Ultra High Speed and High Resolution Separations of Reduced and Intact Monoclonal Antibodies with Agilent ZORBAX RRHD Sub-2 µm 300 Diphenyl UHPLC Column

Application Note

Biopharma

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Abstract

Rapid separations of intact and reduced monoclonal antibodies (mAbs) were achieved with the use of an Agilent ZORBAX Rapid Resolution High Definition (RRHD) 300 diphenyl reversed-phase column and optimized chromatographic conditions. The unique diphenyl phase and robust rapid resolution high definition column technology, in combination with optimized gradient conditions, provided ultra fast separations and delivered excellent peak shapes demonstrating utility for high throughput mAb characterizations. Monoclonal antibodies expressed by both Chinese Hamster Ovary and CDH media cell lines were evaluated and compared with the goal of obtaining high resolution and high efficiency separations during rapid run times. The ZORBAX RRHD 300 diphenyl was also evaluated at elevated operating pressures and temperature, and exhibited high operational tolerance during continuous investigations of reproducibility and lifetime.

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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. Although antibodies can be characterized by many separation techniques, separation by reversed-phase chromatography has been rapidly growing due to the introduction of more efficient and desirable chromatographic materials such as those offered by sub 2 µm columns and the newer phase chemistries.

In this work, we have achieved ultra high resolution separation of intact and reduced monoclonal antibodies (mAbs) during a rapid run time, and demonstrate different selectivity beyond traditional C18, C8, and C3 for mAb characterization. Specifically, we systematically optimized the gradient conditions at elevated temperatures to deliver rapid separation of intact mAb's and reduced, light, and heavy chain mAb variants. The goal of these investigations focused on ultra fast and efficient run-to-run method optimizations that eliminated long equilibration times or, extensive post run washings. We have also evaluated the ZORBAX RRHD 300 diphenyl column for longevity and reproducibility. To evaluate longevity, the columns were tested at elevated operating pressures (> 900 bar) and temperature (75 °C), low pH and high flow for 1,000 injections with a protein standard mix. Run-to-run reproducibility and retention behavior was examined during 200 runs using an mAb standard.

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. Antibody in the amount of 0.5 mL (1.5 mg/mL) was dialyzed with 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 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 a 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. Then 1.0 mL of reduced and alkylated antibody was desalted through a 4 mL 3.5 K MWCO concentrator (p/n 5185-5991) at 3,800 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

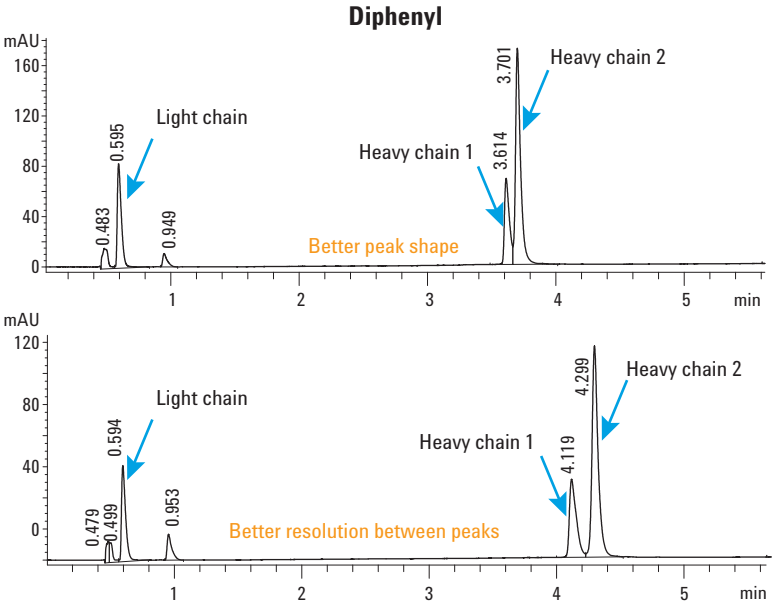
Instrument	Agilent 1290 LC Infinity system with auto injector (ALS), binary pump, thermostatted oven (TLC), and diode array detector (DAD)
Column	Agilent ZORBAX Rapid Resolution High Definition 300 diphenyl, 1.8 µm 2.1 × 100 mm, (p/n 858750-944) 2.1 × 50 mm, (p/n 857750-944)
Mobile phase	(intact mAb) A. 98/2 water/iso-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	1–3 µL
Flow rates	0.5 mL/min (reduced) 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 Monoclonal Antibody

The chromatographic comparisons in Figure 1 show two optimized high speed separations of reduced and alkylated monoclonal antibody. The ZORBAX RRHD 300 diphenyl, 2.1 × 100 mm, column and chromatographic conditions enabled well resolved separations of the reduced mAb light chain and two heavy chain variants. The top panel chromatogram in Figure 1 detail a separation with narrow bands and high resolution of the heavy chains achieved using the gradient conditions shown in Table 1A. In comparison, the separation displayed in the bottom panel of Figure 1 has been optimized for obtaining better resolution of the heavy chains, but with a slight increase in peak width. In this separation, the two heavy chains display near baseline resolution. The optimized conditions for this separation are shown in Table 1B. In contrast between the two separations, the diphenyl phase enabled enhanced separation control for resolving the two heavy chain peaks with minor changes to the gradient slopes. Additionaly, we have observed less dramatic effects towards improving this resolution when the same dimension C3 and C8 columns were used under these identical conditions, suggesting the diphenyl offers a unique selectivity advantage towards this particular antibody beyond the traditional short chain phase chemistries.



Columns	Agilent ZORBAX RRHD 300 Diphenyl, 2.1 × 100 mm, 1.8 µm
Sample	Reduced monoclonal antibody (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
Gradient	1st condition: 0 min–1% B, 2 min–20% B, 5 min–70% B
	2nd condition: 0 min–1% B, 2 min–20% B, 5 min–50% B
Flow rate	0.5 mL/min
Temperature	74 °C
Detection	UV 280

Figure 1. Comparison of two ultra-fast separations of reduced monoclonal antibodies achieved on a Agilent ZORBAX Rapid Resolution High Definition 300 diphenyl (2.1 × 100 mm) under different optimized gradient conditions. The top panel separation delivered narrow peak widths with shorter retention times. The bottom panel separation displays higher resolution between the two heavy chain peaks, but with less efficiency.

Table 1A Gradient A

% Solvent B	Time (min)
1	0
20	2
70	5
90	5.1
1	7

Table 1B Gradient B

% Solvent B	Time (min)
1	0
20	2
50	5
90	5.1
1	7

Optimizing Conditions for Ultra High Resolution and Fast Analysis of Intact mAb's

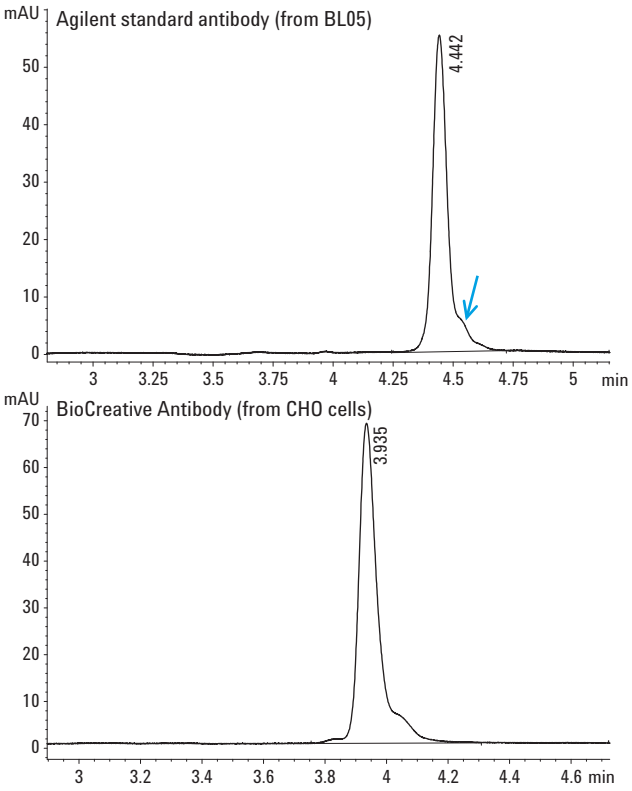
To evaluate separation performance of an intact monoclonal antibody on the ZORBAX 300 diphenyl (2.1 × 50 mm), two mAbs were selected and individually optimized for speed and resolution. Under systematic gradient investigations we identified optimized gradients for each mAb to highlight diphenyl resolving power towards fast intact fast mAb separations. The separation displayed in the top panel of Figure 2, was optimized for the Agilent standard antibody which was expressed from CDH media using gradient conditions shown in Table 2A. The separation was completed in less than 5 minutes and exhibits excellent resolution of the intact peak and shoulder peak identified at 4.5 minutes (arrow). In comparison, the bottom chromatogram shown in Figure 2 was optimized for the mAb expressed from a CHO cell line using gradient conditions shown in Table 2B. In this separation, the gradient was optimized using a slightly modified gradient curve to deliver enhanced resolution at the front and back of the intact mAb peak. In addition to obtaining high resolution-high speed run times, both separations were developed to facilitate run-to-run mAb profiling. Each separation completes with a fast 90% iso-propanol wash and rapid re-equilibration for repeated high throughput injection sequences.

Table 2A Gradient

% Solvent B	Time (min)
15	0
25	2.5
35	4.5
35	4.9
90	5.0
90	5.5
15	6.0

Table 2B Gradient

% Solvent B	Time (min)
10	0
25	2.5
35	4.5
90	4.56
90	5.0
10	6.0

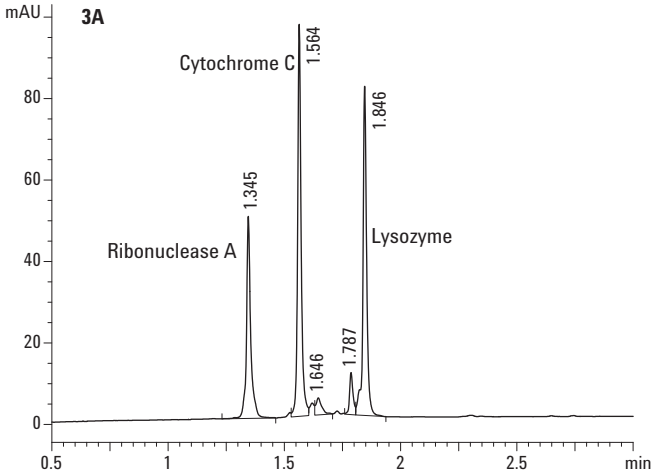


Columns	Agilent ZORBAX RRHD 300 Diphenyl, 2.1 × 100 mm, 1.8 µm
Sample	monoclonal antibody (1.0 mg/mL)- BioCreative and Agilent Standard IgG1
Sample injection	2 µL
Mobile phase A	98/2 water/iso-propyl (0.1% TFA)
Mobile phase B	70/20/10 iso-propyl/ACN/water (0.1% TFA)
Flow rate	1.0 mL/min
Temperature	74 °C
Detection	UV 280

Figure 2. UHPLC separations optimized for two sources of monoclonal antibodies on a 2.1 × 50 mm Agilent ZORBAX RRHD 300SB-diphenyl column. The separations were performed at 1.0 mL/min and 75 °C with %B compositions of 70/20/10 iso-propanol/acetonitrile/water (0.1% TFA). 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 from a CHO cell line. Each separation was completed with a fast 2-minute equilibration post run time.

Column Lifetime

Column packed bed stability and inlet frit performance is critical for continued operation at elevated temperatures and pressures during repeated mAb analysis. ZORBAX RRHD 300 diphenyl column lifetime was evaluated for ruggedness at low pH during repeated injection sequences at 900 bar under the conditions displayed in Table 3. Repeated injections totaling 1,000 of a protein standard mix comprising of Ribonuclease A, Cytochrome C and Lysozyme were performed on a 2.1 × 50 mm column at a flow of 1.25 mL/min. A chromatographic view of this separation can be seen in Figure 3A and details a fast run time under 2 minutes to facilitate the lifetime analysis. During each run, the gradient peak width of each protein was recorded while backpressure was closely monitored. As shown in Figure 3B, 10 runs of peak width performance was plotted at every 60th injection interval. The peak width performance remained stable during the 1,000 injection sequence, while the column backpressure remained unchanged at 900 bar. Steady peak width efficiency and a stable pressure curve indicate an optimally packed column bed with excellent resilience to high flow (relative to 2.1 mm column id), low pH and continuous operation at high pressure. Additionally, after extensive protein injection the ZORBAX RRHD 300 diphenyl column maintained excellent frit flow with high tolerance to inlet plugging from protein or system microparticulates.



Samples	Diphenyl RT (min)	Diphenyl "plates"
Ribonuclease A	1.35	50816
Cytochrome C	1.56	63533
Lysozyme	1.85	92272

Figure 3A. High speed separation of Ribonuclease A, Cytochrome C and Lysozyme for Lifetime stability monitoring using a 2.1 × 50 mm ZORBAX RRHD 300-diphenyl. The separation conditions are described in Table 3.

Table 3

Column	Agilent ZORBAX RRHD 300 2.1 × 50 mm, 1.8 µm	
Sample	Ribonuclease A, Cytochrome C and Lysozyme (3 mg/mL)	
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)	
Temp	ambient	
Gradient	% Solvent B	Time (min)
	10	0
	70	2.5
	90	2.6
	90	3.0
	10	5.0
HPLC instrument	Agilent 1290 Infinity Series	
Detection	UV 280	

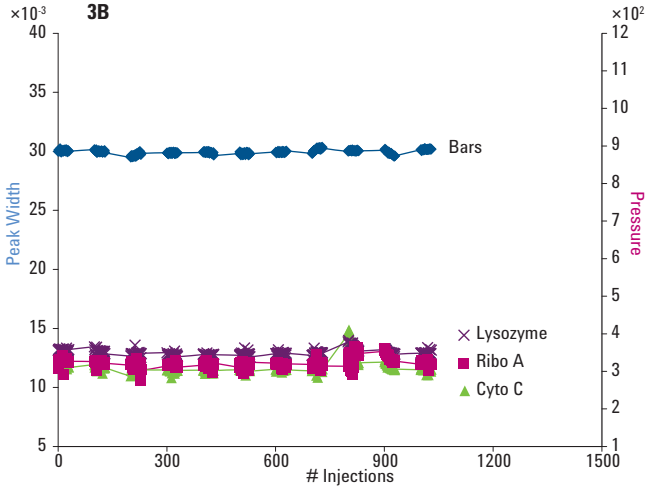
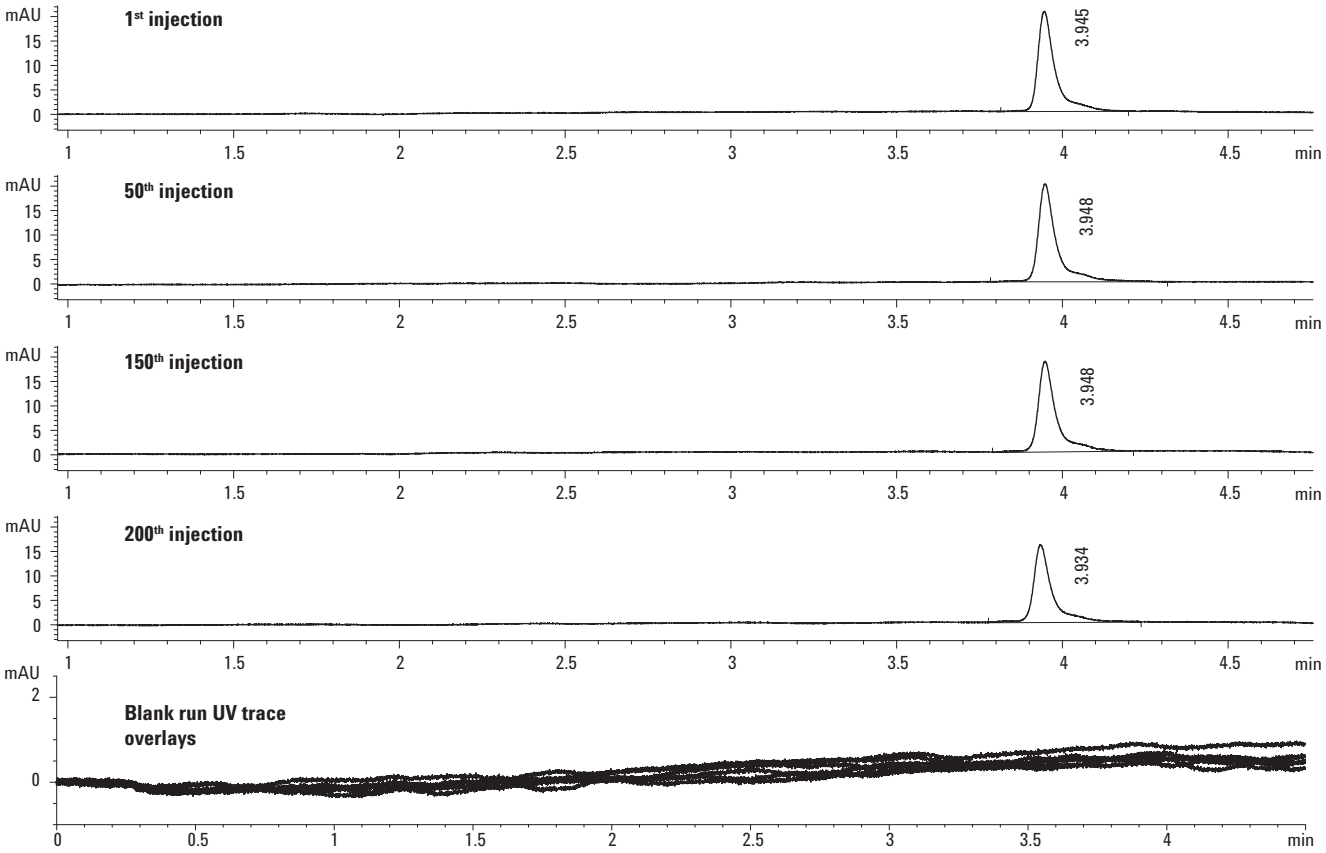


Figure 3B. Agilent ZORBAX RRHD 300-diphenyl 2.1 × 50 mm lifetime plot detailing column lifetime stability curves at elevated pressure and high flow (1.25 mL/min). Ten consecutive protein peak widths of Ribonuclease A, Cytochrome C, and Lysozyme were plotted every 60th run interval during 1,000 injections. Column backpressure was recorded during the intervals and plotted versus the number of injections as shown.

Column Reproducibility

Column reproducibility was examined during repeated injection of an intact humanized mAb during 200 runs. Using a new 2.1 × 50 mm ZORBAX RRHD 300-diphenyl column, run-to-run reproducibility was performed under the gradient compositions and conditions described in Table 2A. To evaluate column reproducibility, and post run recovery, 200 consecutive injections were performed with column blanks collected during every 20th run to monitor the carryover effects from multiple protein injections. The defined separation conditions resulted in repeated high pressure column operation (>700 bar), continued exposure to 75 °C operating temperature and low pH. As detailed in Figure 4, repeated separations of the intact mAb were highly reproducible maintaining retention time and peak shape. Additionally, the post run blanks gave no evidence of run to run mAb carryover (Figure 4 bottom panel).



Columns	Agilent ZORBAX RRHD 300 diphenyl, 2.1 × 100 mm, 1.8 µm
Sample	monoclonal antibody (IgG1) (1.0 mg/mL)– BioCreative IgG1 and Agilent Standard IgG1
Sample injection	1 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
Flow rate	1.0 mL/min
Temperature	74 °C
Detection	UV 280

Figure 4. Details intact mAb profiling during 200 repeated injections using an Agilent ZORBAX RRHD 2.1 × 50 mm 300-diphenyl column at 75 °C. Intact mAb separations shown were collected at 1, 50, 150, and 200th run intervals. The bottom panel displays 5 UV blank run trace overlays collected every 20th run during the column evaluation (note: overlay traces are scaled to 2 mAu). Gradient conditions for the separations are provided in Table 2B.

Conclusions

The Agilent ZORBAX RRHD 300 diphenyl, 1.8 µm, column provided ultra fast and efficient separation of intact and reduced monoclonal antibodies (mAbs) and demonstrated utility for high throughput mAb screening. Through systematic optimization of gradient conditions, tailored to antibody type, high resolution separations of intact and reduced mAb isoforms were achieved in under 5 minutes. The unique diphenyl phase and RRHD column technology enabled robust and repeatable separations while delivering excellent peak shapes, and displayed a unique selectivity advantage during gradient separations than those observed with traditional C3 and C8 phases.

ZORBAX RRHD 300 diphenyl column stability (lifetime) and reproducibility were also demonstrated during repeated injection analyses. During 1,000 injections at 900 bar and low pH, column efficiency performance remained stable and showed high tolerances to backpressure increases from bed instability or frit plugging. Additionally, during 200 consecutive runs at 700 bar, the separations maintained peak shape, resolution and retention position while column blank runs gave no indications of peak carryover or UV trace ghosting.

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