Charge Heterogeneity Analysis of Rituximab Innovator and Biosimilar mAbs

Application Note

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Abstract

This Application Note describes the high-resolution separation of charge variants of innovator and biosimilar rituximab using an Agilent 1260 Infinity Bio-inert Quaternary LC, biocolumns, and an Agilent OpenLAB ChemStation Software tool. An Agilent Bio MAb, 4.6 × 250 mm, 5 µm PEEK ion exchange column features a unique resin designed for the charge-based separation of monoclonal antibodies (mAbs). The optimized salt-gradient showed the differences in acidic and basic charge variant profiles between innovator and biosimilar rituximab. Precision of retention time, height, and area of charge isoforms were well within the acceptable range. C-terminal digestion by Carboxypeptidase B (CPB) revealed the major lysine variant peaks in biosimilar rituximab.

Introduction

Recently, biosimilar products are increasing in popularity in biopharmaceuticals. mAbs can undergo various post-translational modifications (PTMs) including lysine truncation, deamidation, oxidation, glycosylation, and so forth, becoming heterogeneous in their biochemical and biophysical properties. Due to these modifications, charge variants can affect the efficacy, activity, and stability of mAbs as biotherapeutics. Hence, it is very important to characterize the charge heterogeneity in drug development that will serve as a quality control (QC) step in the biopharmaceutical industry. In addition, precise bioanalytical methods are necessary to demonstrate the similarity between a biosimilar and the innovator product.

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Cation exchange chromatography (CEX) is the gold standard for charge-sensitive antibody analysis. In CEX, method parameters often need to be optimized for each protein, as ion exchange depends upon the reversible adsorption of charged protein molecules to immobilized ion exchange groups. This Application Note describes the salt-gradient method for separating the charge variants of innovator and biosimilar rituximab using an Agilent 1260 Infinity Bio-inert Quaternary LC and an Agilent Bio MAb NP5, 4.6 × 250 mm, PEEK ion exchange column. The method compares the CEX profiles of innovator and a rituximab biosimilar. Precision of retention time, height, area, and quantification of acidic, basic, and main forms was determined. Carboxypeptidase B (CPB) digestion was performed to study the contribution of C-terminal lysine variants.

Equipment

Instrumentation

An Agilent 1260 Infinity Bio-inert Quaternary LC, operating to a maximum pressure of 600 bar, was used for the experiments. The entire sample flow path was free of any metal components so that the sample did not come in contact with metal surfaces. Solvent delivery was free of any stainless steel or iron components.

Systems

- Agilent 1260 Infinity Bio-inert Quaternary LC Pump (G5611A)
- Agilent 1260 Infinity Bio-inert High Performance Autosampler (G5667A)
- Agilent 1200 Infinity Series Thermostat (G1330B)
- Agilent 1260 Infinity Thermostatted Column Compartment (TCC) containing bio-inert click-in heating elements (G1316C option 19)
- Agilent 1260 Infinity Diode Array Detector with with 10-mm bio-inert standard flow cell (G1315D)
- Agilent Bio MAb NP5, 4.6 × 250 mm, PEEK (p/n 5190-2407)

Software

- Agilent OpenLAB CDS ChemStation Edition, revision C.01.06
- Agilent Buffer Advisor, Rev. A.01.01

Reagents, samples, and procedure

Innovator and biosimilar rituximab were purchased from a local pharmacy and stored according to the manufacturer's instructions. Sodium phosphate dibasic, sodium phosphate monobasic, sodium chloride, hydrochloric acid (HCl), and sodium hydroxide were purchased from Sigma-Aldrich. All the chemicals and solvents were HPLC grade, and highly purified water was from a Milli Q water purification system (Millipore Elix 10 model, USA). Carboxypeptidase B (C9584) was purchased from Sigma-Aldrich.

Ion exchange chromatography parameters

Table 1 shows the chromatographic parameters for ion exchange chromatography using a 1260 Infinity Bio-inert Quaternary LC. Rituximab (innovator and biosimilar) were diluted to 1 mg/mL in water, and the elution was monitored at 280 nm. Retention time (RT), area, and percent area were used to calculate standard deviation (SD) and relative standard deviation (RSD %) values. Relative percent area was used to quantify the charge variants of mAbs.

Carboxypeptidase B digestion

Biosimilar and innovator rituximab were diluted to 1 mg/mL using 10 mM sodium phosphate buffer, pH 7.5. To these, 0.25 units of CPB was added and incubated at 37 °C. At various time points, the reaction mixture was aliquoted and quenched with acetic acid before analysis.

Table 1. Chromatographic parameters used for IEX chromatography.

Parameter	Conditions				
Mobile phase A	Water				
Mobile phase B	NaCl (850.0 mM)				
Mobile phase C	NaH_2PO_4 (41.0 mM)				
Mobile phase D	Na_2HPO_4 (55.0 mM)				
Gradient	Time (min)	Mobile phase A (%)	Mobile phase B (%)	Mobile phase C (%)	Mobile phase D (%)
	0	30.3	0	59.6	10.1
	2	26.0	5.0	56.9	12.1
	8	21.5	10.0	54.9	13.6
	20	13.3	19.0	51.9	15.8
	21	30.3	0	59.6	10.1
Injection volume	5 µL				
Flow rate	0.75 mL/min				
Data acquisition	280 nm/4 nm, Ref.: 360 nm/100 nm				
Acquisition rate	5 Hz				
TCC	Room temperature				
Sample thermostat	5 °C				
Post run time	10 minutes				

Results and Discussion

The Agilent Buffer Advisor Software is an ideal tool to generate pH or ionic strength gradients for protein charge variant separation. It reduces the time required for method development. In this study, a series of method development scouting runs were carried out using the Buffer Advisor Software for optimal mAb charge variant separation. Figure 1 shows the charge variant profiles of innovator and biosimilar rituximab on a Bio MAb PEEK column, demonstrating high-resolution separation of charge variants in 20 minutes with three distinct peaks in biosimilar (Buffer: 30 mM, pH: 6.3, and NaCl: 0–161.5 mM). The Agilent Bio MAb columns contained a highly uniform, densely packed, weak cation exchange resin. Early and late-eluting peaks were called acidic and basic variants, respectively. The peak at 11.4 minutes was designated as the main peak. The overlay of five replicates of innovator and biosimilar rituximab shows excellent separation reproducibility (Figure 2). The average RTs and area RSDs for main peak are shown in the figure. The RSDs are within the acceptable range, which demonstrates the precision of the system.

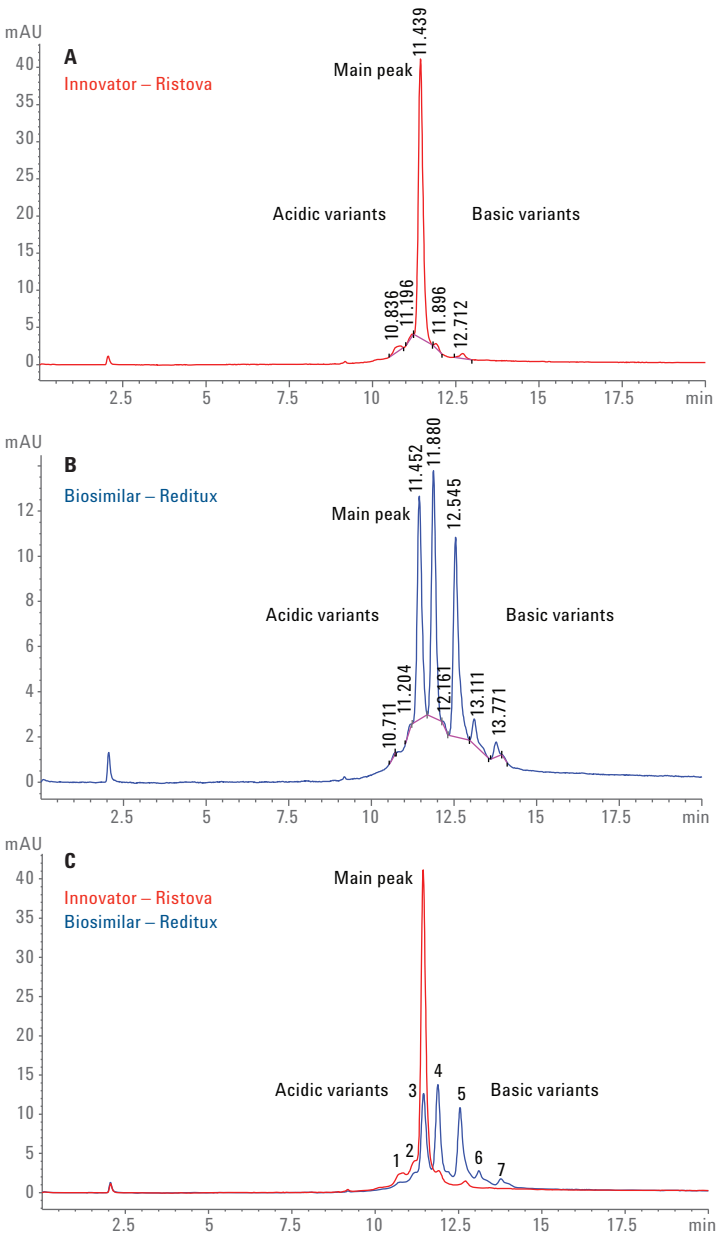

Figure 1. Charge variant profiles of innovator (A) and biosimilar (B) rituximab using an Agilent Bio MAb 5 µm column. C) Overlay of innovator and biosimilar rituximab. Peaks 1 and 2: acidic variants; 3: main form; 4, 5, 6 and 7: basic variants.

The high-resolution separation of mAbs facilitated the quantification of charge variants using peak areas. Table 2 summarizes the area percent of charge variants of five consecutive analyses. There was a significant difference in the area percent of the charge variants between two mAbs. The main form in the innovator rituximab was found to be 93.21 % and 29.78 % in biosimilar rituximab. The major charge variant in biosimilar rituximab was 69.46 % basic variants as compared to the innovator product (3.22 %).

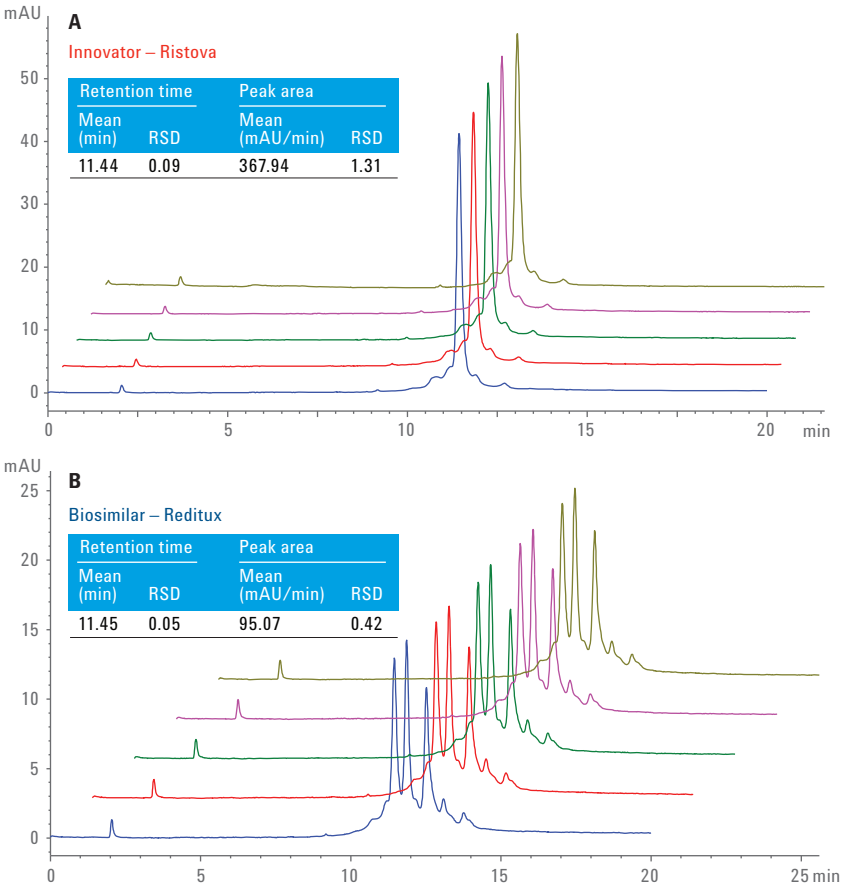

Figure 2. Overlay of five replicates of innovator (A) and biosimilar (B) rituximab on an Agilent 1260 Infinity Bio-inert Quaternary LC using an Agilent Bio Mab, 4.6 × 250 mm, 5 µm, PEEK column. Insert table shows the precision of retention time and area for main peak, n = 5.

Table 2. Charge variants quantification by area %, n = 5.

Innovator – Ristova		
	RT (min)	Area %
Acidic variant	10.84, 11.21	3.56
Main peak	11.44	93.21
Basic variant	11.9, 12.7	3.22
Biosimilar – Reditux		
Acidic variant	10.73, 11.22	0.76
Main peak	11.45	29.78
Basic variant	11.87, 12.15, 12.59, 13.1, and 13.77	69.46

To further characterize the basic variant peaks, both mAbs were subjected to carboxypeptidase B digestion. Figures 3A and 3B show the overlay of the IEX profiles before and after C-terminal cleavage of innovator and biosimilar rituximab, respectively. The disappearance of basic variant peaks after carboxypeptidase B treatment confirmed that the peaks correspond to lysine variants. Figure 4 shows the overlay of the IEX profiles of biosimilar rituximab after CPB treatment and innovator rituximab without CPB treatment, revealing the charge variant similarity between the mAbs.

Conclusion

The salt-gradient method described in this Application Note demonstrates the high-resolution separation of charge variant profiles of mAbs on an Agilent Bio MAb, 4.6 × 250 mm, 5 µm PEEK column. The innovator and biosimilar rituximab had different separation profiles with different degrees of acidic and basic variants. Carboxypeptidase B digestion confirmed that the major basic variant peaks in biosimilar correspond to lysine variants. The Agilent 1260 Infinity Bio-inert Quaternary LC with Bio MAb PEEK columns and reproducible method make this solution particularly suitable for the QA/QC analysis of mAbs for the biopharmaceutical industry.

References

1. Yan, He; *et al. J. Sep. Sci.* **2011**, *34*, 548–555
2. Agilent publication number 5991-0895EN
3. Agilent publication number 5990-6844EN
4. Agilent publication number 5991-0565EN

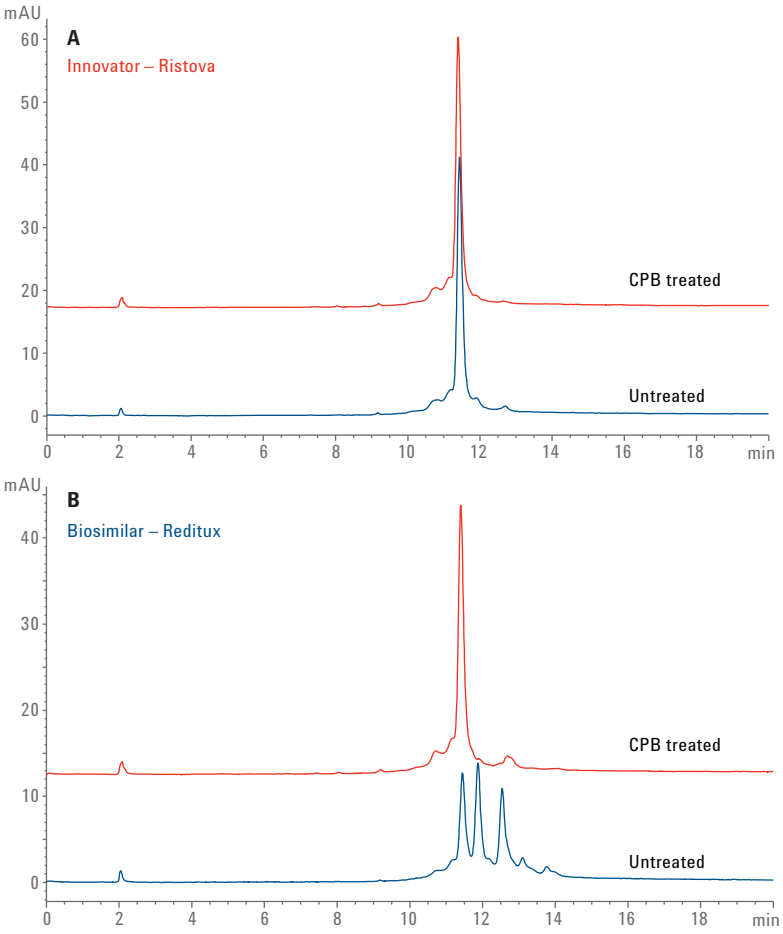

Figure 3. Characterization of basic charge variants. Separation of CPB treated (overnight) and untreated of innovator (A) and biosimilar (B) rituximab on an Agilent 1260 Infinity Bio-inert Quaternary LC using an Agilent Bio Mab, 4.6 × 250 mm, 5 µm, PEEK column.

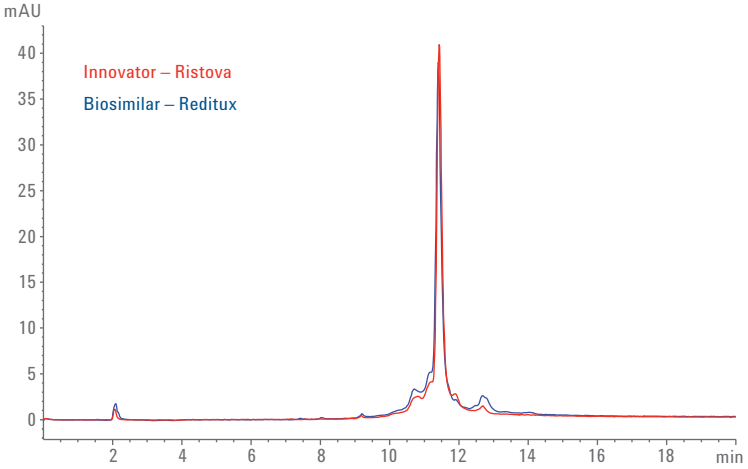

Figure 4. Overlay of innovator rituximab without CPB treatment (red) and biosimilar rituximab after CPB treatment (blue).

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