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Application News

No. AD-0227

Biopharma / Nexera Bio UHPLC / Shim-pack Bio IEX

Exploring Factors That Influence Charge Variant Analysis of mAbs with Shim-pack Bio IEX Columns using Salt Gradient Elution

❑ Introduction

Protein-based therapeutic drugs including monoclonal antibodies (mAbs) have shown to be effective against a variety of diseases. It is critical to characterize and monitor the impurities and variations of the products to ensure the efficacy and safety. Post-translational modifications such as deamidation, glycosylation, oxidation, and C-terminal lysine cleavage can lead to charge variations. These charge variants are usually characterized and quantified using ion-exchange chromatography (IEX).[1]

IEX separates the biomolecules according to their net charge, which is dependent on pH. Cation-exchange chromatography (CEX) with cation-exchange ligands is commonly used to characterize mAbs with basic isoelectric point (pI >7). The acidic variants are eluted before the main peak while the basic variants eluted after. The elution can be performed using salt or pH gradient.

In this study, we describe a charge variant analysis of trastuzumab using salt gradient with Shimadzu strong cation-exchange column, Shim-pack Bio IEX SP-NP. The separation efficiency under different gradient slopes, gradient times and flow rates are discussed.

❑ Experimental

Trastuzumab sample was diluted to 5 mg/mL with water. The sample was directly injected and analyzed by a Shimadzu Nexera Bio UHPLC with a UV detector. The elution was monitored at 280 nm. Relative peak area (%) was used to quantify charge variants of trastuzumab. Sodium phosphate buffer was prepared by mixing 20 mmol/L NaH_2PO_4 and 20 mmol/L Na_2HPO_4 stock solutions to obtain the desired pH. Analytical conditions are shown in Table 1.

Table 1: LC analytical conditions of salt gradient IEX.

LC system	: Nexera Bio UHPLC
Column	: Shim-pack Bio IEX SP-NP (1) 100 mm x 4.6 mm I.D., 5 µm (P/N: 227-31006-03) (2) 100 mm x 4.6 mm I.D., 3 µm (P/N: 227-31005-03) (3) 50 mm x 4.6 mm I.D., 5 µm (P/N: 227-31006-02)
Column temp.	: 25 ℃
Mobile phase	: A: 20 mmol/L (sodium) phosphate buffer, pH 6 B: 20 mmol/L (sodium) phosphate buffer, pH 6 + 250 mmol/L NaCl
Flow rate	: 0.6 mL/min
Elution mode	: Gradient flow
Injection vol.	: 2 µL
Detector	: UV, 280 nm

Table 2: Other consumables

Items
1.5ml Screw-thread amber vial with write on spot, caps with PTFE/white silicone septa (P/N: 226-54111-41)
1L Solvent bottle (P/N: 226-88583-02)
Solvent safety caps kit (P/N: 226-50319-01)
LC solvent waste kit (P/N: 226-50330-00)
PEEK fitting (P/N: 226-50106-02)

Please contact your local Shimadzu representative for more information on these consumables

❑ Results & Discussions

Salt gradient slope

Charge variants of mAbs can be characterized using cation-exchange chromatography (CEX) with a salt gradient elution method. Optimization is often needed for salt gradient slope and gradient time. Analysis started with a steep gradient slope from 0 to 250 mmol/L in 5 min at 0.6 mL/min to estimate the separation profile. As shown in Figure 1, the steep gradient slope resulted in fast elution and impeded the resolution of charge variants from the main peak. Gradient slope became shallower when the starting and ending salt concentration was narrowed down, and the separation of the charge variants was improved. Trastuzumab charge variants were retained longer in column and well separated with a shallower gradient slope.

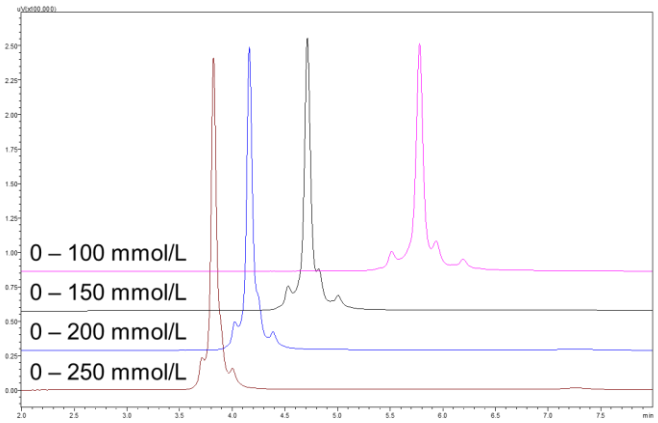

Figure 1: Trastuzumab charge variant separation with different gradient slope (20 mmol/L (sodium) phosphate, pH 6, 5 min gradient time).

Gradient time

The execution time for the salt gradient elution may affect the resolution of mAb charge variants. The impact of gradient time on separation of mAb charge variants was studied (Figure 2). Starting and ending salt concentrations were kept constant while the gradient time was increased from 5 min to 20 min. Results showed that trastuzumab charge variants were better resolved with longer gradient time (Figure 2). Identification of low abundance variants was easier as separation improved with longer gradient time.

Nevertheless, there should be a limit to decrease gradient slope to improve separation. Peak broadening was observed as gradient time was increased (Figure 2). Thus, gradient slope in relation to gradient time should be optimized in order to achieve good resolution without sacrificing sensitivity.

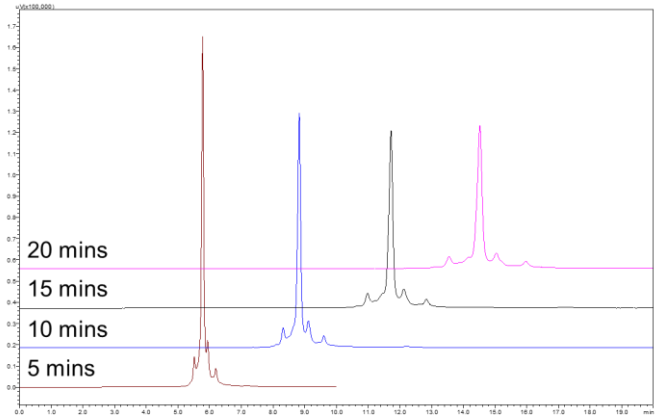

Figure 2: Trastuzumab charge variant separation with different gradient time (20 mmol/L (sodium) phosphate, pH 6, 0 – 100 mmol/L NaCl).

Flow rate

Other than salt concentration and gradient time, gradient slope can also be modified by flow rate. Trastuzumab charge variant analysis was performed with different flow rates. Gradient time was varied to keep the gradient slope constant (Table 3). Results showed that altering flow rate did not significantly change resolution or area percentage of trastuzumab charge variants (Table 4, Figure 3).

Shim-pack Bio IEX SP-NP column is packed with non-porous particles. Further, the particle surface is functionalized with strong cation exchange sulfopropyl group. Protein interactions occur on particle surface without diffusion inside of the particles. Therefore, the interactions are dependent on charge density of protein and interaction area between particle surface and protein, and marginally affected by flow rate. In such cases, the flow rate can be appropriately increased to shorten the analysis with minimal effect on charge variants.

Table 3: Gradient time for different flow rates (constant gradient volume, 3 mL).

Flow rate (mL/min)	0.4	0.6	0.8
Time (min)	7.50	5.00	3.75

Table 4: Resolution and area percentage of trastuzumab charge variants.

Flow rate (mL/min)	Resolution			Area (%)		
	A – M	M – B1	B1 – B2	A	M	B1 + B2
0.4	1.78	0.89	1.23	6.01	77.78	16.21
0.6	1.81	0.92	1.32	6.48	77.47	16.05
0.8	1.72	0.84	1.23	6.81	77.02	16.17

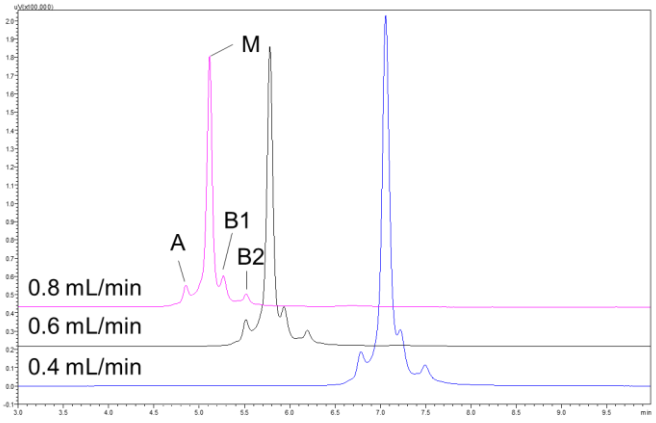

Figure 3: Trastuzumab charge variant separation with different flow rates (20 mmol/L (sodium) phosphate, pH 6, 0 – 100 mmol/L NaCl, constant gradient slope).

Particle size

Particle size is one of the criteria in column selection. Two 100 mm x 4.6 mm I.D. columns with 3 µm and 5 µm particles were used in the analysis. The effect of particle size in trastuzumab charge variant analysis is shown in Figure 4. The improvement brought by particle size was not as significant as in the analysis of small molecules. Thus, minimal improvement in resolution was observed in 3 µm column (Table 5). The smaller particle size resulted in higher backpressure that limited the analysis at high flow rates. Therefore, care must be taken to ensure the analysis is within pressure limit.

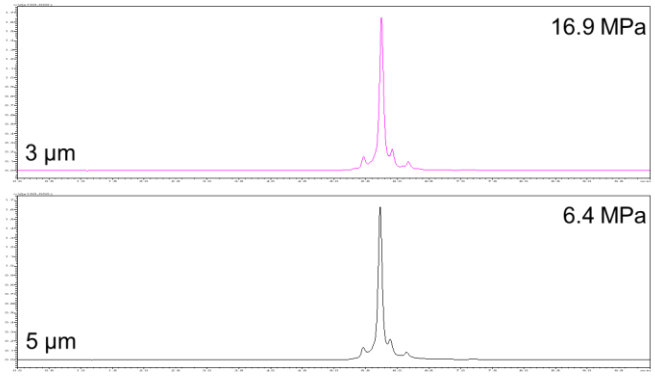

Figure 4: Trastuzumab charge variant separation with different particle size (20 mmol/L (sodium) phosphate, pH 6, 0 – 100 mmol/L NaCl, 5 min gradient time, 0.6 mL/min).

Table 5: Resolution of trastuzumab charge variants with different particle size columns.

Resolution (USP)	3 µm	5 µm
Acidic	--	--
Main peak	2.135	1.813
Basic 1	1.166	0.915
Basic 2	1.688	1.318

Column length

Column length affects peak resolution and retention time. Two 5 µm particle size columns of 50 mm and 100 mm length were used. The effect of column length in Trastuzumab charge variant analysis is shown in Figure 5. Trastuzumab was eluted faster from the shorter column (Figure 5, Table 6). However, the resolution between trastuzumab and its variants was decreased in the shorter column (Table 6). As seen from the result, column length affected peak resolution. Therefore, caution should be taken when transferring method to shorter column.

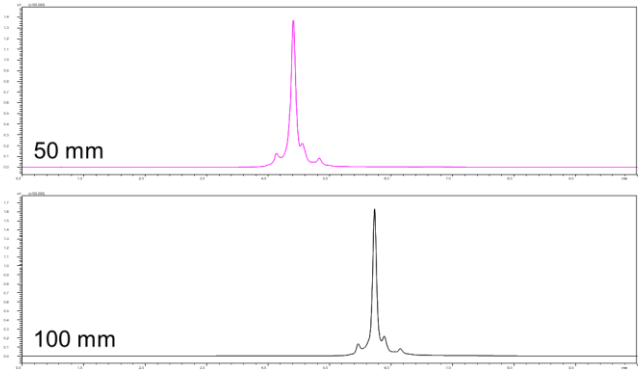

Figure 5: Trastuzumab charge variant separation with different column length (20 mmol/L (sodium) phosphate, pH 6, 0 – 100 mmol/L NaCl, 5 min gradient time, 0.6 mL/min).

Table 6: Retention time and resolution of trastuzumab and charge variants.

Column length (mm)	Retention time (min)	Resolution		
		A – M	M – B1	B1 – B2
50	4.41	1.50	0.62	1.12
100	5.73	1.81	0.92	1.32

❑ Conclusion

Shimadzu Nexera Bio UHPLC system built with a non-metallic flow path provides a platform for charge variant analysis of mAbs. Method development and optimization have been performed on Shim-pack Bio IEX SP-NP cation exchange columns. The results showed that trastuzumab was better separated with shallower salt gradient, longer gradient time, and longer column length while column flow rate and particle size had minimal impact on the separation profile. In short, salt gradient slope, gradient time, flow rates, and column length are among the factors to be optimized for high-resolution separation of charge variants by salt-gradient method.

❑ References

1. Vlasak J., Ionescu R., *Curr. Pharm. Biotechnol.*, **2008**, 9 (6), pp 468-481.

Disclaimer: The data and instruments presented in this Application News are intended for Research Use Only (RUO). Not for use in diagnostic procedures.

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