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アプリケーションノート

Faster SEC-UV Analyses of mAb HMWS Using a 100 mm Sub-2 µm Column

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Abstract

Rapid and uncomplicated single-column size-exclusion chromatography (SEC) methods capable of total run times of 1.6 to 1.4-minutes (37 to 43 analyses/hr) for the analysis of high molecular weight species (HMWS) in monoclonal antibody (mAb) samples are presented. These methods take advantage of a 100 mm length SEC Column packed with 1.7 µm particles for optimal efficiency at high linear velocities in order to provide baseline resolution (R_S > 1.5) between monomer and dimeric HMWS.

The high-throughput (HT) SEC-UV methods presented use an ACQUITY™ Premier Protein SEC (250 Å, 1.7 µm, 4.6 x 100 mm) Column at flow rates of 0.75 or 0.9 mL/min and UV absorbance detection (280 nm). Column performance and robustness (1500 injection cycles) were demonstrated at these flow rates on an ACQUITY Premier UPLC™ System.

Benefits

- Rapid, 1.6- or 1.4-minute run-to-run, SEC-UV analyses of mAb HMW and ~50 KDa LMW size variants in Protein A purified cell culture samples, up-stream purification samples, and formulated stability samples
- Demonstration of column robustness over more than 1500 analyses

Introduction

HMWS mAb impurities can deleteriously impact the safety and efficacy of therapeutic proteins.[1] In a previous study, the ACQUITY Premier Protein SEC 250 Å, 1.7 µm and 2.5 µm Columns 150 mm in length (4.6 mm ID) had been demonstrated to provide robust and high resolution separations and HT SEC analysis capabilities.[2] From that study, it was noted that despite having adequate efficiency for HMWS analysis, maximizing HT capacity was limited for the 1.7 µm particle size column by excessive column pressures.

As a result, we investigated the utility of reducing the column length (100 mm and 50 mm) to increase sample throughput capabilities. It was determined from this work that the 100 mm column length was a reasonable compromise between speed and separation efficiency for the HT analysis of mAb HMWS. The reduced efficiency and greater susceptibility to LC system dispersion of the 50 mm column severely limited its utility for this application. It should be noted that the compromised efficiencies of these HT SEC separations of mAb samples generally preclude the ability to monitor typical levels of the Fc:Fab (~100 KDa) low molecular weight species (LMWS1), however, the Fc and Fab domains (~50 KDa, LMWS2) can still be observed.

The performance of the 1.7 µm (100 mm) column was compared to that of the 1.7 µm (150 mm) and the 2.5 µm (150 mm) columns run at their recommended maximum flow rates. In addition, the 1.7 µm (100 mm) and 2.5 µm (150 mm) columns were evaluated at their maximum operating pressures. Column lifetime data was also generated over 1000 injection cycles at the recommended maximum flow rate (0.75 mL/min) followed by an additional 500 injections at the maximum recommended column pressure (6000 PSI or 414 bar at 0.9 mL/min).

Experimental

Sample Description

NISTmAb RM-8671 neat (10 mg/mL).

LC Method Conditions

LC system:	ACQUITY Premier UPLC with Quaternary Solvent Manager (QSM) and CH-A Column heater
Detection:	ACQUITY UPLC TUV Detector with 5 mm titanium

	flow cell, wavelength: 280 nm, data collection rate: 20–40 points/sec
Vials:	Polypropylene 12 x 32 mm Screw Neck Vial, with Cap and Pre-slit PTFE/Silicone Septum, 300 µL Volume, 100/pk (p/n: 186002639)
Column(s):	ACQUITY Premier Protein SEC 250 Å, 1.7 µm, 4.6 x 100 mm, Column (p/n: 186011018) ACQUITY Premier Protein SEC 250 Å, 1.7 µm, 4.6 x 150 mm, Column (p/n: 186009959) ACQUITY Premier Protein SEC 250 Å, 2.5 µm, 4.6 x 150 mm, Column (p/n: 186009963)
Column temperature:	25 °C
Sample temperature:	6 °C
Injection volume:	0.5 or 0.8 µL as indicated
Flow rate:	0.5 to 1.0 mL/min as indicated
Mobile phase:	Dulbecco’ s phosphate buffered saline (DPBS) calcium and magnesium free, at 1.5X concentration (Sigma-Aldrich Cat. No. D1408) 0.1 µm sterile filtered (Thermo Scientific Mfr. No. 5670010)

Data Management

Chromatography software:	Empower™ 3 (FR 4)

Results and Discussion

Method Development

While previous results have shown that a series of biosimilar mAbs can be analyzed using a Premier SEC Column with DPBS as a mobile phase, low recovery of the HMWS were noted for NISTmAb under that condition.[3] As a result, rapid optimization of the mobile phase conditions for NISTmAb was executed using dilution levels of DPBS ranging from 1X to 2X. Maximum recovery of the HMWS multimeric forms was observed at 1.25X DPBS and higher. As a result, the method was established using 1.5X DPBS as a mobile phase for robustness.

Method Evaluation

The performance of the 1.7 µm (150 mm), 2.5 µm (150 mm), and 1.7 µm (100 mm) columns were evaluated at their recommended maximum flow rates (Figure 1). In addition, the 2.5 µm (150 mm) and 1.7 µm (100 mm) columns were at even higher flow rates that resulted in the column pressures near their respective upper limits. The assessed separation attributes included the peak-to-valley ratio (P/V) between the predominant mAb HMWS1 (primarily dimeric forms) and the mAb monomer. P/V was used instead of half-height resolution (R_{HH}) due to the pronounced confounding effect of HMWS2 (primarily multimeric) size heterogeneity on R_{HH}. It should also be noted that the P/V measured for the 1.7 µm (150 mm) column is of limited value due to the greater than baseline resolution observed between HMWS1 and monomer (R_{HH}=2.26) and presence of trace level size variants that elute between the two peaks. The quantitation of combined HMWS1 and HMWS2 impurities and LMWS2 (~50KDa) was also evaluated, while under these conditions LMWS1 (~100 KDa) cannot be reliably quantified.

The trends in the chromatographic results presented (Figure 1) are consistent with predicted trends of increased SEC resolution with smaller packed particle size, increased column length, and decreased flow rate. In addition, the quantitative results (n=2) were consistent for the relative abundances of total HMWS ranging from 2.92% to 3.04% and a trace level of LMWS2 ranging from 0.16% to 0.20%.

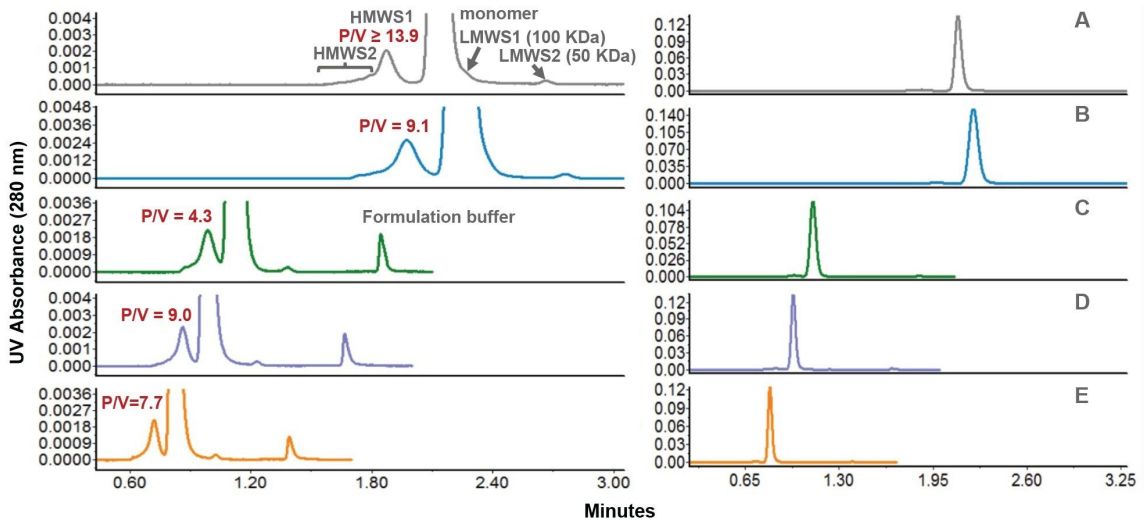

Figure 1. Shown are the HT SEC separations of NISTmAb. Mobile phase was 1.5X DPBS and injection volumes were 0.8 µL for the 150 mm columns and 0.5 µL for the 100 mm column. Columns and flow rates are: A) 1.7 µm (150 mm) at 0.50 mL/min, B) 2.5 µm (150 mm) at 0.50 mL/min, C) 2.5 µm (150 mm) at 1.00 mL/min, D) 1.7 µm (100 mm) at 0.75 mL/min, E) 1.7 µm (100 mm) at 0.90 mL/min. UV absorbance detection was performed at 20 to 40 Hz.

The minimum analysis times for the columns were also compared. The assigned analysis times include an additional allotment of 0.3 min for the autosampler to inject a sample and the use of interlaced SEC runs where the next injection is initiated after the LMWS has eluted and the baseline reestablished. Comparisons of note for the effective HT HMWS analysis capabilities of the 1.7 µm (100 mm) column are summarized in Table 1. Of note is that the analysis times for the 1.7 µm (100 mm) column are less than half that of the 1.7 µm (150 mm) column with a concomitant loss in resolution, and up to 20% faster than the maximum throughput of the 2.5 µm (150 mm) column with significantly greater resolutions.

Premier Protein SEC 250Å Column	Flow rate (mL/min)	Column pressure (PSI)	Interlaced analysis Time[1] (min)	HMWS P/V
1.7 µm, 4.6 x 150 mm	0.50	6000 (7000 max)	3.1	≥ 13.9
2.5 µm, 4.6 x 150mm	1.00	4500 (4500 max)	1.8	4.3
1.7 µm, 4.6 x 100 mm	0.75	5000 (6000 max)	1.7	9.0
1.7 µm, 4.6 x 100 mm	0.90	6000 (6000 max)	1.4	7.7

1. Analysis time is for interlaced injections and includes 0.3 minutes for autosampler to perform an injection

Table 1. Analysis time is for interlaced injections and includes 0.3 minutes for autosampler to perform an injection.

Method Reliability

Lifetime for the 1.7 µm (100 mm) column was demonstrated over 1000 injection cycles at the recommended maximum flow rate (0.75 mL/min) followed by an additional 500 injections at the maximum recommended column pressure (6000 PSI or 414 bar at 0.9 mL/min). For this study the mobile phase was 0.1 µm sterile filtered and samples were centrifuged for one to two minutes at 10000 x g which is recommended to remove subvisible and larger particulates, thereby extending column lifetimes. HMWS resolution was maintained following all 1500 injections (Figure 2). However, following the 500 injections at 0.9 mL/min a modest increase in low level tailing was observed. These results indicate that the column is capable of sustained performance at 0.75 mL/min, while performance is modestly compromised at 0.9 mL/min. This allows for analysis times of 1.4 to 1.6-minutes between injections.

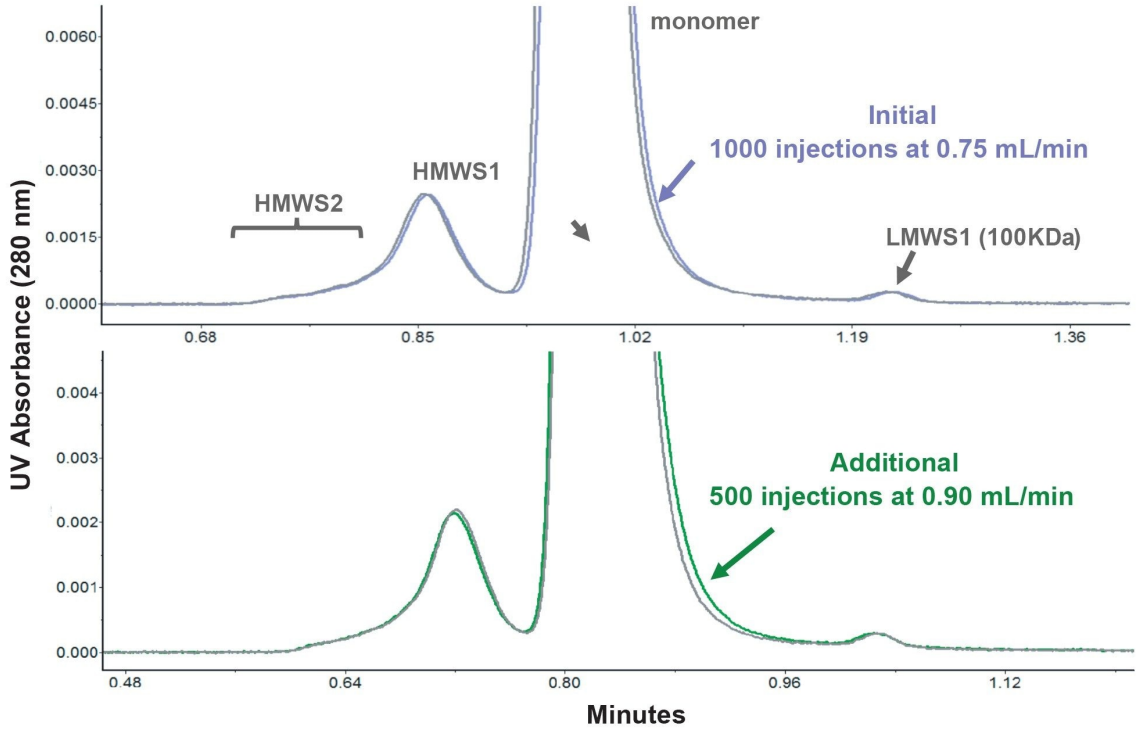

Figure 2. Demonstrated is the robustness of an ACQUITY™ Premier Protein SEC 250 Å, 1.7 µm, 4.6 x 100 mm Column following 1000 injection cycles at 0.75 mL/min (top panel) followed by 500 injection cycles at 0.90 mL/min (bottom panel). Mobile phase was 1.5X DPBS and injection volumes were 0.5 µL. UV absorbance detection was performed at 20 to 40 Hz.

Conclusion

Rapid SEC methods can be useful to support the development of biotherapeutic cell-culture and purification processes, and also the development of drug substance and drug product formulations. A high-throughput (1.6 and 1.4-minute run time) SEC-UV method using an ACQUITY Premier Protein SEC (250 Å, 1.7 µm, 4.6 x 100 mm) Column at flow rates of 0.75 and 0.90 mL/min for the analysis of monoclonal antibodies HMWS and LMWS2 (~50 KDa) fragments has been presented.

In addition, column lifetime performance with 1000 injection cycles at 0.75 mL/min followed by 500 at 0.90 mL/min was also demonstrated. For this evaluation, the mobile phase was 0.1 µm sterile filtered and the

samples were centrifuged for one to two minutes at 10,000 x g, which can greatly reduce the probability of column fouling.

References

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2. Stephan M. Koza and Ying Qing Yu. Rapid Size Variant Analysis of Monoclonal Antibodies Using UPLC™ and HPLC Compatible MaxPeak™ Premier Protein SEC Columns, 720007584, March 2022.
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