thermo scientific

APPLICATION NOTE 21844

Salt gradient analysis of IgG1 monoclonal antibodies using a 5 µm WCX chromatography column

Authors

Shane Bechler, Julia Baek, Shanhua Lin
Thermo Fisher Scientific, Sunnyvale, CA

Keywords

Biopharmaceutical, monoclonal antibody, mAb, IgG1, biosimilar, variants, weak cation exchange, WCX, rituximab, trastuzumab, bevacizumab, infliximab, vedolizumab, secukinimab, pertuzumab, ProPac Elite WCX 5 µm column

Application benefits

- High-resolution separation of mAb charge variants
- Easy, straightforward method development
- Fast analysis times with optimized gradients

Goal

To detail basic method development and demonstrate high-resolution analysis of IgG1 therapeutic mAbs using a salt gradient with the Thermo Scientific™ ProPac™ Elite WCX 5 µm columns

Introduction

Protein therapeutics continue to grow as a major class of treatment for various diseases including cancer, cardiovascular disease, and autoimmune disorders. Monoclonal antibodies (mAbs) are of particular interest due to their ability to target specific tissues for the modulation of cellular activities or delivery of drug payloads (e.g., ADCs). Currently, IgG1 is the most common subclass of mAbs being developed and on the market. The cellular production processes used to manufacture mAbs may result in post-translation modifications (PTMs) including lysine truncation, glycosylation, etc. Additional modifications such as oxidation and isomerization can happen during downstream processing and storage. Together, these modifications

ThermoFisher SCIENTIFIC

result in product heterogeneity that can adversely affect the efficacy and safety of the drug. As such, manufacturers are required by regulatory agencies and internal protocols to verify the fidelity of their products by characterizing the nature and quantity of structural variants.

Charge variants, such as lysine truncation and sialylated glycans alter the overall charge of the mAb. Ion exchange chromatography is a technique commonly employed to separate species based on their accessible surface charge, making it a practical technique for the separation and detection of charge variants using either a pH gradient[1] or salt gradient. For most mAbs, cation exchange chromatography is the standard separation approach since the isoelectric point (pI; pH at which the protein charge is neutral) of most mAbs is typically between 6 and 10. When the buffer pH is less than the protein pI, the mAb will be cationic and readily bind to the anionic stationary phase of cation exchange columns. A gradient from low to high salt concentration is then used to disrupt the ionic interactions of the protein with the solid phase, resulting in protein desorption and elution from the column. To effectively separate charge variants for analysis using this approach, the solid phase must be designed to promote efficient adsorption and desorption of proteins to the stationary phase with minimal secondary interactions.

Here, we report the use of a weak cation exchange (WCX) column for the evaluation of pharmaceutically relevant mAbs and their associated charge variants. The ProPac Elite WCX 5 µm column was selected based on its ability to achieve high-efficiency protein separations. The non-porous 5 µm particle promotes fast convective mass transfer without peak broadening due to sample entrapment in pores. The resin is coated with a hydrophilic layer to minimize secondary interactions of proteins with the solid phase. Anionic acrylate groups grafted to the hydrophilic layer provide the weak cation exchange functionality. We present a practical approach to method development for a specific mAb including the rationale for choosing specific conditions. This development was applied to a wider range of mAbs with the final results presented for a 4 × 150 mm column format.

Experimental

Reagents and consumables

- Deionized water, 18.2 MΩ•cm resistivity
- Polypropylene Vials (P/N C4000-11)
- Vial Screw Thread Caps (P/N C5000-54B)

Sample preparation

All mAb samples were diluted to 5 mg/mL using DI water.

Separation conditions

Instrumentation

- Thermo Scientific™ Vanquish™ Flex Quaternary UHPLC system, including:
  - System Base Vanquish Flex (P/N VF-S01-A)
  - Quaternary Pump (P/N VF-P20-A)
  - Column Compartment H (P/N VH-C10-A)
  - Split Sampler FT (P/N VF-A10-A) with 25 µL (V = 50 µL) sample loop
  - Diode Array Detector HL (P/N VH-D10-A) with Thermo Scientific™ LightPipe™ 10 mm Standard Flow Cell (P/N 6083.0100)
  - VWD-3400RS Rapid Separation Variable Wavelength Detector

Column

- ProPac Elite WCX, 5 µm 4 × 150 mm (P/N 302972)

For mobile phase compositions and gradient conditions including flow rate, column temperature, and injection volume, reference the text and figures in the *Results and discussion* section. Absorbance at 280 nm was used for detection of all samples.

Data processing

The Thermo Scientific™ Chromeleon™ 7.2.7 Chromatography Data System was used for data acquisition and analysis.

Results and discussion

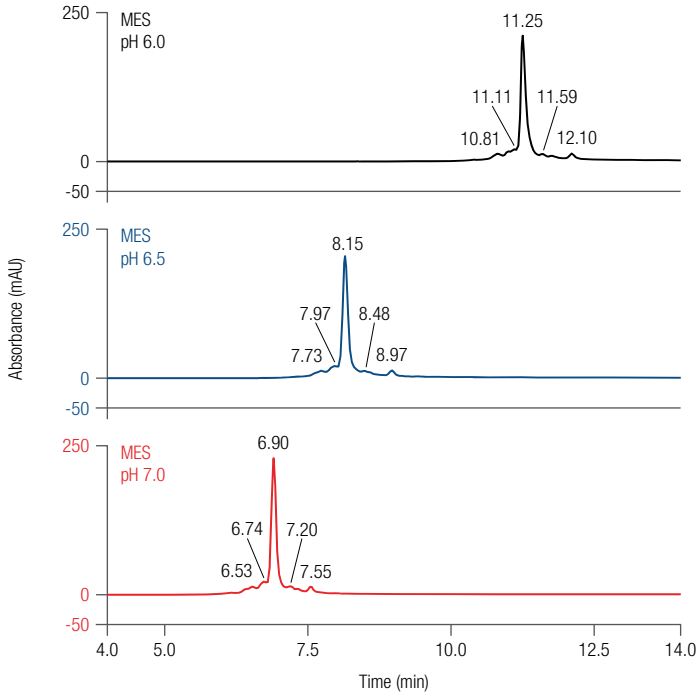
To effectively quantify a mAb and its associated variants, it is important to optimize the method used to analyze the sample. Here, we demonstrate a straightforward approach to the optimization of a salt gradient for the analysis of a mAb. Attention is provided to the mobile phase pH and salt concentration for consistent inter-run separations. The approach for method development is applied to a broad set of innovator mAbs and biosimilars.

Buffer pH effect on chromatography

Buffer pH has a significant effect on the separation of the proteins and associated variants. For cation exchange chromatography, the mobile phase pH should be lower than the isoelectric point (pI) of the protein and variants of interest to promote binding to the solid phase. If the mobile phase pH is higher than the pI, the protein or variants may not be retained nor separated. This effect is particularly pronounced for acidic variants of the mAb. Figure 1 shows the separation of rituximab using MES buffer at pH 6.0 and 6.5 and MOPS buffer at pH 7.0 using a salt gradient from 25 to 175 mM NaCl over 15 minutes. Differences in variant separation are noted with increasing mobile phase pH. The mAb also elutes at lower salt concentrations at higher pH values. This effect is expected as the net charge of the mAb decreases with increasing mobile phase pH, and thus binds more weakly to the anionic solid phase. For rituximab, the best separation of variants was observed using MES pH 6.5 based on the relative separation of both acidic and basic variants from the main peak. This mobile phase composition was chosen for further optimization of the salt gradient.

Determining gradient salt concentrations

For run-to-run consistency of mAb separation, gradient methods should be designed so the mAb is separated solely by the change in salt concentration, without isocratic elution occurring at initial conditions. Comparing the elution of a mAb using a gradient with and without an isocratic hold (the change in time between the injection and start of the gradient) can be used to determine if isocratic elution is occurring during loading. If isocratic elution occurs, the retention time difference for the mAb peaks between no hold and isocratic hold chromatograms will be less than 5 minutes. For the chromatogram with the isocratic hold, the separation of variants from the mAb will increase and broader peaks will be observed compared to the no isocratic hold conditions. Figure 2 demonstrates the isocratic effect by



Column:	ProPac Elite WCX 5 µm	Flow rate:	1.0 mL/min
Format:	4 × 150 mm	Inj. volume:	3 µL
Eluents:	See chromatogram for specific buffer and pH A: 20 mM Buffer B: 20 mM Buffer + 0.5 M NaCl	Temperature:	30 °C
		Detection:	UV, 280 nm
		Sample:	Rituximab, 5 mg/mL
		Peak label:	Retention time

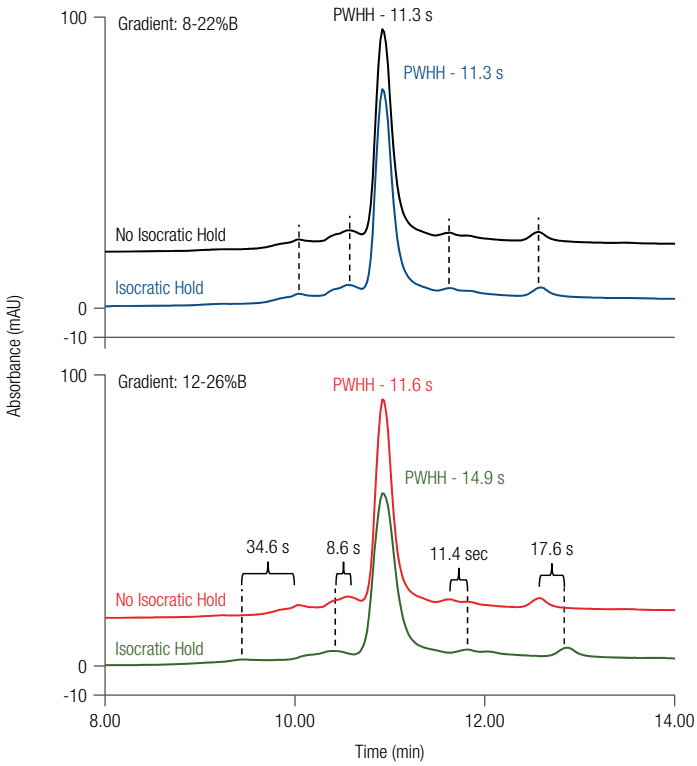
Gradient:

Time (min)	%A	%B
0.0	95	5
15.0	65	35
15.1	50	50
16.0	50	50
16.1	95	5
25.0	95	5

Figure 1. Zoomed in time range showing the effect of buffer pH on rituximab variant separation: (top) 20 mM MES pH 6.0, (middle) 20 mM MES pH 6.5, and (bottom) 20 mM MES pH 7.0

comparing the elution of rituximab when using a loading salt concentration of 40 mM NaCl (top) and 60 mM NaCl (bottom). For each loading concentration, mAb elution with no isocratic hold and a 5-minute isocratic hold are compared. The retention times of the mAb peak with and without the isocratic hold has been normalized to compare the relative separation of variants. At 40 mM NaCl loading, the separations of the variants relative to the main peak are consistent with and without the isocratic hold (dashed lines) and the main peak PWHH is unchanged. At 60 mM NaCl loading, the separation of the variants from the main peak increases with the isocratic hold (brackets – increase in separation) and the PWHH of the main peak increases. Based on these results, isocratic elution is observed at 60 mM NaCl loading but not 40 mM NaCl. A range of starting salt concentrations were evaluated using this approach. Since 40 mM NaCl was determined to be the maximum usable salt concentration without isocratic elution effects,

it was selected for further optimization of the salt gradient method.

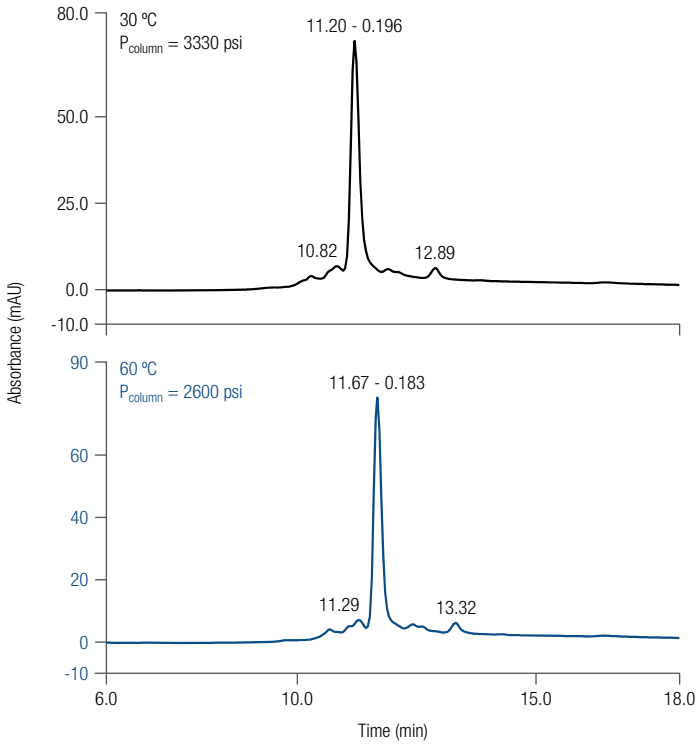


Column:	ProPac Elite WCX 5 µm	No Isocratic hold:	Top 8-22%B (Bottom 12-26%B)
Format:	4 × 150 mm		Time (min) %A %B
Eluents:	A: 20 mM MES pH 6.5		0.0 92 (88) 8 (12)
	B: 20 mM MES pH 6.5		15.0 78 (74) 22 (26)
	+ 0.5 M NaCl		15.1 60 40
Flow rate:	1.0 mL/min		16.0 60 40
Inj. volume:	3 µL		16.1 92 (88) 8 (12)
Temperature:	30 °C		25.0 92 (88) 8 (12)
Detection:	UV, 280 nm		
Sample:	Rituximab, 5 mg/mL	Isocratic hold:	Top 8-22%B (Bottom 12-26%B)
			Time (min) %A %B
			0.0 92 (88) 8 (12)
			5.0 92 (88) 8 (12)
			20.0 67 (74) 33 (26)
			20.1 60 40
			21.0 60 40
			21.1 92 (88) 8 (12)
			30.0 92 (88) 8 (12)

Figure 2. Effect of starting salt concentration on rituximab variant separation with and without a 5-minute isocratic hold: (top) 8–22 %B and (bottom) 12–26 %B. Main peak retention times are normalized to compare relative separation of variants. Dashed lines and brackets assist in comparing the separation of the same variants; see text for details.

Temperature effect on chromatography

The column temperature used during separation can alter the conditions under which the mAb is retained. Higher temperatures generally result in elution of proteins later in the gradient, relative to lower temperature separations. This contrasts with reversed-phase methods, which typically display weakened interactions with the solid phase under higher temperature conditions, thus earlier elution times for the same gradient. This effect is largely due to reduced waters of hydration for ionic groups on both the solid phase and the protein. As such, the ionic interactions between the protein and solid phase are stronger, and higher concentrations of salt are required to disrupt the interactions to elute the protein. Generally, changing the temperature does not significantly alter the selectivity of the separation for mAb variants; the temperature can be modified without changing the relative separation of sample peaks. A benefit of increasing the temperature is reduced column pressure due in part to reduced viscosity of the mobile phase. Figure 3 illustrates the effects of temperature on the analysis of rituximab showing elution of the mAb at higher salt concentrations, with increased temperature and reduced column pressure.



Column:	ProPac Elite WCX 5 µm	Flow rate:	1.0 mL/min
Format:	4 × 150 mm	Inj. volume:	3 µL
Eluents:	A: 20 mM MES pH 6.5	Temperature:	Top – 30 °C
	B: 20 mM MES pH 6.5		Bottom – 60 °C
	+ 0.5 M NaCl	Detection:	UV, 280 nm
Gradient:	Time (min) %A %B	Sample:	Rituximab, 5 mg/mL
	0.0 92 8	Peak label:	mAb - Retention time (min) – PWHH (min)
	15.0 78 22		Variants - Retention time (min)
	15.1 60 50		
	16.0 60 50		
	16.1 92 8		
	25.0 92 8		

Figure 3. Effect of temperature on rituximab variant separation and column pressure

Reducing method time

Generally, the separation of variants from the main mAb peak improves as the gradient slope decreases. Typically, the salt concentration for elution of a mAb and associated variants is appreciably lower than the loading concentration at which no isocratic elution occurs. As such, the use of gradients with low slopes (e.g., < 5 mM NaCl/min) designed for optimal variant separation can result in long method times when using loading conditions that avoid isocratic elution during loading. To minimize this excessive time, the mAb can be loaded at a low NaCl concentration followed by a step increase to a NaCl concentration suitable for mAb separation with a shallow gradient. Chromatograms in the following sections use this approach to limit the analysis time while maintaining a consistent mAb separation from run-to-run.

Method application to mAbs

The methodology discussed above has been applied to a range of mAbs. Each mAb was optimized for salt gradient separation using a non-isocratic eluting salt concentration to load the protein followed by a step change and linear gradient to elute the sample. For simplicity, the temperature and mobile phase buffer were set to 30 °C and 20 mM MES pH 6.5. However, we note that for some mAbs separation of variants may be improved by further optimizing the buffer pH or column temperature.

The following 4 × 150 mm separations in Figures 4 and 5 use the method detailed in Table 1. For each mAb, the %B for loading and initial and final gradient %B values (%A = 100 - %B) are designated in Table 2. Where biosimilar mAbs were available, the same gradient method was used to directly compare the innovator mAb to the biosimilar. After the gradient separation, the salt concentration was stepped to 250 mM to remove any residual analytes electrostatically bound to the column.

Table 1. General mAb gradient for 4 × 150 mm ProPac Elite WCX 5 µm column

Mobile Phase A:	20 mM MES, pH 6.5	
Mobile Phase B:	20 mM MES + 500 mM NaCl, pH 6.5	
Flow Rate:	1 mL/min	
Gradient:	Time (min)	%B (Table 1)
	0	Loading
	0.8	Loading
	1.0	Initial
	16	Final
	16.1	50
	18	50
	18.1	Loading
	25	Loading

Table 2. 4 × 150 mm %B gradient values for loading, initial and final; gradient slope; and time reduced with step change

mAb	Loading %	Initial %	Final %	ΔB/Δt %/min	Minutes Reduced
Rituximab Rituximab biosimilar	8	13	22	0.60	8.3
Trastuzumab Trastuzumab biosimilar	9	12	20	0.53	5.7
Bevacizumab Bevacizumab biosimilar	8	10	17	0.47	4.3
Vedolizumab	5	7	14	0.47	4.3
Infliximab	4	8	18	0.67	6.0
Secukinumab	5	10	16	0.40	12.5
Pertuzumab	9	12	20	0.53	5.7

IgG mAbs and Associated Biosimilars (4 × 150 mm)

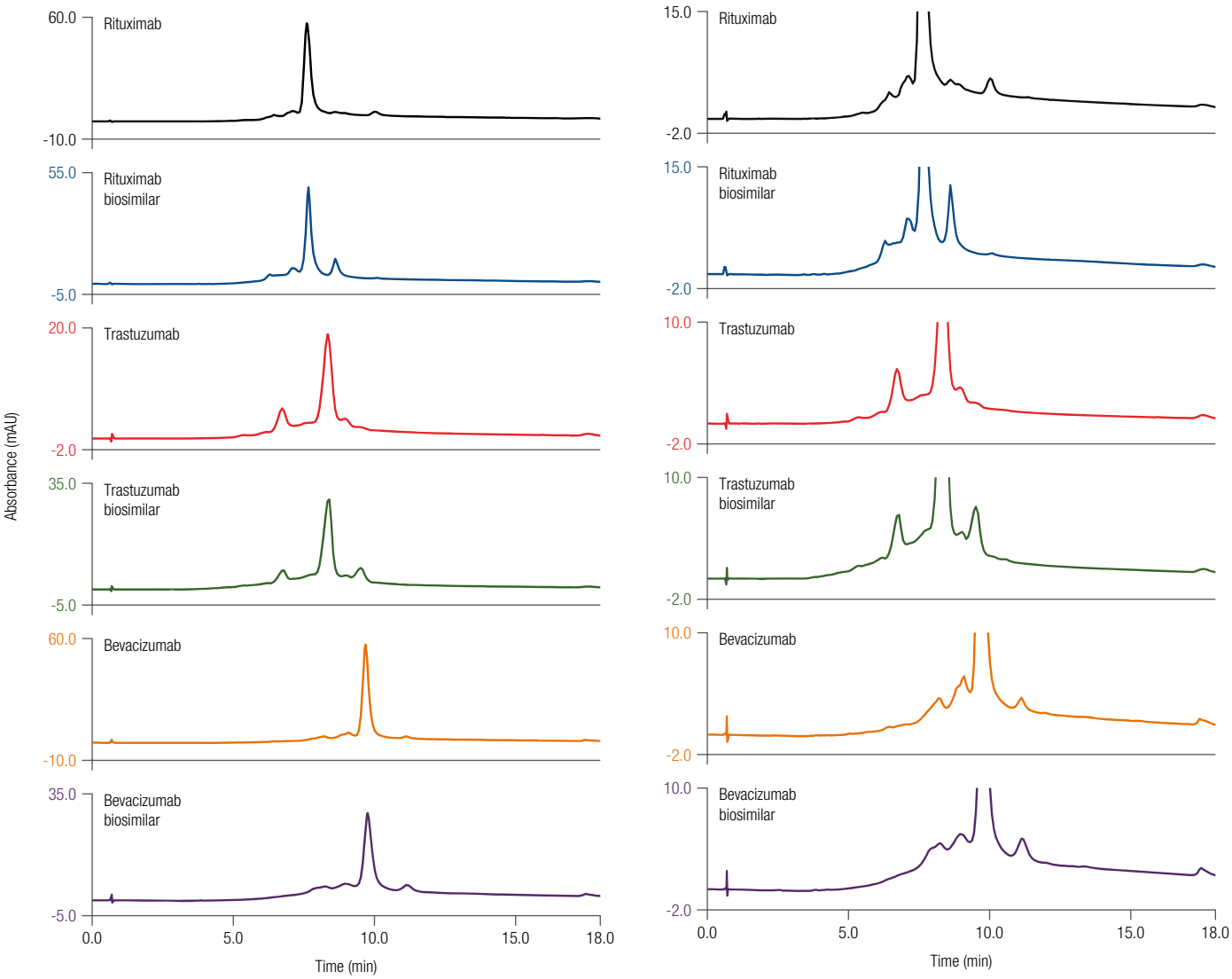


Figure 4. Analysis of innovator mAbs and respective biosimilars on a 4 x 150 mm ProPac Elite WCX 5 µm column using the general mAb gradient for 4 × 150 mm column detailed in Table 2. Left chromatograms show the full signal range, and right chromatograms show a detailed view of the mAb variants.

Additional IgG1 mAbs (4 × 150 mm)

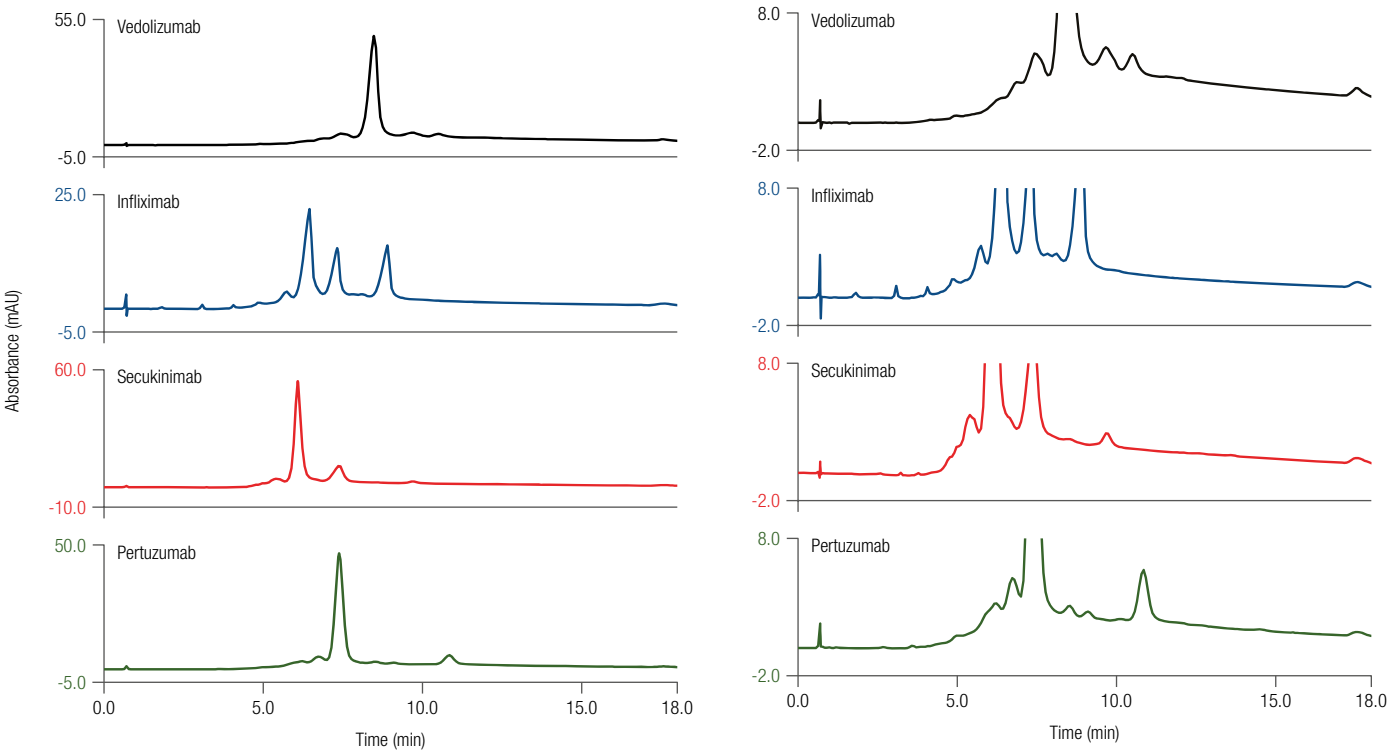


Figure 5. Analysis of mAbs on a 4 × 150 mm ProPac Elite WCX 5 µm column using the general mAb gradient for 4 × 150 mm column detailed in Table 2. Left chromatograms show the full signal range, and right chromatograms show a detailed view of the mAb variants.

Using a gradient tailored for each mAb, the ProPac Elite WCX 5 µm column provides good resolution of charge variants for all mAbs evaluated. Figure 4 shows direct detection of differences in charge variant composition when comparing pharmaceutically relevant innovator mAbs to biosimilar mAbs using the same gradient. The comparison of biosimilar to innovator mAbs is important for demonstrating that biosimilars meet the same stringent requirements of the initial innovator product. Figure 5 shows an additional set of four pharmaceutical mAbs with different charge variant profiles. The results in Figures 4 and 5 illustrate the versatility of the ProPac Elite WCX 5 µm column for analyzing a wide range of different mAb and protein variants.

Conclusions

- The ProPac Elite WCX 5 µm column provides excellent separation of mAb charge variants using a salt gradient for a wide range of pharmaceutical mAbs.
- Buffer composition pH, salt concentration, and temperature can be optimized to provide consistent, high-resolution variant separation.

Reference

1. Thermo Scientific Application Note 21845, pH gradient analysis of IgG1 therapeutic monoclonal antibodies using a 5 µm WCX column (2018), Thermo Fisher Scientific, Sunnyvale, USA.

Find out more at **thermofisher.com/propac**

ThermoFisher SCIENTIFIC