Application Note

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Charge Variant and Aggregation Analysis of Innovator and Biosimilars of Rituximab

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Abstract

Monoclonal antibodies are an important class of biomolecules used for treatment of various diseases. Biosimilars, the copy versions of an innovator molecule, need to be characterized in detail for their critical quality attributes (CQAs) such as aggregates and charge variants. The attributes must fall within a desired range compared to the innovator for approval by regulatory agencies. This study compares two rituximab biosimilars from different manufacturers to the innovator for their aggregation and charge variant profiles by following two analytical workflows using Agilent 1260 Infinity II bio-inert LC and Agilent AdvancedBio columns. The results show the similarities or differences between innovator and biosimilars in their aggregates and charge variant profiles. Biosimilar 1 has more similarities with the innovator than biosimilar 2 in terms of aggregates and charge variants. Excellent intraday and interday reproducibility of the methods was demonstrated. Agilent OpenLab CDS software featuring peak explorer facilitates easy data review at a glance. This work is part of a series of biosimilarity studies of rituximab.

Introduction

Monoclonal antibody (mAb) drugs are one of the fastest growing biotherapeutics in the pharma market. The majority of mAbs are for treatment of cancers.[1] The investment during the discovery, development, manufacturing, and clinical trials is huge for innovator mAb drugs. As a result, the cost of innovator drug treatment is usually high for patients. Therefore, more affordable generic versions of innovator drugs, called biosimilars, are in high demand. The first biosimilar was approved for the European market in 2006, and the U.S. market opened nine years later after the introduction of the Affordable Care Act in March 2010. The development of biosimilars is gaining traction due to patent expiry of innovator molecules.

For biosimilars to be approved by regulatory agencies, manufacturers need to demonstrate that there are no clinically meaningful differences between the biological product and the reference product in terms of the safety, purity, and potency.[2] A critical part in this process is an extensive comparative analytical study to understand the physicochemical similarities between the innovator and biosimilars.

Aggregates, truncation, and other modified forms (deamidation, isomerization, and so forth) are product-related impurities that arise during the manufacturing process or storage. Their presence in the drug negatively impact drug stability, activity, and efficacy. Therefore, they are usually considered CQAs, and are closely monitored and tested throughout the manufacturing process.[3]

This Application Note uses two analytical workflows to demonstrate a comparison between two biosimilars of rituximab and their reference innovator in terms of aggregate and charge variant profiles. Rituximab is a well known biotherapeutic drug for the treatment of rheumatoid arthritis, lupus, vasculitis, and dermatomyositis. The two biosimilars were obtained from two manufacturers in different geographical locations. Both workflows are based on the 1260 Infinity II bio-inert LC system together with advancedBio columns and OpenLab CDS. Charge variants were separated on a weak cation exchange (WCX) column, while aggregates were separated on a size exclusion (SEC) column. Figure 1 shows the two workflow details. Good reproducibility on intraday and interday results ensured reliability of the workflows and demonstrated clear similarities or differences between the innovator and biosimilars.

Experimental

Instrumentation

The systems were composed of the following modules:

- Agilent 1260 Infinity II Bio-inert Pump (G5654A)
- Agilent 1260 Infinity II Bio-inert Multisampler (G5668A) with sample cooler
- Agilent 1260 Infinity II Multicolumn Thermostat (G7116A) with bio-inert heat exchanger
- Agilent 1260 Infinity II Diode Array Detector WR (G7115A) with bio-inert flow cell
- Agilent 1260 Infinity II Bio-inert MultiDetector Suite (MDS) (G7805A) featuring dual-angle static and DLS detection (G7809A)

Columns

- Agilent Bio mAb, nonporous, 2.1 × 250 mm, 5 µm HPLC, PEEK (p/n 5190-2411) for charge variants analysis
- Agilent AdvanceBio SEC 300Å, 7.8 × 300 mm, 2.7 µm (p/n PL1180-5301) for aggregation analysis.

Software

- Agilent OpenLab CDS Version 2.3
- Agilent Buffer Advisor A.01.01 [009]
- Agilent Bio-SEC Software version A.02.01 Build 9.34851[21]

LC instrument control as well as LC data analysis was carried out using Agilent OpenLab CDS Version 2.3. It provides a smooth user interface with customized and interactive reporting with drag-and-drop template creation. The peak explorer feature of the software was used to compare the results between the innovator and biosimilars.

Chemicals and samples

All solvents used were LC grade. Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22 µm membrane point-of-use cartridge (Millipak). Sodium phosphate monobasic, sodium phosphate dibasic, and sodium chloride were purchased from Sigma-Aldrich, St. Louis, USA. The mAb drugs, including the innovator and two biosimilars, were purchased from a local distributor. Before analysis in the DLS system, the mobile phase was triple filtered through a 0.1 µm hydrophilic PTFE membrane filter (Merck Millipore).

Samples were taken from the original container and centrifuged at 13,000 g for two minutes. Supernatant was aliquoted to an LC sample vial for analysis.

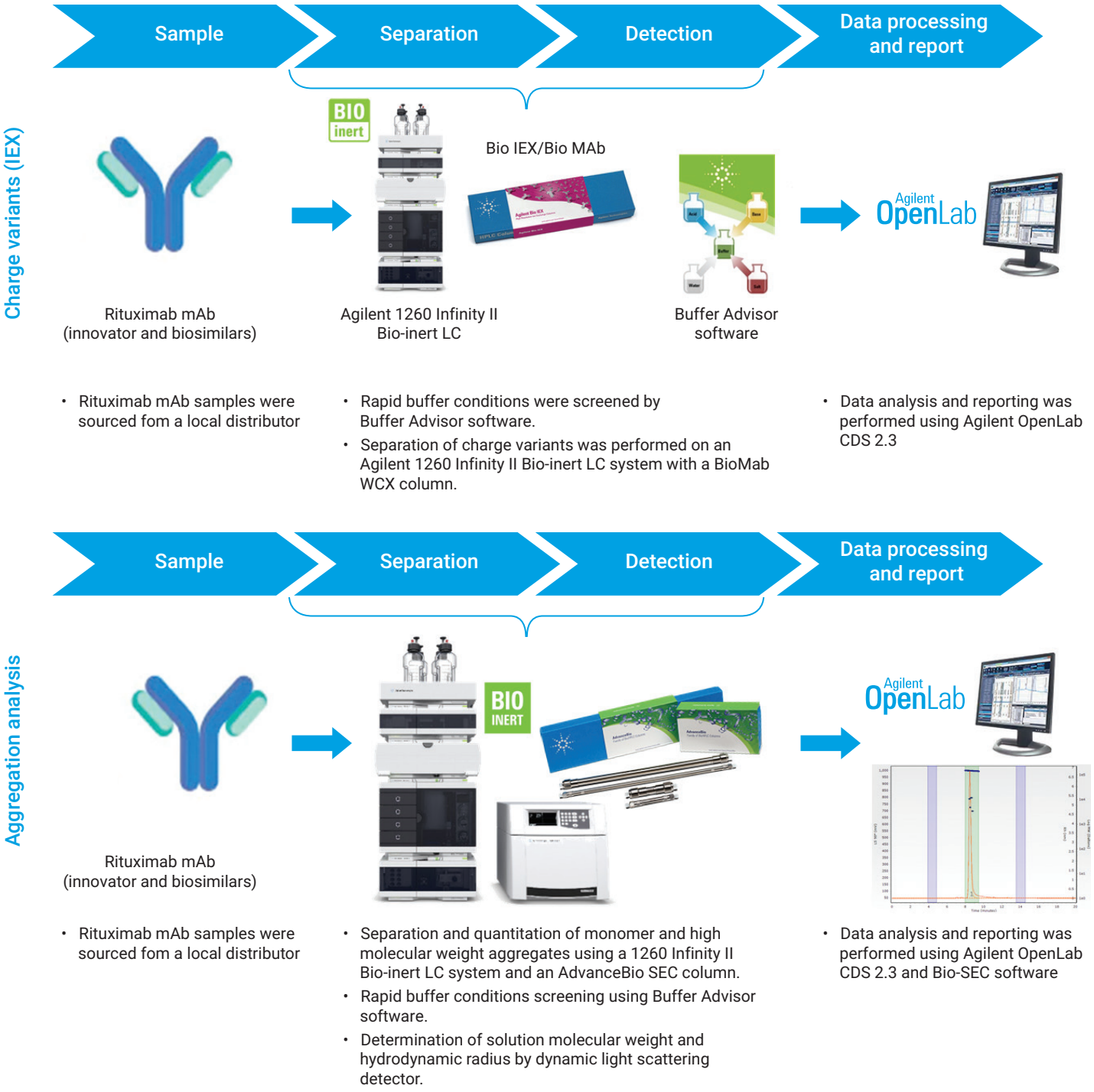


Figure 1. Charge variant and aggregation analysis workflow for the analysis of rituximab innovator and biosimilars.

Experimental methods

Charge variant: Table 1 shows the chromatographic parameters used for ion exchange chromatography of rituximab innovator and biosimilars. The gradient used in the study was calculated from the buffer advisor software. Samples were directly injected without dilution (10 mg/mL). Retention time (RT), area, and area percent were used to calculate relative standard deviation (RSD %) values. Relative percent area was used to quantify the charge variants of the mAbs.

Aggregates analysis: Table 2 shows the chromatographic parameters used for aggregation analysis of rituximab innovator and biosimilars. Samples were directly injected without dilution (10 mg/mL). RT, area, and percent area were used to calculate RSD% values. Relative percent area was used to quantify the high molecular weight species (HMWS) and low molecular weight species (LMWS) in the samples. Average molecular weight and hydrodynamic radius of rituximab were obtained from DLS analysis.

Results and discussion

Charge variant (IEX)

Figure 2 shows the charge variant profiles of innovator and biosimilars on a BioMAb PEEK column, demonstrating high resolution separation of charge variants in 16 minutes. The overlay of six replicates of rituximab innovator and biosimilars shows excellent reproducibility. The RSD of RT and area for main peak and variants are all within 0.3 and 1%, respectively.

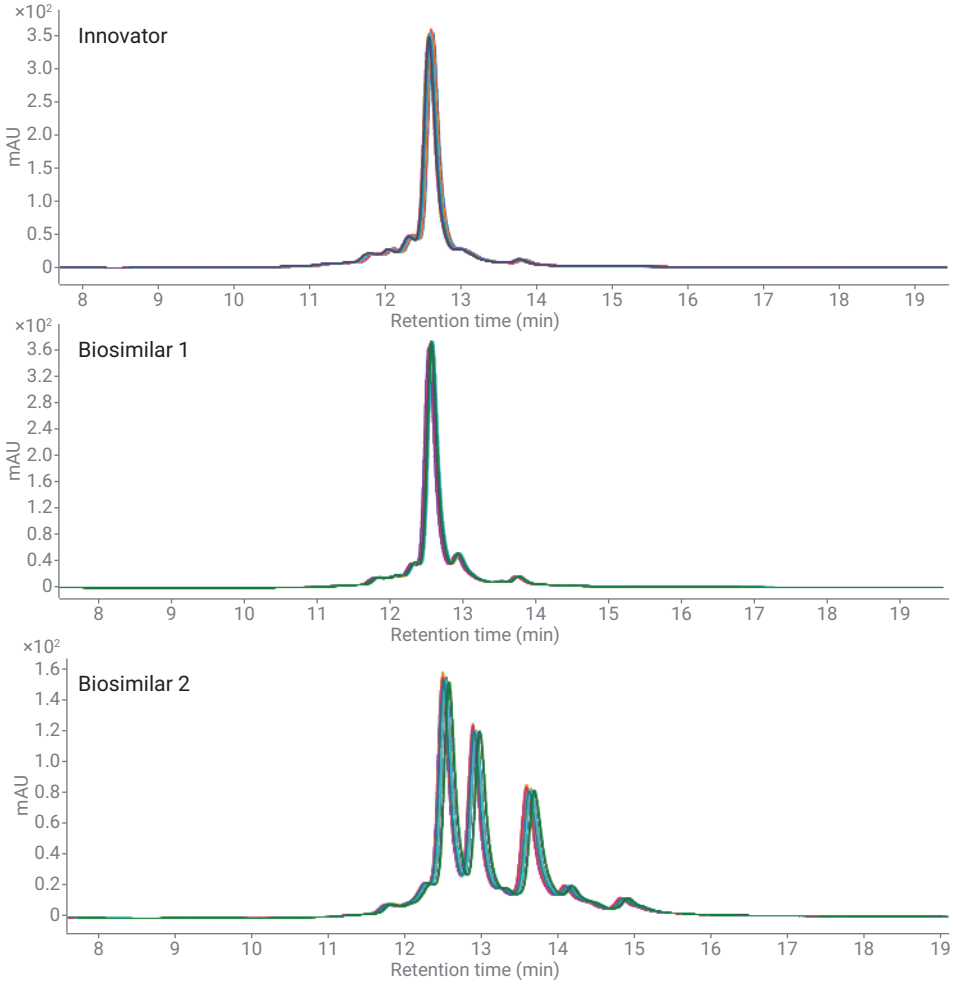
Table 1. IEX chromatographic conditions.

Parameter	Value
Salt Gradient 0 to 200 mM NaCl, 30 mM Sodium Phosphate Buffer, pH 6.8	Time (min) A) Water B) NaCl (1,000 mM) C) NaH_2PO_4 (55 mM) D) Na_2HPO_4 (50 mM) 0.0 43.1 0.0 31.0 25.9 30.0 22.3 20.0 22.7 35.0 35.0 22.3 20.0 22.7 35.0
Stop Time	35 minutes
Post Time	30 minutes
Flow Rate	0.25 mL/min
Injection Volume	2 µL
Sampler Temperature	10 °C
Column Temperature	25 °C
DAD	280 nm/4 nm, Ref:OFF
Peak Width	>0.025 minutes (10 Hz)

Table 2. Aggregation analysis chromatographic conditions.

Parameter	Value
Mobile Phase	100 mM sodium phosphate buffer+150 mM NaCl, pH 7.0
Flow Rate	0.8 mL/min
Stop Time	20 minutes
Injection Volume	10 µL (for UV) / 25 µL (for DLS)
Sampler Temperature	10 °C
Column Temperature	25 °C
DAD	280 nm/4 nm, Ref:off
Peak Width	>0.05 minutes (1.0 second response time) (5 Hz)
LS Detector	25 °C
DLS Operational Parameters	
Correlator Run Time	5 seconds
Correlator Function Clip Time	10 µs
R^2	0.80
Eluent Viscosity	0.0079 (viscosity of water at 30 °C)
Eluent Refractive Index	1.333 (refractive index of water)

Figure 3 shows overlaid chromatograms for comparison between innovator and biosimilars. The peak at ~12.5 minutes is attributed to the main peak, and the peaks to the left and right of the main peak are assigned to acidic and basic charge variants, respectively. The profiles of acidic variants were similar between the innovator and biosimilar 1, while biosimilar 2 shows a slight difference. The profiles of basic variants mainly attributed to lysine truncation showed huge differences between biosimilar 2 and innovator due to the incomplete lysine truncation.[4]



Innovator	Intraday (n = 6)		Interday (n = 6)	
	%RSD RT	RSD% Area	%RSD RT	RSD% Area
Acidic Variants	0.12	0.42	0.31	0.68
Main Peak	0.10	0.15	0.29	0.50
Basic Variants	0.10	0.90	0.28	1.91

Biosimilar 1	Intraday (n = 6)		Interday (n = 6)	
	%RSD RT	RSD% Area	%RSD RT	RSD% Area
Acidic Variants	0.08	0.60	0.16	1.07
Main Peak	0.07	0.32	0.15	0.85
Basic Variants	0.07	0.75	0.14	1.05

Biosimilar 2	Intraday (n = 6)		Interday (n = 6)	
	%RSD RT	RSD% Area	%RSD RT	RSD% Area
Acidic Variants	0.20	0.19	0.26	0.80
Main Peak	0.21	0.52	0.25	0.89
Basic Variants	0.22	0.32	0.26	0.70

Figure 2. Overlay of six replicates of innovator and biosimilars of rituximab on an Agilent 1260 Infinity Bio-inert quaternary LC using an Agilent Bio Mab, 2.1 × 250 mm, 5 µm, PEEK column. The tables in the figure show the precision of retention time and area for main peak and charge variants, n = 6.

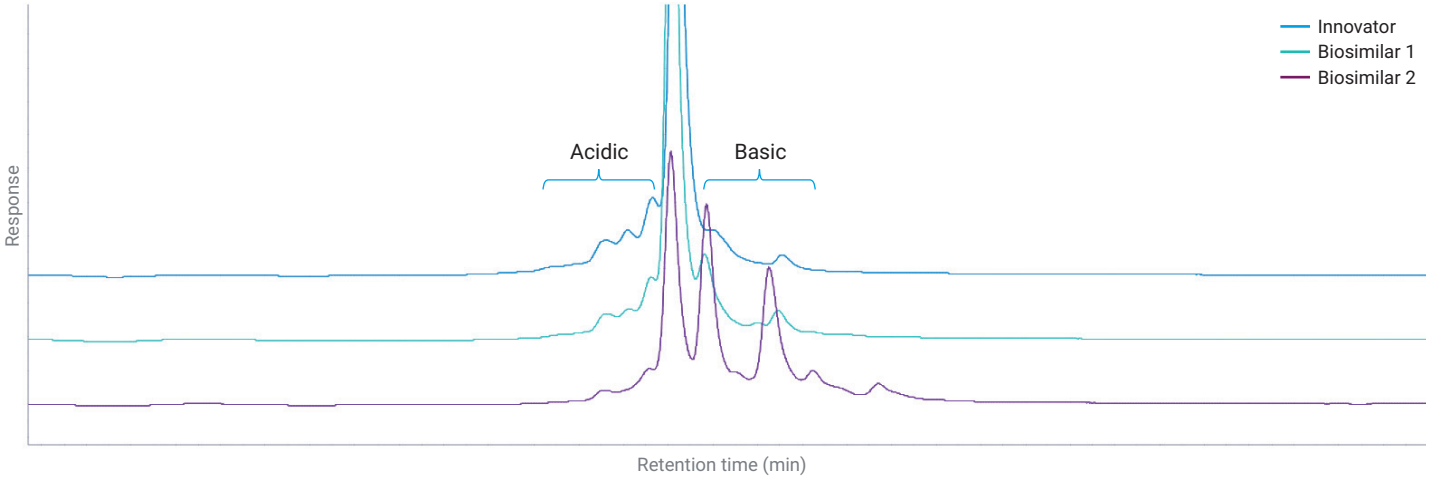


Figure 3. Expanded view of the charge variant profile comparison of innovator and biosimilars of rituximab.

Figure 4 shows the charge variants and main form distribution across innovator and biosimilars. The main form in innovator rituximab was found to be 65.35 ±0.27 %, with 65.14 ±0.10% in biosimilar 1, but only 28.8 ±0.07 % in biosimilar 2. The major charge variant in biosimilar 2 rituximab was 62.99 ±0.06% basic variants compared to the innovator product (12.97 ±0.23%). The innovator and biosimilar 1 are similar in their charge variant profile, except biosimilar 1 showed slightly more basic variants (20.11 ±0.12% versus 12.97 ±0.23%) and fewer acidic variants (14.53 ±0.09% versus 21.68 ±0.13%).

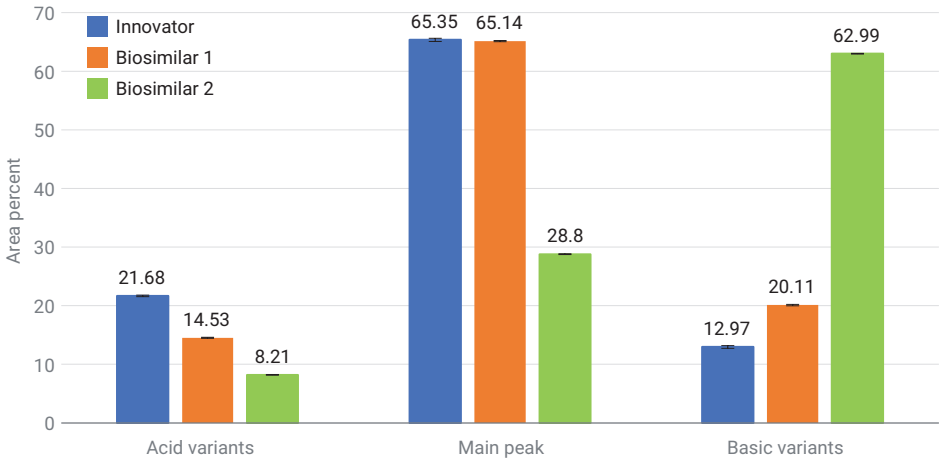


Figure 4. Comparison of the charge variants (acidic, main, and basic) area percentage between innovator and biosimilars.

Another useful data analysis capability feature found in the OpenLab CDS is the Peak Explorer. This feature promotes quick data review for complex samples by visualizing large data sets to discover trends, retention time shifts, outliers, artifacts, and so forth. Peak Explorer was used to examine the charge variant data and compare the innovator and biosimilars. Figure 5 shows the visualization of comparison from Peak Explorer for innovator and biosimilars. Each bubble corresponds to the acid and basic variants and the main peak. The size of the bubble represents the area percent of the variant. The comparison shown with Peak Explorer is in accordance with the Figure 4 conclusion.

Aggregate analysis

Figure 6 shows the aggregates profiles of innovator and biosimilars demonstrating high-resolution separation of aggregates in 20 minutes. The overlay of six replicates of innovator and biosimilars shows excellent reproducibility. The RSD of RT and area for the main peak and variants are all within 0.1 and 2%, respectively.

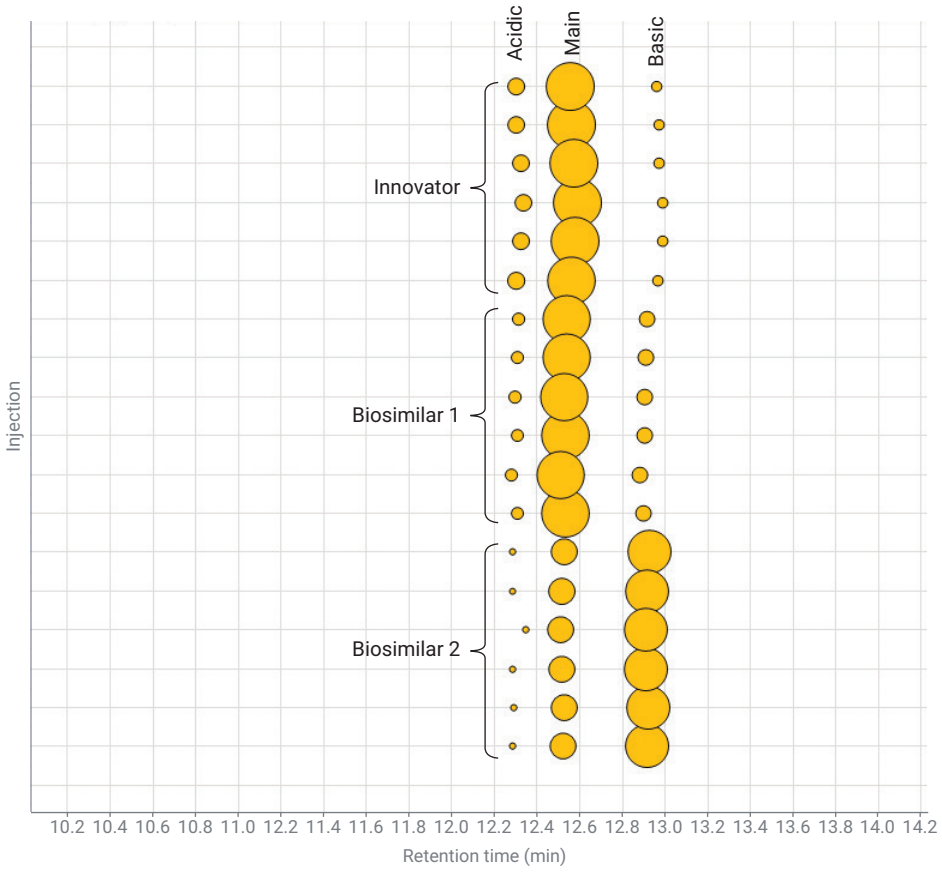
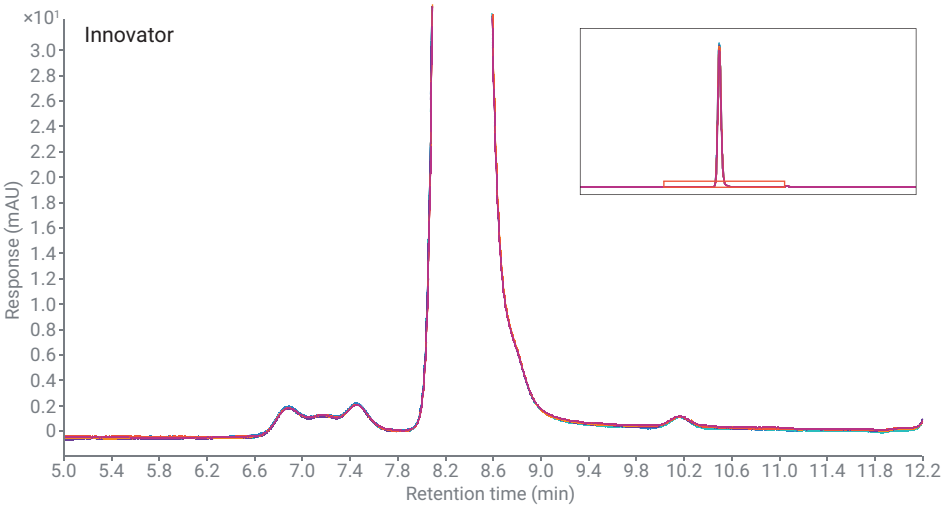
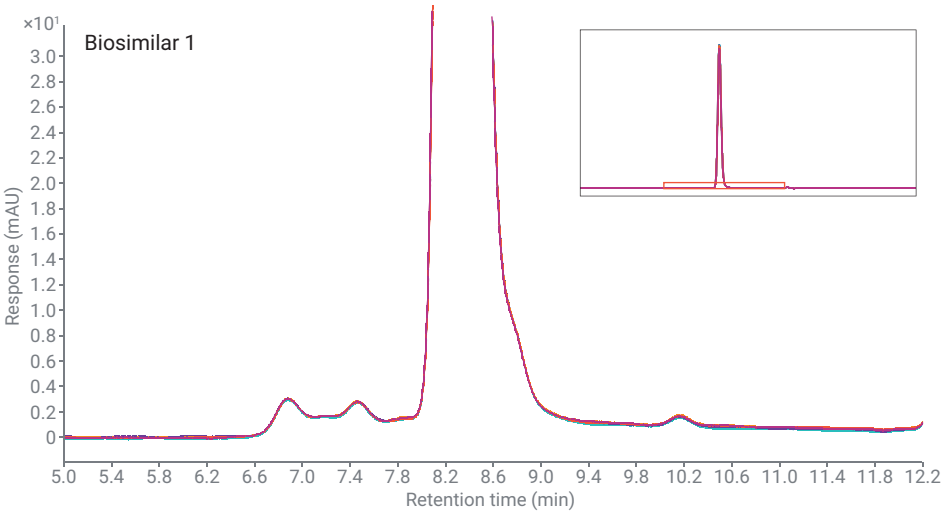


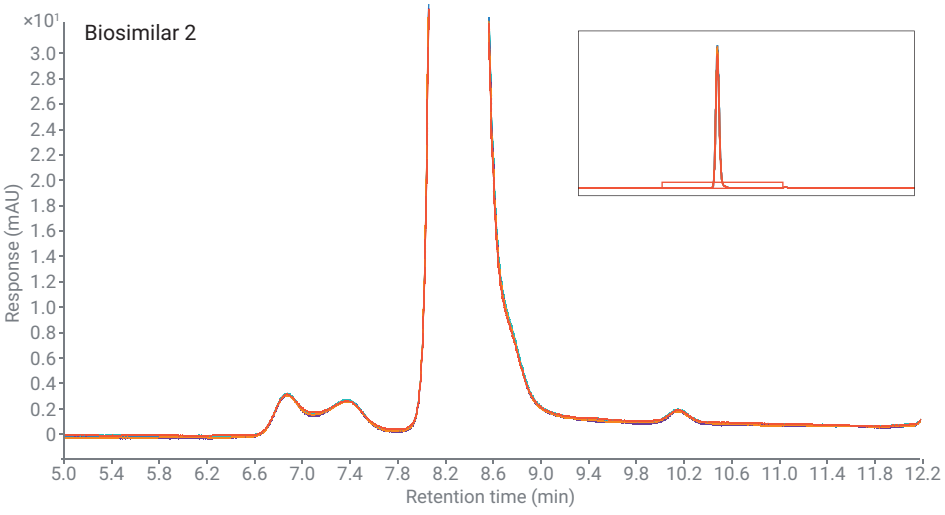
Figure 5. A Peak Explorer data presentation snapshot. The X-axis is retention time, and the Y-axis is the injection number of the data set loaded. Each bubble represents each variant and main peak. The size of the bubble represents peak area percent.



Innovator	Intraday (n = 6)		Interday (n = 6)	
	%RSD RT	RSD% Area	%RSD RT	RSD% Area
HMWS	0.05	0.35	0.05	0.56
Monomer Peak	0.02	0.91	0.04	0.86
LMWS	0.03	1.12	0.06	1.77



Biosimilar 1	Intraday (n = 6)		Interday (n = 6)	
	%RSD RT	RSD% Area	%RSD RT	RSD% Area
HMWS	0.04	0.72	0.08	1.29
Monomer Peak	0.00	0.68	0.02	0.62
LMWS	0.04	1.75	0.04	2.25



Biosimilar 2	Intraday (n = 6)		Interday (n = 6)	
	%RSD RT	RSD% Area	%RSD RT	RSD% Area
HMWS	0.04	0.66	0.04	0.63
Monomer Peak	0.01	0.51	0.01	0.61
LMWS	0.02	2.11	0.05	2.29

Figure 6. Overlay of six replicates of innovator and biosimilars of rituximab on an Agilent 1260 Infinity Bio-inert quaternary LC using an Agilent AdvancedBio SEC, 7.8 × 300 mm, 2.7 µm column. The tables show the precision of retention time and area for HMWS, monomer, and LMWS, n = 6.

Figure 7 shows overlaid chromatograms for comparison between innovator and biosimilars. The peak at 8.4 minutes is attributed to the monomer, and the peaks to the left and right of the main peak are assigned to HMWS and LMWS, respectively. As shown in the figure, the LMWS are similar across samples (0.08, 0.08, and 0.11%) whereas the HMWS shows different profiles; the difference between innovator and biosimilar 2 is more prominent than with biosimilar 1.

Figure 8 shows the HMWS, LMWS, and monomer distribution within the samples. The monomer peak in all three samples was found to be 98 to 99%. Slightly higher levels of HMWS were observed in both biosimilars (0.93 ±0.01%, 0.94 ±0.01%).

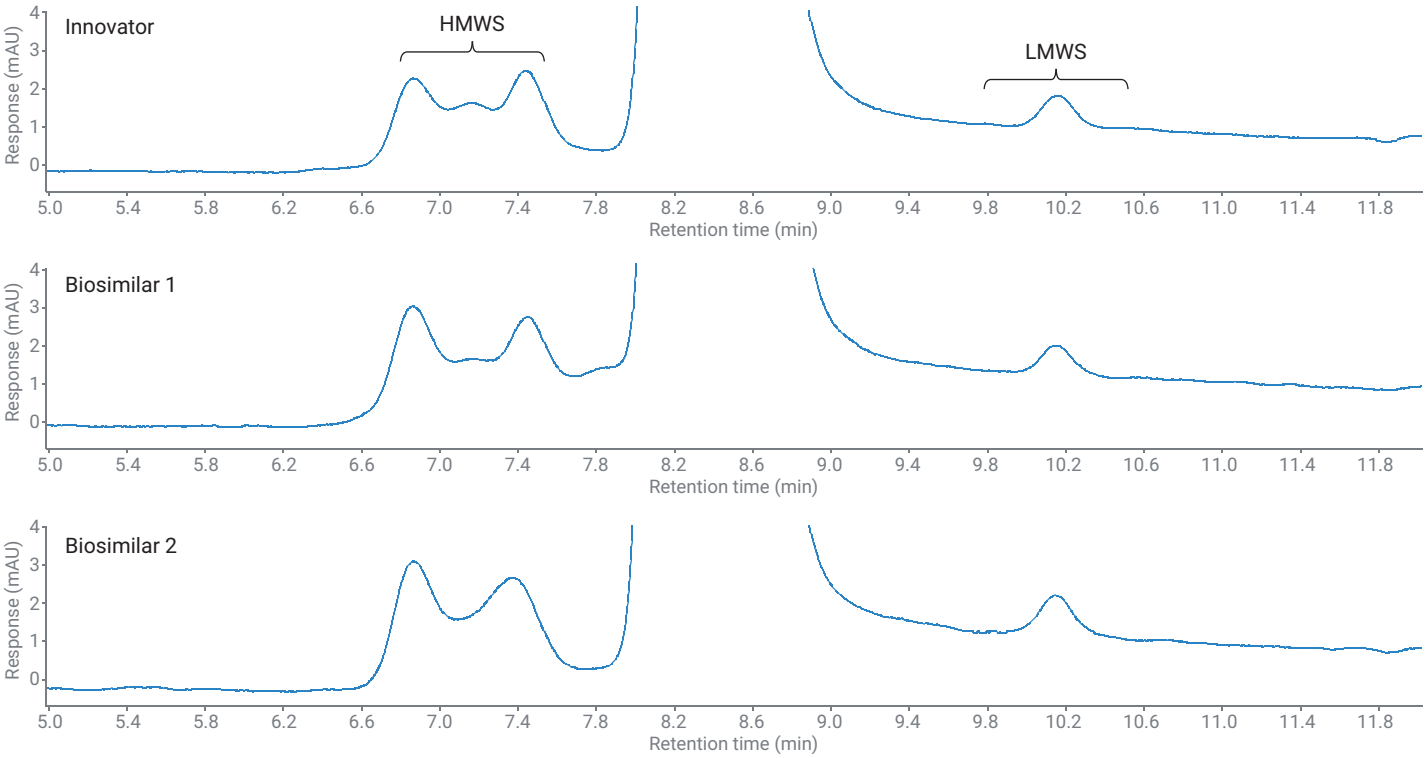


Figure 7. Zoom-in comparison of the aggregate's profiles of innovator and biosimilars.

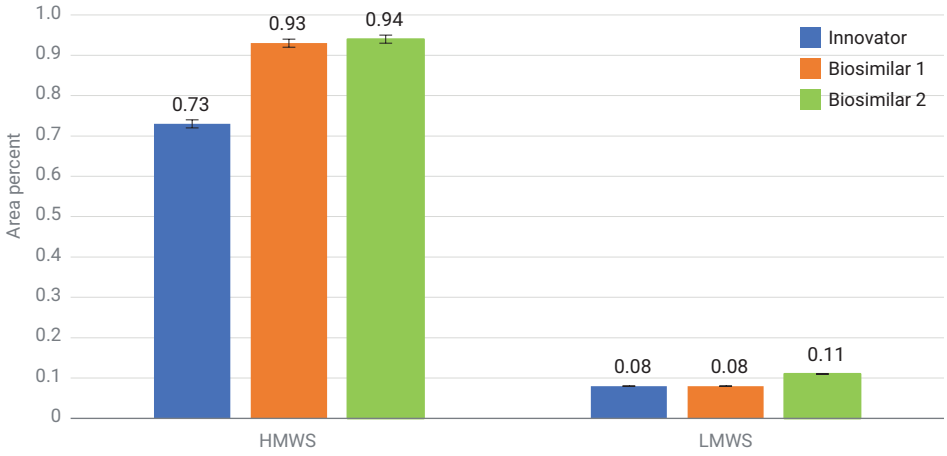
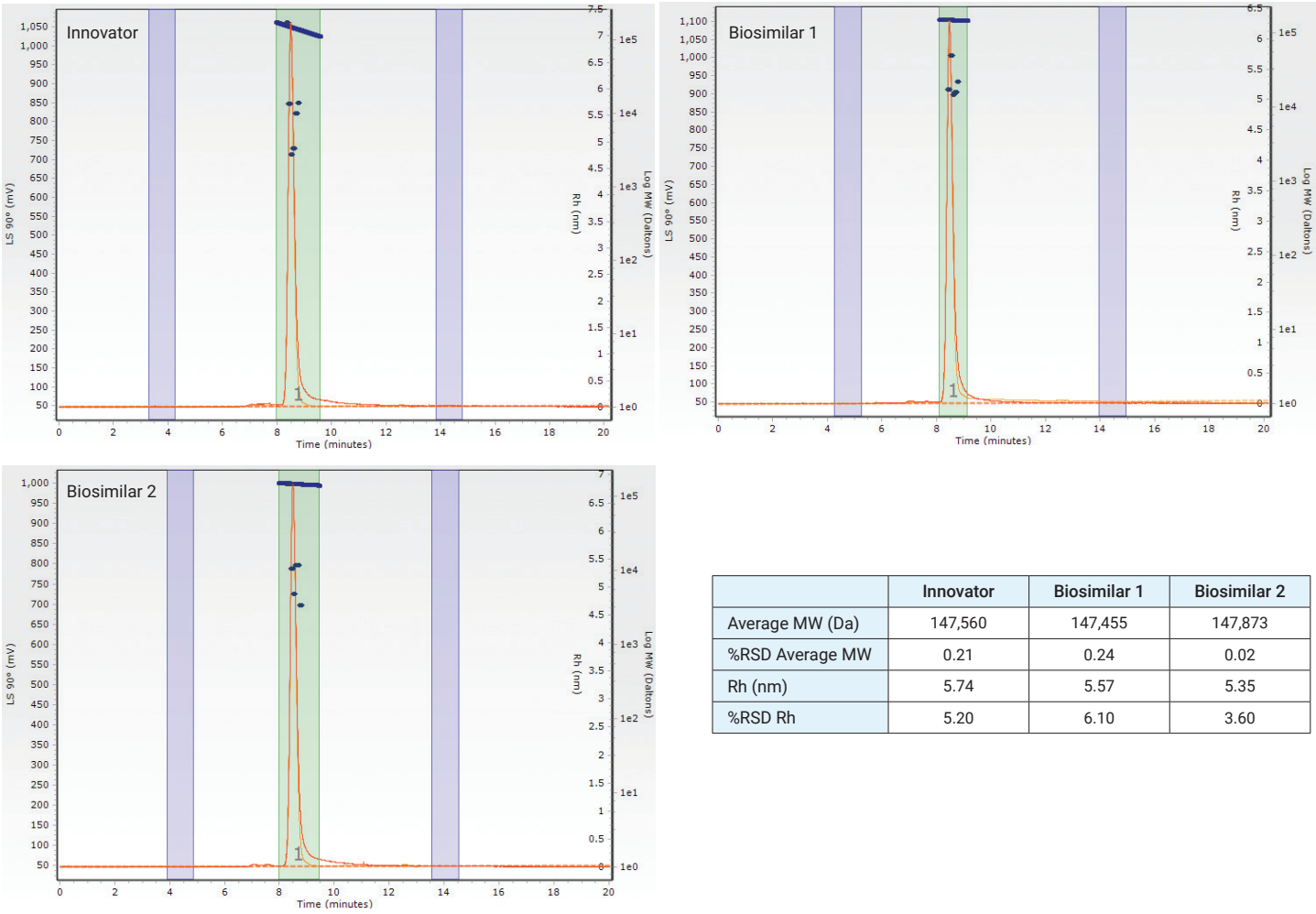


Figure 8. Comparison of the HMWS and LMWS area percentage between innovator and biosimilars.

Biosimilar 2 also presented a higher level of LMWS (0.11%) compared to the innovator (0.08%) and biosimilar 1 (0.08%). However, all the differences are subtle.

Figure 9 demonstrates the results acquired with the bio-MDS system with DLS detection. Absolute average molecular weight can be read directly from the results together with the hydrodynamic radius (Rh) of the mAb monomer. Results showed good reproducibility of DLS analysis and accurate measurement of molecular weight and Rh values.



	Innovator	Biosimilar 1	Biosimilar 2
Average MW (Da)	147,560	147,455	147,873
%RSD Average MW	0.21	0.24	0.02
Rh (nm)	5.74	5.57	5.35
%RSD Rh	5.20	6.10	3.60

Figure 9. Comparison of the average molecular weight and Rh from DLS analysis between innovator and biosimilars.

Conclusion

This Application Note demonstrates two analytical workflows, charge variant analysis and aggregation analysis, to analyze rituximab innovator and its two biosimilars. Good reproducibility in RT and area were achieved for both workflows. In the charge variant analysis, the biosimilar 2 sample presented distinct differences with a high percentage of basic variants that are believed to be lysine truncation variants. In the aggregation analysis, biosimilar 2 presented a slightly different HMWS profile compared to the innovator. In terms of charge and aggregate variants properties of the drug samples, biosimilar 1 demonstrated more similarities to the innovator. The results are also in line with the published data of rituximab characterization.[5] This shows that Agilent charge variant and aggregation analysis workflows are reliable for biosimilar comparability studies. To facilitate easy data review in batch mode, increasing analytical efficiency, Agilent Openlab CDS software offers features such as Peak Explore.

References

1. Dübel, S.; Reichert, J. M. Handbook of Therapeutic Antibodies, second edition.
2. U.S. Public Health Service Act section 351(k).
3. ICH Harmonized Tripartite Guideline Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products Q6B.
4. Suresh Babu, C. V. Charge Heterogeneity Analysis of Rituximab Innovator and Biosimilar mAbs *Agilent Technologies Application Note*, publication number 5991-5557EN.
5. Lee, K. H. *et al.* Analytical Similarity Assessment of Rituximab Biosimilar CT-P10 to Reference Medicinal Product. *Mabs* **2018**, *10(3)*, 380–396.

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Printed in the USA, October 28, 2019
5994-1496EN
DE.0897222222

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