Reversed-Phase Optimization for Ultra Fast Profiling of Intact and Reduced Monoclonal Antibodies using Agilent ZORBAX Rapid Resolution High Definition 300SB-C3 Column

Application Note

Biopharma

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Abstract

Rapid reversed-phase separations of intact and reduced monoclonal antibodies (mAbs) were optimized using Agilent ZORBAX Rapid Resolution High Definition (RRHD) 300SB-C3 columns. The StableBond C3 phase and robust sub-2 µm RRHD column technology enabled high resolution separation of monoclonal antibody structure and has demonstrated utility for high throughput mAb characterizations. Monoclonal antibodies expressed by different cell lines were evaluated and optimized for high resolution separations during rapid run times. The ZORBAX 300SB-C3 columns were evaluated for lifetime stability and reproducibility during continuous run sequences and demonstrated high tolerance to elevated temperature and pressure while delivering robust separation performance.

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Introduction

Drug development of biotherapeutics is rapidly growing among the pharmaceutical industry and reliable antibody drug characterization is an important challenge to this development pipeline. Separation of antibodies can be achieved by various chromatographic techniques and requires multiple modes of separation to detect and measure them. One approach to antibody characterization is by reversed-phase (RP) methods, due to its attractiveness with LC/MS detection. However, separation of large molecules such as monoclonal antibodies (mAbs) by RP, has traditionally suffered from broad, diffuse and poorly resolved peaks and has thus limited its use for analysis. Now with the advent of UHPLC and smaller particle columns, these separations can be achieved during faster run times with increased resolution. Also, with the introduction of newer phase chemistries, RP separations can provide alternate selectivity's with greater sensitivity towards proteins. These alternatives to RP analysis for mAbs are now showing great promise for facilitating biotherapeutic analyses.

In this work, we have achieved ultra high resolution separations of both intact and reduced antibodies during a rapid run time using ZORBAX Rapid Resolution High Definition (RRHD) 300SB-C3 columns. Specifically, we systematically optimized gradient conditions at 75C to deliver ultra fast- high efficiency separations of both intact and reduced light and heavy chain mAbs. We have also evaluated the ZORBAX 300SB-C3 for column lifetime and reproducibility at elevated temperature, high operating pressures and low pH to monitor column changes in retention behavior, peak shape and efficiency. All the work herein demonstrates utility for fast mAb profiling while delivering reproducible high resolution separations.

Experimental

Materials

Two humanized monoclonal antibody lines were used in this study. One line was expressed at Agilent using CDH media (p/n 010774) and the other was expressed from a Chinese hamster ovary (CHO)-cell derived monoclonal antibody purchased from Creative Biolab, Pennsylvania. Triflouroacetic acid was purchased from Sigma-Adrich, St. Louis, MO, and iso-propanol, n-propanol, and acetonitrile were supplied from Honeywell-Burdick & Jackson, Muskegon, MI. The 1-propanol was purchased from VWR (p/n BJ322-4). The dialysis cassettes had a 3,500 MWCO and were purchased from Thermo Scientific (p/n 66330).

Reduction and Alkylation

Reduction and alkylation was performed under denaturing conditions using guanidine hydrochloride (GuHCl) to produce the free light and heavy chains. 0.5 mL (1.5 mg/mL) of antibody was dialyzed against water for preservative removal. Once dialyzed, a 0.5 mL aliquot was diluted to a final concentration of 0.75 mg/mL with 100 mM TRIS-HCl and 4 M GuHCl (Mallinckrodt, Phillipsburg, NJ, USA). The pH was adjusted to 8.0 and a 10-µL of 0.5 M dithiothreitol (DTT, Sigma) stock solution was added for a final concentration of 5 mM. The mixture was placed in a 37 °C water bath and incubated for 30 minutes. The antibody was briefly cooled to room temp and a 26-µL aliquot of 0.5 M iodoacetamide (IAM, Sigma) stock solution was added for a final concentration of 13 mM. The alkylated antibody solution was placed in the absence of light at room temperature for 45 minutes. Once removed, the mixture was quenched with 20 µL of 0.5 M DTT for a final concentration of 10 mM. A 1.0 mL amount of reduced and alkylated antibody was desalted through a 4 mL 3.5 K MWCO concentrator (p/n 5185-5991) at 3800 RPM for 30 minutes using water (0.1% TFA). The concentrating process was repeated two times to a final volume of 0.5 mL (1.5 mg/mL)

UHPLC Conditions

Instrument	Agilent 1290 LC Infinity system with auto injector (ALS), binary pump, thermosttated oven (TLC) and and diode array detector (DAD)
Column	Agilent ZORBAX RRHD 300 SB-C3, 1.8 µm 2.1 × 100 mm (p/n 858750-909) 2.1 × 50 mm (p/n 857750-909)
Mobile phase (intact mAb)	A. 98/2 water/n-propanol (0.1% TFA) B. 70/20/10 iso-propanol/ACN/water (0.1% TFA)
Mobile phase (reduced mAb)	A. H_20 + 0.1% TFA (v/v) B. 80/10/10 n-propanol/ACN/water (0.1% TFA)
Injection	2 µL
Flow rates	0.5 mL/min 1.0 mL/min (intact) 1.25 mL/min (lifetime testing)
Gradient	multisegmented
Temperature	75 °C
Detection	UV, 280 nm

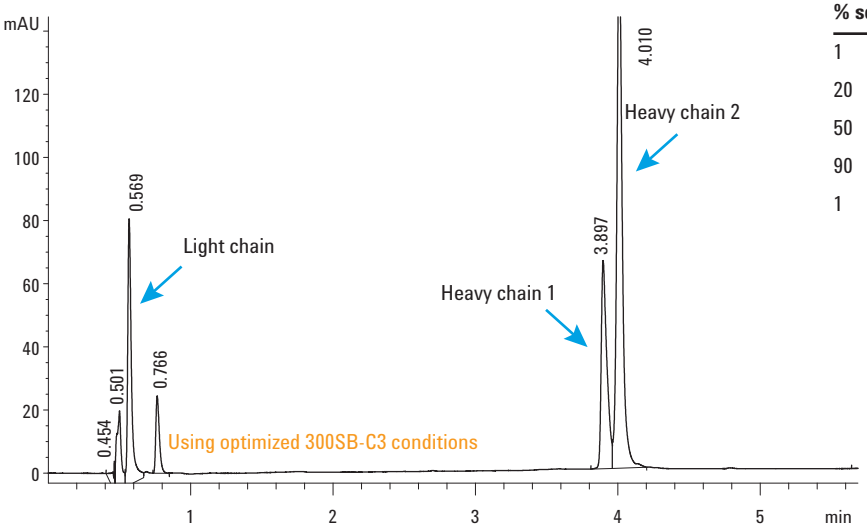
For consecutive chromatographic runs, a 2-minute post run was added to re-equilibrate the column.

Results

Gradient Optimizations for Ultra Fast Analysis of Reduced and Alkylated Monoclonal Antibody

The separation shown in Figure 1 details a high speed separation of reduced and alkylated CHO cell derived mAb. The Agilent ZORBAX RRHD 300SB-C3 2.1 × 100 mm column, 75 °C temperature, and optimized gradient enabled a highly resolved separation of the light chain and two heavy chain variants in under 4.5 minutes. The separation of the two heavy chain peaks, 1 and 2, display narrow band widths and are well resolved. The light chain peak at 0.6 minutes displays excellent separation from the surrounding light chain fragment peaks at 0.5 and 0.7 minutes. The optimized gradient for this separation is shown in Table 1.

Fast separation of reduced monoclonal antibody using an Agilent ZORBAX RRHD 300SB-C3 2.1 × 100 mm, 1.8 µm column



Columns	Agilent ZORBAX RRHD 300SB-C3, 2.1 × 100 mm, 1.8 µm
Sample	Reduced mAb (IgG1) (1.0 mg/ml)-BioCreative IgG1
Sample injection	2 µL
Mobile phase A	0.1% TFA in water
Mobile phase B	80% n-propyl alcohol, 10% ACN, 9.9% water and 0.1% TFA
Temperature	74 °C
Flow rate	0.5 mL/min
Detection	UV 280

Table 1. Agilent ZORBAX RRHD 300 SB-C3 Gradient Conditions

% solvent B	Time (min)
1	0
20	2
50	5
90	6
1	6.1

Figure 1. Rapid reduced monoclonal mAb separation achieved on an Agilent ZORBAX Rapid Resolution High Definition 300SB-C3, 2.1 × 100 mm, 1.8 µm at 75 °C, 0.5 mL/min and monitored at UV 280. Gradient conditions are defined in Table 1.

Optimizing Conditions for High Resolution and Fast Analysis of Intact mAbs from Different Cell Lines

Monoclonal antibodies derived from different cell lines were separated and optimized on an Agilent ZORBAX RRHD 300SB-C3 2.1 x 50 mm, 1.8-µm to demonstrate C3 selectivity towards different mAb expression. Under systematic gradient methods, we identified gradients for each mAb to deliver high speed separations with optimum resolution. The separation displayed in the top panel of Figure 2, was optimized for the Agilent standard antibody expressed from CDH media and using gradient conditions shown in Table 2A. The separation was completed in under 4 minutes and exhibits excellent resolution of the intact peak with a very narrow band width. In comparison, the bottom chromatogram shown in Figure 2 was optimized for the humanized mAb expressed from a CHO cell line using the gradient conditions shown in Table 2B. In this separation, the gradient was optimized with a shallower gradient curve that resolved a front shoulder from the mAb base peak. Both separations in Figure 2 were developed to facilitate high throughput run to run mAb characterization with high efficiency. Each separation finishes with a fast 90% isopropanol wash and rapid re-equilibration for enabling repeated injection sequences.

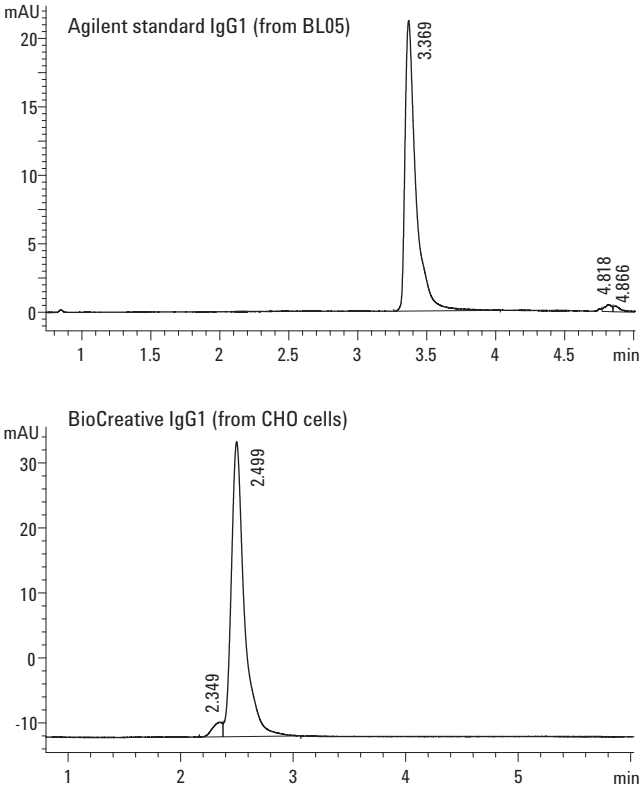
Table 2A Gradient for Agilent Std IgG1

% solvent B	Time (min)
10	0
25	2.5
35	4.5
90	4.56
90	5.0
10	6

Table 2B Gradient for BioCreative IgG1

% solvent B	Time (min)
5	0
25	5
25	7
90	8
5	9

Fast separation Intact IgG1 and their degradation products using an Agilent ZORBAX RRHD 300SB-C3, 2.1 × 50 mm, 1.8 µm column



Columns	Agilent ZORBAX RRHD 300SB-C3, 2.1 × 100 mm, 1.8 µm
Sample	monoclonal antibody (IgG1) 1.0 mg/mL Agilent Standard IgG1 (top) BioCreative IgG1(bottom)
Sample injection	2 µL
Mobile phase A	0.1% TFA in water
Mobile phase B	70% iso-propyl alcohol, 20% ACN, 10% water and 0.1% TFA
Temperature	74 °C
Flow rate	0.5 mL/min
Detection	UV 280

Figure 2. UHPLC separations optimized for two monoclonal antibodies on an Agilent ZORBAX RRHD 300SB-C3 2.1 × 50 mm column. The separations were performed at 1.0 mL/min and 75 °C with iso-propanol/acetonitrile/water. The top panel in Figure 2 was optimized for an mAb expressed from CDH media, while the bottom chromatogram was optimized for a humanized mAb expressed by a CHO cell line. Each separation was followed with a fast 2-minute equilibration post run time.

Column Lifetime

An Agilent ZORBAX RRHD 300SB-C3 column was evaluated for lifetime at low pH during repeated injection sequences at 900 bar. Column packed bed stability, phase stabilization, and inlet frit performance are all critical for continued operation at elevated temperatures and pressures during repeated mAb analysis. To evaluate this performance, 1,000 repeated injections of Ribonuclease A, Cytochrome C, and Lysozyme were performed on a 2.1 x 50 mm column at a flow of 1.25 mL/min. A chromatographic view of this separation can be seen in Figure 3. During each lifetime run, the gradient peak width of the proteins was recorded, while backpressure was closely monitored. As shown in Figure 4A, 10 runs of peak width performance was plotted every 100th injection interval. The peak width performance remained stable during the 1,000 run sequence and column backpressure (Figure 4B) remained unchanged at 900 bar. Maintaining reproducible peak widths and efficiency, while limiting column backpressure increases, are indicators of an optimally packed column bed with excellent resilience to high flow, low pH and repeated high pressure operation. Additionally, maintaining excellent flow dynamics during the lifetime analysis ensured high tolerance to inlet frit plugging from protein or system micro-particulates.

Fast separation standard proteins and their degradation products using Agilent ZORBAX RRHD 300SB-C3 2.1 × 50 mm, 1.8 µm column

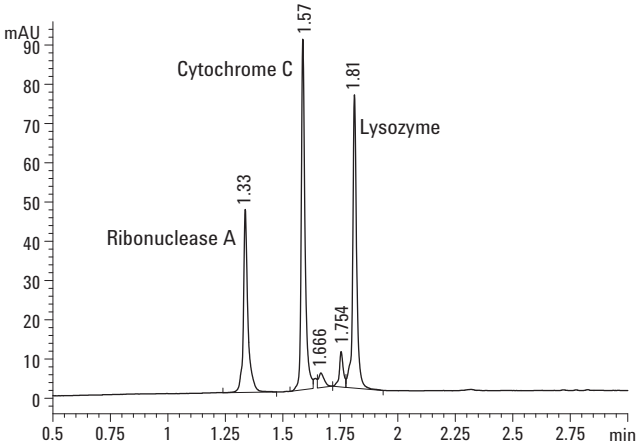


Figure 3. High speed separation of Ribonuclease A, Cytochrome C and Lysozyme for lifetime stability monitoring using an Agilent ZORBAX RRHD 300SB-C3 2.1 × 50 mm. The chromatographic conditions are described in Table 2.

Column	Agilent ZORBAX RRHD 300SB-C3 2.1 × 50 mm, 1.8 µm (p/n 857750-909)	
Sample	Ribonuclease A, Cytochrome C and Lysozyme (3 mg/mL)	
Sample injection	1 µL	
HPLC instrument	Agilent 1290 Infinity Series	
Detection	UV 280	
Mobile phase A	H_20 + 0.1% TFA (v/v)	
Mobile phase B	ACN + 0.1% TFA (v/v)	
Flow	1.25 mL/min	
Injection	1 µL (1 mg/mL)	
Temperature	ambient	
Gradient	% Solvent B	Time (min)
	10	0
	70	2.5
	90	2.6
	90	3.0
	10	5.0

Life test of Agilent ZORBAX RRHD 300SB-C3 2.1 × 50 mm, 1.8 µm

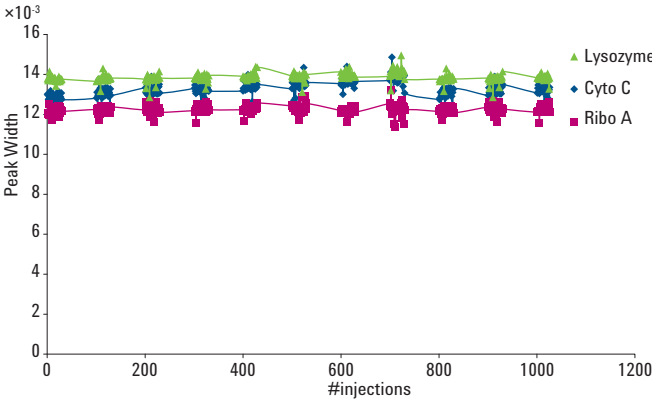


Figure 4A. Agilent ZORBAX RRHD 300SB-C3 2.1 × 50 mm lifetime plot at 900 bar backpressure and high flow (1.25 mL/min). The graph displays a continuous series of protein peak width recordings of Ribonuclease A, Cytochrome C, and Lysozyme plotted during every 100th run interval over the course of 1,000 injections.

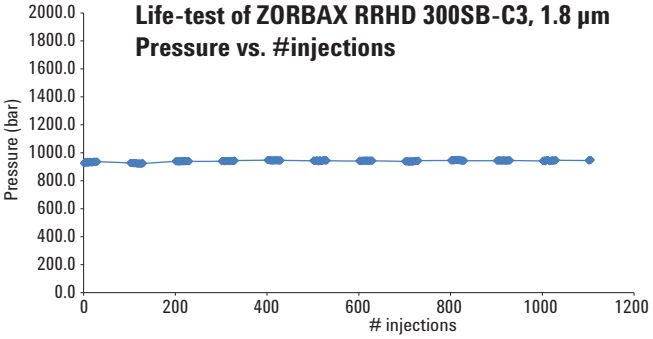
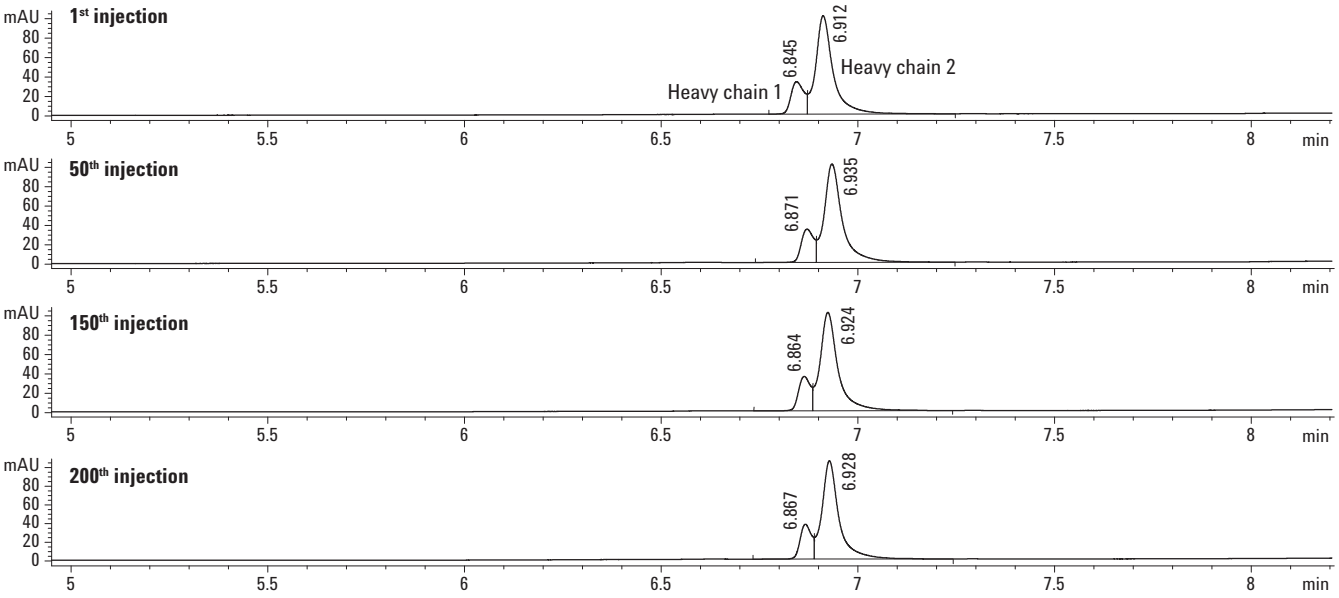


Figure 4B. Agilent ZORBAX RRHD 300SB-C3, 2.1 × 50mm, pressure plot. Consecutive column backpressure readings were recorded and plotted at every 100-injection interval.

Column Reproducibility

An Agilent ZORBAX RRHD 300SB-C3 column reproducibility was examined during 200 repeated injection of a reduced mAb sample. Using a 2.1 x 50 mm column two reduced heavy chain variants were separated, and evaluated for retention time and resolution. As shown in Figure 5, the results of these separations detail excellent run-to-run column reproducibility during continued exposure to 75 °C and low pH. The mAb heavy chain 1 and 2 peaks shown at the 1 st, 50 th, 150 th and 200 th run, maintained retention and peak shape delivering consistent separation performance without any indications of peak deterioration or efficiency loss.

Column reproducibility - 200 injections of reduced monoclonal antibody using Agilent ZORBAX RRHD 300SB-C3, 2.1 × 100 mm, 1.8 µm column



Columns	Agilent ZORBAX RRHD 300SB-C3, 2.1 × 100 mm, 1.8 µm
Sample	Reduced monoclonal antibody (IgG1) (1.0 mg/ml)- Agilent BL05 IgG1
Sample injection	2 µL
Mobile phase A	0.1% TFA in water
Mobile phase B	80% n-propyl alcohol, 10% ACN, 9.9% water and 0.1% TFA
Gradient	0 min–1% B, 2 min–20% B, 5 min–50% B, 7 min–50% B, 8.0 min–90% B, 8.3 min–1% B hold for 2 min
Temperature	75 °C
Flow rate	0.4 mL/min
Detection	UV 280

Figure 5. Reduced and alkylated mAb profiling during 200 repeated injections using an Agilent ZORBAX RRHD 300SB-C3 2.1 × 50 mm column at 75 °C. Sequence runs 1, 50, 150, and 200 are shown. Mobile phase: A: water (0.1% TFA), B: 80/10/10 n-propanol/ACN/ water (0.1% TFA). Gradient: 0 min–1% B, 2 min–20% B, 5 min–50% B, 7 min–50% B, 8.0 min–90% B, 8.3 min–1% B hold for 2 min.

Conclusions

The Agilent ZORBAX RRHD 300SB-C3, 1.8 µm column provided ultra fast and efficient separation of monoclonal antibodies. Optimized chromatographic conditions in combination with the ZORBAX RRHD 300 column technology, enabled rapid and high resolution separation of both intact and reduced mAb's expressed from different sources. The gradient conditions and compositions were systematically optimized to provide complete separation, washing and re-equilibration in a reduced analysis time and demonstrated utility for high throughput mAb characterizations.

ZORBAX RRHD 300SB-C3 column stability (lifetime) and reproducibility were also demonstrated during repeated injection analyses. Throughout 1,000 injections at 900 bar and low pH, the column efficiency performance remained stable and showed excellent tolerance to backpressure increases from bed instability or frit plugging. Additionally, during 200 consecutive runs of two heavy chain mAb variants, the separations displayed consistent peak shape and resolution while they maintained retention position.

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© Agilent Technologies, Inc., 2012
Printed in the USA
February 13, 2012
5990-9667EN

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