Application Note

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SEC Method Transfer from Diol Chemistry to Polymer-Coated Silica

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Abstract

The Agilent AdvanceBio SEC 130 and 300 Å columns contain highly uniform, 2.7-µm particles, with a low-binding, polymer-coated silica stationary phase. These columns provide efficient separations with minimal nonspecific interactions for size exclusion chromatography (SEC). This technology represents an improvement over the older Agilent ZORBAX GF-250 and GF-450, diol-bonded phase columns. To examine these column technologies, a series of experiments was conducted.

Introduction

SEC is considered the gold standard for measuring the amount of aggregates present in monoclonal antibody (mAb) products and other therapeutic proteins[1]. Monoclonal antibody products, therapeutic proteins, and peptides are used to treat many diseases and medical conditions[2,3]. Aggregation of proteins during the manufacture and storage of mAbs and therapeutic proteins and peptides negatively affects drug efficacy and safety, and can result in drug failures[4,5]. Agilent offers diol-bonded phase columns and polymer-coated, silica-based columns for performing SEC.

This Application Note optimizes methods for two of the older diol-bonded phase columns, the Agilent ZORBAX GF-250 and the ZORBAX GF-450. Methods were also optimized for two of the newer, polymer-coated, silica-based Agilent AdvanceBio SEC 130 Å and AdvanceBio SEC 300 Å columns. Three different standards were used during method optimization, the AdvanceBio SEC 300 and 130 Å standards (Tables 1–2) and insulin from bovine pancreas (MW 5.7 kDa). The AdvanceBio SEC standards illustrate the ability of the columns to separate proteins of various sizes. In contrast, the insulin standard is an example of method transfer from the ZORBAX GF-250 to the AdvanceBio 130 Å column.

Materials

- Agilent AdvanceBio SEC 300 Å standard (p/n 5190-9417)
- Agilent AdvanceBio SEC 130 Å standard (p/n 5190-9416)
- Insulin from bovine pancreas, Sigma, I6634
- Agilent vial, screw top, amber, certified, 2 mL, 100/pk vial size: 12 × 32 mm (12 mm cap), (p/n 5188-6535)
- Agilent screw cap, bonded, blue, PTFE/white silicone septa, 100/pk. Cap size: 12 mm (p/n 5190-7021)

Table 1. AdvanceBio SEC 300 Å standard.

Compound	Molecular weight
1. Thyroglobulin	670 kDa
2. Y-globulin	150 kDa
3. Ovalbumin	45 kDa
4. Myoglobin	17 kDa
5. Angiotensin II	1 kDa

Table 2. AdvanceBio SEC 130 Å standard.

Compound	Molecular weight
1. Ovalbumin	45 kDa
2. Myoglobin	17 kDa
3. Aprotinin	6.7 kDa
4. Neurotensin	1.7 kDa
5. Angiotensin II	1 kDa

Columns

- Agilent AdvanceBio SEC 300 Å, 7.8 × 300 mm, 2.7 µm, 300 Å (p/n PL1180-5301)
- Agilent AdvanceBio SEC 130 Å, 7.8 × 300 mm, 2.7 µm, 130 Å (p/n PL1180-5350)
- Agilent ZORBAX GF-250, 9.4 × 250 mm, 4 µm, 150 Å (p/n 884973-901)
- Agilent ZORBAX GF-450, 9.4 × 250 mm, 6 µm, 300 Å (p/n 884973-902)

LC system

- Agilent 1260 Infinity bio-inert quaternary pump (G5611A)
- Agilent 1260 Infinity bio-inert autosampler (G5667A)
- Agilent thermostat for 1200 ALS/fraction collector (G1330B)
- Agilent 1290 Infinity thermostatted column compartment (G1316C)
- Agilent 1260 Infinity DAD (G1315C)

Instrument conditions

Buffers to be specified at a flow rate of 1 and 1.2 mL/min, 25 °C column temperature, chiller set at 4 °C, 220 nm wavelength.

Results and discussion

Method optimization

To optimize the mobile phase conditions for the columns, the following mobile phases were first tested: 50, 100, 150, 200, 400, and 600 mM phosphate buffer, pH 7.0, without NaCl. Next, the phosphate concentration was held constant at 150 mM phosphate buffer pH 7.0, and the salt concentration was varied from 50, 100, 150, 200, and 500 mM NaCl. The flow rate for the AdvanceBio SEC 130 and 300 Å columns was kept at 1.0 mL/min. For the ZORBAX GF-250 and GF-450 columns, the flow rate was kept at 1.2 mL/min (flow rates scaled to achieve the same linear flow velocity for the different column dimensions). The optimized conditions were determined as those that provided the maximum separation and resolution between standard peaks, a lack of peak distortion or shouldering, an absence of shifting retention times, and minimized tailing factors caused by secondary interactions between the samples, mobile phase, and columns.

Evaluation of standards using optimized methods

The parameters used to characterize the performance of the four columns with the AdvanceBio SEC 300 Å standard were:

- The overall chromatography of the standard
- The resolution between the IgG monomer and dimer
- The tailing factors of the angiotensin II peak
- The absence of shifting retention times

The AdvanceBio SEC 300 Å column performed better than the other three columns in separating the AdvanceBio 300 Å standard mixture, which was suited to the larger pore size of the column. The AdvanceBio SEC 300 Å achieved baseline separation of all five standard peaks, the highest resolution between the IgG monomer and dimer, and the lowest tailing factor of the angiotensin II peak (Table 4, Figures 1A–1D).

Table 4. Resolution by column, with optimized mobile phase.

Column	Optimized mobile phase	Res IgG mono/dimer	TF angiotensin II
AdvanceBio SEC 300 Å	150 mM phosphate	1.83	1.07
AdvanceBio SEC 130 Å	N/A	N/A	N/A
ZORBAX GF-250	150 mM phosphate	0.91	1.61
ZORBAX GF-450	200 mM phosphate	0.97	1.92

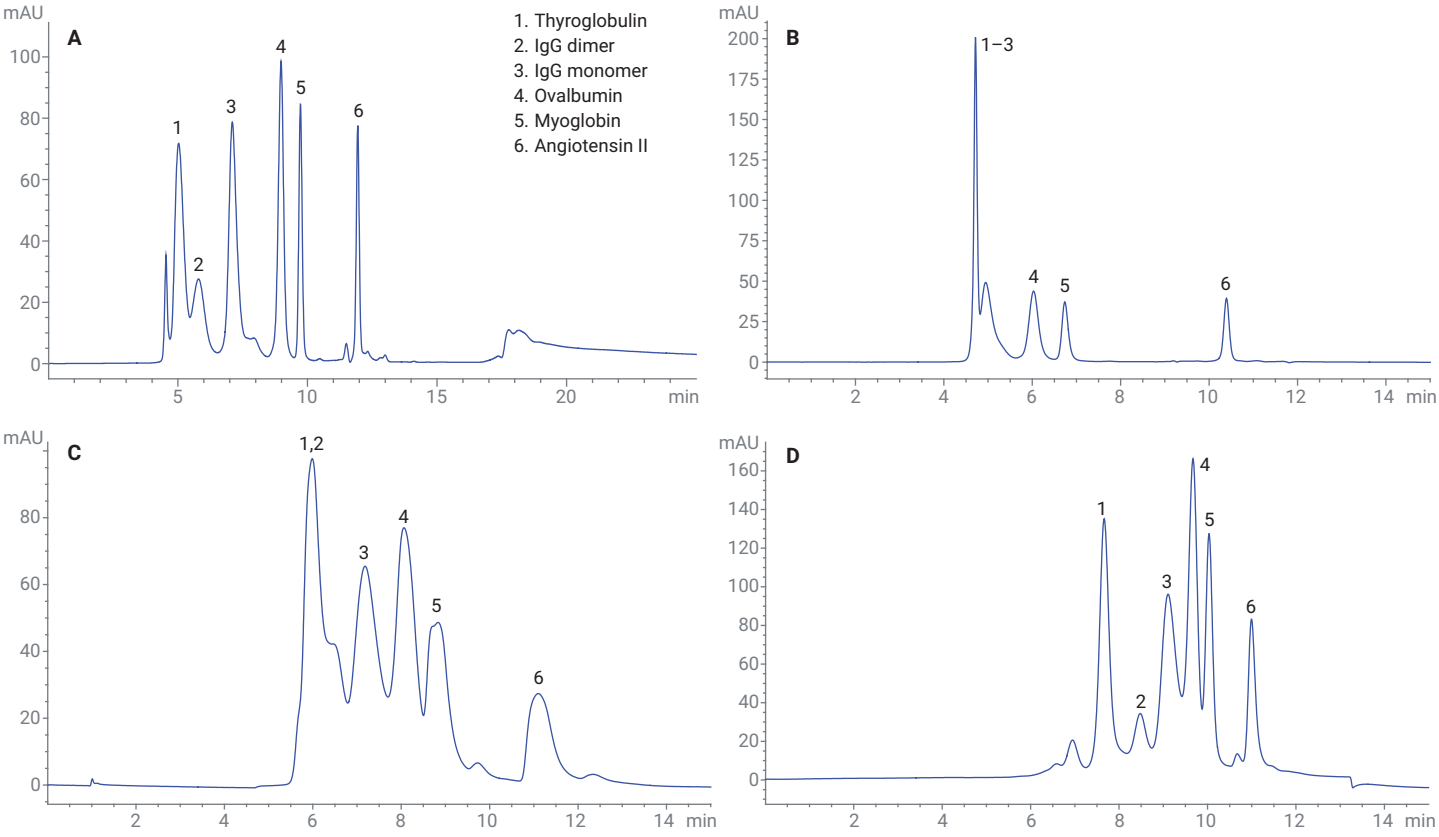

Figure 1. AdvanceBio SEC 300 Å standard run on four different columns each with the optimized mobile phase listed in Table 4. A) AdvanceBio SEC 300 Å; B) AdvanceBio SEC 130 Å; C) ZORBAX GF-250; D) ZORBAX GF-450.

Use of the ZORBAX GF-450 column, which had the same pore size but different chemistry to the AdvanceBio SEC 300 Å column, did not result in baseline separation of all five peaks. In addition, the ZORBAX GF-450 had decreased resolution values between the IgG monomer and dimer compared to the AdvanceBio SEC 300 Å (Table 4, Figure 1D).

The AdvanceBio SEC 130 Å column, with its smaller pore size, did not separate the three largest compounds in the AdvanceBio SEC 300 Å standard under any condition (Figure 1B). The ZORBAX GF-250 column, with its smaller pore size, had low resolution between the IgG monomer and dimer, and a higher tailing factor of the angiotensin II peak (Figure 1C, Table 4).

The AdvanceBio SEC 130 Å standard

The following metrics were used to assess the performance of the selected columns in separating the AdvanceBio SEC 130 Å standard:

- The overall chromatography of the standard
- The resolution between the ovalbumin and myoglobin peaks
- The tailing factors of the angiotensin II peak
- The absence of shifting retention times

The AdvanceBio SEC 130 Å column was the only column to achieve baseline separation of all five peaks of the AdvanceBio 130 Å protein standard (Figures 2A–2D). The AdvanceBio SEC 130 Å column had the lowest tailing factor for angiotensin II of the four columns (Table 5). The AdvanceBio SEC 300 Å had a higher resolution of the ovalbumin and myoglobin peaks, but did not separate the last two peaks in the standard (Table 5, Figure 2A). Therefore, the chromatography using the AdvanceBio SEC 130 Å column was better than the AdvanceBio SEC 300 Å column. This result was expected considering the AdvanceBio 130 Å protein standard was suited to the smaller pore size of the AdvanceBio SEC 130 Å column.

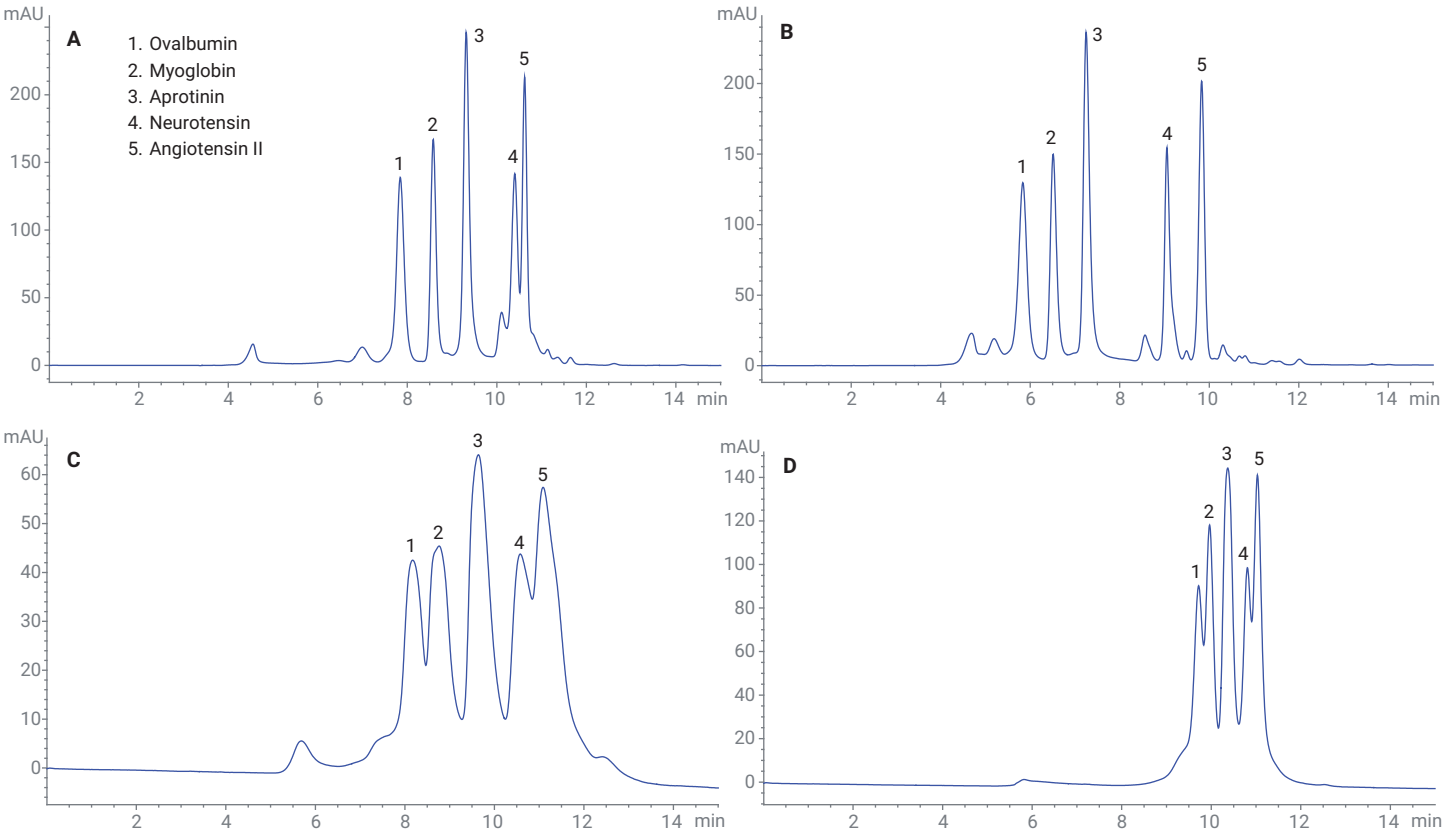

Figure 2. AdvanceBio SEC 130 Å standard run on four different columns each with the optimized mobile phase listed in Table 5; A) AdvanceBio SEC 300 Å; B) AdvanceBio SEC 130 Å; C) ZORBAX GF-250; D) ZORBAX GF-450.

The ZORBAX GF-250 column had significantly lower resolution of the first two peaks and higher tailing factors for the angiotensin II peak than the AdvanceBio SEC 130 Å and 300 Å columns. This lower resolution occurred despite the ZORBAX GF-250 having a similar pore size than the AdvanceBio 130 Å (Table 5). The ZORBAX GF-250 column also required higher phosphate buffer concentrations to separate the standard mixture (Table 5). The ZORBAX GF-450 column performed in a similar manner to the GF-250 column, despite the difference in the pore sizes between the two columns (Table 5).

The smaller particle size (2.7 µm) of the AdvanceBio 130 Å and 300 Å columns also contributed to their improved resolution with their respective standards compared to the larger particle sizes of the ZORBAX GF-250 (4 µm) and GF-450 (6 µm) columns. The AdvanceBio columns average approximately 100 bar higher backpressure than the ZORBAX GF-250 and GF-450 columns. However, the backpressure of the AdvanceBio SEC columns is still under approximately 180 bar, and suitable for HPLC systems.

Insulin standard

This Application Note used insulin from bovine pancreas as the final standard for column comparison. With a molecular weight of 5.7 kDa, the AdvanceBio SEC 130 Å and the ZORBAX GF-250 (150 Å pore size) were selected to determine the best column for this standard. Chromatograms produced using the ZORBAX GF-250 column had a deformed peak shape under every mobile phase tested. Meanwhile, chromatograms produced using the AdvanceBio SEC 130 Å did not have a deformed peak shape. Therefore, the AdvanceBio SEC 130 Å was the better column for the insulin standard (Figures 3A and 3B, Table 6).

Table 5. Resolution by column, with increased mobile phase concentrations.

Column	Optimized mobile phase	Res IgG mono/dimer	TF angiotensin II
AdvanceBio SEC 300 Å	150 mM phosphate, 150 mM NaCl	2.67	1.29
AdvanceBio SEC 130 Å	100 mM phosphate	2.29	0.86
ZORBAX GF-250	600 mM phosphate	0.59	3.03
ZORBAX GF-450	600 mM phosphate	0.62	2.46

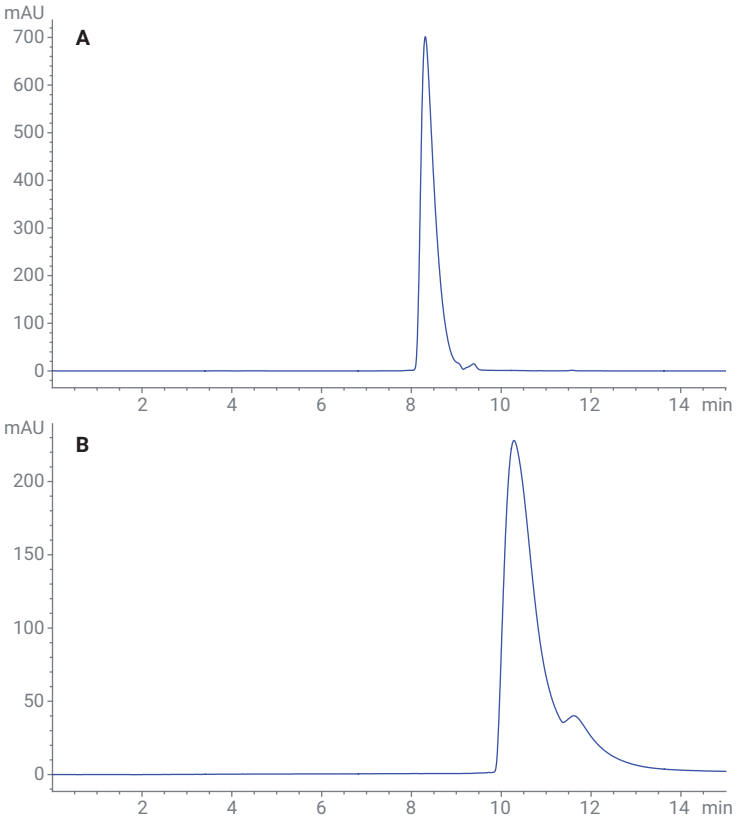

Figure 3. Insulin standard run on two narrow-pore SEC columns, each with the optimized mobile phase listed in Table 5. A) AdvanceBio SEC 130 Å; B) ZORBAX GF-250.

Table 6. Column comparison for the optimization of insulin peak shape.

Column	Optimized mobile phase	RT Insulin	TF Insulin
AdvanceBio SEC 130 Å	200 mM phosphate	8.54	1.74
ZORBAX GF-250	200 mM phosphate	Peak	Peak shouldered

Conclusion

The AdvanceBio SEC 130 Å and 300 Å columns contain the particle technology with a low-binding, polymer-coated silica stationary phase. These AdvanceBio SEC columns provide the superior chromatography of the AdvanceBio SEC 130 Å and 300 Å standards, which cover a large range of protein and peptide standards compared to the ZORBAX GF-250 and GF-450 columns. The AdvanceBio SEC 130 Å and 300 Å columns also provide earlier elution times for all three standards, characterized at 20 % lower flow rates. This earlier elution leads to less time spent making and disposing of mobile phase components, thus improving lab productivity, resulting in fewer materials required for making mobile phases, saving money and labor. This outcome makes the performance improvements a worthwhile investment to transfer methods to a particle technology with a low-binding, polymer-coated silica stationary phase.

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