Characterize mAb Charge Variants by Cation-Exchange Chromatography

Application Note

Biologics and Biosimilars

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Abstract

This application note describes the use of the Agilent Bio MAb weak cation-exchange column in measuring charge variants of a monoclonal antibody, and the subsequent characterization of these variants after peak collection. Analysis was achieved by reversed-phase liquid chromatography and mass spectrometry.

Introduction

Biopharmaceuticals, in particular monoclonal antibodies (mAbs) have grown in importance in recent years. During mAb manufacturing and long-term storage, modifications can arise that can alter the safety and efficacy of the product. Modifications such as deamidation, lysine truncation, and pyroglutamate formation result in charge heterogeneity. Ion-exchange chromatography is an excellent tool to measure and quantify these charge variants. Since most therapeutic mAbs have a higher proportion of basic residues, cation-exchange chromatography (CEX) is commonly used.

We used an Agilent Bio MAb weak cation-exchange (WCX) column to separate charge variants of the monoclonal antibody trastuzumab, marketed as Herceptin, and the subsequent characterization of the variants by reversed-phase liquid chromatography (RPLC) and mass spectrometry (MS). The Agilent Bio MAb weak cation-exchange column is composed of rigid, spherical, highly cross-linked polystyrene divinylbenzene (PS-DVB) nonporous beads grafted with a hydrophilic, polymeric layer, virtually eliminating nonspecific binding of mAbs. The uniform, densely packed, weak cation-exchange layer is chemically bonded to the polymeric coating.

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Experimental

Materials

Acetonitrile and water were obtained from Biosolve B. V. (Valkenswaard, The Netherlands). Trifluoroacetic acid, *tris*(hydroxymethyl)amino methane (*tris*), NaH_2PO_4, $Na_2HPO_4 \cdot 2H_2O$, NaCl, and dithiothreitol (DTT) were purchased from Sigma-Aldrich, Corp. (St. Louis, MO, USA). Gibco UltraPure 1 M *tris*-HCl pH 7.5 was obtained from Invitrogen (Carlsbad, CA, USA). Humanized monoclonal antibody trastuzumab, marketed as Herceptin, was obtained from Roche (Basel, Switzerland).

Sample preparation

Prior to injection, Herceptin was diluted to 2.1 mg/mL in mobile phase A. pH-stressed mAb was obtained by incubating Herceptin at 37 °C for 1 and 3 days in 1 M *tris* pH 9.0. Collected CEX fractions were diluted using DTT at a final concentration of 10 mM.

Instrumentation

Cation-exchange measurements were performed on:

- Agilent 1100 Series Degasser (G1322A)
- Agilent 1100 Series Binary Pump (G1312A)
- Agilent 1100 Series Autosampler (G1367A)
- Agilent 1200 Series Thermostat for autosampler (G1330B)
- Agilent 1100 Series Thermostatted Column Compartment (G1316A)
- Agilent 1100 Series Diode Array Detector (G1315A)
- Agilent 1200 Series Analytical Fraction Collector (G1364C)

LC/MS measurements were performed on an Agilent 6540 Ultra High Definition (UHD) Accurate-Mass Q-TOF with Agilent Jet Stream LC/MS (G6540A) and an Agilent 1290 Infinity LC System equipped with:

- Agilent 1290 Infinity Binary pump (G4220B)
- Agilent 1290 Infinity Autosampler (G4226A)
- Agilent 1290 Infinity Thermostat for autosampler (G1330B)
- Agilent 1290 Infinity Thermostatted Column Compartment (G1316C)
- Agilent 1290 Infinity Diode Array Detector (G4212A)

Software

- Agilent OpenLAB CDS ChemStation revision C.01.05 (35)
- Agilent MassHunter for instrument control (B05.01)
- Agilent MassHunter for data analysis (B06.00)
- Agilent BioConfirm software for MassHunter (B06.00)

Instrumental conditions

Cation-exchange conditions

Column:	Agilent Bio MAb NP5, 2.1 × 250 mm, 5 µm, PEEK (p/n 5190-2411)
Mobile phase:	A) 10 mM phosphate pH 7.65 B) 10 mM phosphate pH 7.65 + 100 mM NaCl
Flow rate:	200 µL/min
Injection volume:	16 µL (32 µg on-column)
Column temperature:	30 °C
Gradient:	Time (min) % B 0 5 36 70 36.5 100 46 100 46.5 5 70 5
Detection:	UV at 214 nm/8 nm
Fraction collection:	Time-based

RPLC-UV/MS conditions

Column:	Agilent ZORBAX RRHD 300SB-C8, 2.1 × 100 mm, 1.8 µm (p/n 858750-906)
Mobile phase:	A) 0.1% TFA in water (v/v) B) 0.1% TFA in acetonitrile (v/v)
Flow rate:	200 µL/min
Injection volume:	20 µL
Needle wash solvent:	Mobile phase B
Autosampler temperature:	7 °C
Column temperature:	80 °C
Gradient:	Time (min) % B 0 30 2 30 25 38.3 25.10 80 29 80 29.1 30 37 30
Detection:	UV at 214 nm/8 nm
Q-TOF source:	Agilent JetStream positive ionization mode
Drying gas temperature:	300 °C
Drying gas flow rate:	8 L/min
Nebulizer pressure:	35 psig
Sheath gas temperature:	350 °C
Sheath gas flow rate:	11 L/min
Nozzle voltage:	1,000 V
Capillary voltage:	3,500 V
Fragmentor voltage:	200 V
Q-TOF detection:	Mass range 3,200 amu
Data acquisition range:	500 to 3,200 *m/z*
High resolution mode (4 GHz)	
Data acquisition rate:	1 spectrum per s
Profile acquisition	

Results and Discussion

Figure 1 shows the overlaid UV 214 nm chromatograms of five replicate measurements of Herceptin on the Agilent Bio MAb WCX column. The precision offered makes the technology attractive for the comparison of different mAb production batches. Herceptin batch-to-batch consistency as revealed by CEX is shown in Figure 2 and Table 1.

Table 1. Relative percentage of cation-exchange pre-peaks, main peak, and post-peaks of three production lots of Herceptin.

Production lot	% CEX pre-peaks	% CEX main peak	% CEX post-peaks
1	27.8	57.7	14.5
2	29.7	59.5	10.8
3	28.6	60.3	11.1

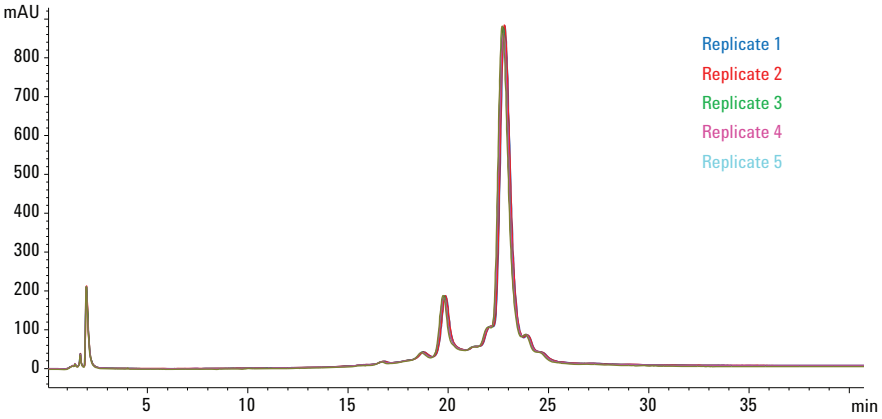

Figure 1. Overlay of UV 214 nm cation-exchange chromatograms of five consecutive replicate injections of Herceptin on an Agilent Bio MAb NP5 column.

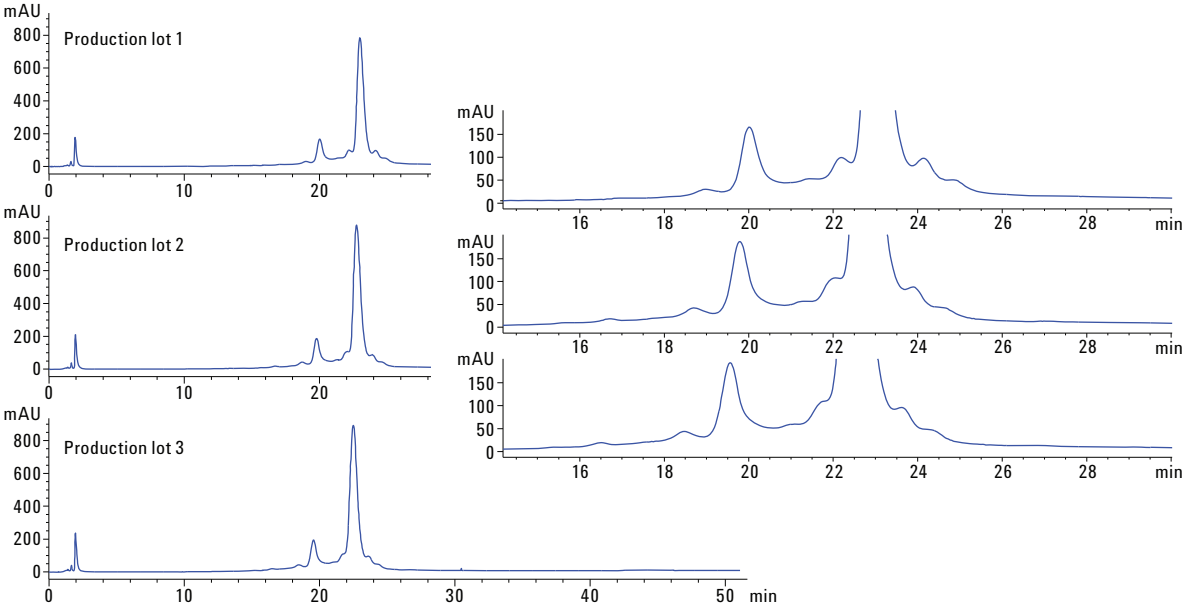

Figure 2. Herceptin batch-to-batch consistency as revealed by cation-exchange chromatography on an Agilent Bio MAb column.

Figure 3 shows the CEX analysis of nonstressed and pH-stressed Herceptin. Stressed samples were obtained by incubating Herceptin at high pH for 1 and 3 days. These conditions are known to induce deamidation of asparagine to aspartic acid. This post-translational modification (PTM) changes the charge of the protein, that is, the mAb becomes more acidic. As shown in Figure 3, the high pH conditions give rise to several acidic mAb variants.

The peaks in Figure 3 were characterized by RPLC-UV/MS using an Agilent ZORBAX RRHD 300SB-C8 column following CEX peak collection and reduction using DTT. The RPLC-UV chromatograms of CEX peaks 1 and 2 of the 1-day pH-stressed mAb, shown in Figure 4, clearly demonstrate the separation of the light-chain variants from the heavy-chain variants, whereby peaks A and B of the light chain are representative for the differences between CEX peaks 1 and 2.

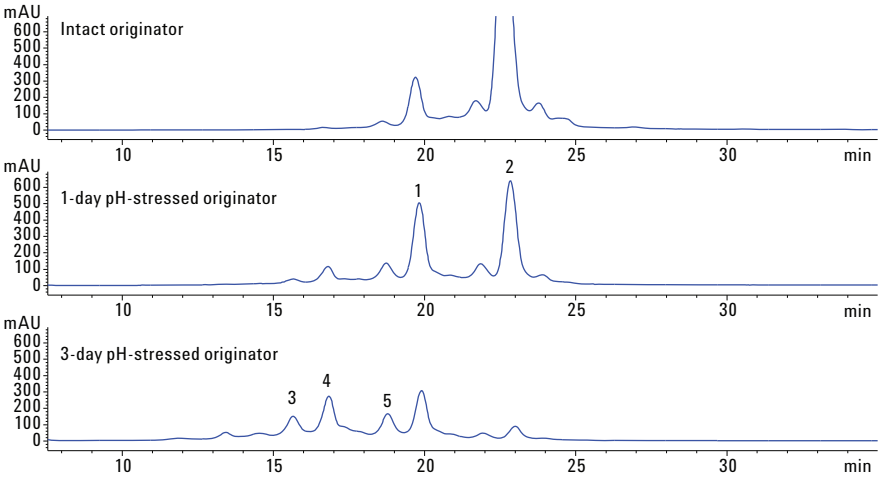

Figure 3. Cation-exchange separation of deamidated variants of intact Herceptin. Comparison between nonstressed and pH-stressed antibodies (1 day and 3 days). The annotated peaks were collected and further characterized using RPLC-UV/MS following reduction.

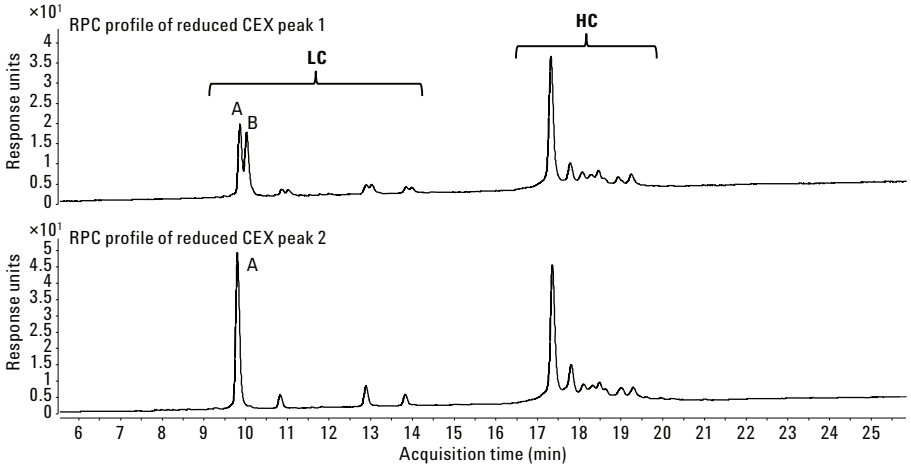

Figure 4. UV 214 nm reversed-phase chromatograms of the two collected and reduced CEX peaks of a 1-day pH-stressed mAb using an Agilent ZORBAX RRHD 300SB-C8 column.

The deconvoluted MS spectra of RPLC peaks A and B reveal a 1 Dalton mass difference between both peaks, which is indicative of a deamidation event taking place (Figure 5). The chromatographic profile of CEX peak 1 reveals an equimolar ratio of peaks A and B, indicating that this charge variant is composed of one deamidated and one nondeamidated light chain.

The same approach was applied for the identification of the additional CEX peaks appearing in the 3-day pH-stressed mAb. Figure 6 shows the RPLC-UV profiles of CEX peaks 3 to 5. Whereas for CEX peaks 1 and 5, both RPLC peaks A and B are present in equimolar quantities, almost the entire peak A is converted into peak B for CEX peaks 3 and 4. From this, it can be concluded that for CEX peaks 1 and 5, only one light chain of the mAb is deamidated, whereas CEX peaks 3 and 4 represent mAb variants where both light chains are deamidated.

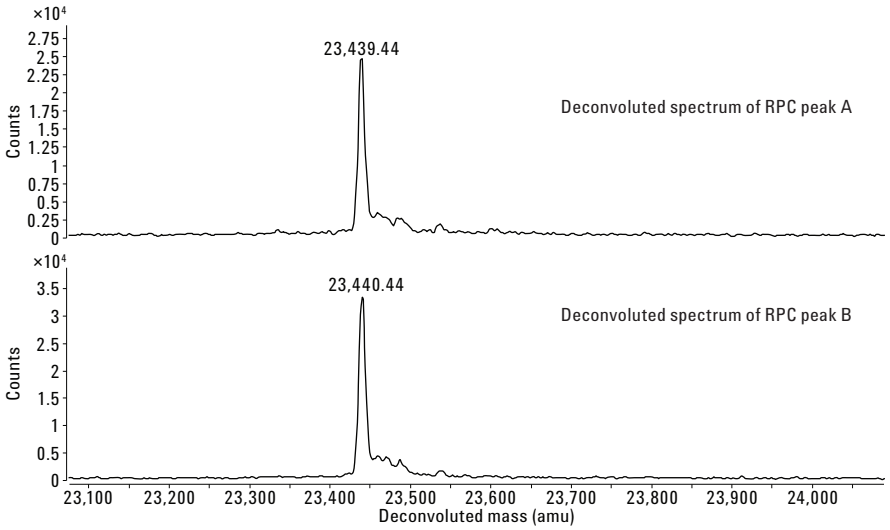

Figure 5. Deconvoluted spectra of the reversed-phase peaks A and B (see Figure 4).

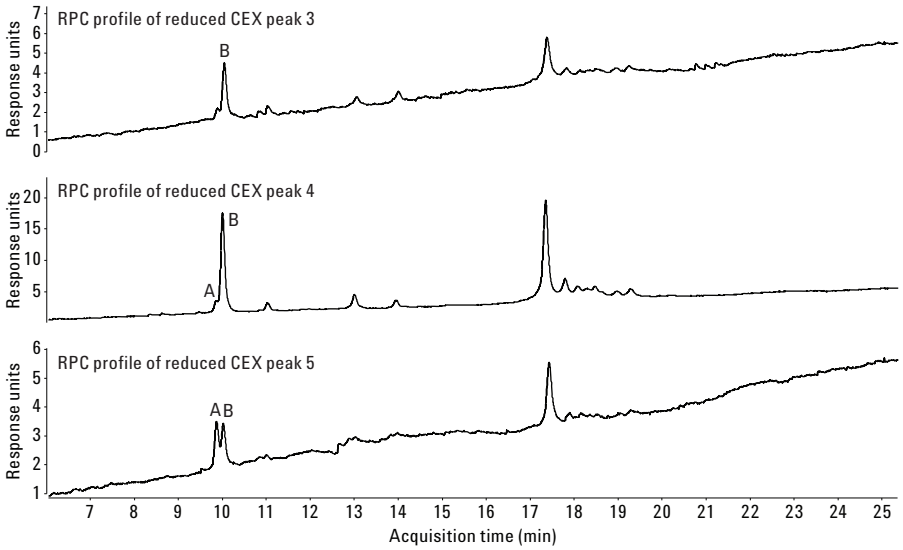

Figure 6. UV 214 nm reversed-phase chromatograms of the three collected and reduced CEX peaks of a 3-day pH-stressed mAb using an Agilent ZORBAX RRHD 300SB-C8 column.

Conclusions

The Agilent Bio MAb weak cation-exchange column successfully separated charge variants of an intact monoclonal antibody. Following peak collection, charge variants could be further characterized by reversed-phase chromatography with UV/MS detection.

For More Information

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