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Application News

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Charge Variant analysis of mAb Biosimilars by Nexera ™ UHPLC with a Shim-pack ™ Bio IEX Column

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❑ Introduction

Monoclonal antibodies (mAbs) are well-established biotherapeutics for a wide range of diagnostic and clinical applications. The newly emerging biosimilars are increasing in popularity in biopharmaceuticals because of the cost savings. However, biosimilars can undergo various post-translational modifications in manufacturing processes, including sialylation, deamidation, C-terminal lysine cleavage, glycosylation, succinimide formation, oxidation and so forth. These modifications can lead to variations of charge distribution that potentially affect the biological activity and stability of the products. Therefore, characterization and monitoring of charge variants of mAb biosimilars are critical quality requirements to ensure the stability and process consistency in drug development.

Conventionally, ion exchange chromatography (IEX) with salt-gradient is the gold standard method for charge-sensitive antibody analysis. A key challenge of salt-gradient is that the method parameters often need to be optimized for each individual protein. In 2009, Genentech first reported the use of IEX with pH-gradient for mAb charge variant separations [1]. It was found that mAbs with isoelectric points in the range of pH 7-9 could be well separated. Moreover, the approach was more generic and could easily be used to separate different charge variants in a wide range of antibodies.

In this study, we describe both salt-gradient and pH-gradient methods for separating the charge variants of bevacizumab biosimilar using Shimadzu Nexera UHPLC and a Shim-pack Bio IEX column (4.6 mm x 100 mm, 3 μm).

❑ Experimental

A bevacizumab biosimilar of 5 mg/mL was prepared in 50 mmol/L Tris-HCl (pH 8.0) buffer. The sample was directly injected and analyzed by a Nexera UHPLC with UV detector. The elution was monitored at 280 nm. Relative peak area (%) was used to quantify charge variants of bevacizumab biosimilar. Tables 1 and 2 show the analytical conditions for salt-gradient and pH-gradient IEX, respectively.

Table 1: LC analytical conditions of salt-gradient IEX

LC system	: Nexera-i UHPLC			
Column	: Shim-pack Bio IEX SP-NP (4.6 mm x 100 mm; 3 μm)			
Temperature	: Ambient			
Mobile phase A	: Water			
Mobile phase B	: 1 mol/L NaCl			
Mobile phase C	: 40 mmol/L NaH$_2$PO$_4$			
Mobile phase D	: 50 mmol/L Na$_2$HPO$_4$			
Gradient	: Time (min)	A (%)	B(%)	C (%)	D (%)
	0	20	0	75	5
	1	15	5	68	12
	5	14	10	58	18
	5.1	20	0	75	5
	7	20	0	75	5
Flow rate	: 0.6 mL/min			
Injection volume	: 5 μL			
Detector	: UV, 280 nm			

Table 2: LC analytical conditions of pH-gradient IEX

LC system	: Nexera Bio UHPLC		
Column	: Shim-pack Bio IEX SP-NP (4.6 mm x 100 mm; 3 μm)		
Temperature	: Ambient		
Mobile phase A	: 10 mmol/L sodium phosphate buffer, pH 6.0, by mixing 10 mmol/L NaH$_2$PO$_4$ and 10 mmol/L Na$_2$HPO$_4$ stock solutions to the desired pH		
Mobile phase B	: 10 mmol/L sodium bicarbonate buffer, pH 10.0, by mixing 10 mmol/L NaHCO$_3$ and 10 mmol/L Na$_2$CO$_3$ stock solutions to the desired pH		
Gradient	: Time (min)	A (%)	B(%)
	0	65	35
	2	45	55
	12	0	100
	16	0	100
	16.1	65	35
	20	65	35
Flow rate	: 0.6 mL/min		
Injection volume	: 5 μL		
Detector	: UV, 280 nm		

❑ Results and Discussion

In this study, both salt-gradient and pH-gradient IEX methods were carried out for optimal bevacizumab biosimilar charge variant analysis. Figure 1 shows the charge variant profile of bevacizumab biosimilar on a Shim-pack Bio IEX column with a salt-gradient elution program, demonstrating high-resolution separation of charge variants in 5 minutes. The peak at 3.448 min was designed as the main peak. The early and late-eluting peaks were called acidic variants and basic variants, respectively. The relative peak area percent of charge variants of bevacizumab biosimilar based on six consecutive analyses is summarized in Table 3. The main peak was found to be around 50.99%. The acidic variants and basic variants were 34.94% and 14.07%, respectively. Also, injection-to-injection variability of the UHPLC-UV system was evaluated based on the six consecutive analyses. As shown in Figure 3, the overlay of six replicates indicates an excellent separation reproducibility. Variations in retention time of six injections were <1% RSD for all the peaks (data not shown). Variations in peak area of six injections were <2% RSD for the main peak as well as acidic and basic variants (Table 3).

The charge variant profile of bevacizumab biosimilar on a Shim-pack Bio IEX column with a pH-gradient elution program is shown in the Figure 3, indicating an excellent separation of charge variants in 12 minutes. The peak at 6.121 min was designed as the main peak. Early and late-eluting peaks were defined as acidic variants and basic variants, respectively. Table 4 shows the peak area percent of charge variants of bevacizumab biosimilar based on six consecutive analyses. The main peak was found to be 67.30%. Acidic variants and basic variants were 23.25% and 9.45%, respectively. Moreover, injection-to-injection variability of UHPLC-UV system was evaluated in Figure 4. The variations in retention time of six injections were <1% RSD for all the peaks (data not shown). Variations in peak area of six injections were <2% RSD for the main peak as well as acidic and basic variants (Table 4).
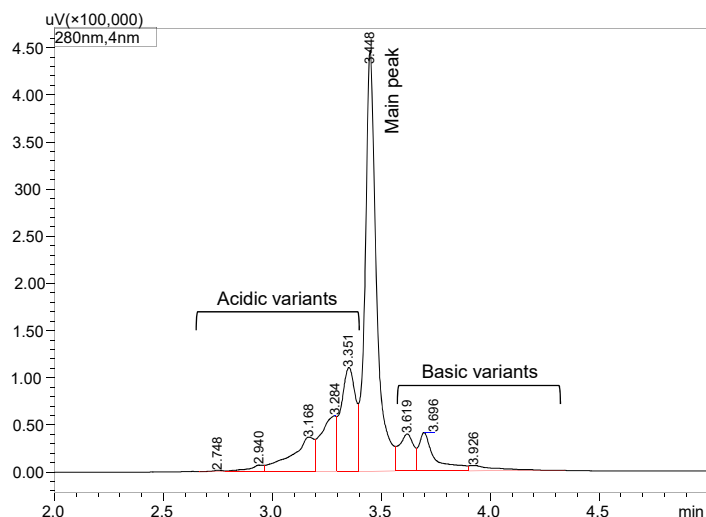

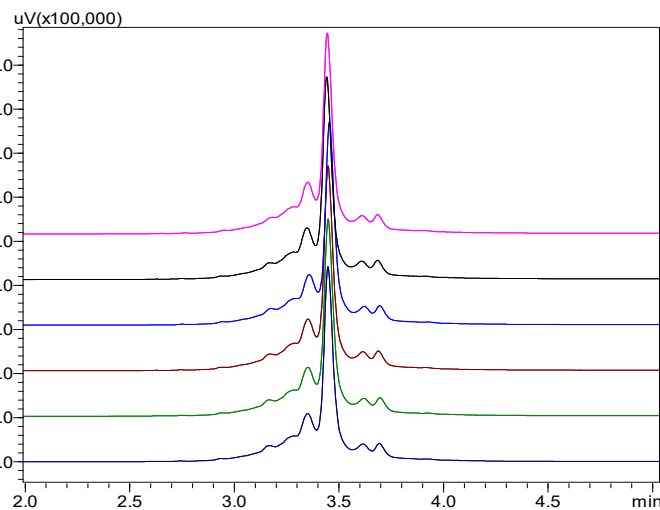**Figure 1: The charge variant profile of bevacizumab biosimilar using salt-gradient**

Figure 2 UHPLC-UV (280 nm) chromatograms of six injections of bevacizumab biosimilar using salt-gradient

Table 3: Injection-to-injection repeatability of peak area (*n* = 6) of charge variants of bevacizumab biosimilar using salt-gradient

	Acidic variants [a]		Main peak		Basic variants [b]		Total
	Area	Area (%)	Area	Area (%)	Area	Area (%)	
Injection _1	1,092,232	34.77	1,606,848	51.15	442,237	14.08	
Injection _2	1,108,765	34.95	1,619,310	51.05	444,149	14.00	
Injection _3	1,149,156	35.00	1,673,033	50.96	460,888	14.04	
Injection _4	1,136,214	34.91	1,662,777	51.09	455,419	13.99	
Injection _5	1,139,549	35.05	1,651,223	50.79	460,176	14.16	
Injection _6	1,125,892	34.92	1,642,164	50.93	456,312	14.15	
Average		34.94		50.99		14.07	100%
%RSD	1.89		1.55		1.78		

[a] Acidic variants is a sum area of peaks at 2.748, 2.940, 3.168, 3.284, 3.351 minutes
[b] Basic variants is a sum area of peaks at 3.619. 3.696, 3.926 minutes

Table 4: Injection-to-injection repeatability of peak area (*n* = 6) of charge variants of bevacizumab biosimilar using pH-gradient

	Acidic variants [a]		Main peak		Basic variants [b]		Total
	Area	Area (%)	Area	Area (%)	Area	Area (%)	
Injection _1	517,010	23.26	1,495,411	67.29	210,035	9.45	
Injection _2	524,489	23.71	1,480,774	66.94	206,779	9.35	
Injection _3	519,527	23.42	1,490,580	67.21	207,808	9.37	
Injection _4	506,853	22.91	1,498,276	67.73	207,115	9.36	
Injection _5	524,504	23.55	1,486,008	66.73	216,506	9.72	
Injection _6	498,975	22.65	1,495,740	67.88	208,671	9.47	
Average		23.25		67.30		9.45	100%
%RSD	1.99		0.45		1.73		

[a] Acidic variants is a sum area of peaks at 4.397, 4.645, 5.214, 5.769 minutes
[b] Basic variants is a sum area of peaks at 6.586, 6.939, 7.550, 8.636 minutes
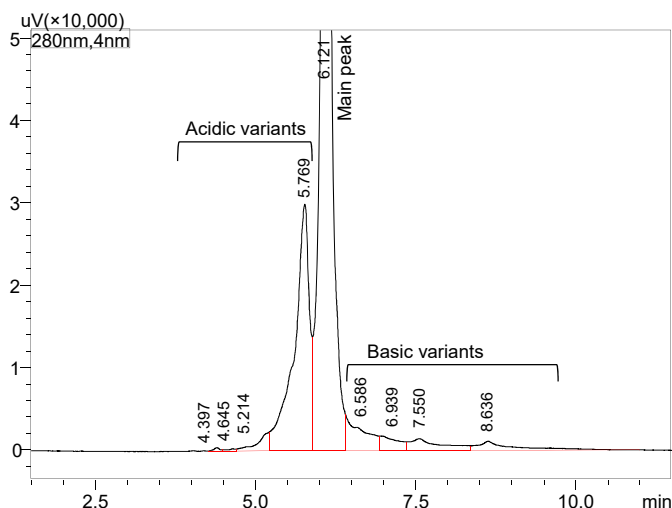
Figure 3 The charge variant profile of bevacizumab biosimilar using pH-gradient
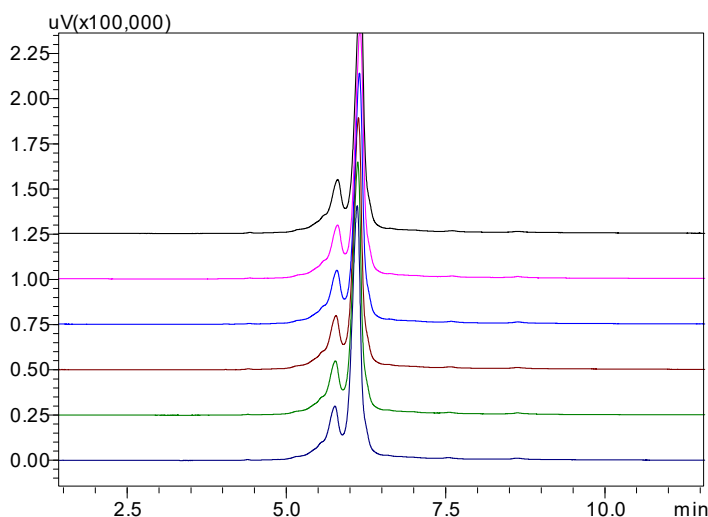
Figure 4 UHPLC-UV (280 nm) chromatograms of six injections of bevacizumab biosimilar using pH-gradient

By comparing the two gradient methods, it was found that the results of relative peak area percent of charge variants of bevacizumab biosimilar are slightly different mainly because the peaks are not fully separated, yet both results showed that the main peak is the major one with >50% of the total peak area, and followed by acidic and basic variants. In addition, the variations might be caused by the different elution programs used here: in the salt-gradient elution, the Na^+ outcompetes the mAb on ion-exchange sites and the mAb begins to elute with increasing salt; in the pH-gradient elution, when the net charge of the mAb becomes neutral at its isoelectric point the mAb begins to elute with increasing pH.

❑ **Conclusions**

Although giving different results on the relative peak area percent for charge variants of bevacizumab biosimilar, both salt-gradient and pH-gradient IEX methods described in current study demonstrate high-resolution separation of charge variants of mAb. More importantly, both methods were validated for stability and repeatability in retention time and peak area with RSDs <2%, demonstrating the precision of the system. In conclusion, both salt-gradient and pH-gradient work equally.

❑ **Reference**

1. Farnan D, Moreno GT, Multiproduct high-resolution monoclonal antibody charge variant separations by pH gradient ion-exchange chromatography, *Anal. Chem.* 2009, 81(21):8846-57.

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