Applying Multiple Orthogonal Analytical Methodologies for Comprehensive Biosimilar Comparability Assessment

バイオシミラーの同等性評価のための包括的LC/MS分析

Kenji Hirose[1], Henry Shion[2], Vera Ivleva[2], Ying Qing Yu[2], Tom Wheat[2], Asish Chakraborty[2], Weibin Chen[2]
[1]Nihon Waters, Osaka, Japan, [2]Late Stage Development, Pharmaceutical and Life Sciences, Waters Corporation, Milford, MA, USA

INTRODUCTION

Comprehensive physicochemical structural characterization of biotherapeutic proteins is required for manufacturers seeking regulatory approval to market a biosimilar drug product. Structural characterization is performed at three distinct stages of development:

- **Early in development, multiple batches of innovator product are tested for certain Critical Quality Attributes (CQA) to establish a range of values of the target biotherapeutic;**
- **During development, in-depth characterization of the candidate biosimilar product to fully understand its structure and establish its degree of similarity to innovator;**
- **During manufacturing scale-up, to ensure that the production process is stable with respect to generating a biosimilar with the acceptable CQA.**

Regulatory agencies have identified a number of structural properties, including presence of charge variants, dimers and higher order aggregates, glycoforms, and glycan composition, that must be understood.

In this study, intact protein (and subunit) mass analysis, SEC and IEX chromatography, peptide mapping, and released glycan analysis were used to characterize multiple batches of an innovator monoclonal antibody (mAb), Infliximab, and a candidate biosimilar. All experiments were performed on a fully integrated LC/UV(FLR)/QTOF-MS instrument orchestrated by the UNIFI Scientific Information System, which coordinates LC and MS instrument control, method and sample management, and reporting.

Figure 1. Biopharmaceutical Platform Solution with UNIFI, comprising ACQUITY UPLC H-Class Bio; Sample Manager-Flow Through Needle; Tunable UV Detector; Fluorescence Detector; Column Manager; XEVO G2-S QTof; and UNIFI v1.6 Scientific Information System.

MATERIALS & METHODS

Experiment	Sample Prep	Separation Column	Detection Method(s)	Quality Attribute(s)
Size Exclusion Chromatography (SEC)	Dilution	BEH200, 1.7 µm, 4.6 X 150 mm	UV	Abundance of multimers/aggregates
Ion Exchange Chromatography (IEX)	Dilution	Protein-Pak Hilres SP, 1.7µm, 4.6 x 100 mm	UV	Charge variants (acidic and basic), post-translational modifications (PTM)
Intact Mass Analysis (+/- deglycosylation)	PNGase, buffer exchange	BEH300 C4, 1.7µm, 2.1 x 50 mm	TUV and MS full scan (Xevo G2S QTof)	Intact mass measurement, major glycoform profile, c-terminal lysine modification
Subunit (reduced) Mass Analysis (+/- deglycosylation)	Reduction (DTT), alkylation (IAA); PNGase	BEH300 C4, 1.7µm, 2.1 X 50 mm	TUV and MS full scan (Xevo G2S QTof)	Heavy and light chain mass measurement, HC glycoform profile, HC c-terminal modification
Peptide Mapping (reduced and non-reduced)	+/- DTT, IAA, buffer exchange, tryptic digestion	BEH130 C18, 1.7µm, 2.1 x 100 mm	TUV and MS (Xevo G2S QTof)	Protein sequence coverage, c-terminal modification, PTM, fucosylation, disulfide bonds
Released N-link Glycan Analysis	PNGase, purification via HILIC plate	GST130 Amide, 1.7 µm, 2.1 mm X 150 mm	FLR and MS full scan (Xevo G2S QTof)	N-linked glycan composition, glycan database search and assignment

Sample Information

- Three batches of Remicade® (Infliximab, Jenssen Biotech Inc., Horsham, PA), produced in a murine cell line (SP2/0).
- Three batches of a candidate biosimilar, produced in an alternative cell line (CHO).

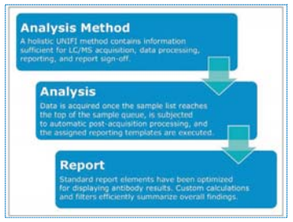


Figure 2. Workflow supported by the Biopharmaceutical Platform Solution with UNIFI

RESULTS AND DISCUSSIONS

SEC for Aggregate Analysis

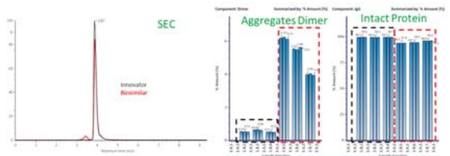


Figure 3. Size exclusion chromatography (SEC) experiment for aggregation assessment of innovator (black) and biosimilar (red) batches. Triplicate injections were run for each batch.

- Overlaid SEC chromatograms (top left) indicate higher percentages of aggregates exist in the biosimilar batch in comparison with the innovator batch.
- Bar charts (top right) display the percentages of mAb monomer and dimer for all three innovator and biosimilar batches, respectively. The biosimilar batches contains more dimer/aggregate than do the innovator batches (4-6% vs. ~0.5%).
- The statistics automatically generated by UNIFI for the innovator and biosimilar samples reveal that the biosimilar displays more batch-to-batch variability in the amount of aggregate present.

IEX Detects Charge Heterogeneity

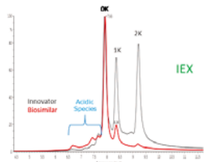
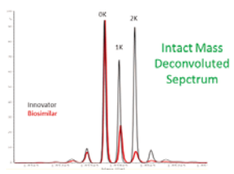


Figure 4.
(A) Cation exchange chromatography comparison between an innovator batch and a biosimilar batch of IgG1 (Infliximab)



(B) Deconvoluted intact mass spectra comparison of deglycosylated innovator and biosimilar proteins. Lysine variants are labeled (0K, 1K, 2K)

Protein charge variants are common in IgG1 and can arise from a post-translational modifications and/or processing variations. The IEX chromatogram (A) shows the innovator batch is quite different from the biosimilar batch in the relative abundance of acidic and basic species. The major peaks are likely due to the presence of a variable number of lysine residues at the C-terminus of the heavy chain.

The mass spectrum of the deglycosylated intact protein (B) further indicates that lysine variants are present in both proteins, but the relative abundance of C-terminal lysine variants are different between the two manufacturers. This is not surprising as the proteins were made in different cell lines.

The UNIFI reporting function automatically generates statistics (Figure 5, below) for purposes of determining the normal heterogeneity of the samples and can ultimately be used to set manufacturing control limits.

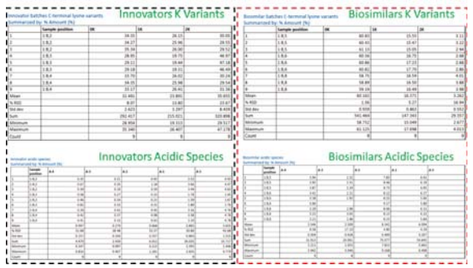


Figure 5. Statistical summary tables for the innovator batches (left) and the biosimilar batches (right). UNIFI automatically calculates the relative abundance of the lysine (top) and acidic (bottom) variant populations.

Verification by Orthogonal Analysis

Characterization of the samples via intact mass analysis, reduced subunit analysis, and peptide mapping shows similar trends between the innovator and biosimilar products. The UNIFI platform automates the analysis of sub-species of interest and easily displays the results. The relative percentages of the C-terminal lysine variants are consistent from sample to sample. Although the IEX method separates species differently, the same general trends are observed. This result demonstrates the power of orthogonal techniques for the characterization of innovator and biosimilar mAbs.

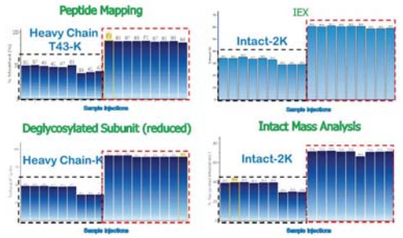


Figure 6. Comparison of four orthogonal methods for assessing lysine variation. The percentage of clipped-lysine variants, automatically calculated in UNIFI, is shown for the innovator (black outline) and biosimilar (red outline).

Glycosylation modification comparison

In addition to lysine heterogeneity, the deconvoluted mass spectrum of the heavy chain reveals variations in fucose–containing glycan moieties. Fucosylation is an important metric for IgG1 heavy chain (HC). The two methods used here to assess the degree of HC fucosylation show similar trends for the percentage of species with the G1F glycan structure.

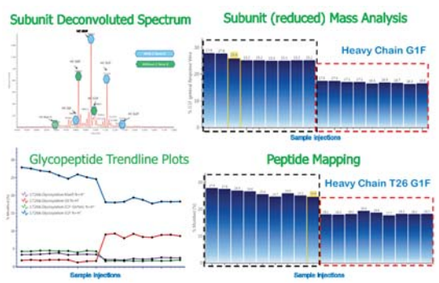


Figure 7. Top, deconvoluted mass analysis of the reduced heavy chain. Bottom, line chart monitoring glycopeptide abundance across the samples. Right, comparison of G1F abundance between innovator and biosimilar as measured by peptide mapping vs. subunit mass analysis.

Released N-Glycan Analysis

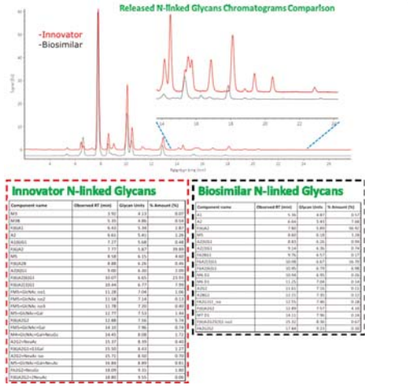


Figure 8. HILIC chromatogram with fluorescence detection (top) of released, 2-AB labeled N-glycans. Based on GU value and mass, 24 glycan species were identified in the innovator sample whereas 18 were identified in the biosimilar sample.

A greater variety of N-glycans was detected in the innovator batch compared to the biosimilar batch (Figure 8). This is not surprising, since the innovator and biosimilar were expressed in different cell lines which is a major determinant of glycosylation pattern. The innovator has several sialic acid-containing glycans (NeuAC and NeuGC) as well as a low level of 1,6 alpha-Galactose. This could be significant since alpha-Gal is a potentially immunogenic glycan.

CONCLUSIONS

- The biopharmaceutical platform solution with UNIFI enabled the thorough analysis of Infliximab from two cell lines (Sp0/2 and CHO), and information on their structural variation was readily generated for the assessment of batch comparability. Experimental results from multiple orthogonal analyses are in good agreement.
- The biosimilar samples have an overall higher degree of aggregates compared to the innovator, as well as more batch-to-batch variability.
- The biosimilar samples display a different profile for C-terminal lysine variants compared to the innovators, with the biosimilar possessing less lysine-containing variants than the innovator.
- As expected, considerable differences in glycosylation exist between the innovator and the biosimilar samples. The biosimilar (produced in CHO) has a lower percentage of the potentially immunogenic glycoform compared to the innovator (produced in SP2/0).
- The study demonstrates that the biopharmaceutical platform solution with Unifi is a powerful, flexible and comprehensive system that can provide high-performance routine analysis for mAb (and biosimilar) development.

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