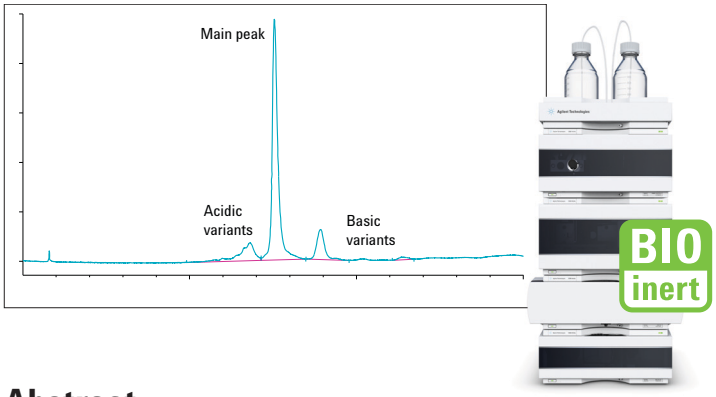
High-resolution Analysis of Charge Heterogeneity in Monoclonal Antibodies Using pH-gradient Cation Exchange Chromatography

Agilent 1260 Infinity Bio-inert Quaternary LC System with Agilent Bio Columns

Application Note

Biopharmaceuticals, Biotherapeutics



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Abstract

Antibody charge variants have gained considerable attention in the biotechnology industry due to their potential influence on stability and biological activity. Subtle differences in the relative proportions of charge variants are often observed during routine manufacture or process changes and pose a challenge when demonstrating product comparability. These changes include differences in glycosylation, deamidation, oxidation, isomerization, incomplete C-terminal processing, and other post-transitional modifications that modify the isoelectric pH (pI) values. In the biotechnology industry, ion-exchange chromatography is widely used for profiling the charge heterogeneity of proteins, including monoclonal antibodies. This Application Note describes a high-resolution, pH-based separation of acidic and basic charge variants for monoclonal antibodies using the Agilent 1260 Infinity Bio-inert Quaternary LC System and an Agilent BiomAb PEEK 4.6 × 250 mm, 5-µm ion exchange column that features a unique resin specifically designed for the charge-based separation of mAbs. The robustness of the method for routine analysis was established by validation studies.

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Introduction

Monoclonal antibodies (mAb) are glycoproteins of the immunoglobulin (Ig) family. MAbs have become the most rapidly growing class of biotherapeutics in the development for many different disease conditions. Novel mAb molecules are entering clinical studies at a rate of almost 40 per year, and the research pipeline includes approximately 250 therapeutic mAbs in clinical studies. There is steadily increasing need for an analytical method that can be used for high-throughput analysis of a large number of samples to support bioprocesses and formulation development. Biotherapeutics, such as mAbs, are complex molecules, and a variety of methods is required to monitor the heterogeneities associated with the mAb to ensure product quality and consistency.[1] Cation exchange chromatography is the gold standard for charge-sensitive antibody analysis. In cation exchange chromatography, method parameters often need to be optimized for each individual protein as ion exchange is dependent on the reversible adsorption of the charged protein molecules to immobilized ion exchange groups. Several authors have made significant progress in demonstrating practical separations using pH changes in the mobile phase to elute the proteins.[2] The Agilent ion exchange column family offers strong cation exchange (SCX), weak cation exchange (WCX), strong anion exchange (SAX) and weak anion exchange (WAX). The Agilent Bio MAb NP 5 (nonporous, 5-µm) PEEK, 4.6 × 250 mm, column is specifically designed to characterize the charge heterogeneity of monoclonal antibodies, including C-terminal lysine variance. The column offers even higher resolution, enabling better peak identification and accurate quantification. This Application Note describes a pH gradient based method for separating the charge variants of IgG1 using a 1260 Infinity Bio-inert Quaternary LC System and a Bio MAb NP 5 PEEK, 4.6 × 250 mm, ion exchange column. Method validation and robustness of an optimized ion exchange method are described.

Equipment

Instrumentation

A completely biocompatible Agilent 1260 Infinity Bio-inert Quaternary LC System operating up to a maximum pressure of 600 bar was used for the experiments (Table 1). The entire sample flow path is free of any metal components so that the sample does not come in contact with metal surfaces. Solvent delivery is free of any stainless steel or iron components.

Description	Model number
Agilent 1260 Infinity Bio-inert Quaternary Pump	G5611A
Agilent 1260 Infinity Bio-inert High Performance Autosampler	G5667A
Agilent 1290 Infinity Thermostat (for autosampler)	G1330B
Agilent 1260 Infinity Thermostatted Column Compartment with bio-inert click-in heating elements (option 019)	G1316C
Agilent 1260 Infinity Diode Array Detector with 60-mm Max-Light high sensitivity flow cell (option 033)	G4212B

Table 1
Configuration of the Agilent 1260 Infinity Bio-inert Quaternary LC System.

Software

Agilent OpenLAB CDS ChemStation Edition, revision C.01.04.

Ion Exchange Chromatography Parameters

Table 2 shows the Chromatographic parameters for Ion Exchange Chromatography using Agilent 1260 Infinity Bio-inert LC System.

Reagents, samples and materials

Human monoclonal antibody IgG1 was a proprietary pharmaceutical molecule. Sodium phosphate dibasic dihydrate, sodium phosphate monobasic dihydrate, sodium chloride, sodium bicarbonate hydrochloric acid (HCl), and sodium hydroxide (NaOH) were purchased from Sigma Aldrich. All the chemicals and solvents used were HPLC grade and high purity water from Milli Q water purification system (Millipore Elix 10 model, USA) was used.

Procedure

Mobile phase A was 10 mM sodium phosphate, pH 6.0 and mobile phase B was 10 mM sodium phosphate, pH 9.5. Monoclonal antibodies were diluted to approximately 2 mg/mL in mobile phase A and elution was monitored at 220 nm and 280 nm. Area and retention time (RT) were used to calculate standard deviation (SD) and relative standard deviation (%RSD). For each elution, the column was pre-equilibrated with at least three column volumes of mobile phase A prior to sample injection. After the injection of the monoclonal antibody sample onto the column, a linear increase in the percentage of mobile phase B was delivered. The linear gradients were run from 0 to 100% B in 27 minutes at 1 mL/min flow rate. After the gradient, the mobile phase was pumped at 100% B until at least one column volume passed before the composition was returned to 100% A in preparation for the subsequent analysis. Relative percent area was used to quantify the charge variants of monoclonal antibodies.

Robustness Study

The four critical method parameters listed below were varied to validate the IEX procedure.

- Variation of injection volume (± 10%)
- Variation of buffer pH (± 0.2)
- Variation of flow rate (± 2%)
- Variation of column temperature (± 5%)

For each robustness parameter, 10 µL of IgG1 was injected six times to calculate average area and RT. The percentage deviation (% accuracy) of area and retention time (RT) was calculated from the optimized method.

Parameter	Conditions	
Column:	Agilent Bio MAb PEEK, 4.6 × 250 mm, 5 µm column (p/n 5190-2407)	
Mobile phase A:	10 mM sodium phosphate buffer, pH 6.0	
Mobile phase B:	10 mM sodium bicarbonate buffer, pH 9.5	
Gradient:	Time (min)	Mobile phase (% B)
	0 minutes	0% B
	25 minutes	100% B
	27 minutes	100% B
	30 minutes	0% B
Injection volume:	10 µL (needle with wash, flush port active for 7 seconds)	
Flow rate:	1.0 mL/min	
Data acquisition:	214 and 280 nm	
Acquisition rate:	20 Hz	
Flow cell:	60-mm path	
Column temperature:	30 °C	
Sample thermostat:	5 °C	
Post time:	5 minutes	

Table 2
Chromatographic parameters used for IEX chromatography.

Results and Discussion

Separation and detection

The Agilent Bio MAb columns are highly uniform, densely packed, weak cation exchange resin. This Application Note used the 5-µm column that features a unique resin specifically designed for high-resolution charge-based separations of monoclonal antibodies. The peaks of the ion exchange profiles were typically denoted into three distinct components[3]. Early and late-eluting peaks were called acidic and basic variants, respectively. The most abundant peak was designated as the main peak. Figure 1 shows the optimized cation exchange elution profile of IgG1 on Bio MAb PEEK column demonstrating excellent separation of IgG1 in 30 minutes into three distinct peaks: basic variants, main peak, and acidic variants. The overlay of six replicates of IgG1 shows excellent separation reproducibility (Figure 2). The high resolution separation of IgG1 facilitated the quantification of charge variants using peak areas (Figure 3). The relative peak areas for the charge variants of IgG1 are shown in Table 3. The IgG1 possessed approximately 9.97% of acidic variants, 76.92% main peak and 13.21% basic variants of the total peak area, respectively. The reproducibility of analysis was tested with six replicates.

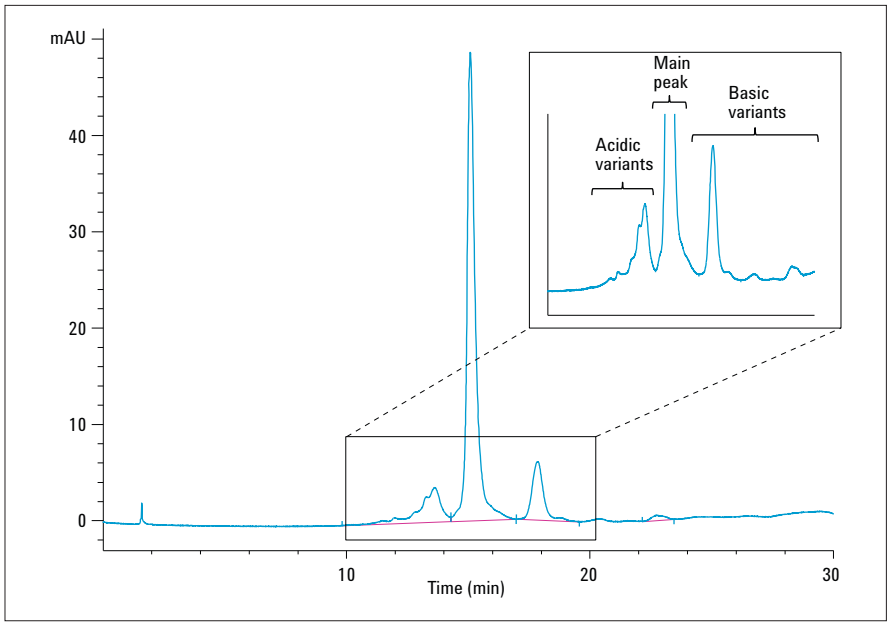

Figure 1
Elution profile of pH based separation of charge variants of IgG1 on an Agilent Bio MAb PEEK, 4.6 × 250 mm, 5-µm column. The acidic, main peak and basic variants are enlarged in the magnified view.

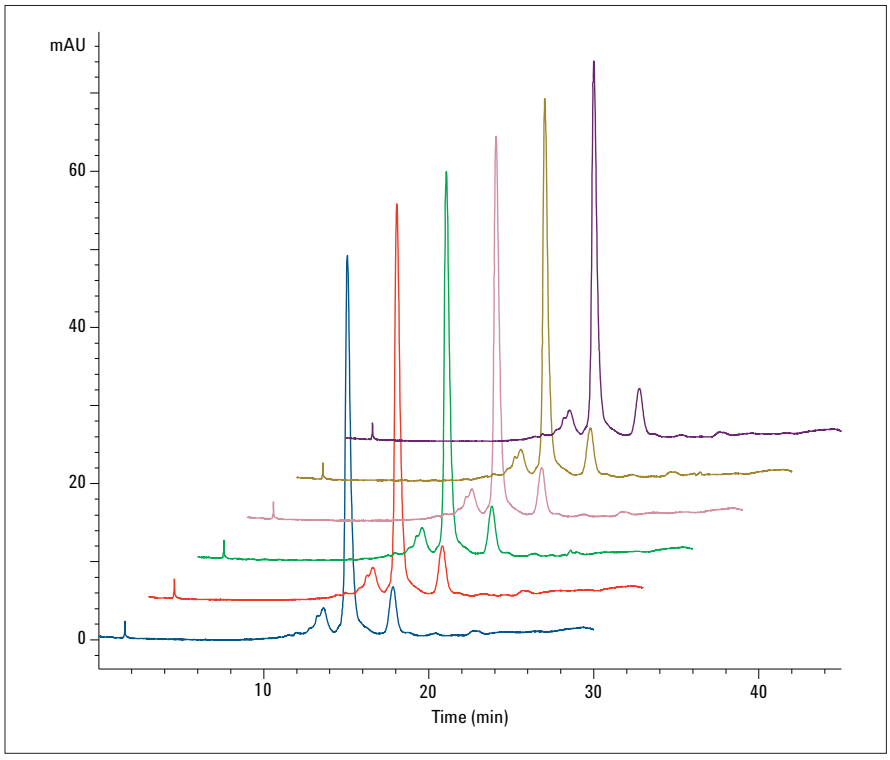

Figure 2
Overlay of six replicates of IgG1 on an Agilent 1260 Infinity Bio-inert Quaternary LC System using an Agilent Bio MAb PEEK, 4.6 × 250 mm, 5 µm column.

Precision of Retention Time and Area

The precision of a procedure expresses the closeness of agreement between a series of measurements obtained from multiple analyses of the homogeneous sample under the prescribed conditions and often expressed as relative standard deviation (RSD). Table 4 shows the average retention times and area RSDs from six replicates of an IgG1 injection. The retention time and peak area RSDs for the main peak were 0.106% and 1.60% respectively which demonstrates excellent reproducibility of the method and thus the precision of the system.

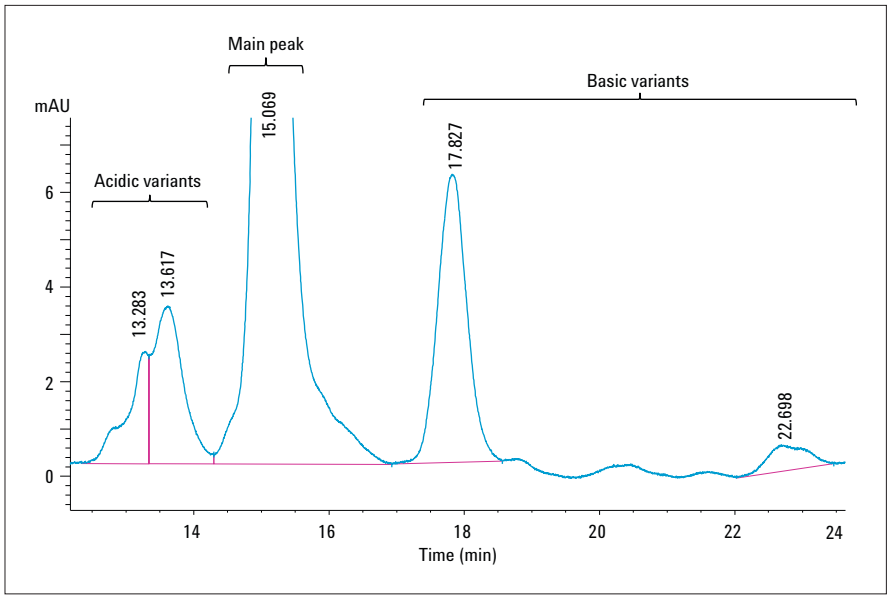

Figure 3
Expanded scale chromatogram of pH gradient-based cation exchange chormatogram of IgG1 separation using an Agilent Bio MAb PEEK, 4.6 × 250 mm, 5-µm column.

	RT (min)	Area %
Acidic variants	13.28	9.87
	13.61	
Main peak	15.058	76.92
Basic variants	17.82	13.21
	22.69	

Table 3
Charge variants quantification by area %, n = 6.

	Retention time	Peak area
Mean (min)	15.058	1172
RSD	0.106	1.60

Table 4
Retention time and area RSD (%), n = 6 for main peak.

Robustness

The robustness of an analytical procedure is the persistence of a method under perturbations or conditions of uncertainty and provides an indication of its consistency during routine use. To evaluate the robustness of the method, four critical parameters of the optimized method were varied (Table 5). Allowed deviations for RT and area RSD were set to ± 3.0% and ± 5% respectively. The red numbers indicate where the result exceeded the allowed deviation. The impact of injection volume, column temperature, buffer pH and flow rate on RT and area RSD was within the acceptable limits. A variation in injection volume by ± 10% compared to the actual method caused the area RSD to deviate significantly; however, this deviation is an expected due to the load on the ion exchange column. There were no further significant changes in the chromatographic pattern when deliberate variations were made in experimental conditions, thus showing the method is robust. Our results show that the method is reliable for routine QA/QC application for manufacturing and storage consistency. However, some parameters such as injection volume are critical and must be carefully controlled.

Parameters	Variations	RT deviation (limit: ± 3.0 %)	Area deviation (limit: ± 5.0 %)
		Main peak	
Variation in injection volume (10 µL ± 10%)	– 1 µL	– 0.19	10.49
	+ 1 µL	0	– 9.89
Variation in column temperature (30 °C ± 5%)	– 5%	– 1.19	2.73
	+ 5%	0.66	2.13
Variation in buffer pH (6.0 ± 0.2)	– 0.2	0.199	– 0.68
	+ 0.2	0.99	– 0.08
Variation of flow rate (1.0 ± 2%)	– 2%	0.66	2.73
	+ 2%	0	– 1.10

Table 5
Robustness (RT and Area % RSD) n = 6.

Conclusion

Cation exchange chromatography has been widely used for separating charge heterogeneity of monoclonal antibodies. This Application Note shows how the Agilent 1260 Infinity Bio-Inert LC System and an Agilent Bio MAb PEEK, 4.6 × 250 mm, 5-µm column were used to perform reproducible and high resolution analysis of charge variants in monoclonal antibodies for biopharmaceutical process development and process monitoring. A simple pH gradient-based cation exchange method for separation and quantification of charge variants was developed. Area, RT precision, and robustness of the method were excellent and show the reliability of the method. There were no significant changes in the chromatographic profile when the modifications were made in experimental conditions, thus showing the method to be robust. The bio-inertness and corrosion resistance of the instrument coupled with simple and reproducible method make this solution particularly suitable for the QA/QC analysis of monoclonal antibodies in the biopharmaceutical industry.

References

1.
Yan He *et al.*, *J. Sep. Sci.*, **2011**, 34, 548–555

2.
Dell Farnan, *Anal. Chem.*, **2009**, 81, 8846–8857

3.
Jennifer C. Rea, *Journal of Pharmaceutical and Biomedical Analysis*, 54 (**2011**) 317–323

www.agilent.com/chem/bio-inert

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