Application Note

Biologics

Size Exclusion Chromatography Method Development of NIST mAb Using an Agilent AdvanceBio SEC 200 Å 1.9 µm Column

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Abstract

This Application Note demonstrates method development with an Agilent AdvanceBio SEC 200 Å 1.9 µm column for size exclusion chromatography (SEC) analysis of the NIST monoclonal antibody (mAb). A wide range of mobile phase combinations can easily be screened with the bio-inert quaternary pump of the Agilent 1260 Infinity II bio-inert LC system and Agilent Buffer Advisor software.

Introduction

SEC is a commonly used technique to characterize and quantify size variants from biotherapeutic proteins. A variety of different mobile phase conditions often need to be evaluated to improve peak shape and resolution for a protein of interest. The AdvanceBio SEC 200 Å 1.9 µm column with its unique bonding chemistry offers reduced secondary interactions under different buffer conditions. SEC method development can be time-consuming, with the requirement of screening a number of different buffer compositions and pH combinations. However, Buffer Advisor software, combined with a bio-inert quaternary LC pump provides a simple way of online mobile phase optimization for SEC analysis. This Application Note presents SEC method development for characterizing the NIST mAb.

Experimental

Materials

NIST mAb (RM 8671) (10 mg/mL) was purchased from NIST SRM standards. Monobasic and dibasic sodium hydrogen phosphate (NaH_2PO_4/ Na_2HPO_4) and sodium chloride (NaCl) were purchased from MilliporeSigma. All chemicals used were ≥99.5 % pure. Water was purified from a Milli-Q A10 water purification system (Millipore). Mobile phases were prepared fresh daily and filtered through a 0.22 µm membrane filter prior to use.

Instrumentation

An Agilent 1260 Infinity II bio-inert LC system with the following configuration was used:

- Agilent 1260 Infinity II bio-inert quaternary pump (G5654A)
- Agilent 1260 Infinity II bio-inert multisampler (G5668A) with sample cooler (option #100)
- Agilent 1260 Infinity II multicolumn thermostat (G7116A) with bio-inert heat exchanger (option #019)
- Agilent 1260 Infinity II variable wavelength detector (G7114A)

Column

Agilent AdvanceBio SEC 200 Å 1.9 µm, 4.6 × 300 mm (p/n PL1580-5201)

Software

- Agilent OpenLab CDS 2.2 software
- Agilent Buffer Advisor software

Instrument conditions

Parameter	Settings
Column Temperature	25 °C
Mobile Phase	A) Water B) 1 M NaCl C) 245 mM NaH_2PO_4 D) 420 mM Na_2HPO_4
Flow Rate	0.35 mL/min
Injection Volume	3 µL
Detection	UV at 280 nm

Results and discussion

To screen a variety of mobile phase conditions for NIST mAb SEC analysis, the following stock solutions were prepared:

- 1 M NaCl
- 245 mM NaH_2PO_4
- 420 mM Na_2HPO_4

Using these stock solutions, it was possible to create sodium phosphate buffer concentrations from 150 to 350 mM without the addition of NaCl, using Buffer Advisor software. In addition, combinations of sodium phosphate buffer with NaCl present at varying concentration were also evaluated. Mobile phase pH was tested at four different values: pH 6.6, 6.8, 7.0, and 7.4. In Buffer Advisor software, we can enter different method development screening conditions including buffer concentration, salt concentration, and pH. The software can then automatically calculate the correct percentage of each stock solution needed to achieve the desired mobile phase conditions. Table 1 shows several selected screening conditions and mobile phase compositions calculated by Buffer Advisor software.

Table 1. Selected screening conditions and mobile phase compositions calculated by Buffer Advisor software.

Buffer (mM)	NaCl (mM)	pH	Software-Calculated Mobile Phase Compositions			
			A%	B%	C%	D%
150	0	6.6	49.9	0.0	34.5	15.6
150	0	6.8	52.6	0.0	27.9	19.5
150	0	7	55.3	0.0	21.5	23.2
150	0	7.4	59.6	0.0	11.3	29.1
50	250	6.6	58.8	25.0	10.2	6.0
50	250	6.8	59.8	25.0	7.9	7.3
50	200	7.4	66.8	20.0	3.1	10.1
25	250	7.0	67.8	25.0	3.0	4.2

A: Water
B: 1 M NaCl
C: 245 mM NaH_2PO_4
D: 420 mM Na_2HPO_4

Figure 1 shows SEC chromatograms of NIST mAb under 150 mM sodium phosphate without the addition of any NaCl at pH ranging from 6.6 to 7.4. As shown in Table 2, at this buffer concentration, a higher pH at 7.4 results in better peak shape and dimer/monomer resolution. In addition, the peak area percentage of high molecular weight species (HMW%) increases gradually from pH 6.6 to 7.4 (Figure 2), and at pH 7.4 the value is close to 3 % as reported by NIST's evaluation of RM 8671[1]. If we set pH at 7.4, and increase the buffer concentration from 150 mM up to 350 mM, peaks have less tailing at 300 and 350 mM, but dimer/monomer resolution continues to drop with increased concentration (Table 3). Under mobile phase compositions without NaCl, 150 mM of sodium phosphate at pH 7.4 gives the best result, with a balance of peak shape and resolution and more accurate HMW%.

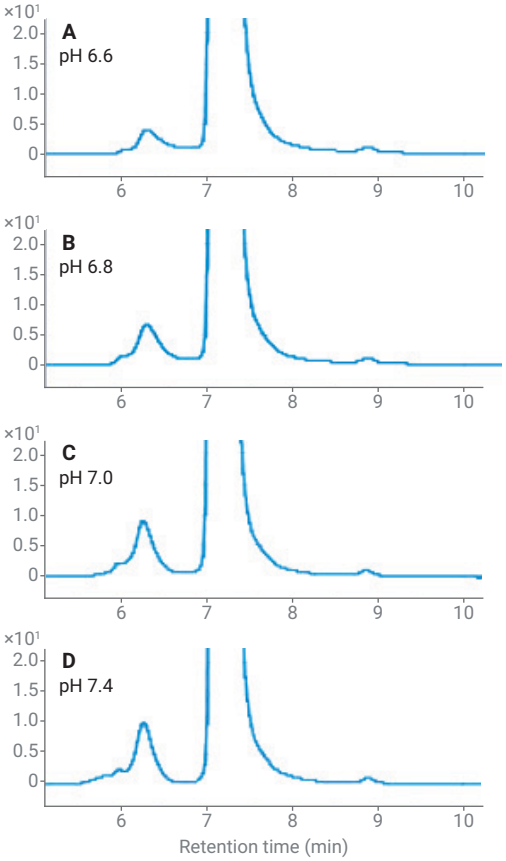

Figure 1. Size exclusion chromatograms of NIST mAb using 4.6 × 300 mm SEC columns running at 0.35 mL/min under 150 mM sodium phosphate at A) pH 6.6, B) pH 6.8, C) pH 7.0, and D) pH 7.4.

Table 2. Effect of pH on peak symmetry and dimer/monomer resolution.

Buffer (mM)	NaCl (mM)	pH	Asymmetry (As)	Resolution (Dimer/Monomer)
150	0	6.6	1.49	2.33
150	0	6.8	1.43	2.35
150	0	7	1.42	2.67
150	0	7.4	1.41	2.78

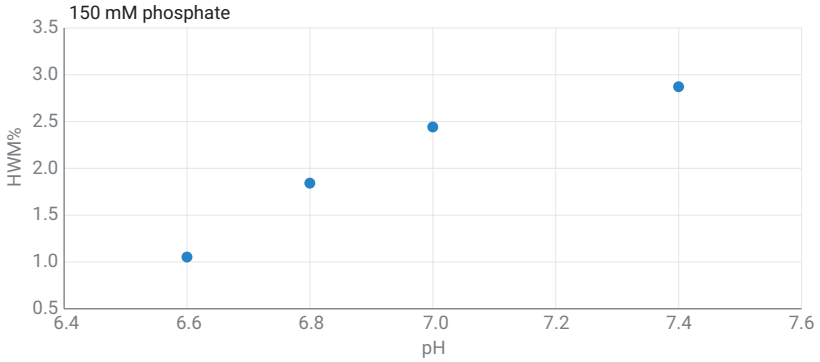

Figure 2. Peak area percentage of HMW of NIST mAb under 150 mM phosphate at different pH values.

In addition, mobile phases consisting of different concentrations (25 to 100 mM) of sodium phosphate and 250 mM NaCl at the four pH values were evaluated by comparing peak symmetry and dimer/monomer resolution (results shown in Table 4). It was shown that 50 mM sodium phosphate and 250 mM NaCl at pH 6.8 gave the best result, with a balance of peak shape and resolution. Using 25 mM phosphate, and 250 mM NaCl, pH at 6.8 or above, gave more accurate HMW% results (Figure 3).

Table 3. Effect of buffer concentration (without NaCl) on peak symmetry and dimer/monomer resolution.

Buffer (mM)	NaCl (mM)	pH	Asymmetry (As)	Resolution (Dimer/Monomer)
150	0	7.4	1.41	2.78
200	0	7.4	1.45	2.60
250	0	7.4	1.42	2.57
300	0	7.4	1.40	2.45
350	0	7.4	1.38	2.33

Table 4. Effect of buffer concentration (with NaCl) and pH on peak symmetry and dimer/monomer resolution.

Buffer (mM)	NaCl (mM)	pH	As	Rs (Dimer/Monomer)
25	250	6.6	1.36	2.73
25	250	6.8	1.36	2.86
25	250	7	1.35	2.83
25	250	7.4	1.37	2.86
50	250	6.6	1.35	2.87
50	250	6.8	1.33	2.86
50	250	7	1.36	2.85
50	250	7.4	1.36	2.84
100	250	6.6	1.36	2.87
100	250	6.8	1.36	2.89
100	250	7	1.35	2.83
100	250	7.4	1.37	2.80

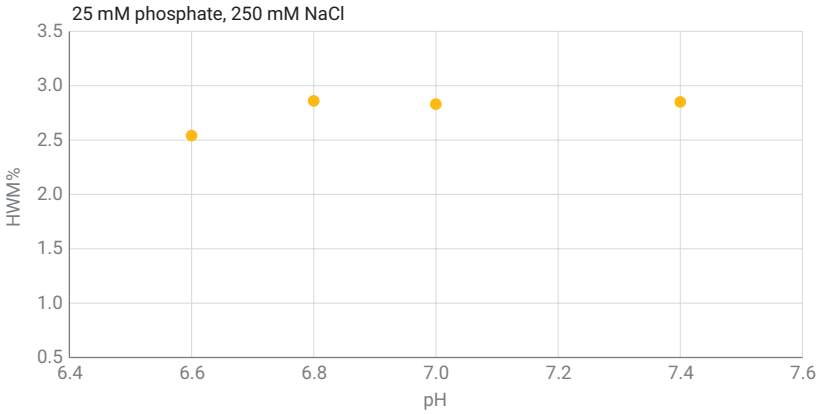

Figure 3. Peak area percentage of HMW of NIST mAb under 25 mM phosphate, 250 mM NaCl at different pH values.

Using 50 mM phosphate and 250 mM NaCl, or 100 mM phosphate and 250 mM NaCl, we obtained accurate HMW% at any of the four pH values. Therefore, with these two mobile phase compositions, pH had negligible effect on HMW% results (Figure 4). Table 5 compares the effect of salt concentration and pH on peak symmetry and dimer/monomer resolution when setting buffer concentration at 50 mM. Among these conditions, 50 mM sodium phosphate with 250 mM NaCl at pH 6.8 gave the best result, with a balance of peak shape and resolution. Using 50 mM phosphate, and 200 mM NaCl, similar to 25 mM phosphate with 250 mM NaCl, pH at 6.8 or above, gave more accurate HMW% results (Figure 5). Overall, considering peak symmetry, dimer/monomer resolution, and HMW% accuracy, the optimum mobile phase composition is 50 mM sodium phosphate, 250 mM NaCl at pH 6.8 (chromatogram shown in Figure 6).

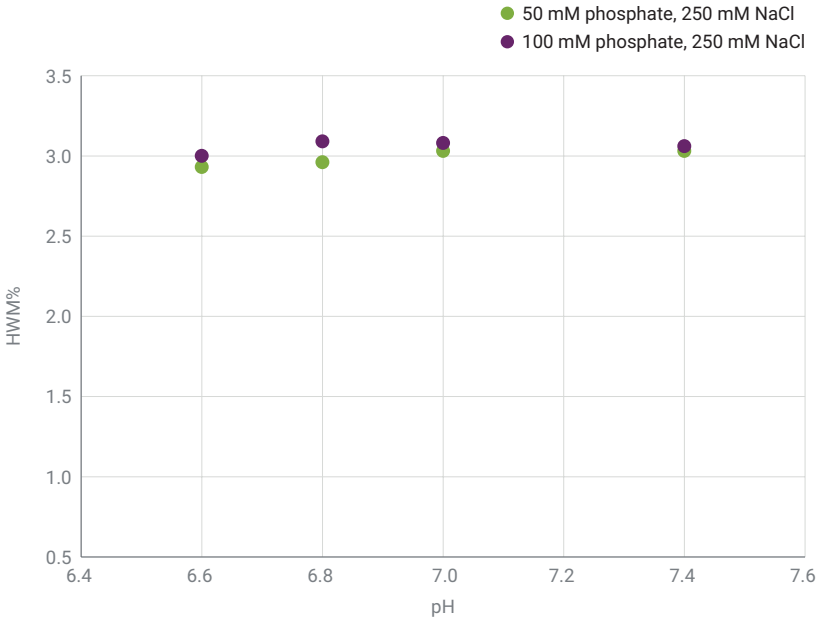

Figure 4. Peak area percentage of HMW of NIST mAb under 50 mM phosphate, 250 mM NaCl or 100 mM phosphate, 250 mM NaCl at different pH values.

Table 5. Effect of salt concentration and pH on peak symmetry and dimer/monomer resolution.

Buffer (mM)	NaCl (mM)	pH	As	Rs (Dimer/Monomer)
50	250	6.6	1.35	2.87
50	250	6.8	1.33	2.86
50	250	7	1.36	2.85
50	250	7.4	1.36	2.84
50	200	6.6	1.36	2.68
50	200	6.8	1.35	2.83
50	200	7	1.36	2.87
50	200	7.4	1.36	2.88

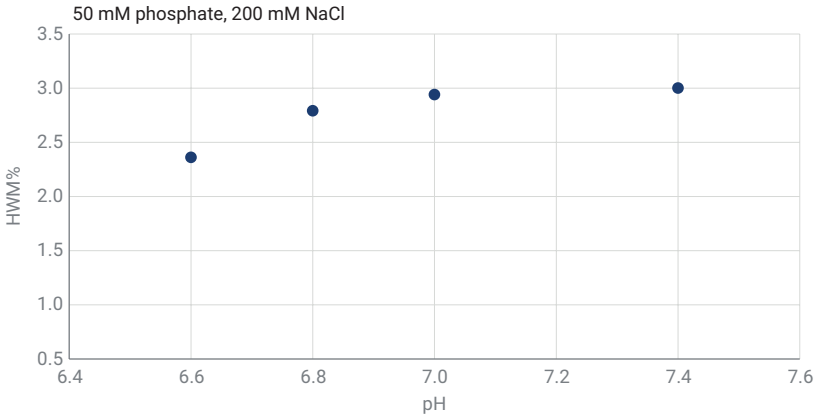

Figure 5. Peak area percentage of HMW of NIST mAb under 50 mM phosphate, 200 mM NaCl at different pH values.

Conclusion

This study demonstrates a simplified approach to mobile phase optimization for SEC analysis of NIST mAb (RM 8671) with the use of an AdvanceBio SEC 200 Å 1.9 µm column together with the bio-inert quaternary pump of the 1260 Infinity II bio-inert LC system, and Buffer Advisor software. Optimized mobile phase combination and pH is selected considering peak symmetry, dimer/monomer resolution, and quantitation accuracy.

Reference

1. Schiel, J. E.; *et al.* The NISTmAb Reference Material 8671 value assignment, homogeneity, and stability, *Anal. Bioanal. Chem.* **2018**, *410*, 2127–2139.

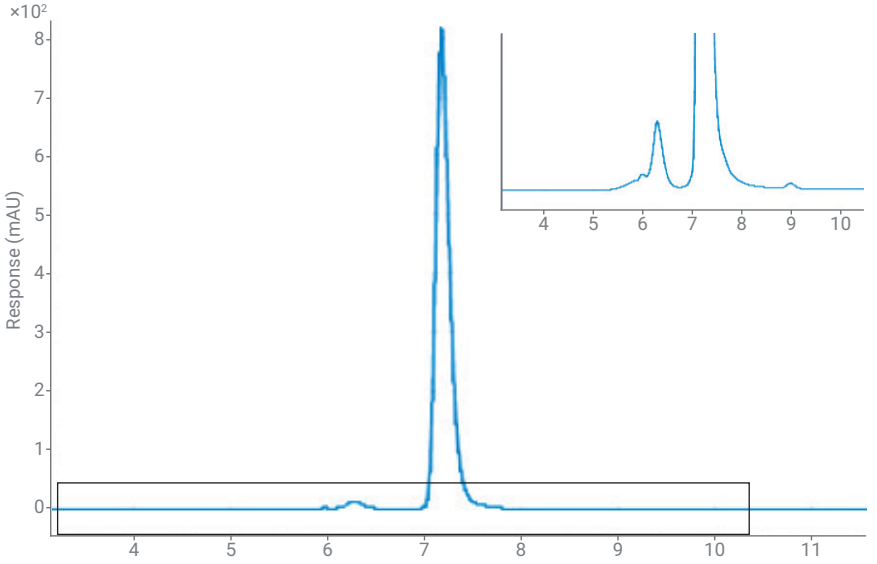

Figure 6. Size exclusion chromatogram of NIST mAb using 4.6 × 300 mm SEC columns running at 0.35 mL/min under 50 mM sodium phosphate, 250 mM NaCl, pH 6.8.

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