

# High Resolution Charge Variant Analysis for Top-Selling Monoclonal Antibody Therapeutics Using Linear pH Gradient Separation Platform?

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## Overview

**Purpose:** To achieve high resolution charge variant analysis for top-selling monoclonal antibody therapeutics.

**Methods:** The charge variants of top-selling mAbs (Rituxan, Herceptin, Humira, and Avastin) are analyzed on a strong cation exchange column with a linear pH gradient separation method. The linear gradient from pH 5.6 to pH 10.2 is generated over time by running a linear pump gradient from 100% Thermo Scientific™ CX-1 pH Gradient Buffer A (pH 5.6) to 100% CX-1 pH Gradient Buffer B (pH 10.2).

**Results:** The pH gradient method is generally applicable to monoclonal antibody charge variant analysis. The data also show that pH gradient method delivers higher resolution power than the traditional salt method.

## Introduction

Charge variants of mAbs are due to modifications such as sialylation, deamidation and C-terminal lysine truncation. Traditionally, salt gradient cation exchange chromatography has been used with some success in characterizing mAb charge variants [1]. However, significant effort is often required to tailor the salt gradient method for each individual mAb. In the fast-paced drug development environment, a fast and robust platform method is desirable to accommodate the majority of the mAb analyses. Thermo Fisher Scientific recently introduced cation-exchange pH gradient buffers which meet the fast and robust generic platform method requirements [2]. This buffer system consists of a low-pH buffer A at pH 5.6 and a high-pH buffer B at pH 10.2. A linear pH gradient from pH 5.6 to pH 10.2 is generated over time by running a linear pump gradient from 100% buffer A to 100% buffer B.

In this study, the charge variants of Rituxan (Rituximab), Herceptin (Trastuzumab), Humira (Adalimumab), and Avastin (Bevacizumab) are analyzed on a Thermo Scientific™ MAbPac™ SCX-10 column with a linear pH gradient separation method. The linear gradient from pH 5.6 to pH 10.2 is generated over time by running a linear pump gradient from 100% CX-1 pH Gradient Buffer A (pH 5.6) to 100% CX-1 pH Gradient Buffer B (pH 10.2). The results demonstrate the general applicability of the pH gradient method on monoclonal antibody charge variant analysis. The data also show that pH gradient method delivers higher resolution power than the traditional salt method. The methods described here can be widely used in the development of the biosimilars of these top-selling mAbs.

## Methods

### Samples

Rituxan/rituximab, 5 mg/mL; Herceptin/trastuzumab, 5 mg/mL; Humira/adalimumab, 5 mg/mL; Avastin/bevacizumab, 1 mg/mL.

### Column and Buffer

MAbPac SCX-10, 10  $\mu$ m, 4  $\times$  250 mm (P/N 074625)  
CX-1 pH Gradient Buffer A (pH 5.6), 125 mL (P/N 083273)  
CX-1 pH Gradient Buffer B (pH 10.2), 125 mL (P/N 083275)

### Liquid Chromatography

Thermo Scientific™ Dionex™ UltiMate™ 3000 BioRS system equipped with:  
SRD-3400 Solvent racks with degasser  
HPG-3400RS Biocompatible Binary Rapid Separation Pump  
WPS-3000TBRS Biocompatible Rapid Separation Thermostatted Autosampler  
TCC-3000RS Rapid Separation Thermostatted Column Compartment  
VWD-3400RS Rapid Separation Variable Wavelength Detector  
PCM-3000 pH and Conductivity Monitor

### pH Gradient Mobile Phases:

Mobile phase A: 1X CX-1 pH Gradient Buffer A, pH 5.6  
Mobile phase B: 1X CX-1 pH Gradient Buffer B, pH 10.2

### Linear pH Gradient Chromatography

The full pH gradient was generated by running a linear gradient from 100% eluent A (pH 5.6) to 100% eluent B (pH 10.2). The half pH gradient was generated by running a linear gradient from 100% eluent A (pH 5.6) to 50% eluent B (pH 10.2).

### Salt Gradient Mobile Phases:

Mobile phase A: 20 mM MES (pH 5.6) + 60 mM NaCl  
Mobile phase B: 20 mM MES (pH 5.6) + 300 mM NaCl

## Salt Gradient Chromatography

The full salt gradient was generated by running a linear gradient from 100% eluent A (60 mM NaCl) to 100% eluent B (300 mM NaCl). The half salt gradient was generated by running a linear gradient from 100% eluent A (60 mM NaCl) to 50% eluent B (300 mM NaCl).

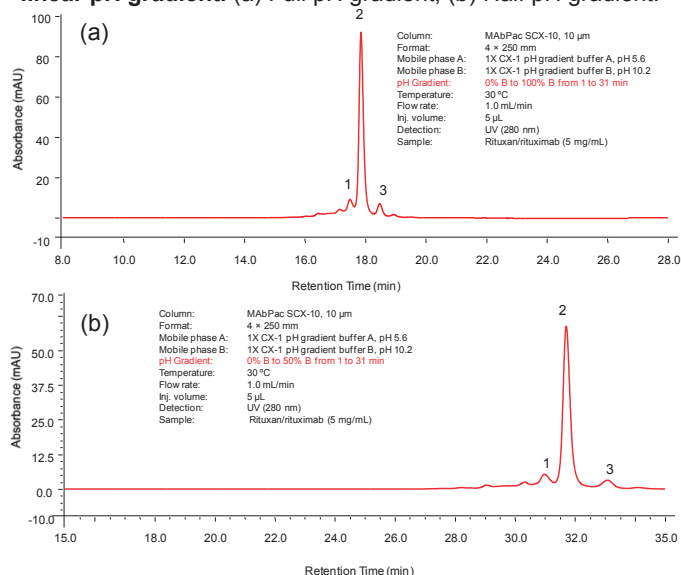
## Results

The CX-1 pH gradient buffer kit is designed to generate a linear pH gradient when a linear pump gradient is run from 100% CX-1 buffer A to 100% buffer B. This pH gradient method serves as a platform method for the mAb charge variant analysis, covering the pH range from 5.6 to 10.2. Most of the therapeutic mAbs have pI values falling within this pH range. Rituximab (Figure 1a) and trastuzumab (Figure 3a) are analyzed on a MAbPac SCX-10 column using the full pH gradient method. Satisfactory separations of multiple variants are observed with all four samples. After the initial survey runs of the full pH gradient, the subsequent runs are aimed at improving resolution by decreasing the pH range and gradient slope. The fact that the pH gradient is linear makes the method optimization simple. Rituximab (Figure 1b) and trastuzumab (Figure 3b), are analyzed using a shallower pH gradient with half the pH range.

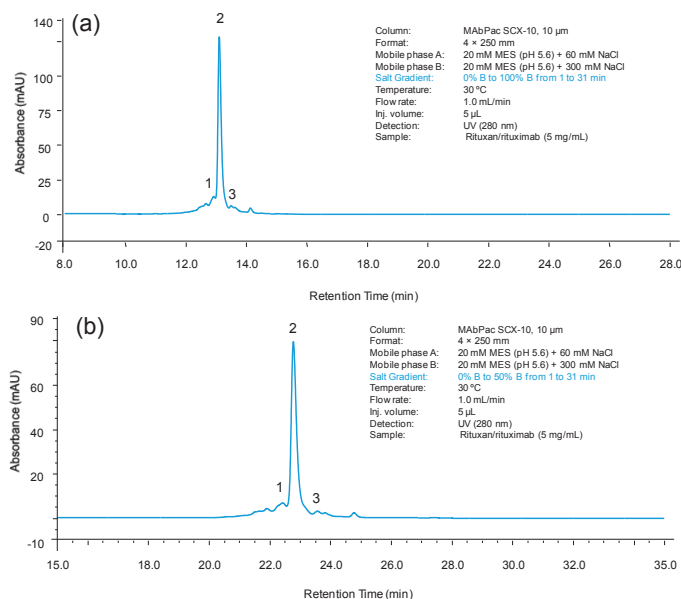
Traditionally, salt gradient method has been used for mAb charge variants analysis. The salt gradient method development usually requires screening at different pH values using different buffers. In addition, the minimum salt concentration required to elute the mAb off the cation exchange column must be individually determined. For comparison and speed, the same initial conditions and buffers (20 mM MES and 60 mM NaCl at pH 5.6) are used for all the samples in this study. Rituximab (Figure 2a and 2b) and trastuzumab (Figure 4a and 4b) are each analyzed by two salt gradient methods: one with steeper gradient slope and the other one with shallower gradient slope.

When comparing the separation profiles obtained by the pH and salt gradient methods, they are similar for the same molecule. In order to simplify the comparison, the acidic variant adjacent to the major variant is labeled as peak 1, the major variant is labeled as peak 2 and the basic variant adjacent to the major variant is labeled as peak 3 for each chromatogram (figures 1–4). Due to the limited space, the chromatographic profiles of adalimumab and bevacizumab are not shown here. In the case of trastuzumab salt gradient chromatogram, the minor acidic variant is very close to the major peak and could be not be determined but this is resolved by the pH gradient. Table 1 lists the retention time of peak 1 (RT1), peak 2 (RT2) and peak 3 (RT3) and the difference between RT1 and RT2 ( $\Delta RT_{1-2}$ ), as well as RT2 and RT3 ( $\Delta RT_{2-3}$ ). In the case of rituximab, trastuzumab, and bevacizumab, it is clear that the delta RTs between variants are greater when using the pH gradient profile. In the case of adalimumab, the delta RTs are similar between the pH gradient profile and the salt gradient profile.

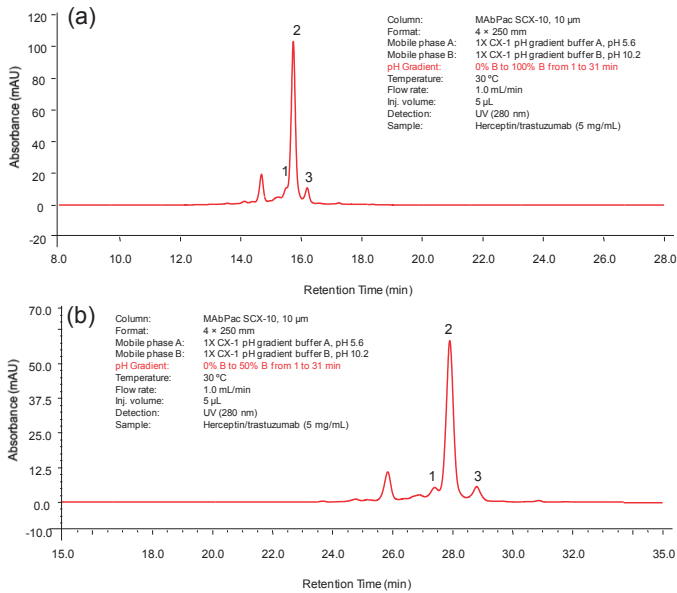
**FIGURE 1. Rituxan/rituximab charge variant analysis using linear pH gradient. (a) Full pH gradient; (b) Half pH gradient.**



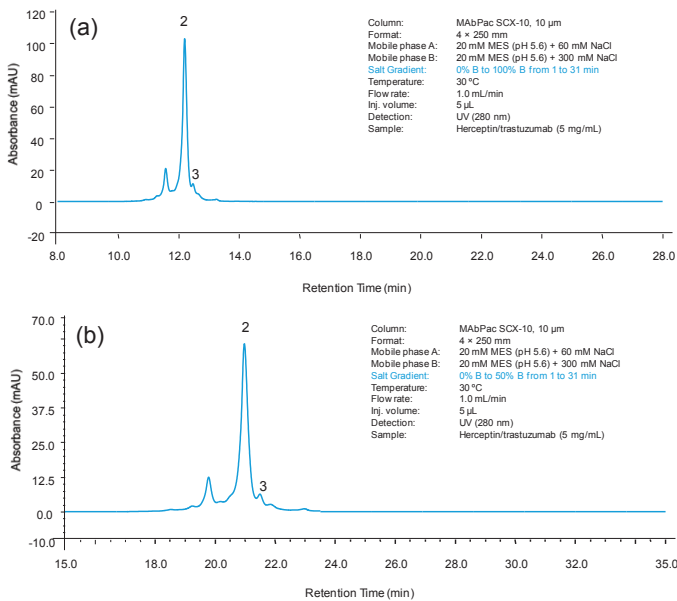
**FIGURE 2. Rituxan/rituximab charge variant analysis using salt gradient. (a) Full salt gradient; (b) Half salt gradient.**



**FIGURE 3. Herceptin/trastuzumab charge variant analysis using linear pH gradient. (a) Full pH gradient; (b) Half pH gradient.**



**FIGURE 4. Herceptin/trastuzumab charge variant analysis using salt gradient. (a) Full salt gradient; (b) Half salt gradient.**



**TABLE 1. Retention time of mAb charge variants analyzed by linear pH gradient and salt gradient methods.**

	Method	Gradient	RT1 (min)	RT2 (min)	RT3 (min)	ΔRT1-2 (min)	ΔRT2-3 (min)
Rituxan/rituximab	pH	full	17.497	17.86	18.477	0.363	0.617
		half	30.98	31.71	33.077	0.73	1.367
	salt	full	12.927	13.11	13.504	0.183	0.394
		half	22.44	22.783	23.567	0.343	0.784
Herceptin/trastuzumab	pH	full	15.257	15.744	16.207	0.487	0.463
		half	27.404	27.914	28.818	0.51	0.904
	salt	full	n.a.	12.204	12.477	n.a.	0.273
		half	n.a.	21.494	21.844	n.a.	0.35
Humira/adalimumab	pH	full	16.063	16.26	16.593	0.197	0.333
		half	28.834	29.244	29.907	0.41	0.663
	salt	full	15.354	15.56	15.9	0.206	0.34
		half	26.877	27.267	27.947	0.39	0.68
Avastin/bevacizumab	pH	full	11.537	11.824	12.264	0.287	0.44
		half	19.977	20.553	21.433	0.576	0.88
	salt	full	13.494	13.724	14.11	0.23	0.386
		half	23.36	23.817	24.587	0.457	0.77

## Conclusion

- Linear pH gradient method is a platform method for mAb charge variant analysis.
- Linear pH gradient method can be easily optimized to improve separation.
- Linear pH gradient method delivers better charge variant separation than salt gradient method.

## References

- Vlasak J. and Ionescu R. Heterogeneity of Monoclonal Antibodies Revealed by Charge-Sensitive Methods, *Current Pharmaceutical Biotechnology*, 2008, 9, 468-481
- Lin, S., Baek, J., Decrop, W., Rao, S., Agroskin, Y., and Pohl, C. Development of a Cation-Exchange pH Gradient Separation Platform. Presented at 39th International Symposium on High Performance Liquid Phase Separations and Related Techniques, Amsterdam, The Netherlands, June 16-20, 2013.

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