TOSOH

Application Note

U/HPLC Workflow Solution for Biosimilar Characterization

Introduction

In 2010, Congress implemented a shortened licensure path for biological products that are proven to be biosimilar to an FDA-approved biological product. Section 351(i) defines biosimilarity to mean "that the biological product is highly similar to the reference product notwithstanding minor differences in clinically inactive components" and that "there are no clinically meaningful differences between the biological product and the reference product in terms of the safety, purity, and potency of the product". The FDA recommends that the analytical similarity evaluation originates with an understanding of the structural/physicochemical and functional characteristics of the innovator reference product. The comprehensive use of various modes of analytical chromatography is useful in this regard.

A series of analytical chromatographic techniques, such as size exclusion chromatography (SEC), reversed phase chromatography (RPC) and hydrophobic interaction chromatography (HIC), are used to characterize the similarity. In this application note, the molecular similarity between Humira® and Yervoy® biosimilars and the corresponding innovator reference products is examined using multiple analytical chromatographic techniques including SEC, RPC and HIC.

Size Exclusion Chromatography

Size exclusion chromatography (SEC) is the method of choice for purity analysis. SEC can be utilized to compare the presence of aggregates and fragments in the innovator and reference product and to confirm or disprove equivalent hydronamic radii between the two molecules.

Experimental HPLC Conditions

Column:	TSKgel® UP-SW3000, 2 µm, 4.6 mm ID × 30 cm
Instrument:	Thermo Fisher Dionex Ultimate® 3000 UHPLC with Chromeleon® v. 6.8
Mobile phase:	100 mmol/L KH_2PO_4/Na_2HPO_4, pH 6.7, 100 mmol/L, Na_2SO_4, 0.05% NaN_3
Gradient:	isocratic
Flow rate:	0.35 mL/min
Detection:	UV @ 280 nm
Temperature:	25 °C
Injection vol.:	5 µL
Samples:	Humira Innovator Reference Product (5 mg/mL) Humira Biosimilar (4 mg/mL) Yervoy Innovator Reference Product (5 mg/mL) Yervoy Biosimilar (3.7 mg/mL)

Results and Discussion

Yervoy and its biosimilar were subsequently injected onto a TSKgel UP-SW3000, 2 µm SEC column in order to analytically assess the similarities between the two products. *Figure 1* shows that Yervoy and its biosimilar display similar retention times at 8.037 minutes and 8.030 minutes respectively. The zoomed-in profile in *Figure 2* provides a closer look at the baseline and impurities present in each sample. Both the innovator and biosimilar molecules were found to be >99% pure.

Figure 1. UHPLC Analysis of Yervoy and Biosimilar with TSKgel UP-SW3000: Retention Time Comparison

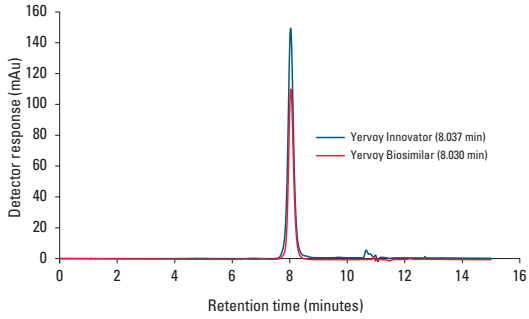
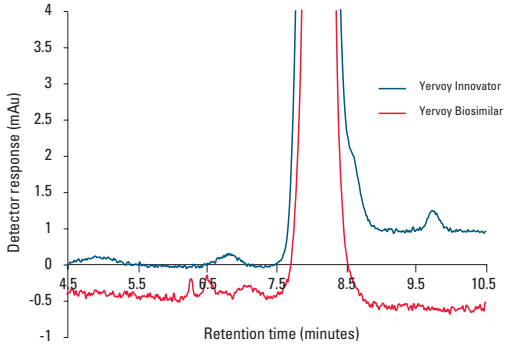


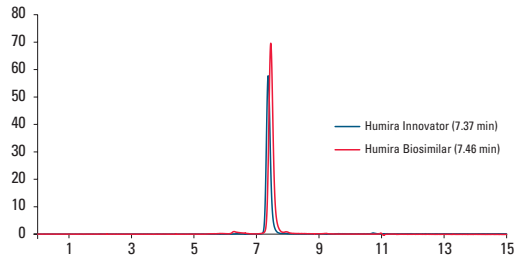
Figure 2. HPLC Analysis of Yervoy Innovator and its Biosimilar Using TSKgel UP-SW3000: Zoomed-in Profile



Retention time (minutes)				
	HMW	Dimer	Monomer	Fragment
Yervoy Innovator	4.837	6.780	8.030	9.707
Yervoy Biosimilar	6.19, 6.223, 6.447	6.987	8.037	-
% Peak area (mAU*min)				
	HMW	Dimer	Monomer	Fragment
Yervoy Innovator	0.18	0.20	99.47	0.15
Yervoy Biosimilar	0.26	0.23	99.56	0

Humira and its biosimilar were then analyzed using a TSKgel UP-SW3000, 2 µm SEC column in order to elucidate and compare the SEC profiles of the two molecules. *Figure 3* shows that Humira and its biosimilar show slight variability in retention times at 7.37 minutes and 7.46 minutes respectively.

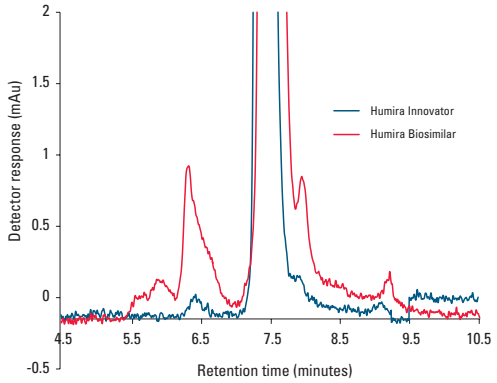
Figure 3. UHPLC Analysis of Humira and Biosimilar with TSKgel UP-SW3000: Retention Time Comparison



The zoomed-in profile depicted in *Figure 4* provides a better look at the aggregates and fragments present in each sample. The relative peak area calculation suggests a larger percentage of impurities, predominantly aggregate, are present in the biosimilar compared to the innovator drug.

The presence of aggregates can reduce the therapeutic efficacy of mAbs and trigger immunogenic responses upon administration. Therefore the increase in aggregation, as seen with the biosimilar, will require further examination before this drug can be released.

Figure 4. UHPLC Analysis of Humira and Biosimilar with TSKgel UP-SW3000: Zoomed-in Profile



Retention time (minutes)				
	HMW	Dimer	Monomer	Fragment
Humira Innovator	0	6.387	7.37	7.883, 9.097
Humira Biosimilar	5.843	6.283	7.46	7.960, 9.217
% Peak area (mAU*min)				
	HMW	Dimer	Monomer	Fragment
Humira Innovator	0	0.27	99.59	0.14
Humira Biosimilar	0.15	1.73	97.66	0.46

Reversed Phase Chromatography

Reversed phase chromatography (RPC) can be used to compare the various peptides present after a tryptic digest of innovator and biosimilar molecules. This serves as an identity test that confirms the primary structure of recombinant proteins and demonstrates process consistency. Differences in the primary structure can thus be elucidated using RPC.

Experimental HPLC Conditions

Column:	TSKgel ODS-100V, 5 µm, 4.6 mm ID × 15 cm
Instrument:	Thermo Orbitrap Q Exactive coupled with Thermo Ultimate 3000 UHPLC
Mobile phase:	A: water with 0.1% formic acid (FA) B: acetonitrile with 0.1% FA
Gradient:	0-2 min: 10% B; 30min: 60% B; 31min: 95% B for 5 min
Flow rate:	0.3 mL/min
Temperature:	40 °C
MS Scan:	The full MS scan was collected at resolution 17,5000 with ESI at capillary voltage 3.5 kV in positive ionization mode, S-Lens RF 75, mass range 400 - 5000 Da
Samples:	Humira Innovator (5 mg/mL)* Humira Biosimilar (4 mg/mL)* Yervoy Innovator (5 mg/mL)* Yervoy Biosmilar (3.7 mg/mL)*

*Each sample underwent a 20 h trypic digest prior to analysis

Results and Discussion

Each sample was subjected to a 20 hour tryptic digest and analyzed by RPC using a TSKgel ODS-100V column. The total ion chromatograms of Humira and Yervoy and their respective biosimilars were compared in order to elucidate differences or confirm similarity between the innovator and biosimilar molecules.

Figure 5 shows that the peptide maps of Humira and its biosimilar show a relatively high degree of similarity under RPC conditions. However, some peaks from the biosimilar are not seen in the innovator profile. These variations in peak profile may be due to variety of reasons; incomplete digestion, some differences in post translational modifications or the absence of a few peptide sequences in the innovator molecule.

Figure 6 shows that the peptide maps of Yervoy and its biosimilar show a high degree of similarity under RPC conditions. Clear peak profile differences could not be seen when comparing the chromatograms of the two biologic molecules.

Figure 5. LC/MS Analysis of Humira Innovator and Humira Biosimilar Using TSKgel ODS-100V

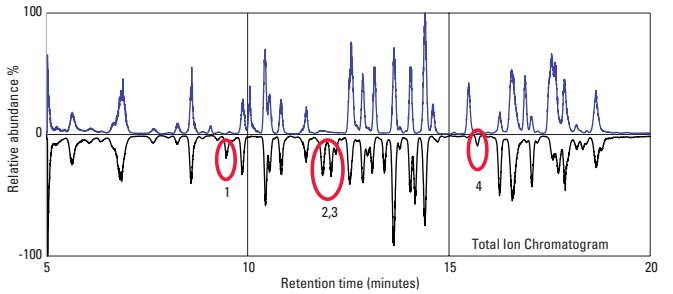
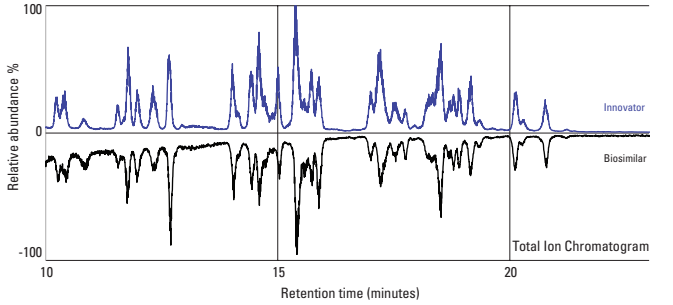


Figure 6. LC/MS Analysis of Yervoy Innovator and Yervoy Biosimilar Using TSKgel ODS-100V



Hydrophobic Interaction Chromatography (HIC)

Differences in cell line and manufacturing processes can result in minor analytical differences in a proposed biosimilar compared with the innovator, resulting in a slight difference in hydrophobicity. Hydrophobic changes may affect the way a drug is processed, thus impacting safety and/or efficacy. Differences can be elucidated using HIC.

Experimental HPLC Conditions

Column:	TSKgel Butyl-NPR™ (non-porous resin), 5 µm, 4.6 mm ID × 10 cm
Instrument:	Agilent 1100 with Chemstation®
Mobile phase:	A: 100 mmol/L phosphate buffer, pH 7.0, + 2 mol/L ammonium sulfate B: 100 mmol/L phosphate buffer
Gradient:	Time %A %B 0 100 0 1 50 100 15.1 stop
Flow rate:	0.5 mL/min
Detection:	UV @ 280 nm
Temperature:	25 °C
Injection vol.:	5 µL
Samples:	Humira Innovator (1 mg/mL) Humira Biosimilar - Source 1 (1 mg/mL) Humira Biosimilar - Source 2 (0.09 mg/mL) Yervoy Innovator (1 mg/mL) Yervoy Biosimilar (0.8 mg/mL)

Results and Discussion

Yervoy and its biosimilar were subsequently injected onto a TSKgel Butyl-NPR, 2.5 µm HIC column in order to analytically assess the similarities between the two products. *Figure 7* shows that Yervoy and its biosimilar display similar retention times and peak profiles. No additional peaks indicating heterogeneity were observed.

The TSKgel Butyl-NPR column was then used to analyze the similarity between Humira innovator and two biosimilars from different sources. *Figure 8* shows that Humira and the biosimilar from source 2 both chromatographically display the presence of a hydrophilic variant. The chromatogram of the biosimilar from source 1 does not indicate that this variant is present. This indicates hydrophobicity differences between the source 1 biosimilar and the innovator drug, which could change its pharmacokinetic properties.

Figure 7. HPLC Analysis of Yervoy Innovator and Its Biosimilar Using TSKgel Butyl-NPR

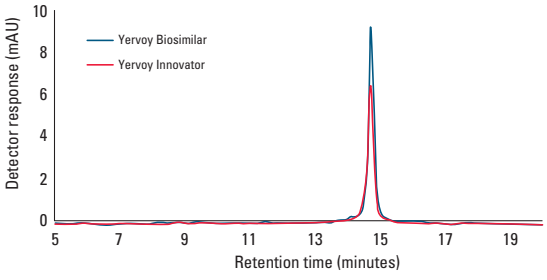
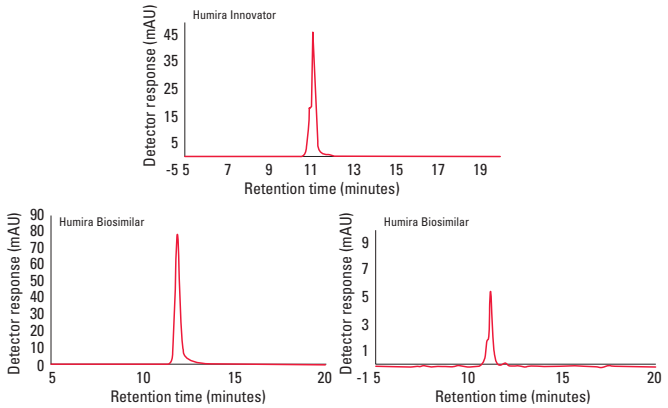


Figure 8. HPLC Analysis of Humira Innovator and Two Biosimilars Using TSKgel Butyl-NPR



Conclusions

This study shows that the following columns from different modes of chromatography can be used for the comparison of an innovator biomolecule with the corresponding biosimilar, as part of a characterization workflow solution for chromatographic analysis.

Mode	Column	Application
SEC	TSKgel UP-SW3000	Purity analysis; examination of impurities such as aggregate and fragments
RPC	TSKgel ODS-100V	Comparative peptide analysis
HIC	TSKgel Butyl-NPR	Hydrophobic heterogeneity

Differences in analytical characterization prompts further investigation of the biosimilar in order to ensure that the safety, purity and potency of the product are not affected.