



# pH gradient analysis of IgG1 therapeutic monoclonal antibodies using a 5 $\mu$ m WCX column

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## Keywords

Biopharmaceutical, QA/QC,  
critical quality attribute, rituximab,  
trastuzumab, adalimumab,  
bevacizumab, infliximab,  
secukinumab, pertuzumab,  
vedolizumab, IgG1, weak cation  
exchange chromatography, pH  
gradient, CX-1 pH Gradient Buffers,  
ProPac Elite WCX column, Vanquish  
Flex UHPLC

## Application benefits

- The combination of the Thermo Scientific™ ProPac™ Elite WCX column and Thermo Scientific™ CX-1 pH Gradient Buffers provides a high-resolution, fast, easy-to-optimize, and reproducible platform method for charge variant characterization of therapeutic mAbs.
- Fast separation can be achieved using the 2 × 50 mm format.
- Comparison of the generic drug and the biosimilar can be easily achieved using ProPac Elite WCX column and CX-1 pH Gradient Buffers.

## Goal

To demonstrate high-resolution, fast separation of IgG1 therapeutic mAbs using CX-1 pH Gradient Buffers and ProPac Elite WCX columns

## Introduction

Monoclonal antibodies (mAbs) are a preferred class of protein therapeutics used for the treatment of various diseases because of their ability to target specific tissues. The structure of monoclonal antibodies is often heterogeneous due to post-translational modifications and degradation during cellular production and downstream processing. Modifications including C-terminal lysine truncation, asparagine deamidation, glycan

sialylation, etc., can impart additional cationic and anionic charges, increasing the charge heterogeneity of the mAb. Since many of these modifications have the potential to affect product efficacy, safety, and stability, thorough characterization of mAbs is performed throughout production to ensure the mAb meets quality and regulatory requirements. These requirements necessitate the need for methods to separate and analyze variants based on their charge.

Ion exchange chromatography (IEX) is a commonly used technique to separate analytes including proteins based on differences in charge. IgG1 antibodies are currently the most common subclass of therapeutic mAbs on the market. The relatively high pI of IgG1 mAbs gives the protein a positive charge when the environmental pH is less than the pI of the protein. As such, cation exchange chromatography, in combination with a buffer with a pH below the mAb pI, is typically employed to separate mAb charge variants. Early methods focused on the use of salt gradients to disrupt ionic interactions of the protein with the solid phase and elute the analyte. More recently, pH gradient methods have been used as an alternative approach. When using a pH gradient, the cationic protein is adsorbed to the anionic solid phase at low pH conditions followed by a gradient of increasing mobile phase pH. As the pH increases the protein charge shifts from cationic to neutral and anionic resulting in desorption from the solid phase and elution from the column. Previously, high-resolution separation of mAbs and their charge variants was demonstrated using CX-1 pH Gradient Buffers formulated with zwitterionic buffer species.<sup>1-4</sup> The CX-1 pH Gradient Buffers generate a linear pH gradient that simplifies method optimization for high resolution separation of the main product and impurities.

The ProPac Elite WCX 5 µm columns possess weak cation exchange functionality designed for the analysis of proteins. The resin is based on a non-porous divinylbenzene particle coated with a hydrophilic layer to limit secondary interactions and ensure high recovery. Acrylate groups grafted to the hydrophilic coating provide weak cation exchange functionality necessary for protein separations. The column chemistry has been developed to be compatible with the CX-1 pH Gradient Buffers.

Here a general method for separating charge variants of IgG1 mAbs using the CX-1 pH gradient buffers and ProPac Elite WCX columns is described. A linear pH gradient ranging from 20% to 70% CX-1 pH Gradient B was used to initially survey the separation of charge variants for nine IgG1 mAbs. A shallower gradient was then tailored to individual mAbs to improve resolution. Using a 4 × 150 mm column, high-resolution separations of charge variants was achieved while a 2 × 50 mm column provided faster separation time with a small loss in resolution.

## Experimental

### Reagents and consumables

- Deionized (DI) water, 18.2 MΩ•cm resistivity
- CX-1 pH Gradient Buffer A, 500 mL (P/N 302779)
- CX-1 pH Gradient Buffer B, 500 mL (P/N 302780)
- 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 equipped with a PCM-3000 pH and Conductivity Monitor

#### Columns

- ProPac Elite WCX column, 4 × 150 mm (P/N 302972)
- ProPac Elite WCX column, 2 × 50 mm (P/N 303028)

Mobile phase A: CX-1 pH Gradient Buffer A  
Mobile phase B: CX-1 pH Gradient Buffer B

## Conditions for 4 × 150 mm format column

**Table 1. Initial survey gradient condition for 4 × 150 mm format**

Time (min)	%A	%B
0.0	80	20
15.0	30	70
15.1	0	100
17.0	0	100
17.1	80	20
24.0	80	20

Flow rate: 1 mL/min  
Column temperature: 30 °C  
Injection details: 2 µL

## Conditions for 2 × 50 mm format column

**Table 2. Initial survey gradient condition for 2 × 50 mm format**

Time (min)	%A	%B
0.0	80	20
5.0	30	70
6.0	30	70
6.1	100	0
7.0	100	0
7.1	80	20
10	80	20

Flow rate: 0.8 mL/min  
Column temperature: 30 °C  
Injection details: 2 µL

For shallower gradient conditions, see tables in the Results and discussion section. Absorbance at 280 nm was used for detection of all samples.

## Data processing

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

## Results and discussion

### High-resolution analysis

Various therapeutic IgG1 antibodies were analyzed using the ProPac Elite WCX, 4 x 150 mm column and CX-1 pH Gradient Buffers on a Vanquish Flex UHPLC system. Separations of mAbs were achieved using a pump gradient from 20% to 70% CX-1 pH Gradient Buffer B over 15 minutes. Although a full 0 to 100% B gradient can be used for specifically lower or higher pI proteins, 20 to 70% B gradient was sufficient to bind and elute all IgG1 mAbs used in this study with good resolution for most charge variants (Figure 1). To further separate acidic and basic charge variants, method optimization was performed by running shallower gradients based on the retention time of each mAb. Table 3 lists the initial and final percent mobile phase B used for individual mAbs. The shallower gradient method resulted in ~10% increase in resolution compared to the 20 to 70% B initial gradient method (Figure 2). The higher resolution allows more accurate quantitation of charge variants.

### Fast analysis

In high-throughput screening environments, fast separations of mAb variants can be performed using a 2 x 50 mm column format with a 5-minute gradient run time. The total cycle time is dependent on the gradient delay as well as the time it takes to re-equilibrate the stationary phase. To minimize the gradient delay and the re-equilibration time, a smaller gradient mixer (150 µL) and a high linear velocity (0.8 mL/min) were used. With these modifications, an 10-minute total cycle time was achieved. As described for the high-resolution analysis, an initial screen was performed by using the 20 to 70% B gradient followed by specific shallower gradients for each mAb (Table 4, Figures 3 and 4).

### Comparison of innovator mAbs and biosimilars

It is important for the developers and manufacturers of biosimilars to prove the similarity of the biosimilar to the original product in terms of structure, function, pharmacodynamics, pharmacokinetic properties, clinical efficacy, and safety. Since charge variants could affect such parameters, cation exchange chromatography is often used to compare the innovator drug and the biosimilar. Figure 5 shows comparisons of three innovator mAbs and their respective biosimilars using the ProPac Elite WCX column and the pH gradient method. For all three innovator mAbs and biosimilar comparisons, there was either a change in the variant profile and/or a change in the intensity of the acidic or basic variants.

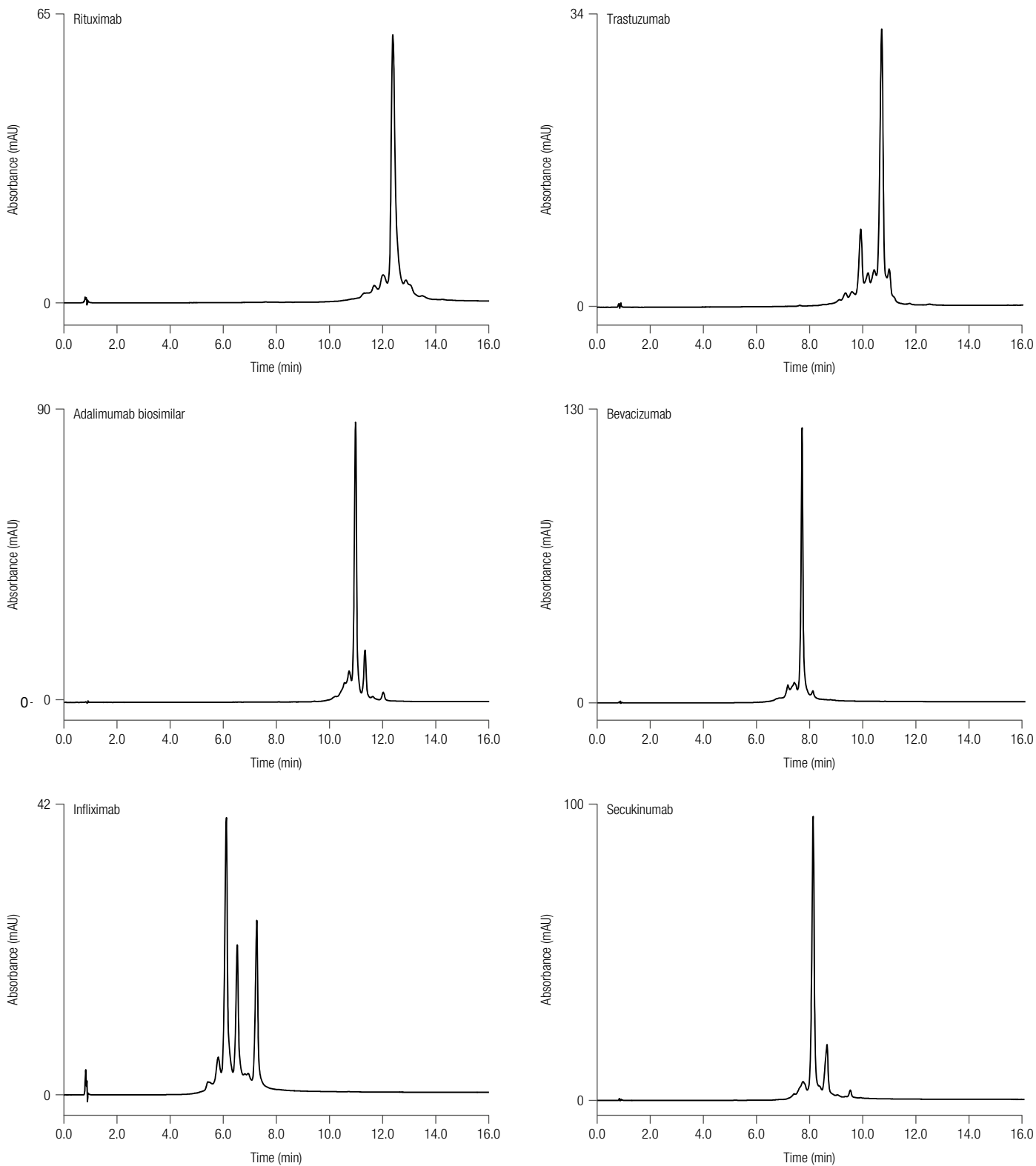
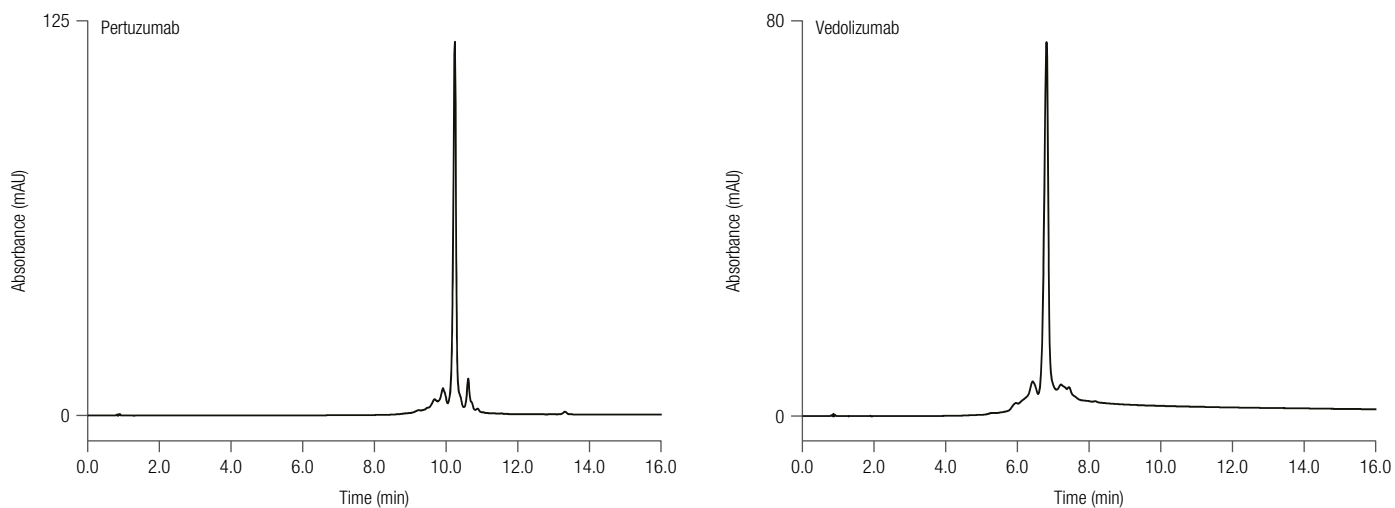


Figure 1. Separation of IgG1 therapeutic mAbs using a 20 to 70% B pH gradient on the 4 x 150 mm column



Column: ProPac Elite WCX, 5  $\mu$ m  
 Format: 4 x 150 mm  
 Mobile phase A: CX-1 pH Gradient Buffer A (pH 5.6)  
 Mobile phase B: CX-1 pH Gradient Buffer B (pH 10.2)  
 Flow rate: 0.8 mL/min  
 Temperature: 30  $^{\circ}$ C  
 Detection: UV (280 nm)  
 Inj. volume: 2  $\mu$ L (5 mg/mL)

Gradient:	Time (min)	%A	%B
	0.0	80	20
	15.0	30	70
	15.1	0	100
	17.0	0	100
	17.1	80	20
	24.0	80	20

Figure 1 (continued). Separation of IgG1 therapeutic mAbs using a 20 to 70% B pH gradient on the 4 x 150 mm column

Table 3. Retention time and elution %B of mAbs using the 20 to 70% B pH gradient condition and initial and final %B for the shallower pH gradients on the 4 x 150 mm format

mAb	Ret. Time (min)	Elution %B*	%B <sub>initial</sub>	%B <sub>final</sub>
Rituximab	11.94	56.6	43	68
Trastuzumab	10.32	51.0	37	62
Adalimumab biosimilar	10.82	51.9	38	63
Bevacizumab	7.06	40.9	27	52
Infliximab	5.45	35.7	22	47
Secukinumab	7.55	42.3	28	53
Pertuzumab	9.72	49.5	35	60
Vedolizumab	6.12	38.0	24	49

\*%B was calculated based on 1.4 minutes of gradient delay that was measured using UV trace of a blank run.

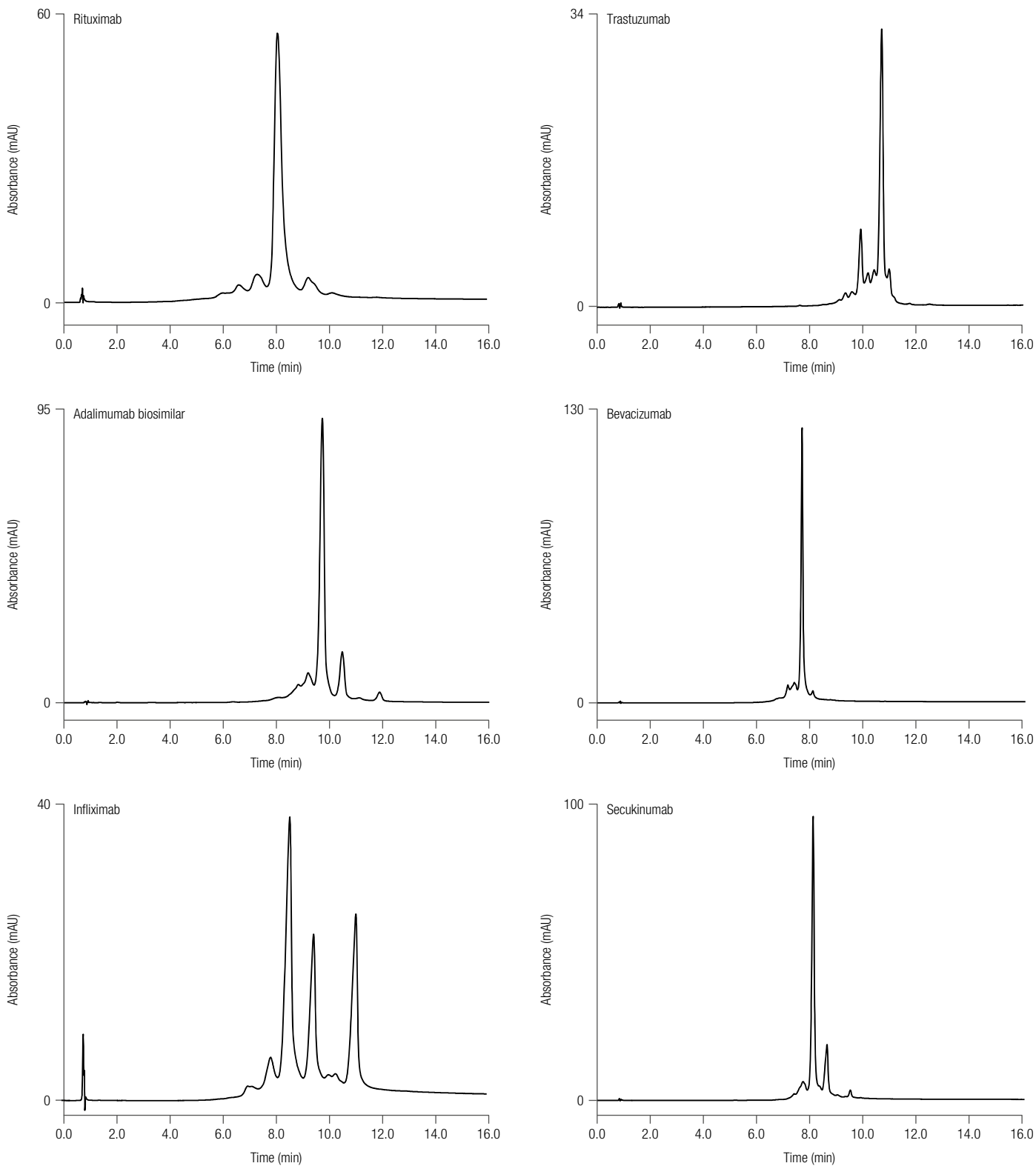
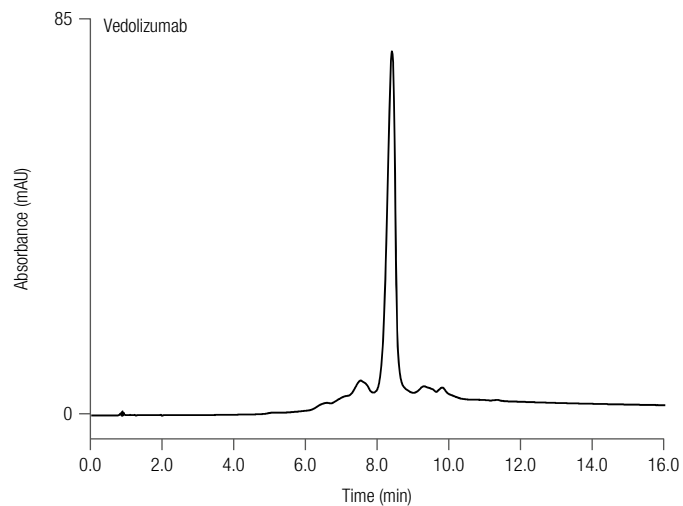
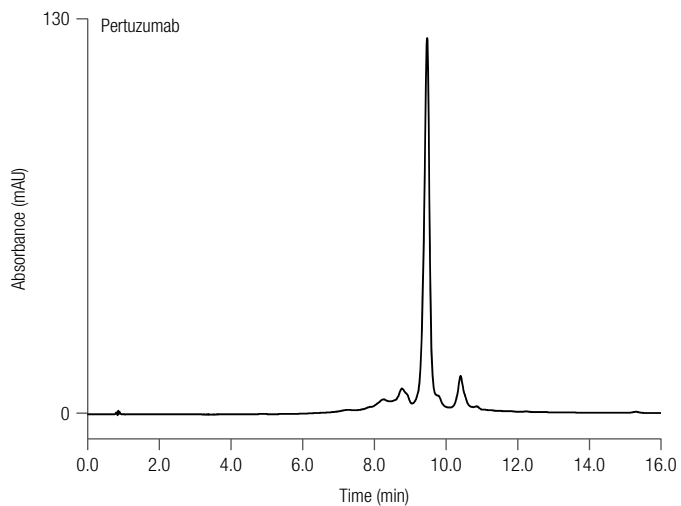


Figure 2. Separation of IgG1 therapeutic mAbs using shallower pH gradients on the 4 × 150 mm column



Column: ProPac Elite WCX, 5  $\mu$ m  
Format: 4 x 150 mm  
Mobile phase A: CX-1 pH Gradient Buffer A (pH 5.6)  
Mobile phase B: CX-1 pH Gradient Buffer B (pH 10.2)  
Flow rate: 0.8 mL/min  
Temperature: 30  $^{\circ}$ C  
Detection: UV (280 nm)  
Inj. volume: 4  $\mu$ L (5 mg/mL)  
Gradient: 25% B change in 15 minutes  
Start and final %B is in Table 3.

Figure 2 (continued). Separation of IgG1 therapeutic mAbs using shallower pH gradients on the 4 x 150 mm column

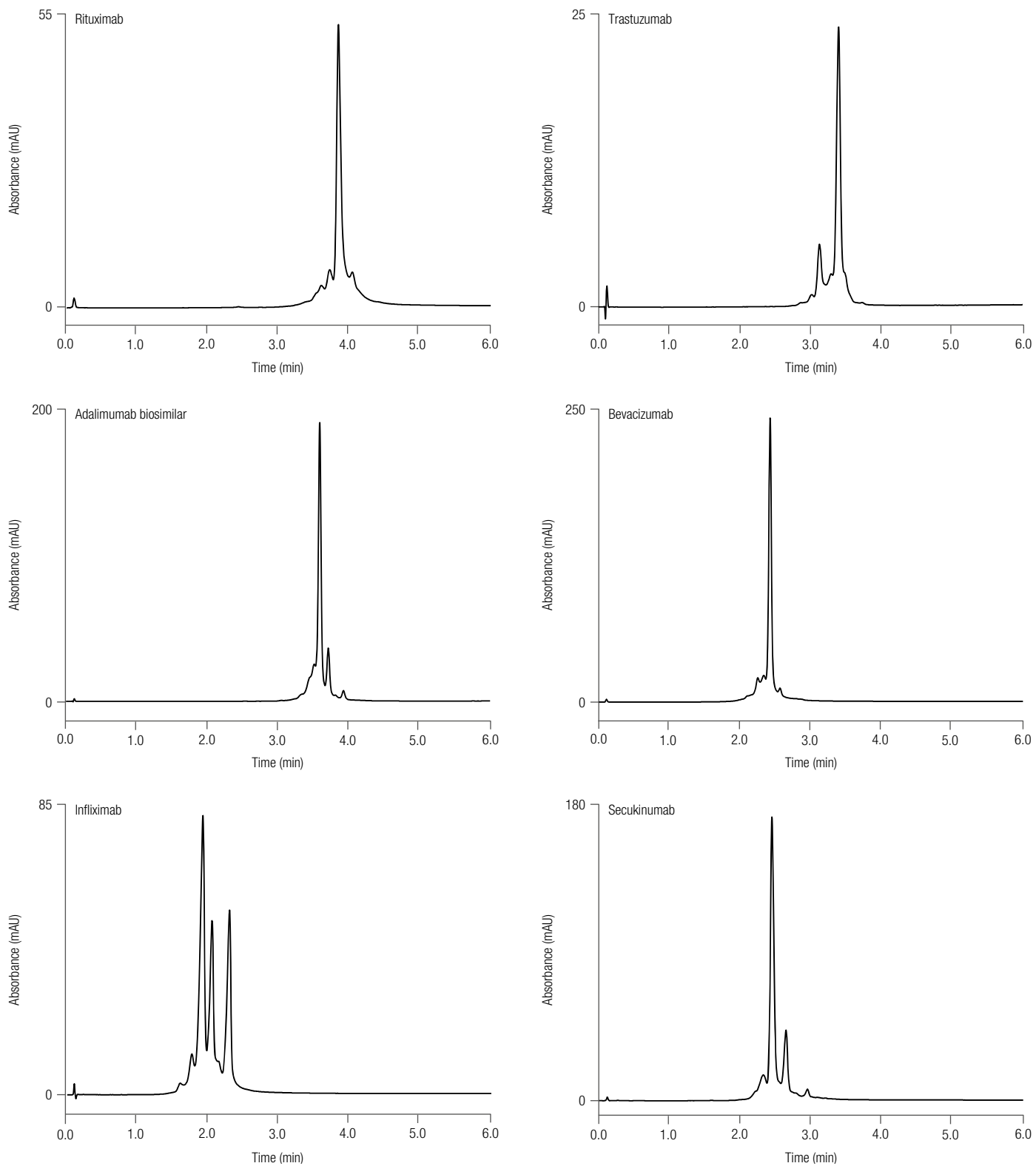
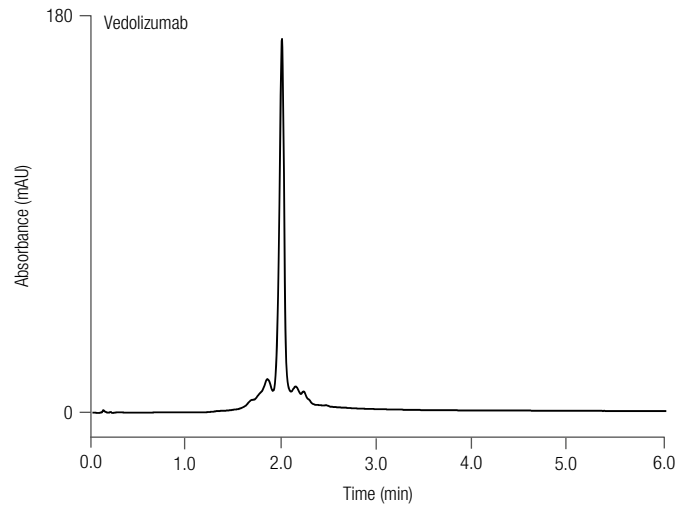
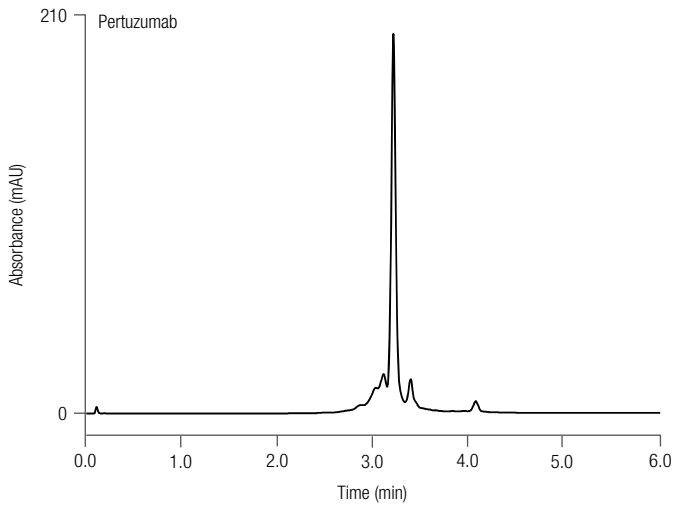


Figure 3. Separation of IgG1 therapeutic mAbs using the 20 to 70% B pH gradients on the 2 x 50 mm column





Column: ProPac Elite WCX, 5  $\mu$ m  
 Format: 2 x 50 mm  
 Mobile phase A: CX-1 pH Gradient Buffer A (pH 5.6)  
 Mobile phase B: CX-1 pH Gradient Buffer B (pH 10.2)  
 Flow rate: 0.8 mL/min  
 Temperature: 30  $^{\circ}$ C  
 Detection: UV (280 nm)  
 Inj. volume: 2  $\mu$ L (5 mg/mL)

Gradient:	Time (min)	%A	%B
	0.0	80	20
	5.0	30	70
	6.0	30	70
	6.1	100	0
	7.0	100	0
	7.1	80	20
	10.0	80	20

Figure 3 (continued). Separation of IgG1 therapeutic mAbs using the 20 to 70% B pH gradients on the 2 x 50 mm column

Table 4. Retention time and elution %B of mAbs using the 20 to 70% B pH gradient condition and initial and final %B for shallower pH gradients on the 2 x 50 mm format

mAb	Ret. Time (min)	Elution %B*	%B <sub>initial</sub>	%B <sub>final</sub>
Rituximab	3.85	51.5	37	62
Trastuzumab	3.40	47.0	32	57
Adalimumab biosimilar	3.59	48.9	34	59
Bevacizumab	2.42	37.2	22	47
Infliximab	1.93	32.3	17	42
Secukinumab	2.45	47.5	22	47
Pertuzumab	3.21	45.1	30	55
Vedolizumab	1.99	32.9	18	43

\*%B was calculated based on 0.7 minute of gradient delay that was measured using UV trace of a blank run.

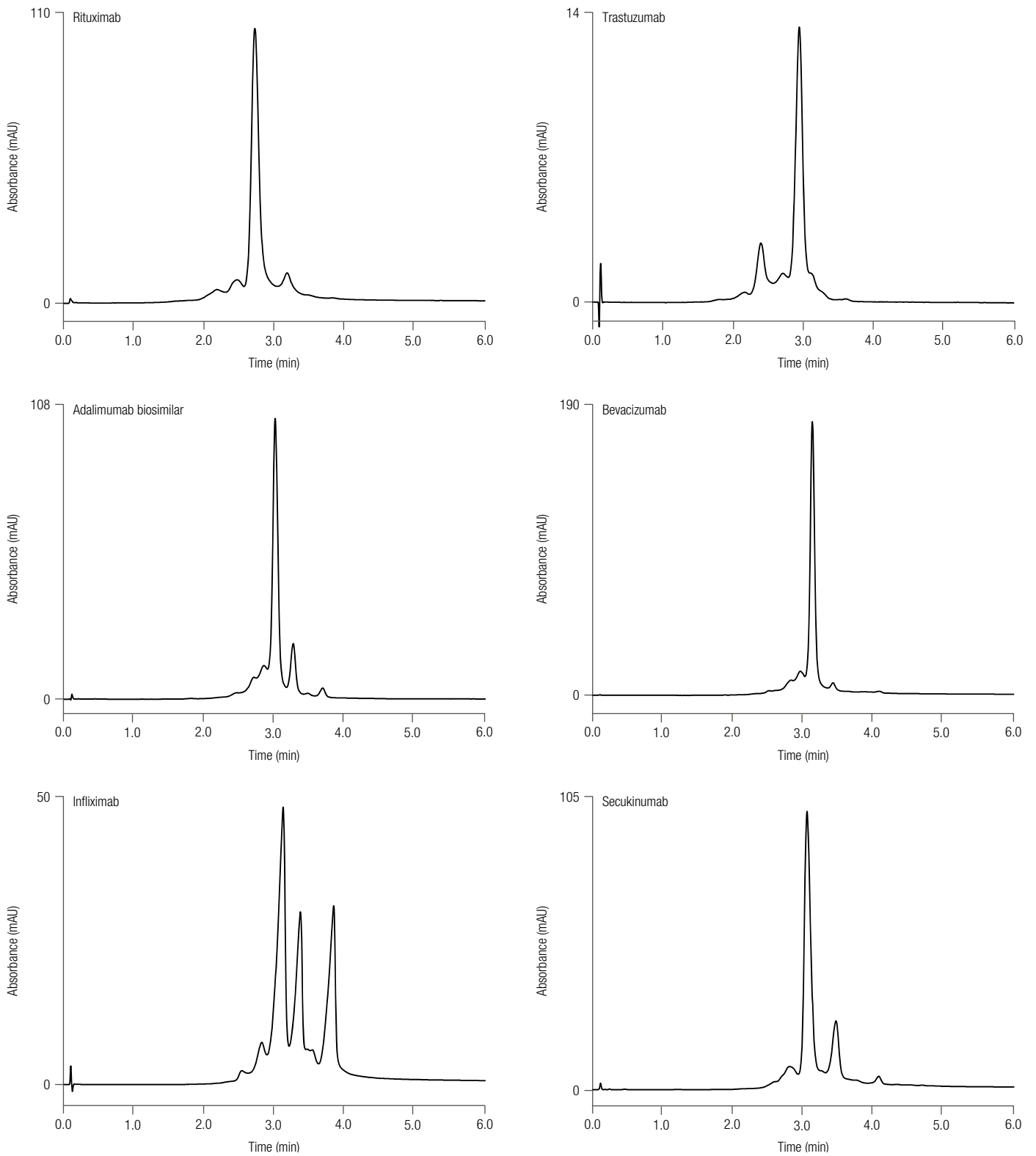
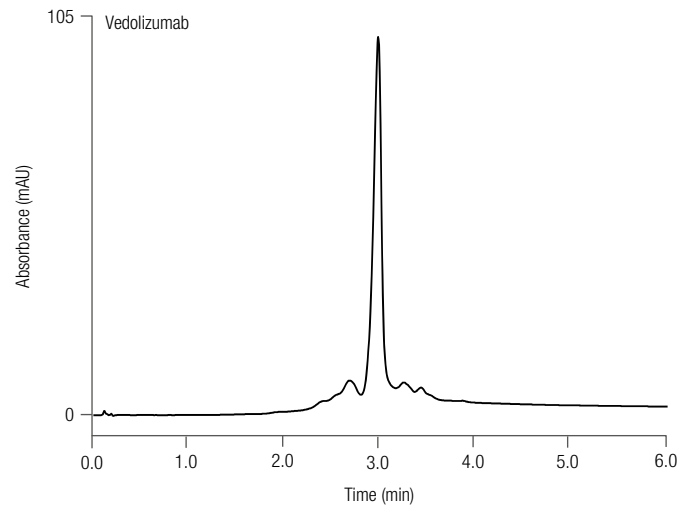
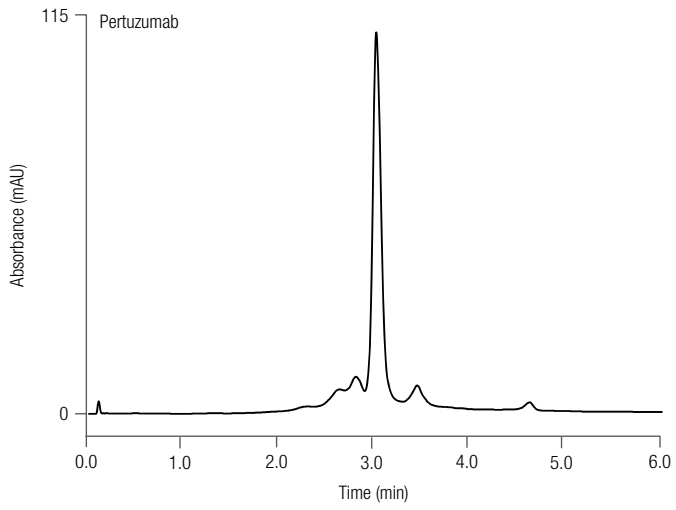
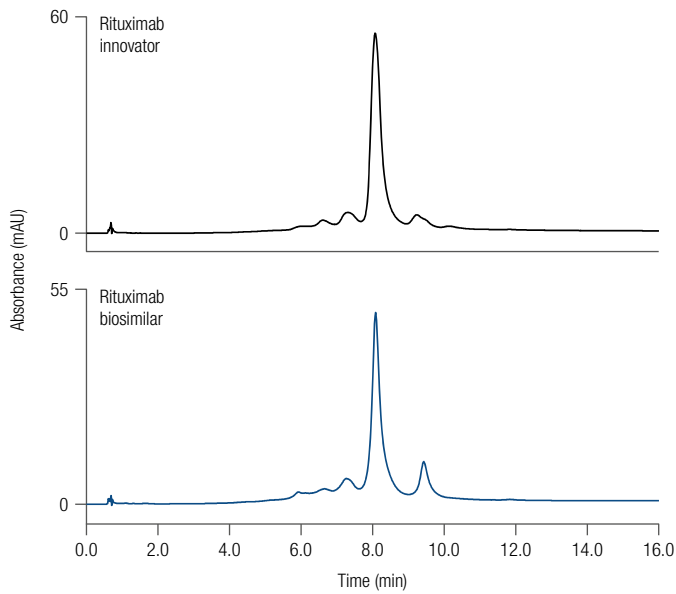


Figure 4. Separation of IgG1 therapeutic mAbs using shallower pH gradients on the 2 x 50 mm column



Column: ProPac Elite WCX, 5  $\mu$ m  
 Format: 2  $\times$  50 mm  
 Mobile phase A: CX-1 pH Gradient Buffer A (pH 5.6)  
 Mobile phase B: CX-1 pH Gradient Buffer B (pH 10.2)  
 Flow rate: 0.8 mL/min  
 Temperature: 30  $^{\circ}$ C  
 Detection: UV (280 nm)  
 Inj. volume: 2  $\mu$ L (5 mg/mL)  
 Gradient: 25% B change in 5 minutes  
 Start and final %B is in Table 4

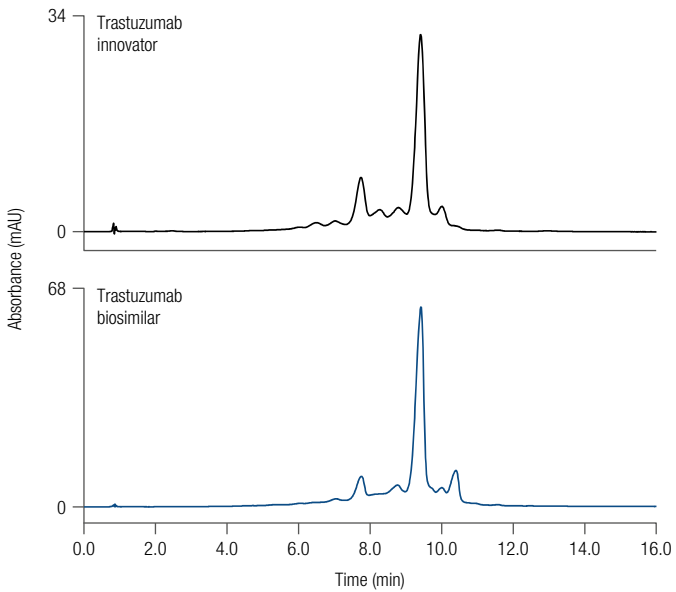
**Figure 4 (continued). Separation of IgG1 therapeutic mAbs using shallower pH gradients on the 2  $\times$  50 mm column**



Column: ProPac Plus WCX, 5  $\mu$ m  
 Format: 4 x 150 mm  
 Mobile phase A: CX-1 pH Gradient Buffer A (pH 5.6)  
 Mobile phase B: CX-1 pH Gradient Buffer B (pH 10.2)

Gradient:	Time (min)	%A	%B
	0.0	57	43
	15.0	32	68
	15.1	0	100
	17.0	0	100
	17.1	57	43
	24.0	57	43

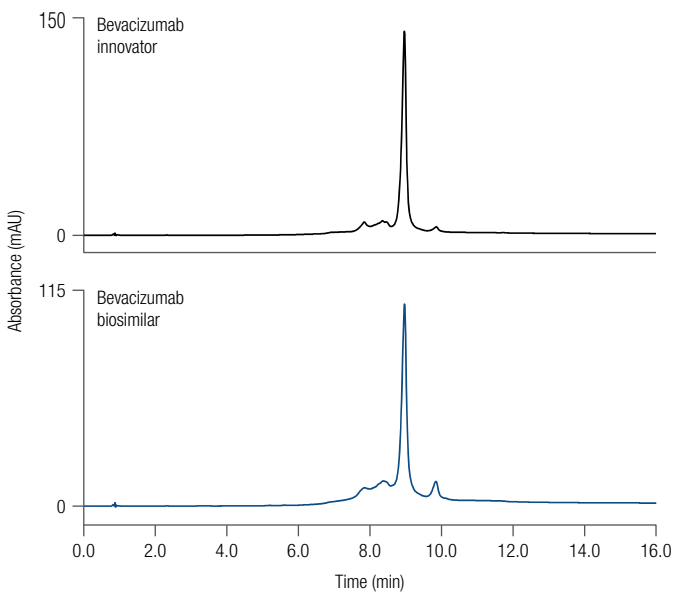
Flow rate: 0.8 mL/min  
 Temperature: 30  $^{\circ}$ C  
 Detection: UV (280 nm)  
 Inj. volume: 4  $\mu$ L (5 mg/mL)



Column: ProPac Plus WCX, 5  $\mu$ m  
 Format: 4 x 150 mm  
 Mobile phase A: CX-1 pH Gradient Buffer A (pH 5.6)  
 Mobile phase B: CX-1 pH Gradient Buffer B (pH 10.2)

Gradient:	Time (min)	%A	%B
	0.0	63	37
	15.0	38	62
	15.1	0	100
	17.0	0	100
	17.1	63	37
	24.0	63	37

Flow rate: 0.8 mL/min  
 Temperature: 30  $^{\circ}$ C  
 Detection: UV (280 nm)  
 Inj. volume: 4  $\mu$ L (5 mg/mL)



Column: ProPac Plus WCX, 5  $\mu$ m  
 Format: 4 x 150 mm  
 Mobile phase A: CX-1 pH Gradient Buffer A (pH 5.6)  
 Mobile phase B: CX-1 pH Gradient Buffer B (pH 10.2)

Gradient:	Time (min)	%A	%B
	0.0	73	27
	15.0	48	52
	15.1	0	100
	17.0	0	100
	17.1	73	27
	24.0	73	27

Flow rate: 0.8 mL/min  
 Temperature: 30  $^{\circ}$ C  
 Detection: UV (280 nm)  
 Inj. volume: 4  $\mu$ L (5 mg/mL)

Figure 5. Comparison of innovator and biosimilar mAbs

## Conclusions

- The combination of a ProPac Elite WCX column and CX-1 pH Gradient Buffers provides excellent separation for charge variants from IgG1 therapeutic mAbs.
- High-resolution separation of mAbs and charge variants was achieved using the 4 × 150 mm format.
- With a narrow and short 2 × 50 mm format, fast separation of mAb charge variants was achieved.
- The combination of a ProPac Elite WCX column and CX-1 pH Gradient Buffers can be used for the comparison of innovator and biosimilar mAbs.

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