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APPLICATION NOTE 21843

Separation of IgG2 and IgG4 therapeutics using weak cation exchange chromatography

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Keywords

Biopharmaceutical, therapeutic mAbs, QA/QC, Critical quality attribute, therapeutic mAbs, IgG2, IgG4, denosumab, panitumumab, nivolumab, weak cation exchange chromatography, pH gradient, CX-1 pH Gradient Buffers, ProPac Elite WCX column, Vanquish Flex UHPLC, charge heterogeneity, charge variants

Application benefits

High-resolution separation of challenging IgG2 and IgG4 molecules

Goal

To demonstrate high-resolution separation of IgG2 and IgG4 therapeutic mAbs using the Thermo Scientific™ ProPac™ Elite WCX column

Introduction

Monoclonal antibodies (mAbs) are used to treat many diseases including rheumatoid arthritis, Alzheimer's disease, and different types of cancers. When developing these therapeutic mAbs, the immunoglobulin G (IgG) subclass is chosen depending on the desired mechanism of action. IgGs consist of four subclasses, named in order of decreasing abundance: IgG1, IgG2, IgG3, and IgG4. Most of the therapeutic mAbs on the market are based on the IgG1 subclass. The IgG1 and IgG3 subclasses have a greater ability to activate antibody-dependent cell mediated cytotoxicity (ADCC) and complement-dependent cytoxtoxicity (CDC) than the IgG2 and IgG4 subclasses. This feature is often desired for treatment of cancers. Although both IgG1 and IgG3 are capable of ADCC and CDC effector functions, IgG3 has not been utilized due to its shorter circulation half-life.[1] IgG2 and IgG4 subclasses are chosen when the goal is to neutralize soluble antigens and effector functions are not desired nor necessary. In addition to the difference

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in their ability to trigger effector functions, IgG2 and IgG4 antibodies have different *in vivo* properties and structural features that need to be considered when developing therapeutic mAbs. For instance, both have shorter hinge length and different exposed residues on the constant domains compared to the IgG1 subclass. IgG2 has four disulfide bonds, while IgG1 and IgG4 have two. IgG2 forms covalent dimers between identical or different IgG2 molecules with intermolecular disulfide bonds, whereas IgG4 has the capability to form a half-molecule with intra-chain disulfide bonds *in vivo*.[1] These properties of IgG2 and IgG4 may contribute to the effectiveness of these subclasses, but also cause greater challenges for the quality control characterization of structure and charge heterogeneity.

Ion exchange chromatography has been widely used to detect charge variants of mAbs. Most IgGs have a pI from 6 to 10 resulting in a positive charge for the protein under most buffer conditions. This makes cation exchange chromatography an effective method for mAb charge variant analysis. In addition, cation exchange chromatography has been shown to be useful for studying aggregation and degradation in mAbs.[2] Analysis of charge variants of IgG1 mAbs using weak cation exchange chromatography is discussed in other application notes.[3,4] Here we demonstrate the use of weak cation exchange chromatography to separate variants of IgG2- and IgG4-based therapeutic mAbs. Since it has been reported that variants of IgG2 molecules are challenging to separate,[5,6] we used the ProPac Elite WCX column, which is based on a 5 µm particle size. The nonporous divinylbenzene resin of the ProPac Elite WCX column is coated with a proprietary hydrophilic layer. This hydrophilic layer ensures high recovery and minimal secondary interactions. The hydrophilic resin is then grafted with acrylate groups, which provide weak cation exchange functionality. The column chemistry has been designed for high-resolution separation of charge variants of proteins including mAbs.

Experimental

Reagents and consumables

- Deionized (DI) 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 equipped with a PCM-3000 pH and Conductivity Monitor

Column

- ProPac Elite WCX column, 4 × 250 mm (P/N 303025)

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

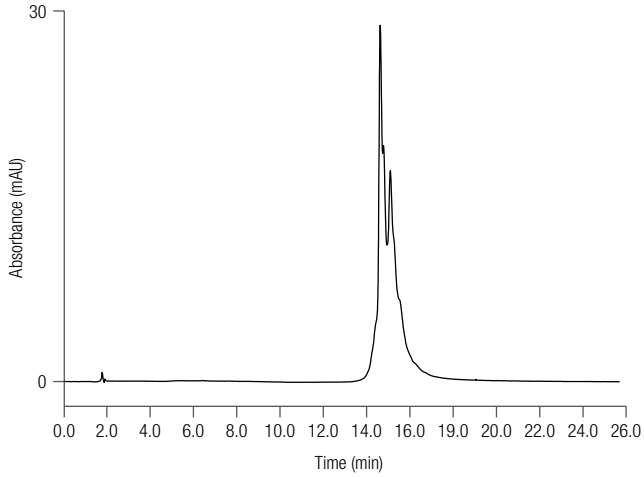
The Thermo Scientific™ Chromeleon™ Chromatography Data System was used for data acquisition and analysis.

Results and discussion

Charge heterogeneity of two IgG2 and one IgG4 therapeutic mAbs—panitumumab, denosumab, and nivolumab—was analyzed using the ProPac Elite WCX column. When developing a salt gradient method, the pH of the mobile phase should be lower than the pI value of the protein to promote binding of the analyte to the stationary phase. However, the pH should not be lower than the pKa of the carboxylic acid functional groups (~4.5) on the stationary phase. Denosumab and nivolumab have been reported to have pI values of 8.9 and 8.0, respectively, while panitumumab has a relatively lower pI of 6.8.[7] Therefore, panitumumab was analyzed at pH 5.6, whereas denosumab and nivolumab were analyzed at pH 6.5. First a 20 to 200 mM NaCl gradient was performed for all mAbs to determine the salt concentration for potein elution. From these results a shallower gradient was developed and used to maximize the resolution of charge variants (Figures 1, 2, and 3).

The ProPac Elite WCX column was able to separate more than 15 variants of panitumumab (Figure 1b) when the shallower gradient was applied. IgG2 antibodies have been shown to have three structural isoforms—IgG2-A, IgG2-B, and IgG2-A/B—resulting from different disulfide bond formations between light chains and heavy chains from the cysteine residues in the hinge region.[6] Multiple peaks observed in the CEX separation for panitumumab are likely a combination of different structural isoforms and charge variants. Although denosumab is also an IgG2-based therapeutic mAb, it separated into fewer peaks when analyzed on the ProPac Elite WCX column. The separation of the main basic variant improved significantly with the optimized shallower gradient (Figure 2). In addition, the main acidic variant is partially separated. Nivolumab, which is based on an IgG4 backbone, showed more distinct peaks that resemble IgG1 separations compared to the IgG2 chromatograms. These results demonstrate the high resolving power of the ProPac Elite WCX column suitable for analysis of IgG2 and IgG4 mAbs.

a

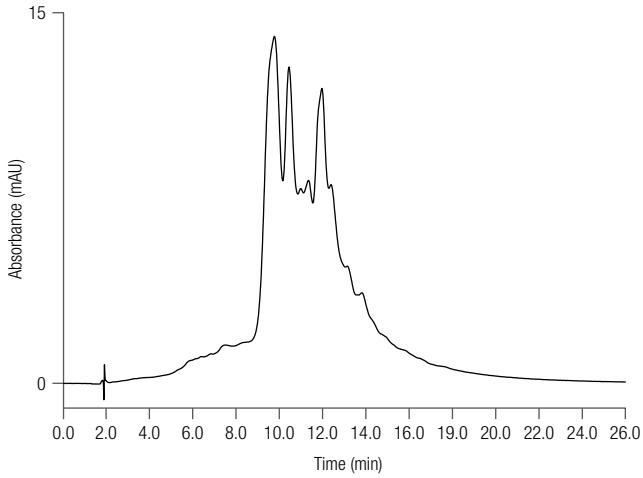


Column:	ProPac Elite WCX, 5 µm	Flow rate:	0.6 mL/min
Format:	4 × 250 mm	Temperature:	30 °C
Mobile phase A:	20 mM MES, pH 5.6	Detection:	UV (280 nm)
Mobile phase B:	20 mM MES, 0.5 M NaCl, pH 5.6	Inj. volume:	2 µL (5 mg/mL)

Gradient:

Time (min)	%A	%B
0.0	96	4
1.0	96	4
26.0	60	40
26.1	0	100
28.0	0	100
28.1	96	4
40.0	96	4

b



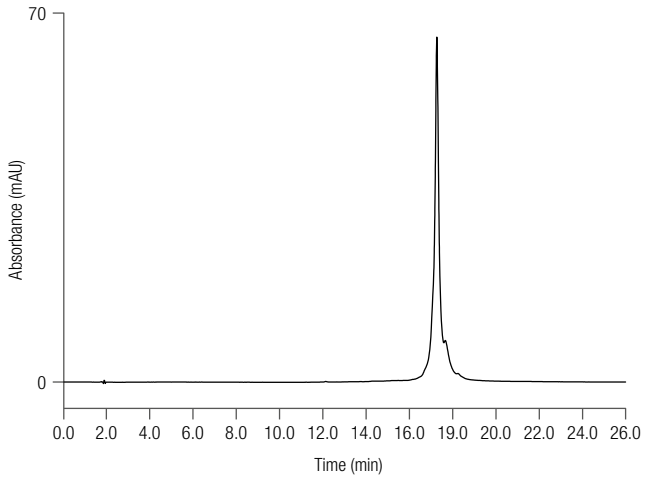
Column:	ProPac Elite WCX, 5 µm	Flow rate:	0.6 mL/min
Format:	4 × 250 mm	Temperature:	30 °C
Mobile phase A:	20 mM MES, pH 5.6	Detection:	UV (280 nm)
Mobile phase B:	20 mM MES, 0.5 M NaCl, pH 5.6	Inj. volume:	5 µL (5 mg/mL)

Gradient:

Time (min)	%A	%B
0.0	88	12
1.0	88	12
26.0	76	24
26.1	0	100
28.0	0	100
28.1	88	12
40.0	88	12

Figure 1. Analysis of panitumumab. a) 20 to 200 mM NaCl gradient, b) 60 to 120 mM NaCl gradient

a

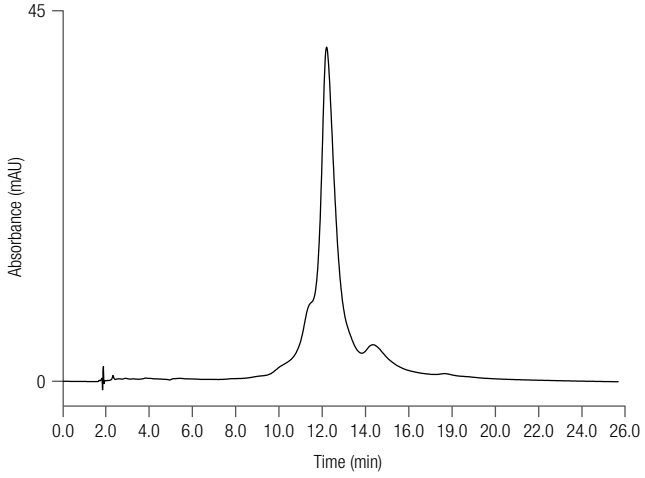


Column:	ProPac Elite WCX, 5 µm	Flow rate:	0.6 mL/min
Format:	4 × 250 mm	Temperature:	30 °C
Mobile phase A:	20 mM MES, pH 6.5	Detection:	UV (280 nm)
Mobile phase B:	20 mM MES, 0.5 M NaCl, pH 6.5	Inj. volume:	2 µL (5 mg/mL)

Gradient:

Time (min)	%A	%B
0.0	96	4
1.0	96	4
26.0	60	40
26.1	0	100
28.0	0	100
28.1	96	4
40.0	96	4

b



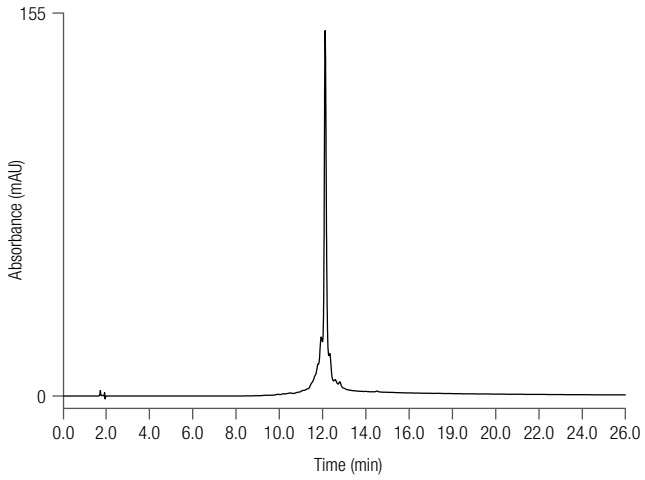
Column:	ProPac Elite WCX, 5 µm	Flow rate:	0.6 mL/min
Format:	4 × 250 mm	Temperature:	30 °C
Mobile phase A:	20 mM MES, pH 6.5	Detection:	UV (280 nm)
Mobile phase B:	20 mM MES, 0.5 M NaCl, pH 6.5	Inj. volume:	5 µL (5 mg/mL)

Gradient:

Time (min)	%A	%B
0.0	83	17
1.0	83	17
26.0	78	22
26.1	0	100
28.0	0	100
28.1	83	17
40.0	83	17

Figure 2. Analysis of denosumab. a) 20 to 200 mM NaCl gradient, b) 95 to 120 mM NaCl gradient

a

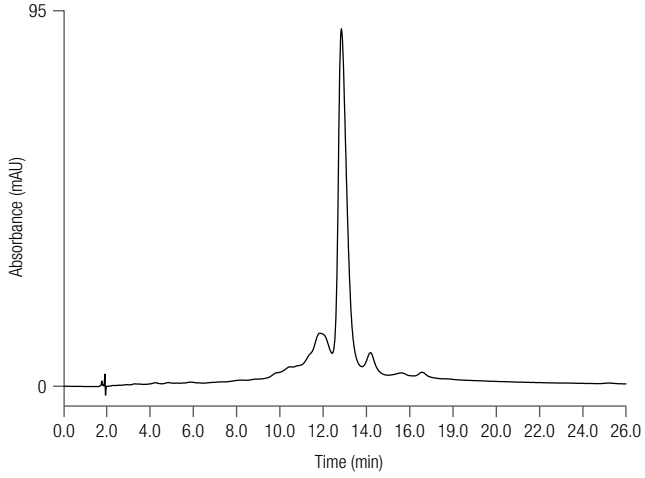


Column:	ProPac Elite WCX, 5 µm	Flow rate:	0.6 mL/min
Format:	4 × 250 mm	Temperature:	30 °C
Mobile phase A:	20 mM MES, pH 6.5	Detection:	UV (280 nm)
Mobile phase B:	20 mM MES, 0.5 M NaCl, pH 6.5	Inj. volume:	2 µL (5 mg/mL)

Gradient:

Time (min)	%A	%B
0.0	96	4
1.0	96	4
26.0	60	40
26.1	0	100
28.0	0	100
28.1	96	4
40.0	96	4

b



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

Gradient:

Time (min)	%A	%B
0.0	90	10
1.0	90	10
26.0	85	15
26.1	0	100
28.0	0	100
28.1	90	10
40.0	90	10

Figure 3. Analysis of nivolumab. a) 20 to 200 mM NaCl gradient, b) 50 to 75 mM NaCl gradient

Conclusion

The ProPac Elite WCX column was able to separate acidic and basic variants of challenging IgG2 and IgG4 therapeutic mAbs.

References

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