Simple Method Optimization in mAb Charge Variant Analysis using pH Gradients Generated from Buffer Advisor with Online pH and Conductivity Monitoring

Application Note

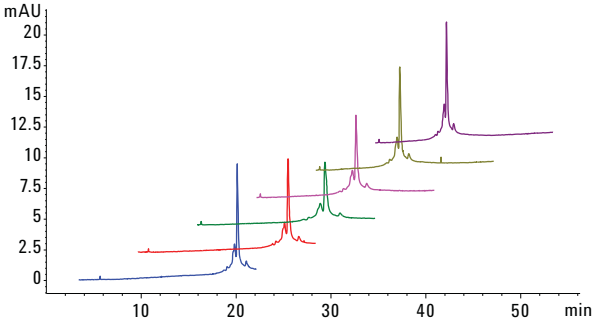
Biotherapeutics & Biosimilars

Author

Sonja Schneider
Agilent Technologies, Inc.
Waldbronn, Germany

Abstract

This Application Note shows method development for charge variant profiling of monoclonal antibodies using pH gradient elution. The combination of the Agilent 1260 Infinity Bio-inert Quaternary LC System and the calculations from Agilent Buffer Advisor Software enables easy method development for optimal charge variant separation. Online pH and conductivity monitoring during method development becomes possible by the connection of the Monitor pH/C-900 from GE Healthcare to the 1260 Infinity Bio-inert LC System.



Agilent Technologies

Introduction

During development and production of pharmaceutical biomolecules such as monoclonal antibodies (mAbs), it is essential to monitor the product stability. Post translational modifications of the proteins such as glycosylation, phosphorylation, deamidation, and many others lead to charge heterogeneity of the protein. To ensure safety and efficacy of the therapeutic protein, it is necessary to characterize and quantify the charge variant profile of the protein.

Ion-exchange chromatography (IEX) separates molecules, for example protein mixtures, according to their net surface charge. With this separation technique, the charge heterogeneity of biotherapeutic proteins can be analyzed either by salt gradient or pH gradient elution. The elution principle in both gradient types is to decrease the net charge of the proteins. At a pH where the protein has no net charge, called the isoelectric point (pI), there is no interaction between protein and the charged medium of the column and the protein elutes from the column.

Compared to salt-gradients elution, proteins are focused in narrower bands during the pH gradient elution, which results in higher resolution[1]. Further advantages of pH gradients are the lowered salt concentration of the eluted fractions which puts less stress on the LC instrument and the potential possibility to determine the pI of the proteins. For the separation of charge variants of monoclonal antibodies, the application of pH gradients demonstrated significantly higher resolving power and peak capacities than achieved with salt gradients[2].

With the Agilent 1260 Infinity Bio-inert Quaternary LC System and the calculations from Agilent Buffer Advisor Software, highly linear pH gradients can be performed[3]. Online pH and conductivity monitoring during method development becomes possible by the connection of the Monitor pH/C-900 from GE Healthcare to the 1260 Infinity Bio-inert LC System and Agilent OpenLAB CDS ChemStation[4].

This Application Note shows the optimization of a linear pH gradient method for the separation of charge variants of monoclonal antibodies. Method development is performed using calculations from Buffer Advisor Software with online pH and conductivity monitoring for optimal results.

Experimental

The Agilent 1260 Infinity Bio-inert Quaternary LC system consisted of the following modules:

- Agilent 1260 Infinity Bio-inert Quaternary Pump (G5611A)
- Agilent 1260 Infinity High performance Bio-inert Autosampler (G5667A)
- Agilent 1290 Infinity Thermostat (G1330B) for sample cooling
- Agilent 1290 Infinity Thermostatted Column Compartment (G1316C) with bio-inert solvent heat exchangers
- Agilent 1260 Infinity DAD (G1315C with a 10-mm bio-inert standard flow cell)
- Two Agilent 1200 Infinity Universal Interface Boxes (G1390B), one for each signal (pH and mS/cm)
- Monitor pH/C-900 from GE Healthcare
- Two signal cables (6-pin mini DIN-open, GE Healthcare)
- Two C-Grid connectors
- Agilent LC driver revision: A.02.05 SI349

All modules must have the same firmware version. The firmware versions can be validated with Agilent Lab Advisor software.

Software

OpenLAB CDS ChemStation Edition for LC and LC/MS Systems, Rev. C.01.05 [35]

Samples

Anti-c-Myc Monoclonal Antibody (Clone 9E10, mouse IgG 1), RAT Anti-DYKDDDDK Tag Antibody

Chromatographic conditions

The column used was the Agilent Bio MAb PEEK, 4.6 × 250 mm, 5 µm (p/n 5190-2407). Table 1 shows the chromatographic conditions.

Table 1. Chromatographic conditions.

Chromatographic conditions					
Salt gradient 10–100 mM NaCl, 30 mM sodium phosphate buffer (gradient calcluated from Buffer Advisor)	Time (min)	A: Water	B: 500 mM NaCl	C: NaH_2PO_4 (70 mM)	D: Na_2HPO_4 (75 mM)
	0.00	55.8	2.0	32.8	9.4
	20.00	38.0	20.0	29.8	12.2
	21.00	28.1	30.0	28.5	13.4
	25.00	28.1	30.0	28.5	13.4
pH gradient 6.4–7, 30 mM sodium phosphate buffer (example, all gradients were calculated from Buffer Advisor)	Time (min)	A: Water	B: 500 mM NaCl	C: Na_2HPO_4 (70 mM)	D: Na_2HPO_4 (75 mM)
	0.00	57.7	0.0	33.3	9.0
	20.00	58.7	0.0	19.3	22.0
	21.00	29.1	30.0	14.3	26.6
	25.00	29.1	30.0	14.3	26.6
Stop time	25 minutes				
Post time	15 minutes				
Flow rate	1 mL/min				
Injection volume	7 or 20 µL				
Thermostat autosampler and FC	6 °C				
Temperature TCC	RT				
DAD	280 nm/4 nm, Ref.: Off				
Peak width	> 0.05 minutes (1.0 seconds response time) (5 Hz)				
UIB II (pH)					
Peak width	> 0.013 minutes (0.25 seconds response time) (50 Hz)				
Analog In settings	0 V: 1 pH, 1 V: 14 pH				
UIB II (conductivity)					
Peak width	> 0.013 minutes (0.25 seconds response time) (50 Hz)				
Analog In settings	0 V: 0 mS/cm, 1 V: 100 mS/cm				

Results and Discussion

When attempting to obtain the optimum resolution for the individual antibodies, method development for salt-gradient elution can be very difficult[2]. Even with relatively flat salt gradients (for example from 10 to 100 mM NaCl), the resolution of the mAb charge variants can be nonsatisfying. With optimized pH gradients, the resolution can be significantly higher, in comparison to salt gradients (Figure 1).

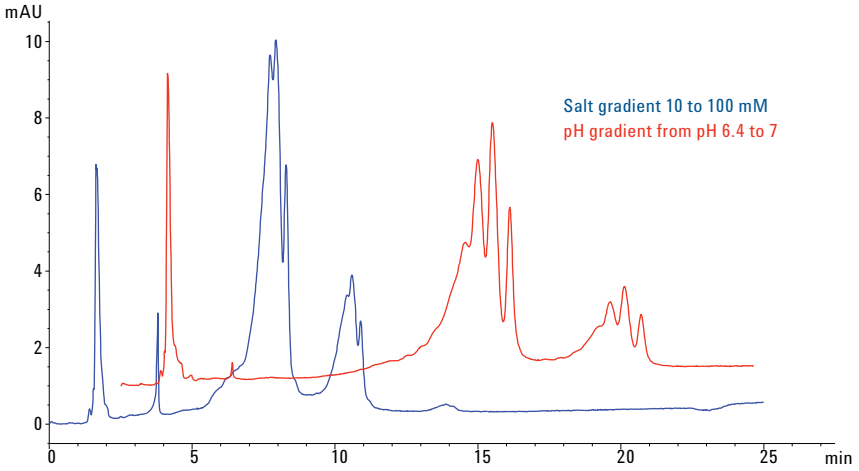


Figure 1. Comparison of a flat salt gradient (10 to 100 mM NaCl) to optimized pH gradient elution from pH 6.4 to 7.

Easy and reliable method development for pH gradients was achieved using the calculation from Buffer Advisor software to create different pH ranges for optimal resolution of mAb charge variants for two different mAbs (Figures 2A and B). Linear pH gradients were calculated from pH ranges of 6 to 7.8 to find the optimal resolution for the mAb Anti-DYKDDDDK (Figure 2A). The pH ranges for the mAb Anti-c-Myc (Figure 2B) ranged from pH 6 to 7.6.

Figure 3 displays the pH traces monitored using the Monitor pH/C-900 from GE Healthcare. This was recorded by the ChemStation software using the Agilent 1200 Infinity Universal Interface Boxes. Online monitoring of pH traces and conductivity enables the user to verify system performance or the calculations from Buffer Advisor for reliable method development.

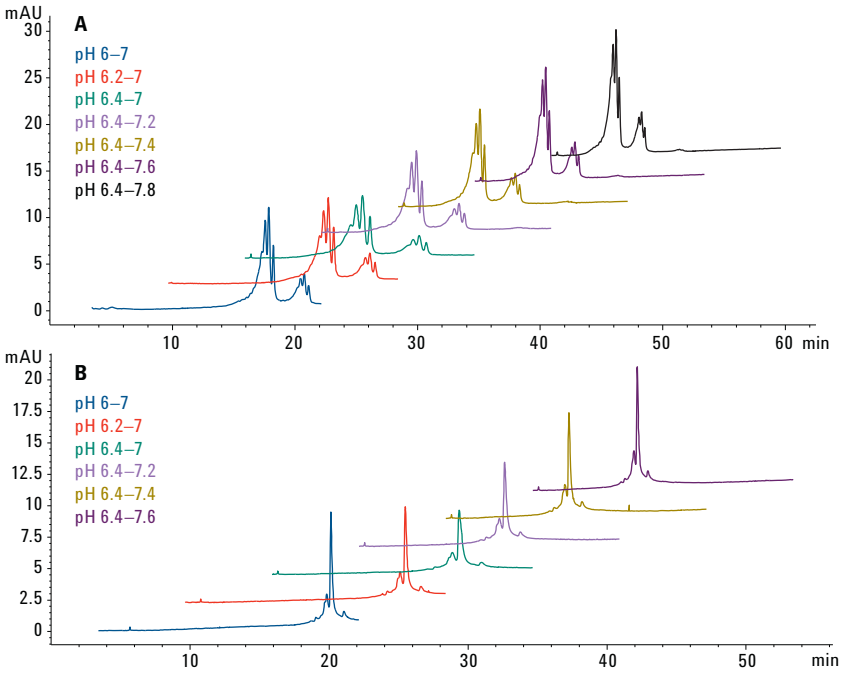


Figure 2. Method development for optimal pH gradient elution for mAb Anti-DYKDDDDK (A) and Anti-c-Myc (B) ranging between pH 6 to 7.8 and pH 6 to 7.6, respectively.

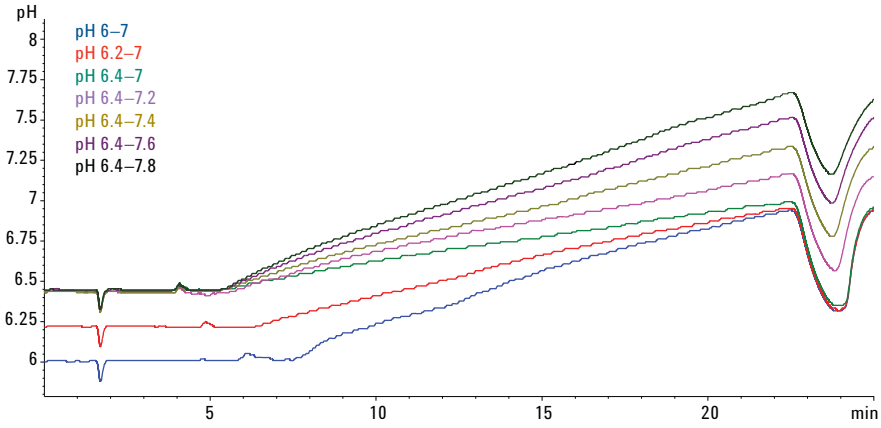


Figure 3. pH traces, visible in ChemStation, monitored using the Monitor pH/C-900 from GE Healthcare in combination with the Agilent 1260 Infinity Bio-inert LC System and an Agilent OpenLAB CDS ChemStation.

For mAb Anti-DYKDDDDK, the optimal charge variant separation was achieved between pH 6.4 to 7. Figure 4 displays, in addition to the UV-Chromatogram, the pH (red trace) and conductivity (green trace) traces. At the end of the pH gradient, at pH 7, a salt gradient step was added from 0 to 150 mM NaCl to ensure complete elution of all sample constituents from the column. This step is displayed in the conductivity trace (green). The pH sensor reacted to the sudden increase of salt with a drop in pH detection, but quickly recovered back to pH 7.

Conclusion

Optimized pH gradients are much easier to develop and enable much better resolution compared to salt gradients. With the combination of the Agilent 1260 Infinity Bio-inert Quaternary LC System and the calculations from Agilent Buffer Advisor Software, easy method development was enabled to find the optimal resolution for two monoclonal antibodies: mAb Anti-DYKDDDDK and mAb Anti-c-Myc. To verify system performance and pH conformity of the Buffer Advisor calculations, pH and conductivity traces were monitored online using the Monitor pH/C-900 from GE Healthcare.

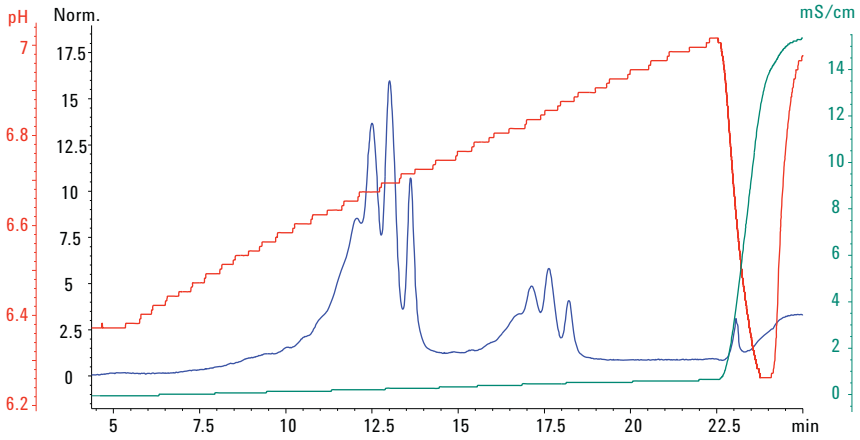


Figure 4. Optimized pH gradient elution for mAb Anti-DYKDDDDK. In addition to the UV-chromatogram, pH (red) and conductivity (green) traces are also displayed.

References

1. T. Ahamed, *et al.* "pH-gradient ion exchange chromatography: An analytical tool for design and optimization of protein separations", *Journal of Chromatography A*, 1164: 181-188, **2007**.

2. D. Farnan and G.T. Moreno "Multiproduct High-Resolution Monoclonal Antibody Charge Variant Separations by pH Gradient Ion-Exchange Chromatography" *Anal. Chem*. 81, 8846–8857, **2009**.

3. "Protein Separation with pH Gradients Using Composite Buffer Systems Calculated by the Agilent Buffer Advisor Software" Application Note, Agilent Publication Number 5991-1408EN, **2012**.

4. "Online pH and Conductivity Measurement with an Agilent 1260 Infinity Bio-inert LC System and a GE Monitor pH/C-900" Technical Overview, Agilent Publication Number 5991-2354EN, **2013**.

www.agilent.com/chem

Published in the USA, November 1, 2013
5991-3365EN

Agilent Technologies