# High Resolution Separation of Charge Variant Profiles of Monoclonal Antibodies: Rituximab Innovator and Biosimilar

Suresh Babu C.V., Agilent Technologies India Pvt Ltd, Bangalore, India

This article describes the high-resolution separation of charge variants of innovator and biosimilar rituximab using a Bio-inert Quaternary LC, and OpenLAB ChemStation software. A Bio MAb, 4.6 × 250 mm, 5 µm PEEK ion-exchange column was used to obtain a separation. The column features a unique resin designed for the charge-based separation of monoclonal antibodies (mAbs). The optimised salt gradient revealed differences in acidic and basic charge variant profiles of innovator and biosimilar rituximab. Precision of retention time, peak height, and peak area of the charged isoforms were well within the acceptable range. C-terminal digestion by carboxypeptidase B (CPB) revealed the major lysine variant peaks in biosimilar rituximab.

Recombinant monoclonal antibodies (mAbs) are important biotherapeutics with a wide range of diagnostic and clinical applications. 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 important to characterise the charge heterogeneity during drug development, which serves as a quality control (QC) step for the biopharmaceutical industry. In addition, precise bioanalytical methods are necessary to demonstrate the similarity between a biosimilar and the innovator product.

Cation-exchange chromatography (CEX) is the gold standard for charge-sensitive antibody analysis [1,2]. This article describes a salt-gradient method [1, 3] for separating the charge variants of innovator and biosimilar rituximab. The method compares the CEX profiles of innovator and rituximab biosimilar. Precision of retention time, peak height, peak 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, with diode array detector fitted with a bio-inert flow cell, operating to a maximum pressure of 600 bar, was used. The Agilent 1260 Infinity Bio-inert Quaternary LC system is a dedicated solution for large bio-molecule analysis. Solvent delivery was free of any stainless steel or iron components. All the capillaries and fittings throughout the autosampler, column compartment and detector are metal free so that bio-molecules come in contact with ceramics or PEEK. This will ensures the integrity bio-molecule, minimises unwanted surface interactions and avoid the pitfalls of peak tailing, low recovery, and decreased column life. The column was an Agilent Bio MAb, 4.6 × 250 mm, 5 µm PEEK.

### Software

Agilent OpenLAB CDS ChemStation Edition, revision C.01.06, and Agilent Buffer Advisor, revision A.01.01, software for instrument control and data analysis was used.

### 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, and sodium hydroxide were purchased from Sigma-Aldrich (St. Louis, MO). All the chemicals and solvents were HPLC grade, and distilled water (18 M-ohm grade) was from a Milli Q water purification system (Millipore Elix 10). Carboxypeptidase B was also purchased from Sigma-Aldrich.

### Ion-exchange chromatography parameters

*Table 1* shows the chromatographic parameters for ion-exchange chromatography. 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.

*Table 1. Chromatographic parameters used for IEX chromatography*

| Parameter | Conditions | | | | |
|---|---|---|---|---|---|
| Mobile phase A | Water | | | | |
| Mobile phase B | NaCl (850.0 mM) | | | | |
| Mobile phase C | NaH2PO4 (41.0 mM) | | | | |
| Mobile phase D | Na2HPO4 (55.0 mM) | | | | |
| Gradient | Time (min) | Mobile phase A (%) | Mobile phase B (%) | Mobile phase C (%) | Mobile phase D (%) |
| | 0 min | 30.3 | 0 | 59.6 | 10.1 |
| | 2 min | 26.0 | 5.0 | 56.9 | 12.1 |
| | 8 min | 21.5 | 10.0 | 54.9 | 13.6 |
| | 20 min | 13.3 | 19.0 | 51.9 | 15.8 |
| | 21 min | 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 | Ambient | | | | |
| Sample thermostat | 5°C | | | | |
| Post run time | 10 min | | | | |

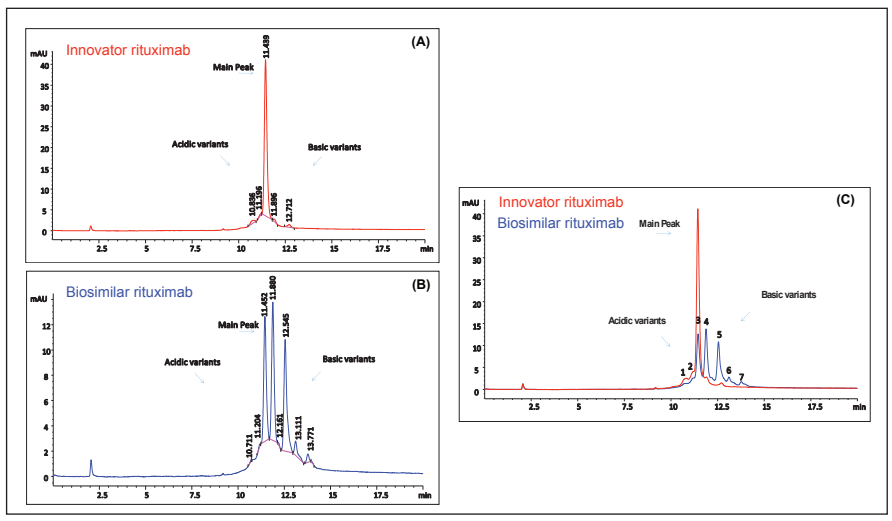
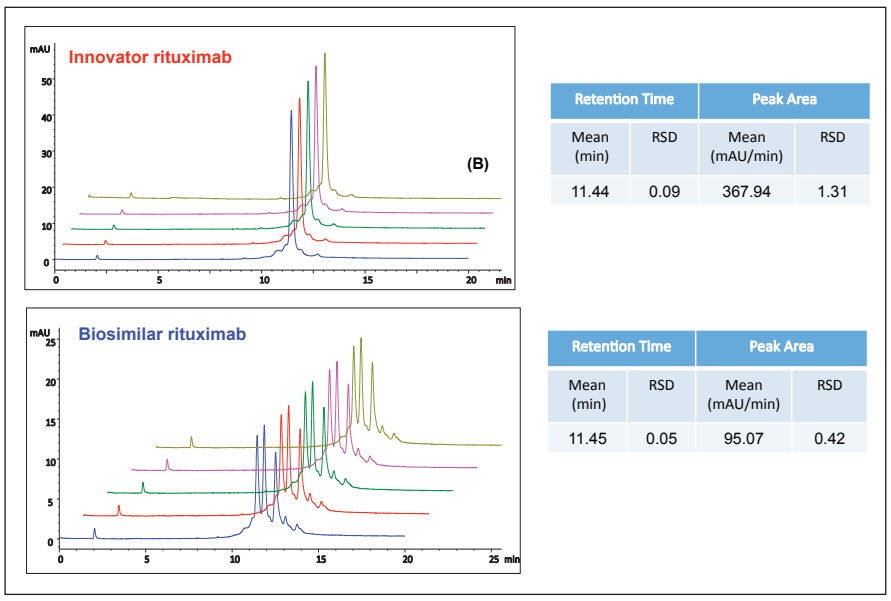



*Figure 1. Charge-variant profiles of innovator (A) and biosimilar (B) rituximab using an Bio MAb 5 µm column. (C) Overlay of innovator and biosimilar rituximab. Peaks 1 and 2 are acidic variants, peak 3 is the main form, peaks 4, 5, 6, and 7 are basic variants.*

### Carboxypeptidase B digestion

Biosimilar and innovator rituximab were diluted to 1 mg/ml using 10 mM sodium phosphate buffer, pH 7.5. CPB (0.25 units) was added and incubated at 37°C. At the various time points (0 min, 30 min, 1 hr, 2hr and overnight), the reaction mixture was aliquoted and quenched with acetic acid before analysis.




| Retention Time | | Peak Area | |
|---|---|---|---|
| Mean (min) | RSD | Mean (mAU/min) | RSD |
| 11.44 | 0.09 | 367.94 | 1.31 |

| Retention Time | | Peak Area | |
|---|---|---|---|
| Mean (min) | RSD | Mean (mAU/min) | RSD |
| 11.45 | 0.05 | 95.07 | 0.42 |

*Figure 2. Overlay of five replicates of innovator (A) and biosimilar (B) rituximab on an Bio-inert Quaternary LC using an Bio Mab, 4.6 × 250 mm, 5 µm PEEK column.*

## Results and Discussion

The Buffer Advisor Software is an ideal tool to generate pH or ionic strength gradients for protein charge-variant separations. The Buffer Advisor software enables the development of a robust method through design-of experiment principles. The automatic blending facilities and dynamic mixing of solvents from stock solutions simplifies IEX workflows and can save considerable time and solvent cost. In this study, a series of method development scouting runs were carried out using Buffer Advisor for optimal mAb charge-variant separation. *Figure 1* shows the charge-variant profiles of innovator and biosimilar rituximab on the 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 to 161.5 mM). The Bio MAb column contains 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 min was designated as the main peak. The overlay of five replicates of innovator and biosimilar rituximab shows excellent separation reproducibility *(Figure 2)*. The average RT's, and area RSD's for the main peak are shown in the figure. The RSD's are within the acceptable range, which demonstrates the precision of the system.

The high-resolution separation of mAbs facilitated the quantification of charge variants using peak areas. *Table 2* summarises 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 compared to the innovator product (3.22%). The difference in amount of acidic and basic variants between innovator and biosimilar may affect efficacy profiles.

To further to characterise the basic variants peaks, both mAbs were subjected to carboxypeptidase B digestion. *Figure 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 corresponded 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.

*Table 2. Charge-variant 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, 13.77 | 69.46 |

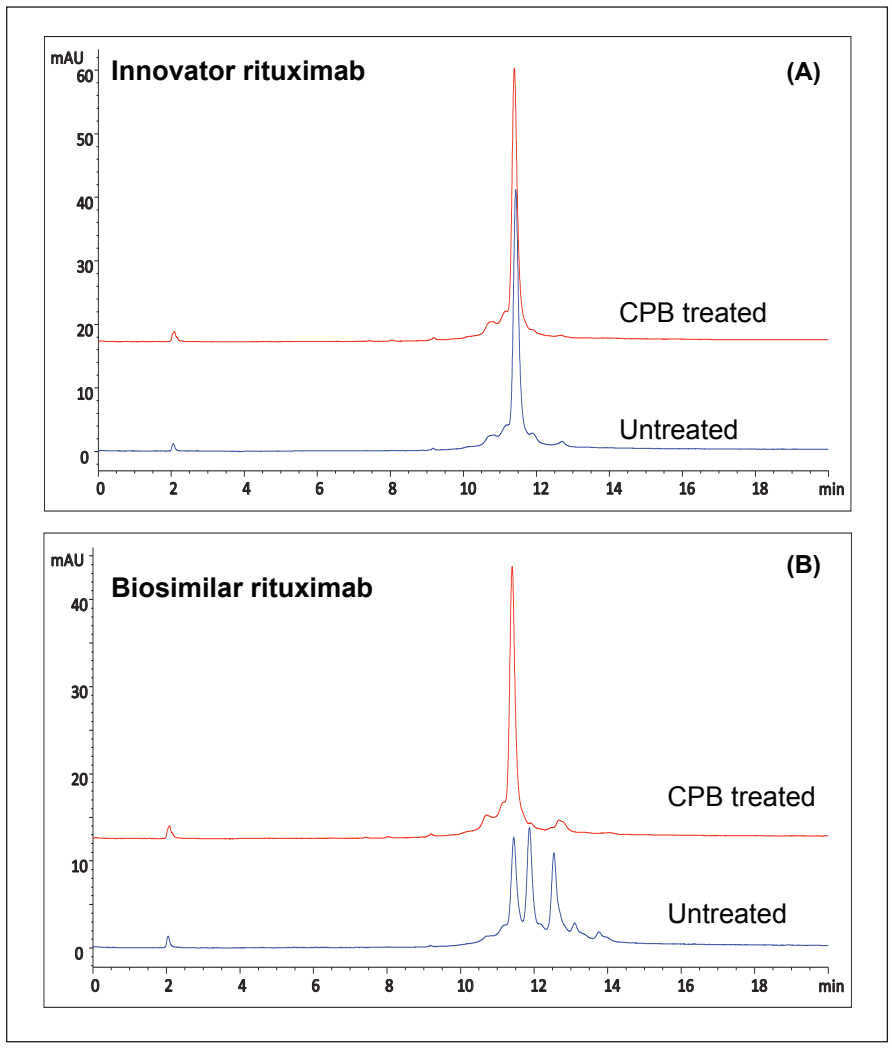



*Figure 3. Characterisation of basic charge variants. Separation of carboxypeptidase-treated (overnight) and untreated, of innovator (A) and biosimilar (B) rituximab on an Bio-inert Quaternary LC using an Bio Mab, 4.6 × 250 mm, 5 µm PEEK column.*

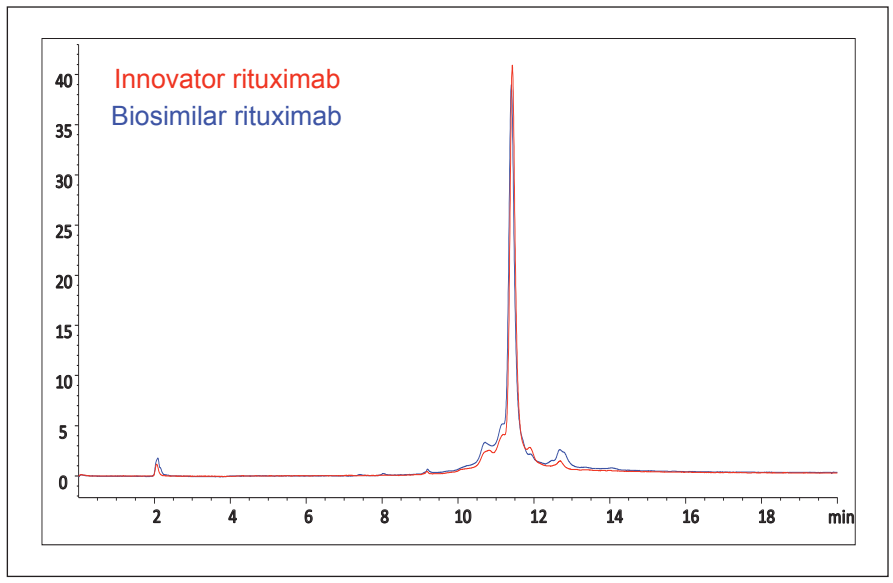



*Figure 4. Overlay of innovator rituximab without carboxypeptidase treatment (red) and biosimilar rituximab after carboxypeptidase treatment (blue).*

## Conclusions

The salt-gradient method described demonstrates the high-resolution separation of charge-variant profiles of mAbs. 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 corresponded to lysine variants. The columns used and the reproducible method make this solution suitable for the QA/QC analysis of mAbs for the biopharmaceutical industry.

## References

*1.Szabolcs Fekete et al. Method development for the separation of monoclonal antibody charge variants in cation exchange chromatography, Part I: Salt gradient approach. J. Pharm. Biomed. Anal. 102, 2015, 33–44.*

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