Analysis of Charge Heterogeneity in Rituximab Innovator and Biosimilar Monoclonal Antibodies Using Salt-Gradient Cation Exchange Chromatography

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Introduction

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 modifi cations (PTMs) including lysine truncation, deamidation, oxidation, glycosylation, and so forth, becoming heterogeneous in their biochemical and biophysical properties. Due to these modifi cations, charge variants can affect the effi cacy, 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.

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 poster describes the salt-gradient method for separating the charge variants of innovator and biosimilar rituximab using an Agilent 1260 Infi nity Bio-inert Quaternary LC and an Agilent Bio mAb NP5, 4.6 × 250 mm, PEEK ion exchange column. The method compares the CEX profi les of innovator and a rituximab biosimilar. Precision of retention time, height, area, and quantifi cation of acidic, basic, and main forms was determined. Carboxypeptidase B (CPB) digestion was performed to study the contribution of C-terminal lysine variants.

Experimental

Reagents, samples, and procedure

Innovator and biosimilar rituximab were purchased from a local pharmacy and stored according to the manufacturer's instructions. All the chemicals and solvents were HPLC grade, and highly purifi ed water was from a Milli Q water purifi cation system (Millipore Elix 10 model, USA). Carboxypeptidase B (C9584) was purchased from Sigma-Aldrich.

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 Carboxypeptidase B (CPB) was added and incubated at 37 °C. At various time points, the reaction mixture was aliquoted and quenched with acetic acid before analysis.

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.

- 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

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)				
Injection volume	5 µL				
Flow rate	0.75 mL/min				
Data acquisition	280 nm/4 nm, Ref.: 360 nm/100 nm				
TCC	Room temperature				
Sample thermostat	5 °C				
Post run time	10 minutes				
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

Results and Discussion

Agilent Buffer Advisor software

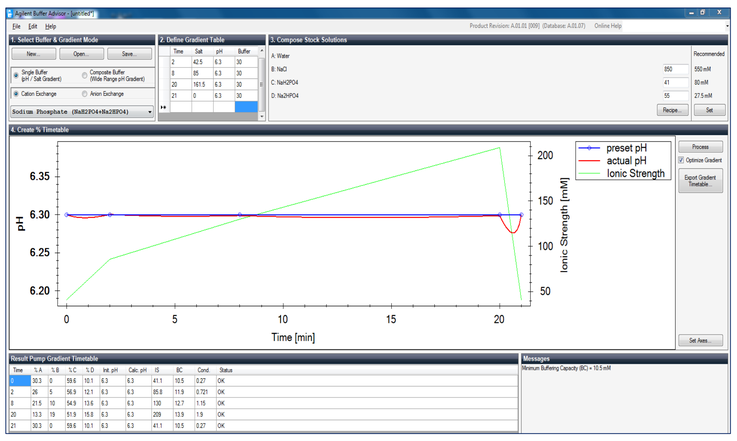


Figure 1: Screen image from Agilent's Buffer Advisor software. Agilent's Buffer Advisor software is an ideal tool to generate pH or ionic strength gradients for protein charge variant separation. It reduces the time required for method development. In this study, a series of method development scouting runs were carried out using the Buffer Advisor Software for optimal mAb charge variant separation

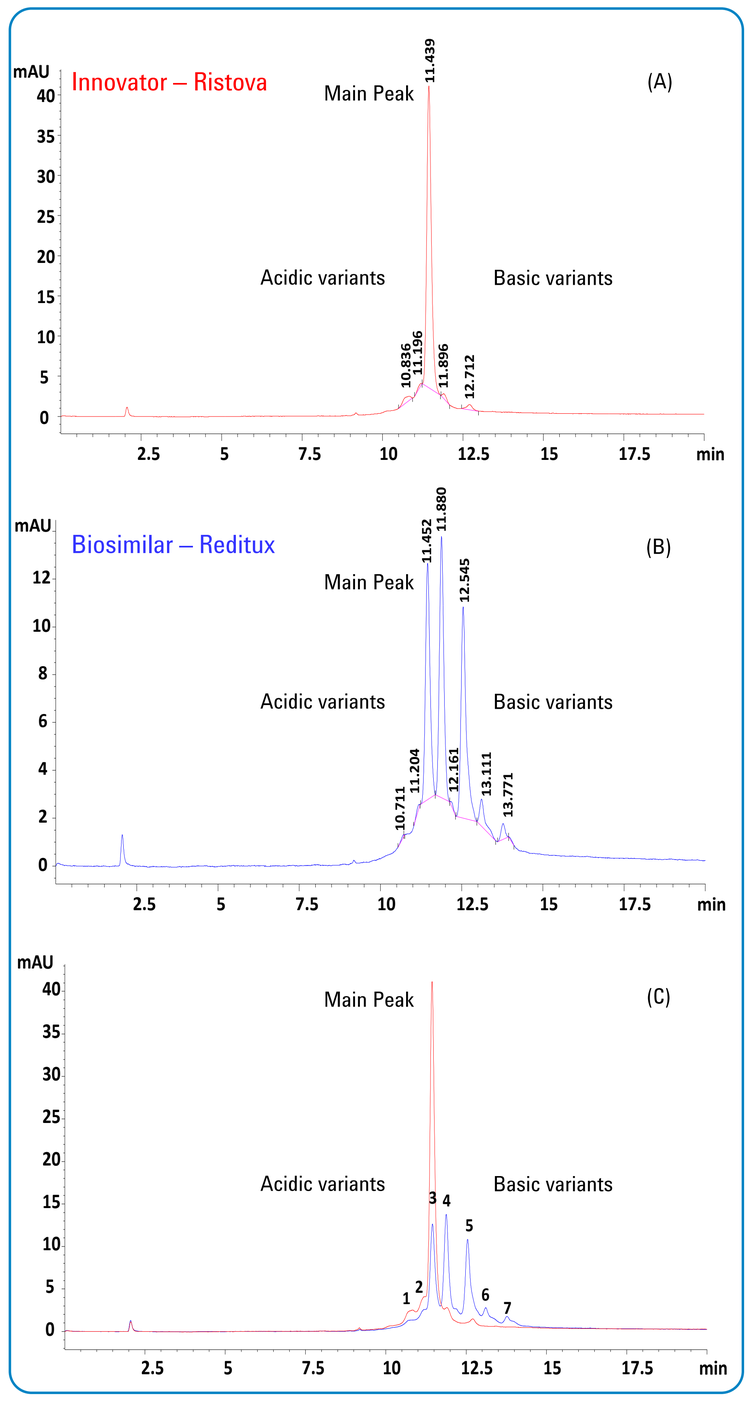
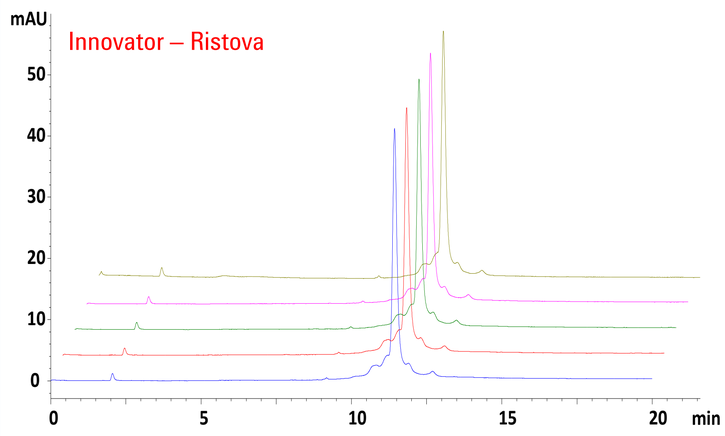


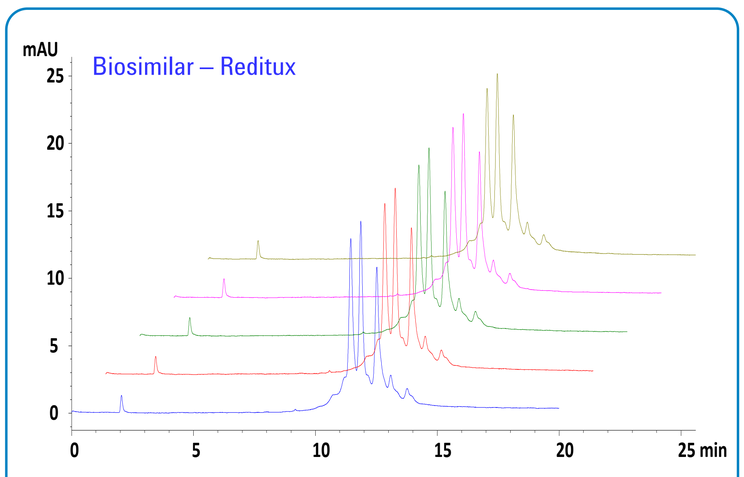
Figure 2: Charge variant profiles of innovator (A) and biosimilar (B) rituximab using an Agilent Bio MAb 5 µm column. (C) Overlay of innovator and biosimilar rituximab. Peaks 1 and 2 : acidic variants; 3: Main form; 4,5,6 and 7 : basic variants



Retention time		Peak area	
Mean (min)	RSD	Mean (mAU/min)	RSD
11.44	0.09	367.94	1.31

Figure 3: Overlay of five replicates of innovator – rituximab on an Agilent 1260 Infinity Bio-inert Quaternary LC using an Agilent Bio Mab, 4.6 × 250 mm, 5 µm, PEEK column. Table shows the precision of retention time and area for main peak, n = 5.

Results and Discussion



Retention time		Peak area	
Mean (min)	RSD	Mean (mAU/min)	RSD
11.45	0.05	95.07	0.42

Figure 4: Overlay of five replicates of biosimilar rituximab on an Agilent 1260 Infinity Bio-inert Quaternary LC using an Agilent Bio Mab, 4.6 × 250 mm, 5 µm, PEEK column. Table shows the precision of retention time and area for main peak, 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

Table: Charge variants quantification by area %, n = 5

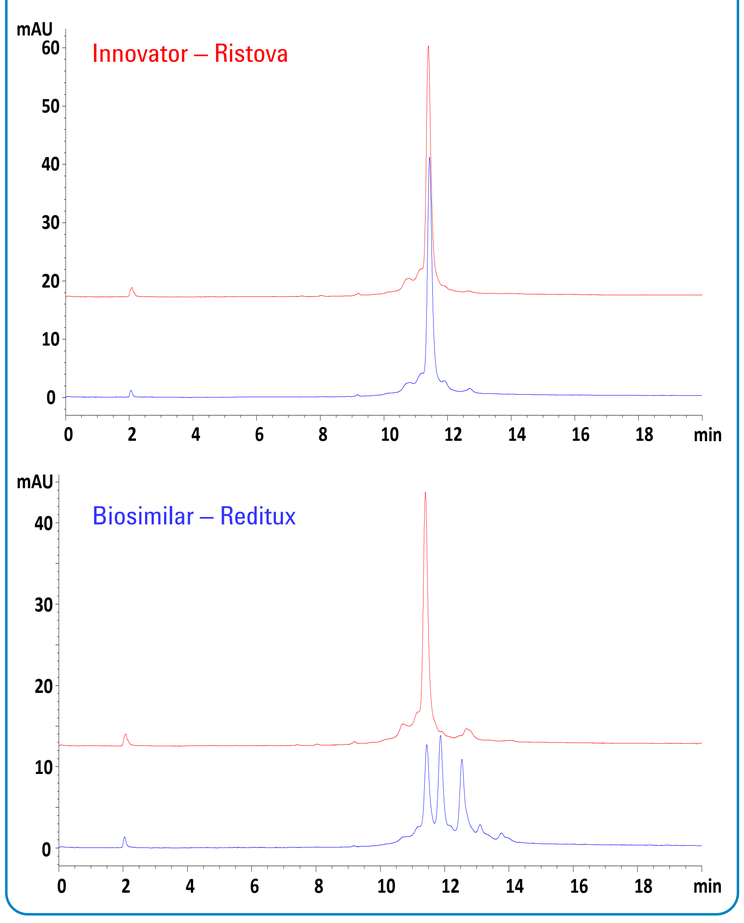


Figure 5: Characterization of basic charge variants. Separation of CPB treated (overnight) and untreated of innovator (A) and biosimilar (B) rituximab using an Agilent Bio Mab, 4.6 × 250 mm, 5 µm, PEEK column

Conclusions

- The salt-gradient method 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
- 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