Reversed-phase Separation of Intact Monoclonal Antibodies Using Agilent ZORBAX Rapid Resolution High Definition 300SB-C8 1.8 µm Column

Application Note

Biopharmaceuticals

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Abstract

Analysis and separation of monoclonal antibodies (mAb) by reversed-phase chromatography presents many challenges for maximizing resolution, recovery and run-to-run reproducibility. Additionally, adapting UHPLC methods to these separations for increasing analysis times and throughput, requires robust column technologies and specific bonded phase selection for complete optimization. The Agilent ZORBAX Rapid Resolution High Definition (RRHD) 300SB-C8 1.8 µm column is designed to address complex protein challenges, such as mAbs, by providing increased sensitivity in reduced analysis times, enhanced resolution and long column lifetimes with high reproducibility. The stable bonded short chain C8 proprietary phase offers an alternate selectivity to C18 and is an ideal choice for resolving large hydrophobic proteins, such as monoclonal antibodies. The 300SB-C8 coating technology also provides exceptional thermal and low pH stability allowing mobile phase eluents with trifluoroacetic acid or formic acid to be routinely used at temperatures up to 80 °C. This application note highlights these beneficial column characteristics in the analysis of a derived intact monoclonal antibody produced from Chinese hamster ovary.

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Introduction

Monoclonal antibodies (MAbs) currently represent the largest class of therapeutic drugs made by the biotechnology industry and will play a significant role in the future of pharmacological interventions of disease. Purification, characterization, and monitoring of mAbs are all critically important to drug development, with a variety of analysis techniques routinely used. Due to the heterogeneity in hydrophobic structure of mAbs, reversed-phase separation is thus becoming an option for monitoring purity and stability during manufacturing, formulation and storage. However, too many reversed-phase methods fall short in providing robust separation performance, with fast analysis times, to consider this technique mainstream for mAb impurity characterization. Additionally, there are limited column choices which can provide reproducible, high resolution separations.

We used an Agilent ZORBAX Rapid Resolution High Definition (RRHD) 300SB-C8 1.8 µm column for intact monoclonal antibody separations to demonstrate utility for fast analysis during mAb screening and optimization of critical separation parameters. The ZORBAX StableBond C8 coating technology, in combination with an optimized packing process, enabled high resolution mAb separations during faster run times. The columns displayed exceptional tolerance to back pressure increases beyond 1,000 bar and ensured reproducible column operation under acidic conditions and elevated temperatures. What's more, the RRHD 300SB-C8 1.8 µm column achieved greater sensitivities with enhanced peak shapes and greater resolution when compared to ZORBAX 300SB-C8 3.5 µm columns.

Materials and Methods

The Chinese hamster ovary (CHO)-cell derived monoclonal antibody was purchased from Creative Biolab, Pennsylvania. Triflouroacetic acid was purchased from Sigma-Adrich, St. Louis, MO, and iso-propanol and acetonitrile were supplied from Honeywell-Burdick & Jackson, Muskegon, MI

Conditions

Instrument	Agilent 1290 LC Infinity system with auto injector (ALS), binary pump and thermostatted oven and diode array detector (DAD)
Column	Agilent ZORBAX Rapid Resolution High Definition 300SB-C8, 2.1 × 50 mm, 1.8 µm (p/n 857750-906)
Mobile Phase	A. H_20:IPA (98:2) + 0.1% TFA (v/v) B. IPA:ACN:H_20 (70:20:10) + 0.1% TFA (v/v)
Injection	1 µL (2 mg/mL)
Flow rates	Between 0.5 mL/min and 1.0 mL/min
Gradient	Multi-segmented and linear elution
Temperature	80 °C
Detection	UV, 225 nm

For consecutive chromatographic runs, a one-minute post run was added to re-equilibrate the column.

Results and Discussion

Elevating column temperature to enhance peak shape performance and decrease retention

Solvent viscosity, protein diffusivity and mobile phase polarity depend strongly on temperature. The manipulation of column temperature is a crucial variable in the separation of hydrophobic peptides and proteins [1]. The expanded chromatographic overlays in Figure 1 show the effect of column temperature on a mAb separation as temperature is increased from 23 °C to 80 °C. Employing a gradient of 25% to 40% B (1.5% B/min), the mAb shoulder peak (highlighted in yellow) shows improved resolution from the base peak, while the pressure and retention time of both peaks decreases, thus making the 80 C separation more amendable for a fast and highly efficient analysis.

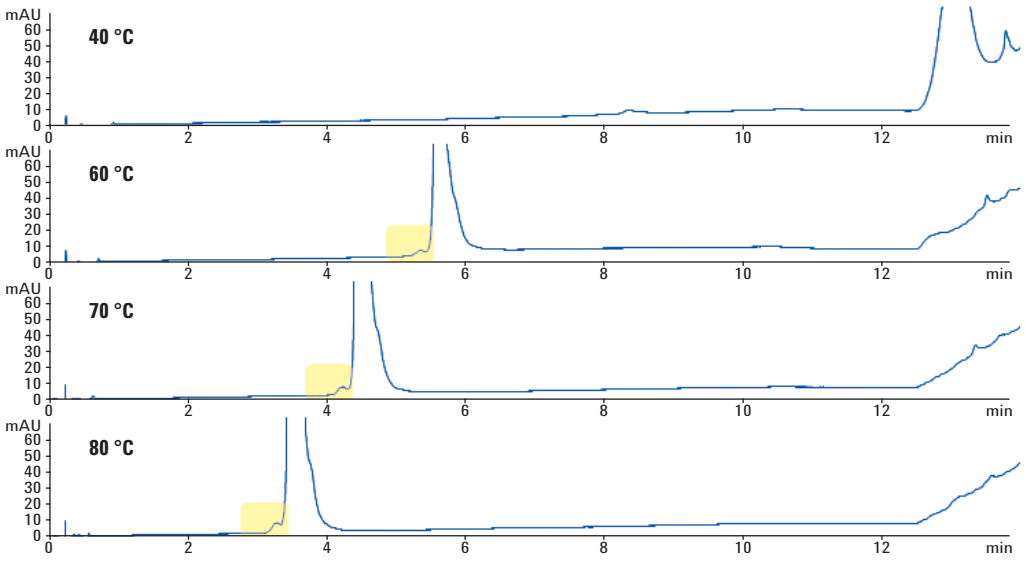

Figure 1. Temperature-dependant performance of intact monoclonal antibody separations on an Agilent ZORBAX Rapid Resolution High Definition 300SB-C8 1.8 µm column. During each chromatographic run temperature was increased (top to bottom) while flow rate remained constant at 0.5 mL/min. The shoulder peak, contrasted in yellow, details the resolution improvement and decrease in retention as temperature is increased.

Gradient flow rate optimization for improving sub-2 µm resolution

Changes in flow rate for optimizing protein separations, particularly with mAb's, can have dramatic outcomes in terms of resolution, efficiency and peak shapes due to mass transfer constraints with large protein diffusion. Protein dynamic size, hydrophobicity, polarity and eluent environment can all create separation difficulties in obtaining adequate peak shapes at various flow rates. Thus flow rate determinations for larger proteins, such as mAbs, become critical to the gradient optimization. Ideally, fast gradient run times are desired for 2.1 × 50 mm columns; however, this requires careful evaluation and optimization of the gradient slope at different flows. Flow rates of 0.5 mL/min, 0.75 mL/min, and 1.0 mL/min were evaluated under steep gradient conditions (Table 1, gradient B). As shown in Figure 2, a fast flow rate of 1.0 mL/min (relative to a 2.1 mm column id) produced enhanced mAb peak resolution in a shorter retention window. The intact mAb peak shape displayed better resolution of the intact peak at faster flow, while the increase in subsequent back pressures was well tolerated.

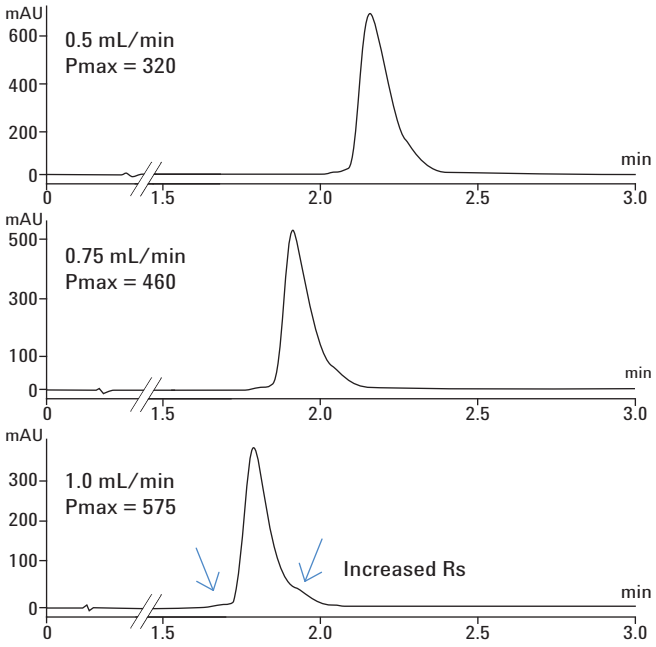

Figure 2. Three chromatographic overlays comparing increasing flow rates using fast gradient analysis conditions at 80 °C on an Agilent ZORBAX Rapid Resolution High Definition 300SB-C8 1.8 µm column. Increasing flow rate from 0.5 mL/min (top), 0.75 mL/min (middle) to 1.0 mL/min (bottom) improved intact mAb resolution and decreased retention.

Optimizing gradient conditions for high resolution and fast intact mAb analysis

Systematic gradient optimizations were performed under various column flow velocities to evaluate separation speed and the subsequent resolution effects towards intact mAb separation. We identified two gradients to highlight C8 separation efficiency for very fast mAb monitoring or for achieving ultra high resolution in a longer run time. To obtain a highly resolved mAb separation, a shallow gradient was identified and optimized at 0.5 mL/min (Table 1–Gradient A). The top chromatogram in Figure 3 details the resolution and highlights the base peak shoulder profile obtained when employing Gradient A. To obtain higher sensitivity and less band broadening in a much shorter run time, useful for mAb screening, a steeper gradient slope was used at a faster flow rate of 1.0 mL/min (Table 1–Gradient B). The bottom chromatogram and inset in Figure 3 displays a faster mAb separation and details an increase in sensitivity and earlier elution time compared to the top chromotagram, while still enabling adequate resolution for peak profiling.

Table 1. Optimized Gradient A and B Conditions for Figure 3

Gradient A, 0.5 mL/min	% solvent B	Time (min)
	25	0
	35	10
	35	12
	90	14
	25	18

Gradient B, 1.0 mL/min	% solvent B	Time (min)
	25	0
	35	3
	90	4
	25	5

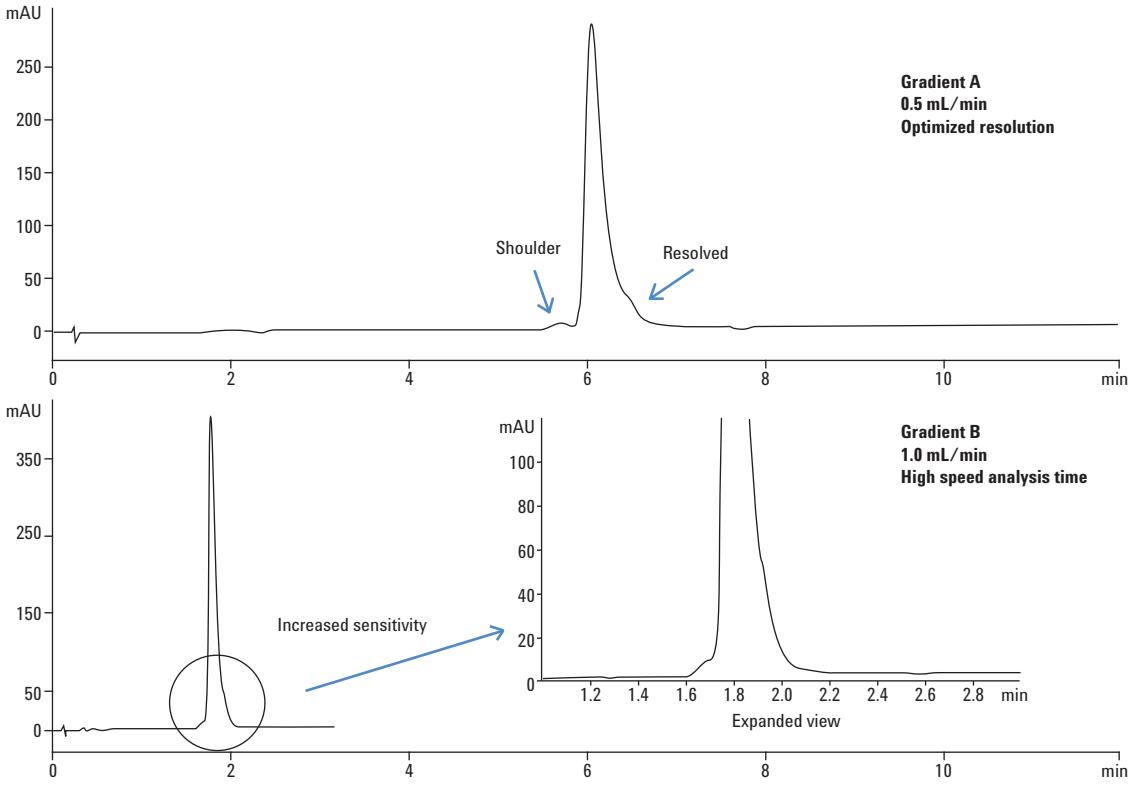

Figure 3. Two chromatographic comparisons showing optimized gradient separations of intact mAb's on an Agilent Rapid Resolution High Definition ZORBAX 300SB-C8 1.8 µm column. The top chromatogram details ultra high resolution obtained during a longer run time and slower flow rate, while the bottom chromatogram ,with expanded view, shows increased sensitivity with adequate resolution in a very fast analysis time, useful for mAb screening.

Performance comparison of Agilent ZORBAX RRHD 300SB-C8 1.8 µm and 300SB-C8 3.5 µm columns

Using optimized gradients A and B in Table 1, a ZORBAX RRHD 300SB-C8 1.8 µm column was compared directly to a ZORBAX 300SB-C8 3.5 µm column. The results of these comparisons are shown in Figure 4a and Figure 4b. Figure 4a is a comparison using the longer gradient time for obtaining higher resolution of the intact mAb. Under these gradient conditions (gradient A), the 1.8 µm 300SB-C8 outperforms the 3.5 µm 300SB-C8 column, delivering better resolution and higher sensitivity with a slightly shorter retention factor. In this comparison, the 3.5 µm front shoulder peak has been reduced and the subsequent base peak resolution has been diminished. Alternatively, in Figure 4b, employing the steeper B gradient with faster flow rate, the 1.8 µm 300SB-C8 shows greater resolution, enhanced peak shape and almost 2× higher sensitivity.

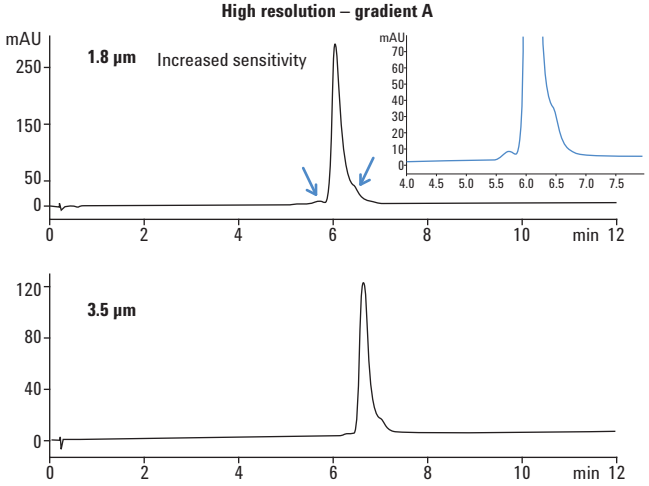

Figure 4a. Gradient A (Table 1) comparison of 1.8 µm (top) and 3.5 µm (bottom) Agilent ZORBAX Rapid Resolution High Definition 300SB-C8, 2.1 × 50 mm columns at 80 °C. The top 1.8 µm chromatogram inset details the improved resolution.

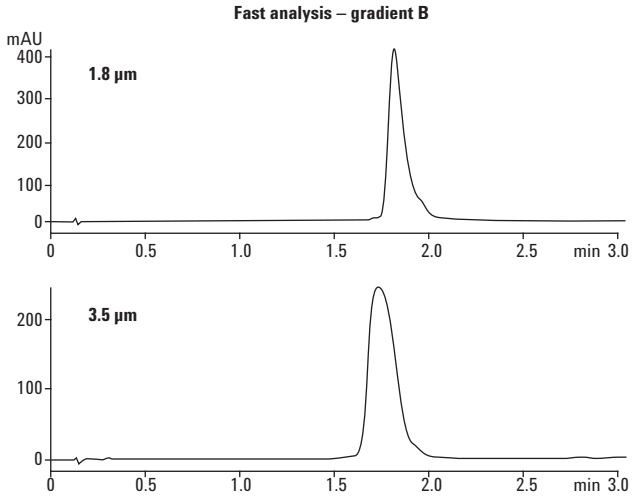

Figure 4b. Gradient B (Table 1) comparison of 1.8 µm (top) and 3.5 µm (bottom) Agilent ZORBAX Rapid Resolution High Definition 300SB-C8, 2.1 × 50 mm at 80 °C. The top 1.8 µm chromatogram shows improved peak shape, resolution and sensitivity.

Determining column performance for run-to-run reproducibility and mAb recovery

Column reproducibility and recovery were investigated at 80 °C using Gradient A conditions (Table 1). A faster separation time, requiring a quicker equilibration time, is the preferred method to fully evaluate the column for run-to-run reproducibility and total protein recovery. High protein recovery is a critical attribute for intact mAb analysis [2]. Although specific post run wash regimes can be employed for column cleanup between injections, it is more desirable to develop in-run conditions that allow high mass balance transfer and the absence of peak ghosting from run-to-run. To evaluate column reproducibility and recovery of the ZORBAX 300SB-C8 1.8 µm, 150 consecutive runs were performed. The repeated intact mAb separations gave no indications of retention time shifts, peak broadening or changes in symmetry (Table 2). The bottom chromatogram in Figure 5 displays the pre- and post-150 injection blank runs (0 µL injected) and gradient pressure curves. The UV trace at 225 nm shows no apparent peak ghosting or baseline disturbance after 150 injections, and the pressure remained stable indicating no fouling of the inlet frit or potential shifts in bed stability.

Table 2. Retention Time, Peak Width, and Symmetry Calculated During Reproducibility Runs at Every 50th Run Beginning From Run #1 to Run #150

Reproducibility injection #	Ret. time (min)	Peak width (W)	Symmetry	Reproducibility injection #	Ret. time (min)	Peak width (W)	Symmetry
1	1.975	0.0938	0.518	100	1.990	0.0999	0.539
50	1.980	0.0969	0.540	150	1.977	0.0828	0.546

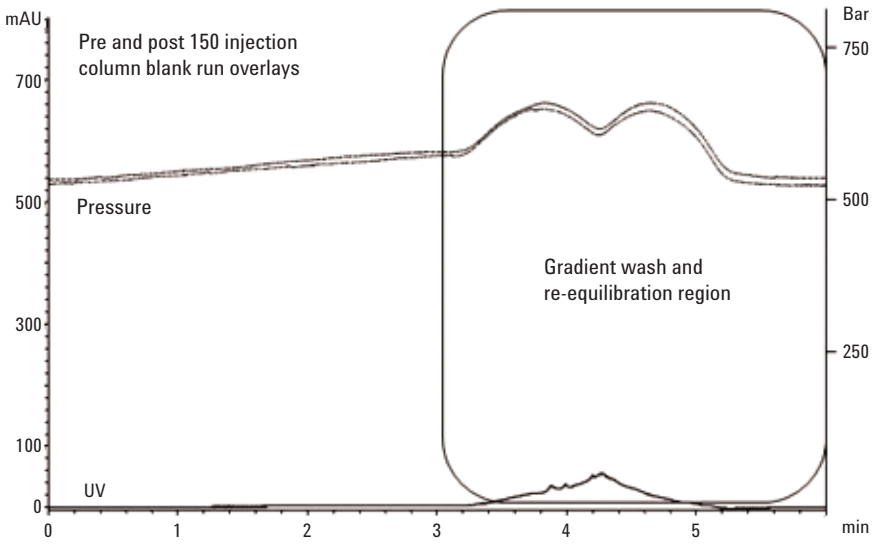

Figure 5. Chromatographic pressure and UV trace overlays for the 1st and 150th injection of the intact mAb on an Agilent ZORBAX Rapid Resolution High Definition 300SB-C8 1.8 µm column. All injections from 1 to 150 were consecutive with total run-to-run injection times at six-minute intervals.

Conclusions

The Agilent ZORBAX RRHD 300SB-C8 1.8 µm column was investigated under gradient optimized conditions at 80 °C for separation of an intact monoclonal antibody. Employing the use of two preferred gradients, separations were optimized to efficiently resolve an intact mAb for very fast mAb screening or ultra high resolution of the mAb and its constituents. In addition, temperature, recovery and flow rate optimizations were demonstrated and established, and compared to a 3.5 µm ZORBAX RRHD 300SB-C8 column. The 1.8 µm ZORBAX RRHD 300SB-C8 delivered fast-highly efficient separations for intact mAb analysis and performed reliably at elevated temperatures and low pH. In combination with the optimized gradient conditions and elevated temperature, the RRHD 300SB-C8 column delivered reproducible separations, during very fast run times, for 150 consecutive injections.

References

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2. C. T. Mant, L. H. Kondejewski, P. J. Cachia, O. D. Monera, R. S. Hodges, Methods Enzymol., 1997, 289, 426-429.

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