows for quantifying the attached glycans on the chains (21-22). Recent studies demonstrated that intact and reduced mass workflows had been preferred over released *N*-glycan and glycopeptide analysis for monitoring mAb glycosylation profiles (23-25). Schilling et al. (23) introduced a novel reduced mass analysis platform method as an in-process control test for monitoring mAb glycosylation. The authors reported that the proposed method could provide early-stage glycosylation characteristics from cell culture harvest upon process development (23). Similarly, a study by Lanter et al. (24) showed that the intact mass-based multi-attribute method (MAM) could be used to determine the *N*-linked glycosylation profile during upstream process development. The authors highlighted that the glycoform profile revealed by intact mass MAM is highly comparable with released *N*-glycan and glycopeptide mapping analyses. Moreover, Martelet and co-workers utilized MS-based MAM workflows at the intact and subunit levels to comprehensively characterize antibody-drug conjugates (ADCs) (25). These reports have shown that intact and reduced mass workflows are today preferred for glycoform characterization over released *N*-glycan and glycopeptide analysis due to the assays' rapid, simple, and robust features. In recent publications, hyphenated mass spectrometric techniques, namely capillary electrophoresis-mass spectrometry (CE-MS) and supercritical fluid chromatography-tandem mass spectrometry (SFC-MS/MS), were demonstrated for high-throughput screening of mAb glycosylation (26-27) with high sensitivity.

In the current study, the glycoform profiles of the biosimilar and the four originator mAb batches were analyzed and compared side-by-side via intact and reduced protein levels by using an ultra-performance liquid chromatography (UPLC) coupled to electrospray ionization-quadrupole time of flight mass spectrometry (ESI/Q-TOF MS). This hybrid system offers a high mass accuracy and mass resolution for the intact and reduced mass analyses of mAbs.

2. EXPERIMENTAL SECTION

2.1. Chemicals and Reagents

All chemicals and reagents were of analytical grade. HPLC-grade acetonitrile, methanol, isopropanol, and formic acid (>99%) were purchased from Merck (Darmstadt, Germany). Ammonium bicarbonate (≥99.0%), sodium iodide (NaI, ≥99.5%), and dithiothreitol (DTT, >99%) were obtained from Sigma-Aldrich (St.Louis, MO, USA). Leucine enkephaline (YGGFL–OH) was purchased from Waters Corporation (Milford, MA, USA). Ultra-pure water (18.2 MΩ.cm) was prepared in-house using a Milli-Q water purification system (Merck-Millipore, Darmstadt, Germany).

The biosimilar candidate was developed and produced by Turgut Pharmaceuticals (Istanbul, Turkey). Four originator batches (2 batches from the European Union-EU and 2 from the United States-US) were procured and stored per the manufacturer's recommendations until further use. The biosimilar and originators are recombinant humanized immunoglobulin G (IgG1) monoclonal antibodies expressed by Chinese hamster ovary (CHO) cells. Both products have only one N-glycosylation site in the Fc region. The biosimilar is purified using standard chromatographic steps (capture step with Protein A chromatography, purification with cation exchange and hydrophobic interaction chromatography, followed by buffer exchange and concentration) in Turgut Pharmaceuticals. The composition of the formulation buffer of biosimilar and originator products is identical.

2.2. Sample Preparation

Sample preparations were performed as previously described (20, 24) with slight modifications. Briefly, for intact mass analysis, antibody samples were diluted to a concentration of 1 mg/mL with 50 mM ammonium bicarbonate solution, and 100 µL sample solution was transferred into UPLC vials for LC-MS analysis. For reduced mass analysis, antibody samples were diluted

to a concentration of 1 mg/mL with 50 mM ammonium bicarbonate solution, and then 1 µL of 500 mM DTT solution (prepared in ultrapure water) was added for reduction. The mixture was then incubated on a thermomixer at 80 °C for 60 minutes for a complete reduction. After incubation, the samples were allowed to cool for 5 minutes on the bench, and then 100 µL of sample solution was transferred into UPLC vials for LC–MS analysis.

2.3. Liquid Chromatography – Mass Spectrometry (LC–MS)

All LC–MS experiments were conducted on ultra-performance liquid chromatography (ACQUITY H-Class Bio UPLC) coupled online to a Xevo G2–XS QTOF hybrid mass spectrometer (Waters Corporation, Milford, MA, USA). The MS instrument was equipped with an electrospray ionization (ESI) source operating in positive ion mode with full MS scan functionality over a mass range of 400 – 4000 *m/z*. LC–MS settings were set to the same parameters for intact and reduced mass analysis. The capillary and cone voltages were set at 3000 V and 30 V, respectively. The source temperature was kept at 150°C, and the desolvation temperature was set at 500°C. The sampling cone and desolvation gas flows were 50 L/h and 1000 L/h, respectively. The autosampler temperature was maintained at 10 °C during the analyses. Before analysis, the instrument was calibrated externally with a sodium iodide solution (2 µg/µL) in a mass range of 400 – 4000 *m/z*. Leucine enkephaline, a pentapeptide (YGGFL–OH, $[M+H]^+$ *m/z* 556.2766) was continuously applied during data acquisition as a reference standard to correct mass accuracy drift.

For each analysis, 3 µg of antibody samples (biosimilar or originator) were injected onto the column separately (Waters Acquity UPLC Protein BEH C4, 2.1 x 50 mm, 1.7 µm particle size, 300 Å pore size) for chromatographic separation. At least three blank injections were performed between sample runs to eliminate any carryover problems. Mobile phase A was ultrapure water, mobile phase B was acetonitrile, and mobile phase C was 1% formic acid in ultrapure water. The mAb sample was eluted off from the column with increasing acetonitrile with a constant level of mobile phase C. Total gradient time 5 minutes with mobile phase B from 5% to 90% in 2.7 min at a flow rate of 0.2 – 0.5 mL/min. Mobile phase C was used as a weaker ion-pairing modifier in the column, and the column temperature was maintained at 80 °C during the analysis. The eluted antibody samples were detected by UV absorbance at 280 nm before entering the MS. Triplicate injections were performed for each sample, and the average abundance for each glycoform pair was reported.

The instrument control and data processing were achieved using UNIFI (v1.9.4) Scientific Information System software. The deconvolutions of the ESI mass spectra of intact and reduced antibody samples were done by applying the maximum entropy deconvolution (MaxEnt1™) algorithm with a maximum of 20 iterations. The start and end peak widths were set to 2.4 and 3.5, respectively, and the charge carrier was selected as hydrogen. The minimum intensity ratio was selected as 80%. The output mass ranges of deconvolutions were set to 140,00 – 150,000 Da and 20,000 – 60,000 Da for intact and reduced protein analyses, respectively. The raw data were extracted and further analyzed with the Igor Pro software package (WaveMetrics, Lake Oswego, OR).

3. RESULTS AND DISCUSSION

3.1. Intact Mass Analysis

The molecular masses and glycoform profiles of the biosimilar and four originator batches were determined at the intact protein level by LC–MS analysis. Throughout the study, the glycoform pair term was used to represent different combinations of two glycan residues attached to the HC of the antibody. Briefly, monosaccharides (galactose (G), fucose (F), and mannose (M)) and amino sugar (N-acetylglucosamine (GN)) are linked together via glycosidic bonds to form a glycan structure. For instance, G0F, G1F, and G2F represent core-fucosylated and 0, 1, and 2 terminal galactose-containing glycan types, respectively. Similary, G0 displays afucosylated and agalactosylayed glycan, and G0F-GN glycan shows a core-fucosylated agalactosylated structure without one terminal GN. Figure 1 shows the overlaid full mass spectra of the biosimilar and originator batches over the m/z 2000 – 4000 mass range. The envelopes of charge distribution were symmetrical and centered on 51+ and 50+ charge states, ranging from 38+ to 70+ in all mass spectra (see Figure 1). As a rule of thumb, ESI generates a series of multiply charged states for all tested samples.

Each full mass spectrum was automatically deconvoluted (zero charged) using the MaxEnt1 deconvolution algorithm (processed by UNIFI) to yield its intact mass spectrum. The deconvoluted intact mass spectra for biosimilar and originator mAbs are compared in Figure 2. The assignments of each paired glycoform peak were established by comparing the observed molecular mass against its theoretical molecular mass via high-resolution mass spectrometry. It is apparent that the intact mass spectra of the biosimilar and originator batches possess a high degree of similarities in terms of having the same glycoform pairs together with comparable relative peak abundances (see Figure 2).

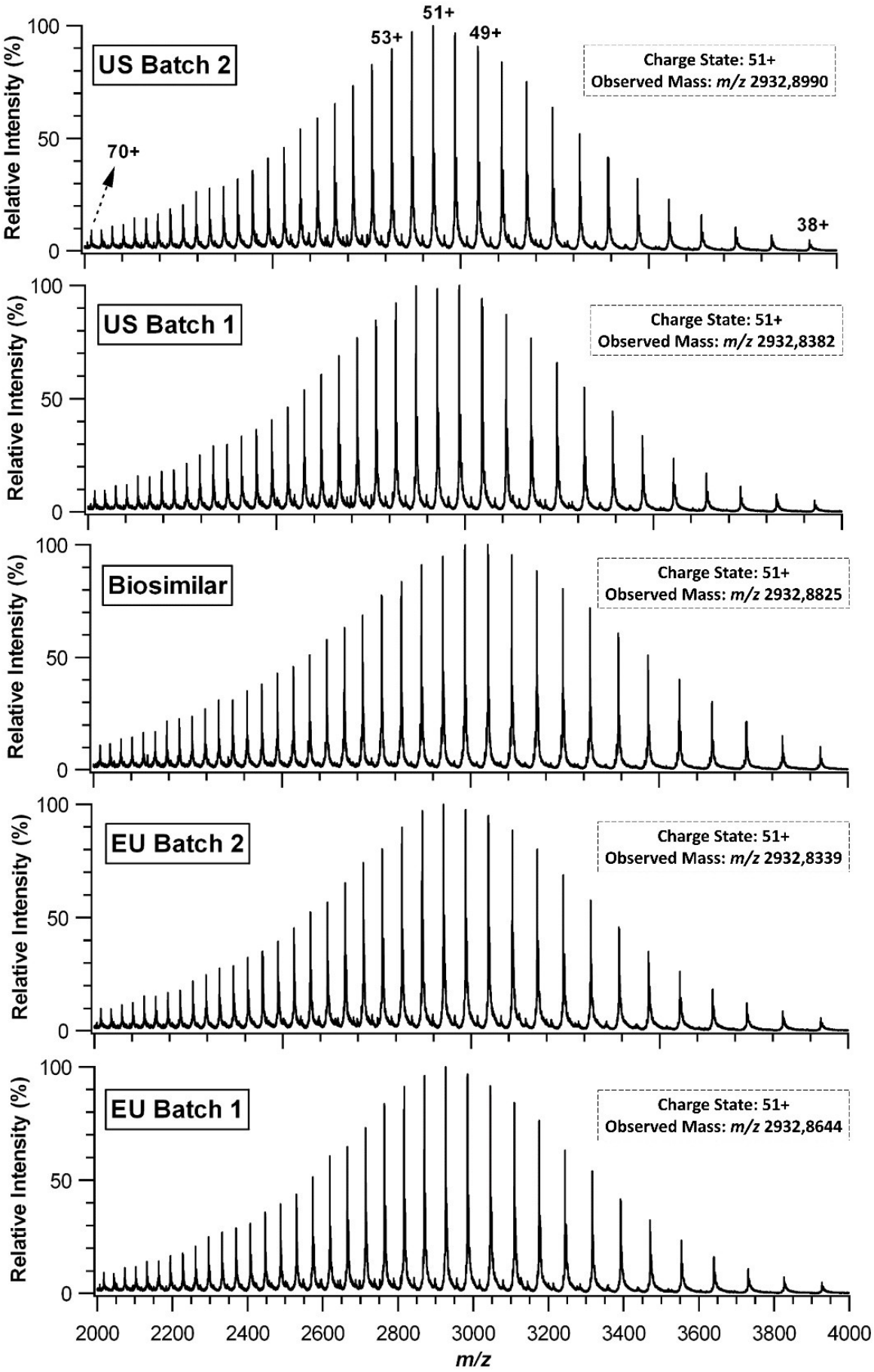

Figure 1: Comparison of overlaid full mass spectra for biosimilar and four originator batches.

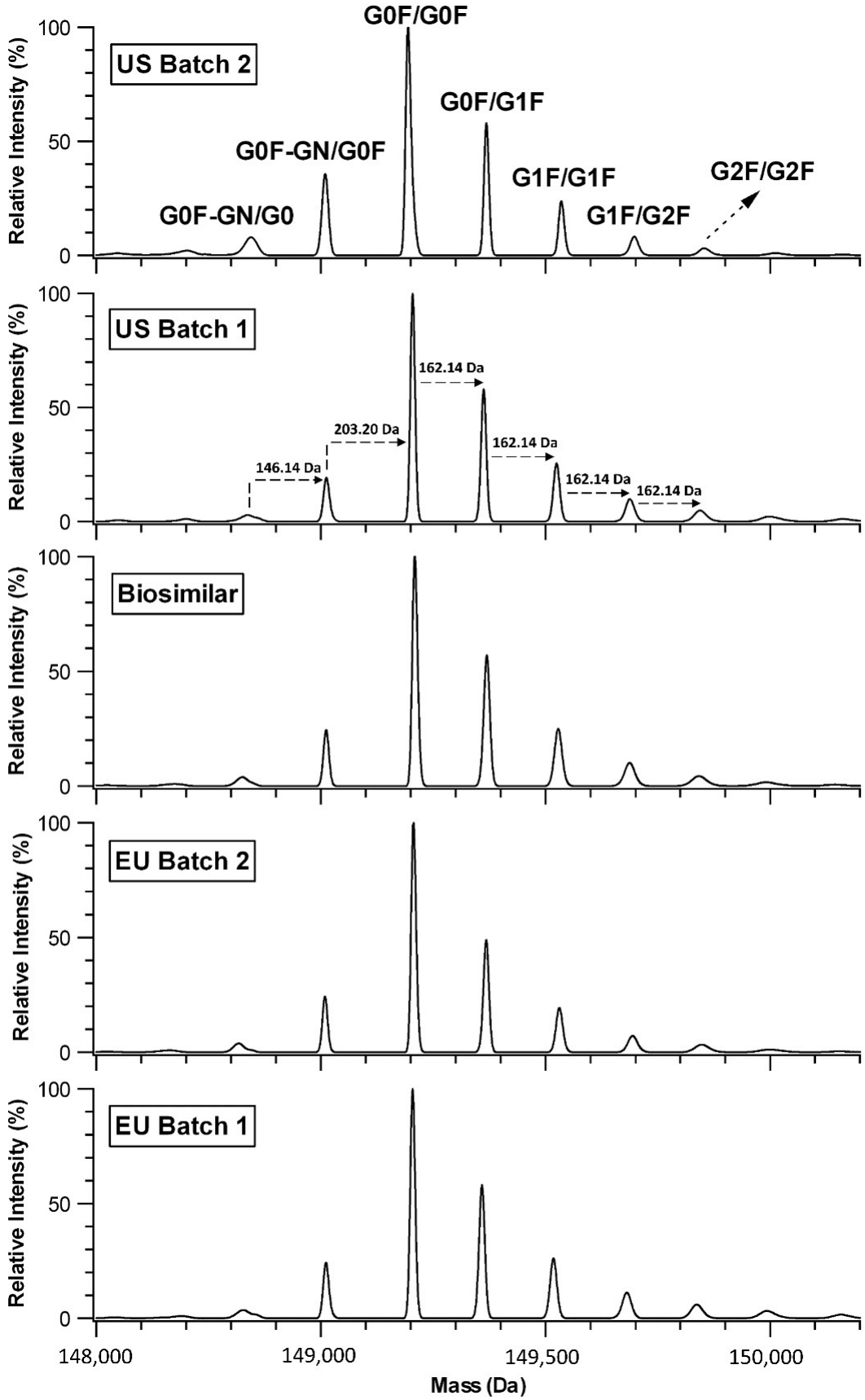

Figure 2: Comparison of deconvoluted intact molecular mass spectra for biosimilar and four originator batches.

Seven different glycoform pairs were detected for biosimilar and originator batches. Briefly, the major glycoforms (>50% of maximum MS peak height) were identified as G0F/G0F and G0F/G1F pairs, together with moderate contributions (20% to 40% of maximum MS peak height) from G0F–GN/G0F and G1F/G1F paired glycoforms. Moreover, G0F–GN/G0, G1F/G2F, and G2F/G2F glycoform pairs were detected at low levels (<10%) in the deconvoluted mass spectra.

The theoretical molecular mass of an aglycosylated antibody (an antibody that lacks glycans on its heavy chain) is calculated as 146,306.15 Da based on its theoretical amino acid sequence. As expected, the addition of glycans to the structure inherently increases the total molecular mass of the antibody (18, 20). The theoretical masses of the antibody that contain different combinations of glycoform pairs are 148,847.48 Da (G0F–GN/G0), 148,993.62 Da (G0F–GN/G0F), 149,196.82 Da (G0F/G0F), 149,358.96 Da (G0F/G1F), 149,521.10 Da (G1F/G1F), 149,683.24 Da (G1F/G2F), and 149,845.38 Da (G2F/G2F). Theoretical masses were calculated assuming all cystines are paired and C-terminal lysine on both heavy chains is truncated. Table 1 compares the observed and theoretical masses for each glycoform pair from biosimilar and originator batches.

Briefly, the observed intact molecular mass of the biosimilar containing two G0F residues (G0F/G0F pair) is 149,202.13 Da, where it ranges from 149,194.29 to 149,204.32 Da for four originator batches. Similarly, the observed intact molecular masses for the other paired glycoforms of biosimilar were within the range of four originator batches, as tabulated in Table 1. It is apparent that the observed intact molecular masses of each glycoform pair were matched entirely with their theoretical molecular masses (see Table 1). Furthermore, the observed molecular mass of the biosimilar with G0F/G0F glycoform (149,202.13 Da) agrees well with its theoretical molecular mass (149,196.82 Da), with a 36 ppm mass error. All glycoform pair identifications were achieved with a mass error of 100 ppm or less for all tested samples (the mass errors were listed in parenthesis in Table 1). Nevertheless, due to the limitations of the QTOF system, the isotopic peaks at each charge state cannot be resolved. The only instruments are orbitrap or FTMS systems that are capable of resolving isotopic spaces for multiply-charged states.

Table 1: Comparison of theoretical masses of each glycoform pair with observed masses for biosimilar and originator batches.

Glycoform Pair Type	Theoretical Mass (Da)	Observed Mass (Da) (Mass Error)				
		EU Batch 1	EU Batch 2	Biosimilar	US Batch 1	US Batch 2
G0F–GN/G0	148,847.48	148,834.08 (-90 ppm)	148,854.65 (48 ppm)	148,851.71 (28 ppm)	148,840.48 (-47 ppm)	148,843.59 (-26 ppm)
G0F–GN/G0F	148,993.62	149,003.45 (66 ppm)	149,008.20 (98 ppm)	149,004.93 (76 ppm)	149,006.75 (88 ppm)	149,007.13 (91 ppm)
G0F/G0F	149,196.82	149,203.63 (46 ppm)	149,194.29 (-17 ppm)	149,202.13 (36 ppm)	149,204.32 (50 ppm)	149,194.65 (-15 ppm)
G0F/G1F	149,358.96	149,357.13 (-12 ppm)	149,365.85 (46 ppm)	149,362.65 (25 ppm)	149,361.51 (17 ppm)	149,368.07 (61 ppm)
G1F/G1F	149,521.10	149,515.25 (-39 ppm)	149,528.19 (47 ppm)	149,524.79 (25 ppm)	149,523.25 (14 ppm)	149,535.04 (93 ppm)
G1F/G2F	149,683.24	149,677.62 (-38 ppm)	149,687.54 (29 ppm)	149,689.23 (40 ppm)	149,686.75 (23 ppm)	149,697.32 (94 ppm)
G2F/G2F	149,845.38	149,833.26 (-81 ppm)	149,840.94 (-30 ppm)	149,841.48 (-26 ppm)	149,842.72 (-18 ppm)	149,853.64 (55 ppm)

The relative abundances of each glycoform pair were calculated based on the peak area of individual glycoform pairs relative to the total peak areas of all identified glycoform pairs. Table 2 summarizes the average relative abundances of each glycoform pair detected in biosimilar and originator batches (averages of three runs were reported for each glycoform pair, and data were shown as mean ± standard deviation (SD, n = 3)). The obtained data demonstrated that the relative abundances of all paired glycoforms of biosimilar fall within the originator's paired glycoform range (see Table 2). For instance, the G0F/G0F type has an abundance of 39.79% for the biosimilar, where EU-batches and US-batches have ranged from 38.45 to 43.43% and 39.64 to 40.16%, respectively. The calculated relative standard deviation (RSD) of G0F/G0F pair was 0.35% for biosimilar and varied from 0.22% to 0.47% for originators, showing a high repeatability among injections. The overall data revealed that the intact molecular masses and relative abundances of each glycoform pair of the biosimilar were within the range of four originator batches. It can be concluded that the biosimilar candidate is highly similar to the originator in terms of glycoform pair content as well as their intact molecular masses.

Manufacturing of a biosimilar candidate comprises a multistep process and unique production parameters, starting from the cell line to the final drug product. Therefore, each biosimilar has its own product quality

characteristics. An intact mass analysis has recently been preferred as the first assay to demonstrate the similarity between biosimilar and originator products (28-30). Montacir et al. (28) demonstrated a comparability study of the original rituximab and its biosimilar through intact mass analysis. Four abundant glycoforms (G0F/G0F, G1F/G0F, G1F/G1F, and G2F/G1F) were found on the heavy chains of both the biosimilar and the original with almost the same relative abundances (28). In another study by Hutterer and co-workers, similarities in the glycoform profile of trastuzumab biosimilar have been reported compared to several US- and EU-source originators (29). The authors emphasized that intact mass analysis revealed the same type of glycoforms in both biosimilar and originators. Seo et al. (30) studied the analytical and functional similarity of bevacizumab biosimilar to several originators' batches. It was shown that the predominant glycoforms for biosimilars were totally in agreement with the originators via high-resolution MS analysis. These literature studies have revealed that the most common glycoform species observed in mAbs are G0F/G0F, G0F/G1F, and G1F/G1F, and the data collected in our study were consistent with those findings in the literature.

Table 2: Comparison of relative glycoform pair abundances for biosimilar and originator batches. Data are shown as mean ± SD (n = 3).

Glycoform Pair Type	%Glycoform (n=3)				
	EU Batch 1	**EU Batch 2**	**Biosimilar**	**US Batch 1**	**US Batch 2**
G0F–GN/G0	4.28 ± 0.04	4.15 ± 0.05	4.40 ± 0.10	4.96 ± 0.12	7.34 ± 0.10
G0F–GN/G0F	10.81 ± 0.08	11.16 ± 0.05	11.61 ± 0.10	9.85 ± 0.11	14.50 ± 0.10
G0F/G0F	38.45 ± 0.11	43.43 ± 0.20	39.79 ± 0.14	39.64 ± 0.13	40.16 ± 0.09
G0F/G1F	22.11 ± 0.13	21.12 ± 0.10	22.20 ± 0.14	21.78 ± 0.17	19.32 ± 0.05
G1F/G1F	11.44 ± 0.22	10.54 ± 0.11	11.32 ± 0.11	11.45 ± 0.06	10.91 ± 0.12
G1F/G2F	7.62 ± 0.10	5.82 ± 0.12	6.43 ± 0.26	7.40 ± 0.09	5.56 ± 0.22
G2F/G2F	5.29 ± 0.10	3.78 ± 0.10	4.25 ± 0.08	4.92 ±0.06	2.21 ± 0.17

3.2. Reduced Mass Analysis

In reduced mass analysis, the biosimilar and four originator batches were chemically reduced using DTT to their light chain (LC, ~ 25 kDa) and heavy chain (HC, ~ 50 kDa) subunits. In comparison to intact mass analysis, a more accurate mass measurement was achieved (mass error of ± 25 ppm) with reduced mass analysis. Figure 3 shows a comparison of the deconvoluted mass spectra of LCs originating from biosimilar and originator batches.

The deconvoluted mass spectra of LCs comprise a single major peak at 23,451.02 Da for biosimilars, where the originator batches have a mass range of 23,450.98 – 23,451.05 Da, as listed in Table 3. Peaks labeled with asterisks were presumably loss of water (-18 Da) from LC and sodium adduct (+22 Da) of LC. The observed masses of each LC were comparable across all tested materials, with no meaningful differences among samples (see Table 3). The calculated mass error was below 15 ppm, which shows that all LCs have the same amino acid sequence order. The single peak was assigned to the mass of LC based on the known amino acid sequence of the target mAb. Additionally, the observed masses of LCs were in full agreement with their theoretical masses (23,450.74 Da), with a mass error of below 13 ppm (see Table 3). The observation of a single peak in the deconvoluted mass spectra indicates no glycan attachment site on the LC.

Figure 4 demonstrates the comparison of deconvoluted mass spectra of the HCs obtained from biosimilar and originator batches. It is obvious that the deconvoluted mass spectra of HC is more complicated than LC's mass spectra. This complexity is explained by having a N-glycosylation site in the Fc region of the HC.

As Figure 4 illustrates, biosimilar and originator batches have the same types of glycans (*e.g.,* G0F–GN, G0, G0F, G1F, and G2F) along with similar relative abundances. Peaks labeled with asterisks were loss of water (-18 Da) from HC and sodium adduct (+22 Da) of HC. For biosimilar products, the observed molecular masses of 50,960.15 Da, 51,017.11 Da, 51,163.70 Da, 51,325.84 Da, and 51,487.60 Da represent G0F–GN, G0, G0F, G1F, and G2F glycan containing HC, respectively. The most abundant peak is HC+G0F, with an observed mass of 51,163.70 Da for the biosimilar, closely matched with its calculated theoretical mass (51,163.79 Da) with a mass error of -2 ppm. The originator batches have a mass range of 51,163.60 – 51,163.76 Da for G0F comprising HC. In addition, G0F–GN, G0, G1F, and G2F glycans were also identified with minor intensities (<15% of maximum MS peak height) in the deconvoluted mass spectra. The calculated theoretical masses of HC containing G0F–GN,

G0, G1F, and G2F glycans were 50,960.60 Da, 51,017.65 Da, 51,325.93 Da, and 51,488.07 Da, respectively. Table 3 demonstrates that the observed masses of HC with different glycans for biosimilar and originator batches were totally in agreement with their theoretical molecular masses. The calculated mass errors for all samples were below 25 ppm.

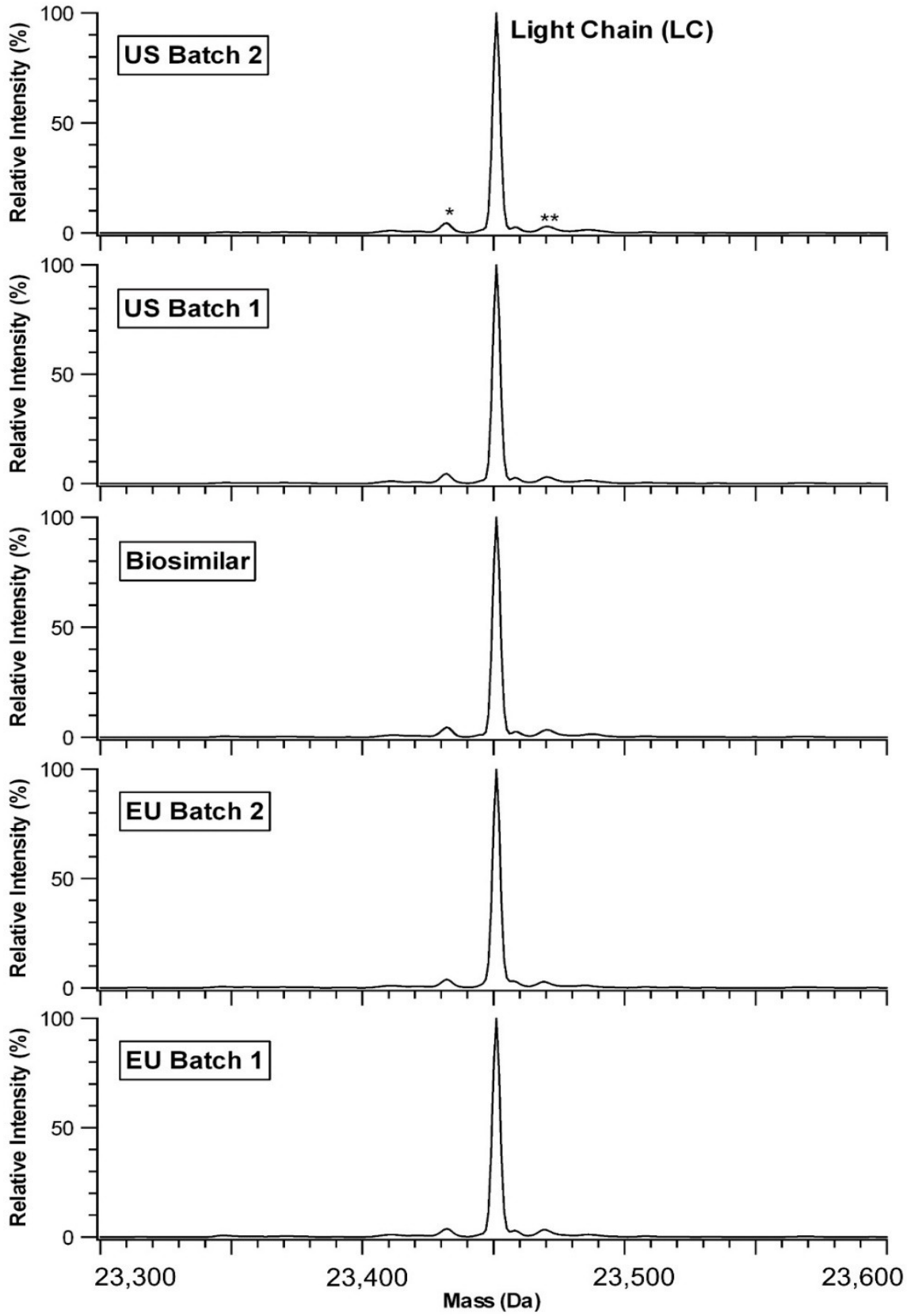

Figure 3: Comparison of deconvoluted mass spectra of light chains of the biosimilar and the four originator batches.

Table 3: Comparison of theoretical mass of light chain (LC) and heavy chain (HC) with observed masses for biosimilar and originator batches.

Chain Name + Glycan Type	Theoretical Mass (Da)	Observed Mass, Da (Mass Error)				
		EU Batch 1	EU Batch 2	Biosimilar	US Batch 1	US Batch 2
LC	23,450.74	23,450.98 (10 ppm)	23,451.04 (13 ppm)	23,451.02 (12 ppm)	23,451.04 (13 ppm)	23,451.05 (13 ppm)
HC + G0F–GN	50,960.60	50,960.27 (-6 ppm)	50,960.07 (-10 ppm)	50,960.15 (-9 ppm)	50,959.83 (-15 ppm)	50,959.92 (-13 ppm)
HC + G0	51,017.65	51,017.17 (-9 ppm)	51,017.19 (-9 ppm)	51,017.11 (-11 ppm)	51,017.06 (-12 ppm)	51,016.52 (-22 ppm)
HC + G0F	51,163.79	51,163.72 (-1 ppm)	51,163.76 (-1 ppm)	51,163.70 (-2 ppm)	51,163.60 (-4 ppm)	51,163.62 (-3 ppm)
HC + G1F	51,325.93	51,325.81 (-2 ppm)	51,325.91 (-0 ppm)	51,325.84 (-2 ppm)	51,325.85 (-2 ppm)	51,325.87 (-1 ppm)
HC + G2F	51,488.07	51,487.80 (-5 ppm)	51,487.24 (-16 ppm)	51,487.60 (-9 ppm)	51,487.41 (-13 ppm)	51,486.88 (-23 ppm)

The calculated relative abundances of glycan-containing peaks are summarized in Table 4 for all tested samples (averages of three runs were reported for each glycoform pair, and data were shown as mean ± SD (n = 3)). The relative abundances were calculated by considering the peak intensities of each identified peak in the deconvoluted mass spectra of the heavy chain. For instance, G0F comprising HC has an abundance of 76.73% for the biosimilar, whereas EU-batches and US-batches have ranged from 77.44 to 77.90% and 75.21 to 76.92%, respectively. The data showed that the abundances of each biosimilar glycan were within the range of four originator batches (see Table 4). The predominant *N*-linked glycan identified in all samples was core-fucosylated biantennary complex type without galactose (G0F), along with a minor contribution of core-fucosylated biantennary type with one galactose residue (G1F). In addition to these forms, minor contributions of core-fucosylated biantennary type glycan with two galactose residues (G2F), afucosylated biantennary type (G0), and lacking the terminal N-acetylglucosamine (G0F–GN) glycans were also detected in the deconvoluted mass spectra. As represented in Figure 4, the mass difference between HC+G0F and HC+G1F peaks was 162.14 Da, indicating that they differ from each other by one galactose residue. Similarly, HC+G1F and HC+G2F peaks differ by only 162.14 Da, the mass of one galactose residue. On the other hand, HC+G0 and HC+G0F peaks have a mass difference of 146.14 Da, showing the mass of one fucose (F) residue, and the mass difference between HC+G0F and HC+(G0F–GN) was 203.20 Da, representing the mass of one terminal N-acetylglucosamine residue.

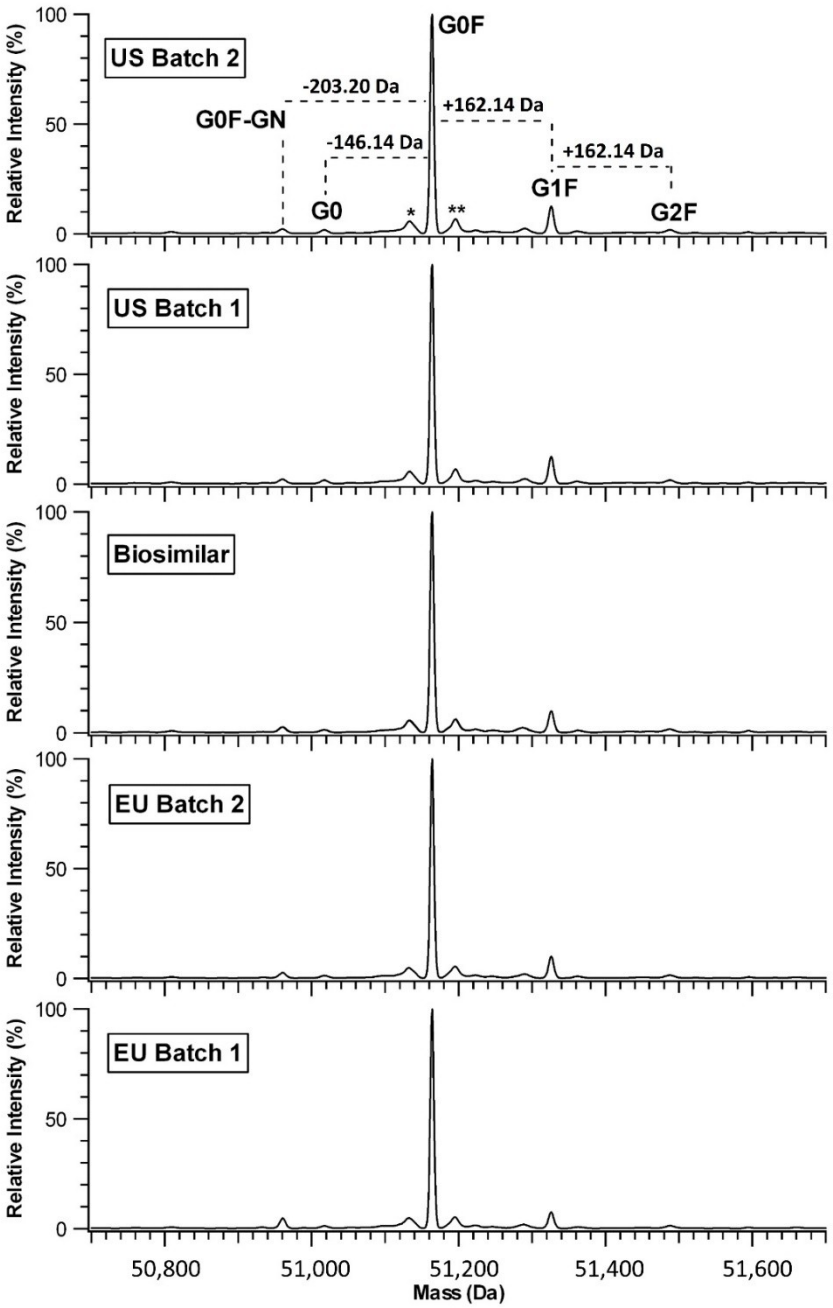

Figure 4: Comparison of deconvoluted mass spectra of heavy chains of the biosimilar and the four originator batches.

Table 4: Relative glycan abundances of biosimilar and originator batches. Data are shown as mean ± SD (n = 3).

Glycan Type	% Glycan (n=3)				
	EU Batch 1	EU Batch 2	Biosimilar	US Batch 1	US Batch 2
G0F–GN	6.51 ± 0.09	4.48 ± 0.06	5.64 ± 0.08	4.17 ± 0.07	4.65 ± 0.16
G0	2.81 ± 0.08	2.93 ± 0.04	2.97 ± 0.06	3.30 ± 0.08	3.03 ± 0.09
G0F	77.90 ± 0.17	77.44 ± 0.10	76.73 ± 0.16	75.21 ± 0.09	76.92 ± 0.12
G1F	9.26 ± 0.15	11.25 ± 0.23	10.58 ± 0.12	13.18 ± 0.08	11.19 ± 0.08
G2F	3.52 ± 0.05	3.90 ± 0.06	4.08 ± 0.12	4.14 ± 0.04	4.21 ± 0.08

Together with intact mass analysis, a reduced mass assay has also provided comprehensive data for comparability assessment studies. Ayoub et al. (31) have conducted a combination of intact and reduced mass analyses for the routine structural assessment of marketed cetuximab products. They monitored and compared biosimilar and originator glycoforms and glycosylation profiles using ESI and matrix-assisted laser desorption ionization (MALDI) mass spectrometry techniques. Xie et al. (32) demonstrated that intact and reduced mass analyses combined with peptide mapping and released glycan assays are accepted as routine techniques for detailed comparability studies. Liu and colleagues (33) have reported reduced and deglycosylated mass data for the adalimumab biosimilar and originators via electrospray ionization time-of-flight mass spectrometer. With the help of a reverse-phase column, buffer components were removed from the samples prior to the introduction to the interface of the mass spectrometer. The authors have shown that both products have the same polypeptide compositions with the help of reduced mass analysis (33). These literature studies have highlighted that accurate mass measurement via reduced mass analysis provides fast and reliable mass data for biosimilarity demonstration. In this study, we implemented both intact and reduced mass analyses for the comparison of glycoform entities of biosimilar and originator through high-resolution MS. Therefore, these assays can be used as appropriate tests for comparability studies in quality control laboratories.

4. CONCLUSION

This manuscript compares biosimilar and four originator batches' molecular masses and glycoform profiles via intact and reduced mass analyses. The deconvoluted intact mass spectra revealed that the relative abundances of the detected glycoform entities were comparable for all tested samples. G0F/G0F and G0F/G1F pairs comprise the highest abundance compared to the other glycoform pairs. Additionally, reduced mass analysis demonstrates that the same types of glycans (namely G0F, G1F, G2F, G0, and G0F-NG) are localized on the HC of biosimilar and four originator batches. No differences between the biosimilar and originators were observed regarding LC and HC masses, while the mass errors were below 15 ppm and 25 ppm, respectively. Overall data have revealed that the results are consistent with the expected amino acid sequence of the antibody. Additionally, the results showed that intact and reduced mass analyses can be implemented to monitor the glycosylation heterogeneity of mAbs during the manufacturing process. These assays require no or minimum sample preparation step and also the analysis time is short compared to the other assays. The data also provided that EU-sourced and US-approved originator batches have a batch-to-batch variability in terms of glycoform contents as well as their molecular masses.

5. CONFLICT OF INTEREST

There are no conflicts of interest declared by the author.

6. ACKNOWLEDGMENTS

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