Charge Variant Analysis of Therapeutic Proteins Using a Novel Weak Cation Exchange Stationary Phase

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ABSTRACT

Purpose: Demonstrate high resolution separation of IgG1, IgG2 and IgG4 monoclonal antibodies (mAbs) and an antibody-drug conjugate (ADC) on a new weak cation exchange stationary phase.

Methods: Charge variants of various therapeutic mAbs and an ADC sample were separated using ProPac Elite WCX, 5 µm columns. Both salt gradient and pH gradient methods were used for the IgG1 mAbs. Salt gradient methods was used for IgG2 and IgG4 mAbs and pH gradient method was used for the ADC sample.

Results: Charge variants of three different subclasses of mAbs—IgG1, IgG2, and IgG4—were successfully separated using the new 5 µm weak cation exchange stationary phase. In addition, the column was able to separate charge variants of an NISTmAb derived ADC standard as well as the unmodified NISTmAb.

INTRODUCTION

Sales of protein biotherapeutics, such as monoclonal antibodies, have grown rapidly over the past two decades. The increasing complexity of these large biomolecules has required the analytical field to keep pace with better techniques for separating, detecting and characterizing the different variant structures that can arise during production. Chromatographic methods are commonly employed to separate and characterize structural heterogeneity. Specifically, cation-exchange chromatography has become a method of choice for separating charge-related heterogeneity, such as variants formed due to deamidation and lysine truncation.

The diverse structure of monoclonal antibodies (such as IgG1, IgG2, and IgG4) and monoclonal antibody derived therapeutics (such as ADC and bi-specifics) presents a challenge in designing cation-exchange chemistries that can accommodate a wide range of analyses. Here, we report the development of a 5 µm weak cation-exchange stationary phase designed for high resolution charge variant separation of therapeutic proteins. This 5 µm divinylbenzene-based resin particle is coated with an hydrophilic polymer layer, followed by grafting of acrylate groups to provide weak cation-exchange functionality.

Separation of acidic and basic variants of ten therapeutic proteins among three different classes of mAbs were performed on the ProPac Elite, 5 µm column. Seven IgG1 (rituximab, trastuzumab, infliximab, bevacizumab, secukinumab, pertuzumab, and vedolizumab), two IgG2 (panitumumab, denosumab) and one IgG4 (nivolumab) were separated using salt gradient and linear pH gradient methods. Furthermore, we examined the charge variant analysis of NISTmAb and NISTmAb derived ADC standard. These examples demonstrate that the new WCX stationary phase provides unique selectivity and high resolution for therapeutic protein charge variant separation.

MATERIALS AND METHODS

Sample Preparation

IgG1, IgG2 and IgG4 therapeutic mAb products were donated by biotech companies. These mAb samples were diluted to 5 mg/mL with DI water. The NISTmAb derived ADC standard was prepared as described previously.[1]

Liquid Chromatography

HPLC experiments were carried out using a Thermo Scientific™ Dionex™ Vanquish™ F system equipped with:

- Quaternary Pump F (P/N VF-P20-A)
- Split Sampler FT (P/N VF-A10-A)
- Column Compartment H (P/N VH-C10-A) equipped with an active pre-column heater (P/N 6732.0110) and a post-column cooler (P/N 6732.0510).
- Diode Array Detector HL (P/N VH-D10-A)

Columns: ProPac Elite WCX, 5 µm 4×150 mm (P/N 302972)
ProPac Elite WCX, 5 µm 4×250 mm (P/N 303025)

Mobile Phases

	Salt Gradient Method	pH Gradient Method
Mobile phase A	20 mM MES, pH 6.5	CX-1 pH Gradient Buffer A
Mobile phase B	20 mM MES, 0.5 M NaCl, pH 6.5	CX-1 pH Gradient Buffer B

Data Analysis

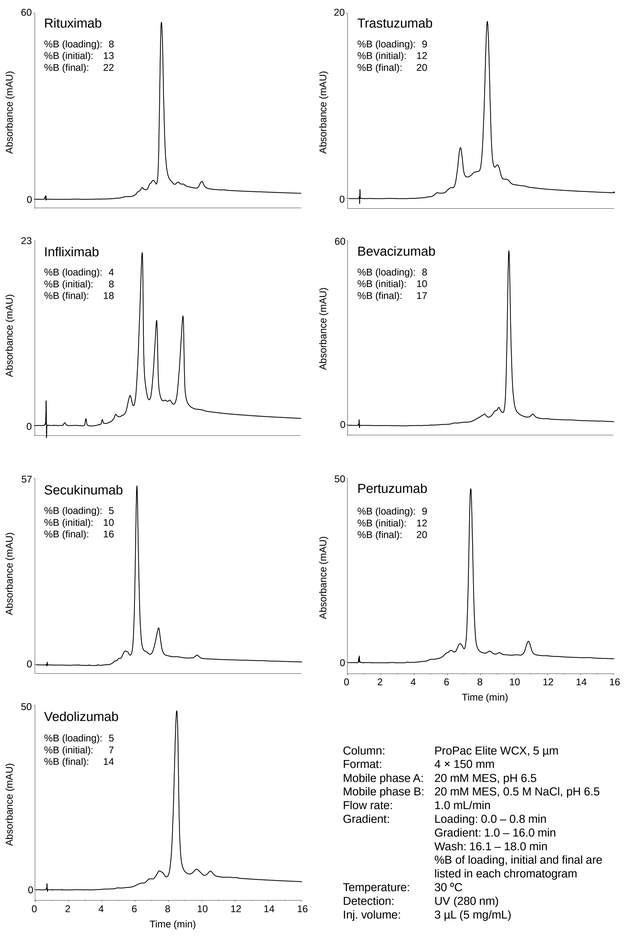
Chromatography data collection and analysis was performed using Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System Version 7.2.

RESULTS

Separation of IgG1 mAbs using Salt Gradient Method

Salt gradient methods have been widely used with weak cation exchange columns to separate charge variants of therapeutic mAbs. Salt gradient analysis typically requires method optimization to achieve the best separation. Based on preliminary experiments, MES buffer at pH 6.5 and sodium chloride were used as a general mobile phase system for the analysis of various IgG1 mAbs. The initial and final salt concentrations were optimized for each mAb. To avoid isocratic elution occurring during the loading of the sample, a short lower salt concentration loading period (0.8 minute) was added before the main salt gradient. With this approach all seven IgG1 mAbs showed high resolution separation of multiple acidic and basic variants.

Figure 1. Separation of IgG1 therapeutic mAbs using salt gradient method on the ProPac Elite WCX, 5 µm column.

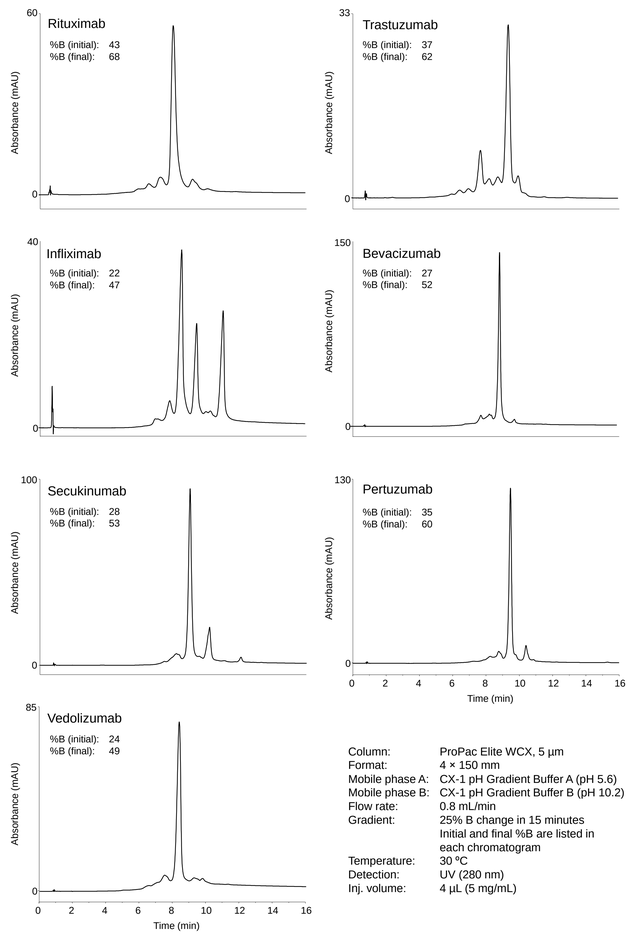


Column: ProPac Elite WCX, 5 µm
Format: 4 × 150 mm
Mobile phase A: 20 mM MES, pH 6.5
Mobile phase B: 20 mM MES, 0.5 M NaCl, pH 6.5
Flow rate: 1.0 mL/min
Gradient: Loading: 0.0 – 0.8 min
Gradient: 1.0 – 16.0 min
Wash: 16.1 – 18.0 min
%B of loading, initial and final are listed in each chromatogram
Temperature: 30 °C
Detection: UV (280 nm)
Inj. volume: 3 µL (5 mg/mL)

Separation of IgG1 mAbs using pH Gradient Method

pH gradient methods have been increasingly used as an alternative approach to salt gradient method when analyzing basic proteins on cation exchange columns. The CX-1 pH Gradient Buffers generate a linear pH gradient that simplifies method optimization for high resolution separation between the main product and impurities. Initially, separations of mAbs were achieved using a gradient from 20% to 70% CX-1 pH Gradient Buffer B over 15 minutes (data not shown). To further separate acidic and basic charge variants, method optimization was performed by running shallower gradients based on the retention time of each mAb (Figure 2). Initial and final percent mobile phase B used for individual mAbs are shown in each chromatogram. The pH gradient method showed higher resolution separation of trastuzumab while the salt gradient method showed higher resolution separation of pertuzumab. Other mAbs showed similar resolution when comparing the two gradient methods.

Figure 2. Separation of IgG1 therapeutic mAbs using pH gradient method on the ProPac Elite WCX, 5 µm column.



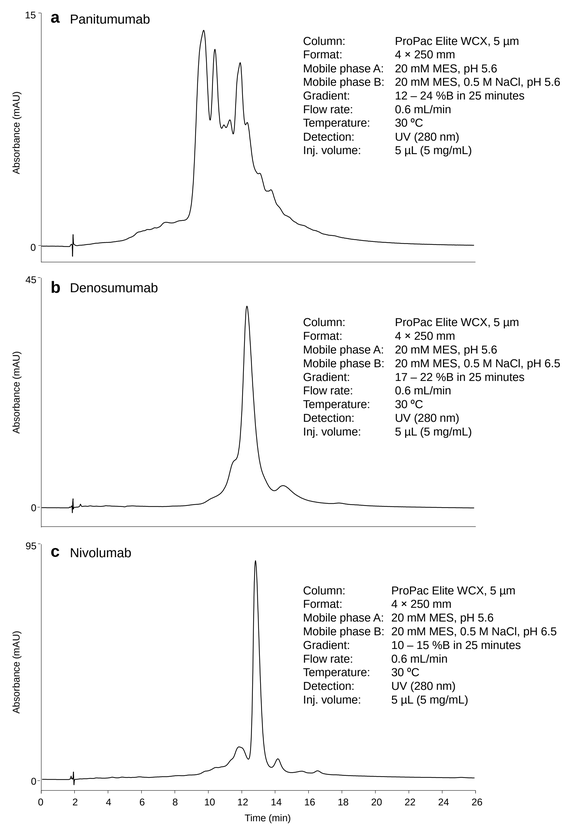
Column: ProPac Elite WCX, 5 µm
Format: 4 × 150 mm
Mobile phase A: CX-1 pH Gradient Buffer A (pH 5.6)
Mobile phase B: CX-1 pH Gradient Buffer B (pH 10.2)
Flow rate: 0.8 mL/min
Gradient: 25% B change in 15 minutes
Initial and final %B are listed in each chromatogram
Temperature: 30 °C
Detection: UV (280 nm)
Inj. volume: 4 µL (5 mg/mL)

Separation of IgG2 and IgG4 mAbs using Salt Gradient Method

Charge heterogeneity of two IgG2 and one IgG4 therapeutic mAbs—panitumumab, denosumab and nivolumab—was analyzed using the ProPac Elite WCX column. For salt gradient methods, the pH of the mobile phase was adjusted to be lower than the pI value of the protein and higher than the pKa of the acrylate groups (pKa ~4.5) to promote binding to the stationary phase. The pI of denosumab and nivolumab have been reported to be 8.9 and 8.0 respectively, while panitumumab has relatively lower pI of 6.8.[2] Therefore, panitumumab was analyzed at pH 5.6 and denosumab and nivolumab were analyzed at pH 6.5.

IgG2 antibodies have been shown to have three structural isoforms—IgG2-A, IgG2-B and IgG2-A/B—resulting from different cysteine disulfide bond formations between the light and heavy chains the hinge region.[3] Multiple peaks observed in the CEX separation are potentially a combination of different structural isoforms and charge variants. For panitumumab (Figure 3a), the ProPac Elite WCX column was able to separate more than 15 variants. Although denosumab is also an IgG2 based mAb, it separated into fewer peaks (Figure 3b). A distinct main basic variant was separated from the product peak while the main acidic variant was partially separated. Nivolumab, which is based on an IgG4 backbone, showed more distinct peaks compared to the IgG2 chromatograms. These results demonstrate that the high resolving power of the ProPac Elite WCX column is suitable for analysis of IgG2 and IgG4 mAbs.

Figure 3. Separation of IgG2 and IgG4 therapeutic mAbs using salt gradient method on the ProPac Elite WCX, 5 µm column.



Column: ProPac Elite WCX, 5 µm
Format: 4 × 250 mm
Mobile phase A: 20 mM MES, pH 5.6
Mobile phase B: 20 mM MES, 0.5 M NaCl, pH 5.6
Gradient: 12 – 24 %B in 25 minutes
Flow rate: 0.6 mL/min
Temperature: 30 °C
Detection: UV (280 nm)
Inj. volume: 5 µL (5 mg/mL)

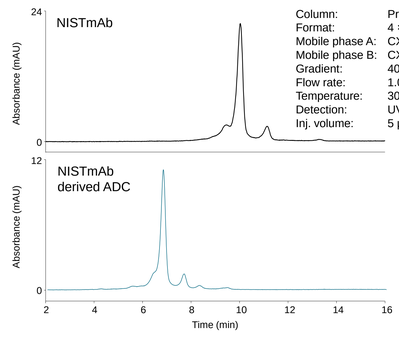
Column: ProPac Elite WCX, 5 µm
Format: 4 × 250 mm
Mobile phase A: 20 mM MES, pH 5.6
Mobile phase B: 20 mM MES, 0.5 M NaCl, pH 6.5
Gradient: 17 – 22 %B in 25 minutes
Flow rate: 0.6 mL/min
Temperature: 30 °C
Detection: UV (280 nm)
Inj. volume: 5 µL (5 mg/mL)

Column: ProPac Elite WCX, 5 µm
Format: 4 × 250 mm
Mobile phase A: 20 mM MES, pH 5.6
Mobile phase B: 20 mM MES, 0.5 M NaCl, pH 6.5
Gradient: 10 – 15 %B in 25 minutes
Flow rate: 0.6 mL/min
Temperature: 30 °C
Detection: UV (280 nm)
Inj. volume: 5 µL (5 mg/mL)

Separation of NISTmAb Derived ADC Standard using pH Gradient Method

ADCs have gained tremendous interest among pharmaceutical companies due to their significantly improved clinical efficacy over unmodified mAbs. Recently a NISTmAb derived ADC standard was developed to support the multitude of analytical analyses required for ADC characterization.[1] This standard was synthesized using the SiteClick™ site-specific antibody labeling technology, which results in the conjugation of two drugs per mAb. Figure 4 shows the separation of native NISTmAb and the Alexa Flour 488 (AF488) conjugated NISTmAb ADC on the ProPac Elite WCX column using the CX-1 pH Gradient Buffers. The acidic and basic variants of both NISTmAb and the NISTmAb derived ADC were separated from the main peak. In addition, the column showed high resolution separation of the ADC from the unmodified NISTmAb.

Figure 4. Separation of NISTmAb and NISTmAb derived ADC standard using pH gradient method on the ProPac Elite WCX, 5 µm column.



Column: ProPac Elite WCX, 5 µm
Format: 4 × 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: 40 – 65 %B in 15 minutes
Flow rate: 1.0 mL/min
Temperature: 30 °C
Detection: UV (280 nm)
Inj. volume: 5 µL (2.5 mg/mL)

CONCLUSIONS

- Wide range of pharmaceutical mAbs including IgG1, IgG2, IgG4 and ADC, were successfully separated on the new 5 µm weak cation exchange stationary phase using salt and/or pH gradient methods.
- When evaluating a new therapeutic protein sample, both salt gradient and pH gradient methods should be evaluated to determine the method that provides the best separation.

REFERENCES

1. Lin, S. et al. Development of NISTmAb-derived Antibody-drug Conjugate (ADC) Standards, ASMS (2018) Poster.
2. Goyon, A. et al. Determination of isoelectric points and relative charge variants of 23 therapeutic monoclonal antibodies, *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* **2017,** *1065–1066,* 119–128.
3. Wypych, J. Human IgG2 Antibodies Display Disulfide-mediated Structural Isoforms, *J. Biol. Chem.* **2008,** *283,* 16194.

TRADEMARKS/LICENSING

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