Rapid UHPLC Analysis of Reduced Monoclonal Antibodies using an Agilent ZORBAX Rapid Resolution High Definition (RRHD) 300SB-C8 Column

Application Note

BioPharma

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Abstract

An Agilent ZORBAX sub-2 µm Rapid Resolution High Definition (RRHD) 300SB-C8 reversed-phase column was used under optimized chromatographic conditions for delivering highly resolved ultra fast separations of reduced and alkylated monoclonal antibodies. The StableBond C8 phase and RRHD column technology, in combination with robust gradient elution conditions enabled efficient, fast, and effective separation of light chain and two heavy chain monoclonal antibody variants in under 4 minutes. Additionally, alternate mobile phase compositions were employed, making separations flexible for ultra fast LC/MS analysis. At elevated operating pressures and temperature, the stable C8 phase and rugged column packing technology delivered highly reproducible separations and delivered excellent chromatographic run-to-run results.

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Introduction

Biotherapeutic drug development is rapidly growing among the pharmaceutical industry and 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 limited due to inefficient protocols, poor resolution, and loses in total protein recovery. Structurally, monoclonal antibody is a glycoprotein comprised of two identical copies of heavy chains (50 kDa) and two identical copies of light chains (25 kDa) attached through disulfide bridges. Fast and efficient characterization of these chains and isoforms are becoming increasingly desired and critical, for high throughput monitoring of purity and stability during manufacturing, formulation and storage.

We have used an Agilent ZORBAX Rapid Resolution High Definition (RRHD) 300SB-C8, 1.8 µm column in combination with optimized chromatographic conditions for reduced monoclonal antibody analysis to demonstrate utility for ultra fast mAb characterization and screening. The ZORBAX StableBond C8 phase, elevated temperature (75 °C), and optimized gradient conditions enabled high resolution separation of the light and heavy chain mAbs in an extremely short elution time that to our knowledge, has not previously been demonstrated. The separations were performed under various mobile phase compositions with mass spectrometer (MS) friendly ion pairing additives to provide flexibility for different LC/MS user preferences. The columns displayed exceptional tolerance to high backpressure increases beyond 900 bar and ensured reproducible column operation under acidic mobile phase conditions and elevated temperatures. Additionally, protein recovery was repeatedly evaluated during 250 runs and gave no indications of peak ghosting or changes in retention behavior.

Experimental

Materials

The human monoclonal antibody used in this study was produced using CDH media at Agilent (p/n 010774) and stored at pH 7.2 in 10 mM phosphate buffer and 0.09% (w/v) sodium azide at 4 °C. Antibody concentration was 10 mg/mL. Triflouroacetic acid was purchased from Sigma-Adrich, St. Louis, MO, and 1-propanol and acetonitrile were supplied from Honeywell-Burdick & Jackson, Muskegon, MI. The dialysis cassettes had a 3,500 MWCO and were purchased from Thermo Scientific (p/n 66330).

Reduction and Alkylation

Reduction and alkylation were performed under denaturing conditions using guanidine hydrochloride to produce the free light and heavy chains for reversed-phase analysis. A 0.5 mL (1.5 mg/mL) aliquot of antibody was dialyzed against water for preservative removal. Once dialyzed, the 0.5 mL aliquot was diluted to a final concentration of 0.75 mg/mL with 100 mM TRIS-HCl and 4M guanidine hydrochloride (Mallinckrodt, Phillipsburg, NJ, USA). The solution pH was adjusted to pH 8.0. A 10 µL aliquot of 0.5 M dithiothreitol (DTT, Sigma) stock solution was added to obtain a final concentration of 5 mM. The mixture was placed in a 37 °C water bath and incubated for 30 minutes. The antibody solution was then briefly cooled to room temperature 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 antibody solution was placed in the absence of light at room temperature for 45 minutes. Once removed, the solution was quenched with 20 µL of 0.5 M DTT for a final concentration of 10 mM. The 1.0 mL of reduced and alkyklated antibody was then 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 for 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, and diode array detector (DAD)
Column	Agilent ZORBAX Rapid Resolution High Definition 300SB-C8, 2.1 × 100 mm, 1.8 µm (p/n 858750-906)
Mobile Phase	(Various) A. H_20 + 0.1% TFA (v/v) B. n-propanol:ACN:H_20 (80:10:10) + 0.1% TFA (v/v) A. H_20 + 0.05% TFA/0.5% acetic acid (v/v) B. n-propanol:ACN:H_20 (80:10:10) + 0.05% TFA/0.5% acetic acid (v/v) A. H_20 + 0.05% TFA/0.05% formic acid (v/v) B. ACN + 0.05% TFA/0.05% formic acid (v/v)
Injection	1-3 µL
Flow rate	0.5 mL/min
Gradient	multi-segmented
Temperature	75 °C
Detection	UV, 225 nm

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

Results and Discussion

Separation optimization for ultra fast analysis of reduced and alkylated monoclonal antibody

The chromatograms in Figures 1A and 1B detail two optimized chromatographic profiles for ultra fast separation of reduced and alkylated monoclonal antibody. The Agilent ZORBAX RRHD 300SB-C8, 1.8 µm column, elevated temperature of 75 °C and optimized mobile phase compositions enabled two well resolved, high speed separations. The faster separation profile displayed in the top chromatogram (1A) details a reduced antibody separation completing in less than 4 minutes. The separation exhibits narrow light and heavy chain bands with high efficiency. In contrast, the chromatogram shown in Figure 2B also delivers a fast separation, but during a longer run time. In this separation, the light and heavy chain bands are slightly broadened, however the two heavy chain peaks 1 and 2 are now fully resolved. For each separation, mobile phase A contained water (0.1%TFA) and mobile phase B contained an 80/10/10 solvent mixture of 1-propanol, acetonitrile and water (0.1% TFA). Alternate eluent compositions of mobile phase B were tried, including 100% acetonitrile, however they were unable to deliver satisfactory peak shapes as those displayed in Figures 1 and 2. The optimized segmented gradient conditions (Table 1A) used in the top panel separation were critical to resolve the light and heavy chains in under 4 minutes, while the shallower gradient conditions (Table 1B) were optimized to enable baseline resolution of the two heavy chain variants. For both separations, each gradient completed with a fast column washing step that facilitated faster column equilibration for high throughput analysis and continued run-to-run testing.

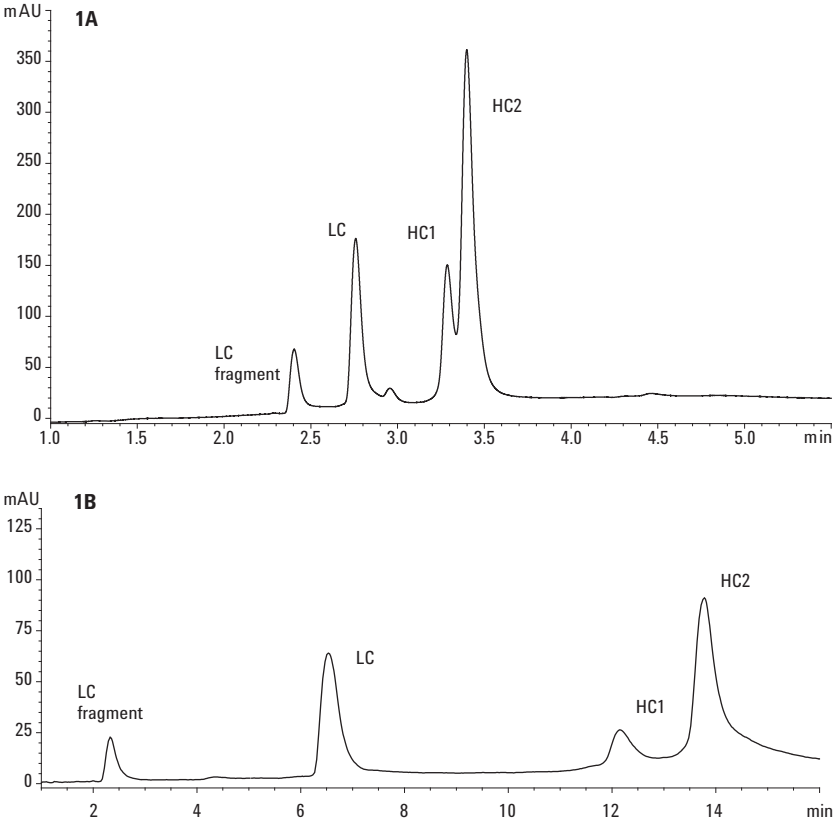


Figure 1. Comparison of two optimized gradients for the ultra fast separation of reduced and alkyklated monoclonal antibodies on an Agilent ZORBAX Rapid Resolution High Definition 300SB-C8, 1.8 µm column, 2.1 × 100 mm. The top panel details a rapid separation of the light and heavy chain variants in a shortened run time of less than 4 minutes. The bottom panel displays complete baseline resolution of the two heavy chain variants during a longer runtime using a shallower gradient profile. Both separations were performed at 75 °C and completed with a fast 90% 1-propanol wash step (UV not shown).

Table 1A. Optimized for Speed

Gradient	% Solvent B	Time (min)
	20	0
	35	3
	40	4
	40	5
	90	5.1
	90	5.5
	25	6

Table 1B. Optimized for Resolution

Gradient	% Solvent B	Time (min)
	25	0
	32	15
	32	16
	90	17
	90	17.5
	25	18

Mobile phase compositions for fast enhanced LC/MS analysis

MS electrospray ionization in positive ion mode requires reduced quantities of trifluoroacetic acid (TFA), which can greatly affect signal suppression. Additionally, MS analysis benefits from the addition of volatile mobile phase additives that increase signal intensity. Small organic acids such as formic acid (FA) or acetic acid (AcOH) are therefore the preferred ion pairing reagents for reversed-phase chromatography coupled with MS detection. We have evaluated several combinations of organic mobile phase B with various ion pair additives for providing higher signal intensity alternatives to conventional ACN/TFA only systems for LC/MS analysis. The concentration amounts of the ion pair reagents were adjusted between 0.5% and 5% while the B mobile phases included ACN, 1-propanol and iso-propanol or combinations thereof. Figures 2A and 2B below display the top two separation results from this study. Under the defined gradient conditions (Tables 2A and 2B), separation of light and two heavy chain variants were fully optimized for fast analysis with each separation completing in under 5 minutes. The top panel separation has been optimized using the same mobile phase compositions used in Figures 1A and 1B, however the TFA has been reduced from 1.0% to 0.05%, while 0.5% AcOH has been added. The gradient for this separation is shown in Table 2A. The bottom panel (Figure 2B) displays the separation from a more conventional water/ACN gradient but with TFA reduced to 0.05% and 0.05% FA added. In this separation, the two heavy chain variants show different selectivity compared to the 2A separation, which used n-propanol, where HC1 and HC2 have switched retention positions. Additionally, it was observed that using the same gradient slope and mobile phase compositions for 2B, but eliminating FA, did not provide sufficient resolution between HC1 and HC2. Conditions for each separation, using reduced amounts of TFA with FA or AcOH, are very amendable for ultra fast analysis of mAb and suggest profiling alternatives for LC/MS analysis.

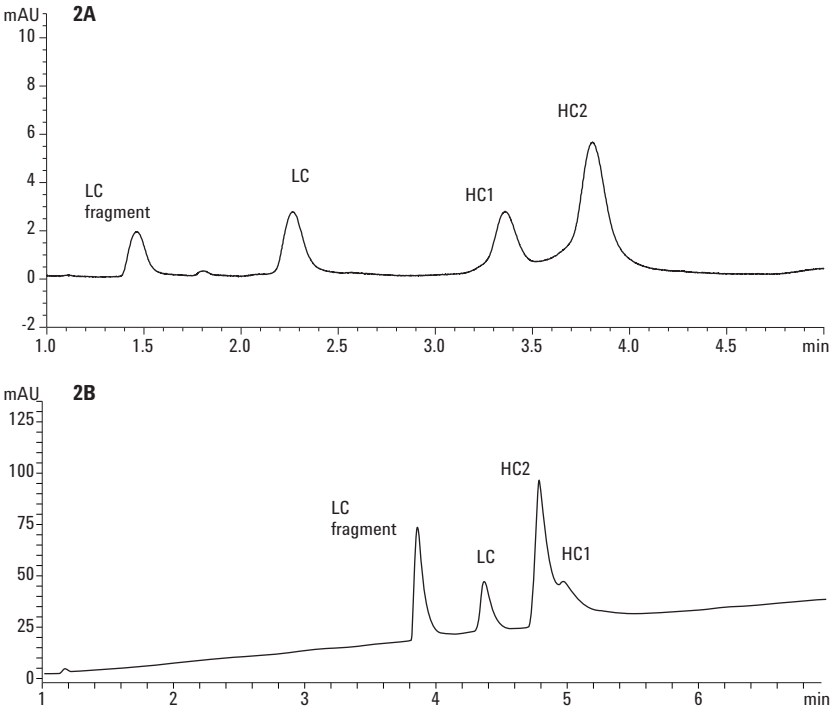


Figure 2. MS friendly mobile phase compositions for ultra fast LC/MS characterization of reduced and alkylated antibodies using an Agilent ZORBAX RRHD 300SB-C8, 2.1 × 100 mm column. The top panel (2A) separation uses the same solvent composition as noted in Figures 1A and 1B, however with the addition of acetic acid for enhancing MS signal intensity. The bottom panel (2B) was performed with a more common water/acetonitrile mobile phase, but with a reduced amount of TFA and addition of formic acid to aid in less signal suppression.

Table 2A. A: H_2O + 0.5% AcOH/0.05% TFA (v/v)
B: n-propanol:ACN:H_2O (80:10:10) + 0.5% AcOH/0.05% TFA (v/v)

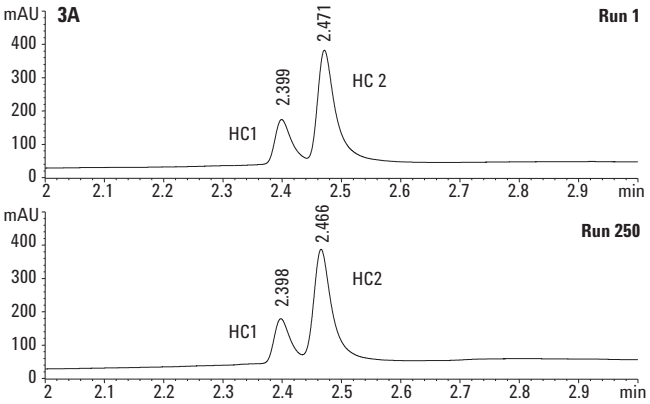
Gradient	% Solvent B	Time (min)
	25	0
	35	10
	35	12
	90	14
	25	18

Table 2B. A: H_2O + 0.05% FA/0.05% TFA (v/v)
B: n-propanol:ACN:H_2O (80:10:10) + 0.05% FA/0.05% TFA (v/v)

Gradient	% Solvent B	Time (min)
	25	0
	50	7
	90	8
	25	9

Reproducibility and Recovery during repeated mAB analysis

Using a new ZORBAX RRHD SB C8, 2.1 × 100 mm, 1.8 µm column, run-to-run reproducibility and recovery of reduced monoclonal antibodies were investigated under the gradient composition and conditions described in Table 3. To evaluate column reproducibility and recovery, 250 consecutive column runs were performed, while post run blanks and column back-pressures were evaluated every 20th injection. During the lifetime sequence, the column was exposed to repeated high pressure operation (>700 bar) and 75 °C temperature. As detailed in Figure 3A, repeated separations of heavy chain variants 1 and 2 were highly reproducible with no changes in retention time or peak shapes. The blank run UV overlap traces shown in the bottom of chromatogram of Figure 3B indicate consecutive runs do not exhibit carryover of residual mAb, while the top pressure overlays in 3B demonstrate rugged column longevity for repeated analysis and thus provided high tolerance to internal column fouling and frit blinding.



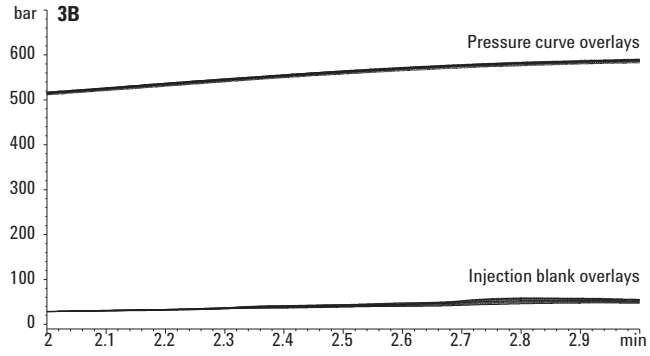


Figure 3A. Run 1 (top) and run 250 (bottom) comparison of heavy chain 1 and heavy chain 2 peak shape and retention repeatability.

Figure 3B. Thirteen overlay comparisons of blank injection UV and pressure traces during 250 repeated injections. Blank traces were collected after every 20 injection during the lifetime sequence.

Table 3. Mobile Phase and Gradient Used During Repeated Analysis of mAb

Mobile phase	A. H_2O + 0.1% TFA (v/v)	
	B. n-propanol:ACN:H_2O (80:10:10) + 0.1% TFA (v/v)	
Gradient	% solvent B	Time (min)
	25	0
	35	10
	35	12
	90	14
	25	18

Conclusions

The Agilent ZORBAX RRHD 300SB-C8, 1.8 µm column provided ultra fast and efficient separation of reduced and alkylated monoclonal antibodies. Optimized gradient conditions and elevated operating temperature (75 °C), in combination with the rugged ZORBAX C8 coating chemistry, enabled high resolution separations of antibody light chain and two heavy chain variants in under 4 minutes. Gradient conditions and compositions were systematically optimized to provide complete separation, washing and re-equilibration of antibody in a greatly reduced analysis time. Additionally, separations were optimized in ultra fast run times with use of alternate mobile phase additives (for example, formic acid and acetic acid), to provide different separation options for obtaining better MS sensitivity.

Excellent reproducibility and recovery results were obtained after 250 consecutive runs of reduced monoclonal antibody separations. At elevated pressures greater than 700 bar, repeated separations maintained peak shape and retention time, and gave no indications of early column failure due to frit plugging or a packed bed instability. The 1.8 µm ZORBAX RRHD 300SB-C8 column displayed excellent recovery as indicated by the absence of peak carryover and demonstrates robust column operation for the repeated analysis of reduced and alkylated antibodies. All separations provided high resolution and ultra fast separation of the reduced light and heavy chains and thus demonstrated utility for high throughput mAB screening.

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