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Rapid SEC-UV Analysis of Monoclonal Antibodies Using Ammonium Acetate Mobile Phases

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Abstract

A 4 minute high resolution size-exclusion with UV absorbance detection (SEC-UV) method for the analysis of aggregation in monoclonal antibody (mAb) samples using an ammonium acetate mobile phase is presented. Rapid non-denaturing SEC-UV methods can be useful to support biotherapeutic cell culture, purification, and formulation development. In addition, a rapid SEC-UV method using an ammonium acetate (AMA) mobile phase can also be more efficiently deployed on an LC system dedicated for LC-MS analysis versus using a traditional size exclusion chromatography (SEC) mobile phase comprised of non-

volatile salts and buffers (NaCl, phosphate, *etc.*). It is noted that while ESI-MS detection could also be used for this SEC method, the high AMA concentration and flow rate resulted in low quality MS data.

In this application, SEC was effectively used to monitor self-associated or aggregated mAb impurities, which can impact product safety and efficacy, in Protein A purified mAb samples. The automated Protein A based mAb purification was performed using an Andrew+™ pipetting robot. The high-throughput SEC-UV method uses an ACQUITY™ Premier Protein SEC (250 Å, 1.7 µm, 4.6 x 150 mm) Column, a 200 mM ammonium acetate mobile phase at a flow rate of 0.50 mL/min and UV absorbance detection (280 nm). Method performance and robustness were evaluated on stand-alone ACQUITY Premier UPLC™ Systems in addition to a BioAccord™ LC-MS (ESI-Tof) System.

Benefits

- Rapid, 4 minute, SEC-UV analyses of mAb HMW and LMW size variants in Protein A purified cell culture samples, up-stream purification sample, and formulated stability samples
- Effective UV-SEC analysis of mAb samples at concentrations of 0.5 mg/mL or greater that is also compatible with UPLC-MS systems
- Demonstration of real-use column lifetime over 3 months usage and more than 700 analyses

Introduction

SEC is frequently used to monitor self-associated or aggregated mAb impurities, which can impact product safety and efficacy of therapeutic proteins.[1] In previous studies, a Waters ACQUITY Premier Protein SEC 250 Å, 1.7 µm Column had been demonstrated to provide robust and high resolution separations at low ionic strengths with rapid and high-throughput (HT) size exclusion chromatography (SEC) analysis capabilities.[2,3] In this study, these capabilities were applied toward the development of a HT SEC-UV analysis of Protein A purified mAb samples while using a ammonium acetate (AMA) mobile phase compatible with LC-MS instruments.

While AMA is ubiquitously used in native SEC-MS analyses we selected it as a mobile phase salt for SEC-UV analysis due to its significantly greater compatibility with LC-MS instrumentation versus use of traditional SEC buffer components such as sodium phosphate and sodium chloride. In addition, AMA has been reported to have bacteriostatic properties at high concentrations.[4] Although this was not evaluated specifically for AMA this may be a potential benefit versus phosphate and other biological buffer systems that are more prone to microbiological contamination.

In addition, column lifetime data generated as part of a parallel study focused on automating a Protein A purification method for over 700 analyses generated over the course of more than 3 months is also presented along with column-to-column reproducibility results.

Experimental

Sample Description

mAbs were obtained from various sources and diluted in phosphate buffered saline (PBS) to indicated concentrations. These included infliximab, rituximab, natalizumab, cetuximab, tocilizumab, and NISTmAb RM-8671, and two samples of trastuzumab, trastuzumab (Herceptin™), and trastuzumab-anns (Kanjinti™).

LC Conditions

LC system:	ACQUITY Premier UPLC with Binary or Quaternary Solvent Manager (BSM or QSM) and CH-A column heater or a BioAccord™ LC-MS (ESI-ToF) System
Detection:	ACQUITY UPLC TUV Detector with 5 mm titanium flow cell, wavelength: 280 nm
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, 2.5 µm, 4.6 x 150mm, Column plus mAb Size Variant Standard (p/n: 176004783)
Column temperature:	25 ˚C
Sample temperature:	6 ˚C
Injection volume:	5 µL or as indicated
Flow rate:	0.5 mL/min
Mobile phase:	ammonium acetate, LC-MS grade (Supelco LiChropur™, eluent additive for LC-MS, 73594), 0.1 µm sterile filtered, 200 mM or as indicated

Data Management

Chromatography software:	Empower™ 3 (FR 4) and UNIFI™ Version 2.1.2.14

Results and Discussion

Method Development

The goal of this study was to develop a HT non-denaturing SEC-UV method for the analysis of mAb aggregation that could be more readily deployed on UPLC systems dedicated for LC-MS analyses. This target was met using a 200 mM AMA mobile phase, which delivered an effective separation for all 7 of the mAbs tested. A flow rate of 0.5 mL/min was also selected resulting in an analysis time of 4 minutes.

AMA, which has a sublimation point of 120 °C and is available at high purity for LC-MS use, was selected as a volatile, MS compatible salt. AMA was used un-titrated at a pH of approximately 7.0, where its buffering capacity is negligible, although acetate (pKa 4.75) and ammonium (pKa 9.25) would be predicted to provide at least 5% of their maximum buffering capacities below pH 6.0 or above pH 8.0, respectively. AMA concentrations ranging from 100 mM to 300 mM were evaluated for a series of mAbs (Figure 1). The mAb samples were diluted to 1.0 mg/mL in PBS based on their original labeled concentrations. Injection volumes of 5 µL were used for mAb samples with relative HMWS abundances of approximately 1% or less and reduced injection volumes were used for mAb samples with greater HMW levels in order to maintain comparable mass loads of HMWS for each mAb.

At AMA concentrations of 150 mM and greater comparable profiles and HMWS peak areas were observed for all of the mAbs evaluated. However, for the 100 mM AMA mobile phase reduction in peak areas for the HMWS forms were

observed for both trastuzumab samples with the loss of HMWS2 being most obvious for trastuzumab and to a lesser extent HMWS peak areas were also reduced for tocilizumab, and NISTmAb. Based on these results 200 mM AMA was selected as the optimal mobile phase as it provides for a robust separation for all of the mAbs tested. Low molecular weight mAb fragments (LMWS1 and LMWS2) were also observed. While for the majority of the mAb samples, the LMWS were present at trace levels and challenging to resolve (<0.7 % LMWS1 and <0.1% LMWS2) the separations were consistent between 150 mM and 250 mM AMA.

A flow rate of 0.5 mL/min was selected resulting in an analysis time of 4 minutes. At this flow rate effective resolution was achieved, in addition, when deployed on an LC-MS system excessive pressure on the UV flow cell that is caused by connection to the MS divert valve was avoided.

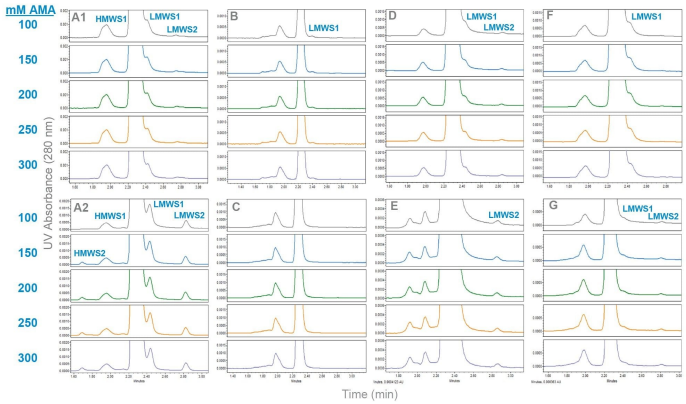

Figure 1. Shown are the SEC-UV chromatograms for several mAb samples with AMA mobile phase at concentrations ranging from 100 mM to 300 mM. Injection volumes were 5 µL unless noted otherwise. Sample identities are: (A1) trastuzumab-anns, (A2) trastuzumab, (B) cetuximab (0.5 µL) , (C) natalizumab (1 µL) , (D) tocilizumab, (E) rituximab, (F) infliximab, and (G) NISTmAb RM-8671 (1.5 µL). All preparations were analyzed past expiry. These data were collected on an ACQUITY Premier QSM UPLC.

Method Evaluation

Consistent relative peak areas were observed for HMWS and LMWS size variants at mAb sample concentrations from 0.5 µg/µL to 2.0 µg/µL. To assess the effect of sample concentration trastuzumab was evaluated at concentrations of 0.5, 1.0, and 2.0 µg/µL. These results are highlighted in Figure 2. Consistent chromatographic profiles relative peak areas were observed for the three HMWS size variants (HMWS2, HMWS1, and HMWSs) and both LMWS size variants (LMWS1 and LMWS2). HMWS1 and HMWS2 are primarily dimeric and multimeric self-associated mAb. The peak identified as HMWSs was not definitively identified,

however it is suspected to be the result of self-association involving LMWS mAb fragments. LMWS1 is primarily mAb with one missing Fab domain (~100 KDa) and LMWS2 is primarily a mixture of Fab and Fc (~50 KDa).

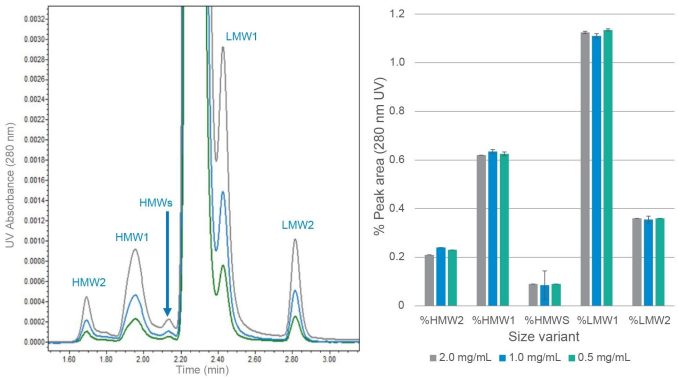

Figure 2. Shown are the SEC-UV chromatograms and quantitative results for trastuzumab evaluated at concentrations of 0.5, 1.0, and 2.0 mg/mL. Error bars represent the range of values obtained (n=3). Injection volumes were 5 µL and all preparations were analyzed past expiry. These data were collected on an ACQUITY Premier QSM UPLC.

Injection volumes were 5 µL for this study, and although while not investigated further in this work, lower detection limits may be achieved by increasing injection volumes, using UV absorbance at a more sensitive wavelength (e.g., 214 nm), or using intrinsic protein fluorescence detection.

The effectiveness of the method for the relative quantification of size variants was also confirmed using mixtures of the two trastuzumab samples (Figure 3). For this evaluation the two preparations of trastuzumab shown

in Figure 1, trastuzumab-anns and trastuzumab were analyzed separately and as a 1:1 (volume: volume) mixture. The trastuzumab sample used, which had been stored primarily at 80 °C for approximately ten years, had a higher total mAb concentration than the trastuzumab-anns sample based on SEC analysis of the neat samples. As a result, the 1:1 (v: v) mixture was determined to be comprised of 56.1% trastuzumab and 43.9% trastuzumab-anns on a weight: weight basis. Functional response curves were obtained for the HMWS2, HMWS1, LMWS1, and LMWS2 size variants at relative levels below or near 1%.

For this study a drop-baseline integration was used for partially resolved peaks (Figure 3). While this approach generally over-estimates the percentage of peaks such as LMW1, which is partially resolved from the far more abundant (100X larger) main (monomer) peak that precedes it, the drop baseline approach generally tends to better facilitate reliable automated integration algorithms. However, this is only the case when a valley can be detected between the two peaks. The presence of this valley is predicated upon both the quality of the chromatographic separation, which among other variables can be impacted by mobile phase effectiveness, column resolution, LC system dispersion, and the relative abundance of the low- level component.[5] In these studies, the 5 σ UPLC Ssystem dispersions were lower than 12 µL. In this method for example, quantification of the LMW1 variant is not predicted to extend below the level (0.6%) observed in the trastuzumab-anns sample.

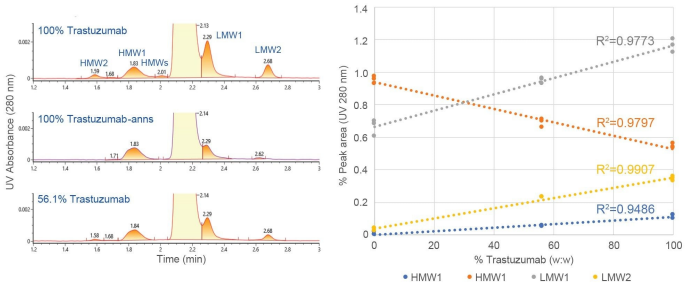

Figure 3. Shown are the SEC-UV chromatograms and quantitative results for trastuzumab, trastuzumab-anns, and a mixture of the two samples. Injection volumes were 5 µL and all preparations were analyzed past expiry. These UV data were collected on an BioAccord LC-MS (ESI-Tof) System. Chromatographic conditions are provided in the text.

One of the primary intended uses of this HT method, the testing of mAb samples purified by Protein A affinity, was also demonstrated. For this evaluation, a sample of trastuzumab-anns was prepared at 1 µg/µL in PBS and spiked 1 µg/µL into a clarified non-transfected CHO cell media (NTM). NTM was prepared by Syd Labs, Inc. using non-transfected CHO-K1 cells in a spinner flask where spent media was collected from the flask on days 2 through 15 (~90% average cell viability), pooled, and 0.2 µm filtered. The two spiked samples were then Protein A purified using an Andrew+ pipetting robot (Andrew Alliance™). Briefly, samples (120 µL each) were captured using Cytiva MabSelect™ resin (~15 µL) that was dispensed into a 96-well Pall AcroPrep™ 96-well 0.2µm Supor™ plate. The sample was allowed to bind for 20 minutes on an orbital plate shaker (1200 rpm), washed with PBS, and eluted three

times (5-min static hold times) with 30 µL of 100 mM glycine pH 3 which was instantly neutralized with a 10 µL volume of 1.0 M TRIS, pH 7.5. Recoveries were 94.4% (6.1 %RSD, n=8) for the NTM-spiked samples and 90.8% (6.9 %RSD, n=8) for the PBS-spiked samples.

The effectiveness of the SEC method is demonstrated from the relative abundances of the size variants determined for a control trastuzumab-anns sample and the Protein A purified samples (Figure 4). With respect to the SEC method, the %RSD values (n=3) for HMWS1 (0.9% RSD), LMWS1 (2.4% RSD), and trace level of LMWS2 (7.9% RSD) indicate reproducible relative abundance determinations of the size variants in the control sample. The results obtained for the two sets of Protein A purified samples showed greater maximum variances (n=8) for HMWS1 (3.5% RSD), LMWS1 (2.8% RSD), and LMWS2 (24.7% RSD). These higher variances are likely due to the combined variances introduced by the Protein A purification and the SEC method.

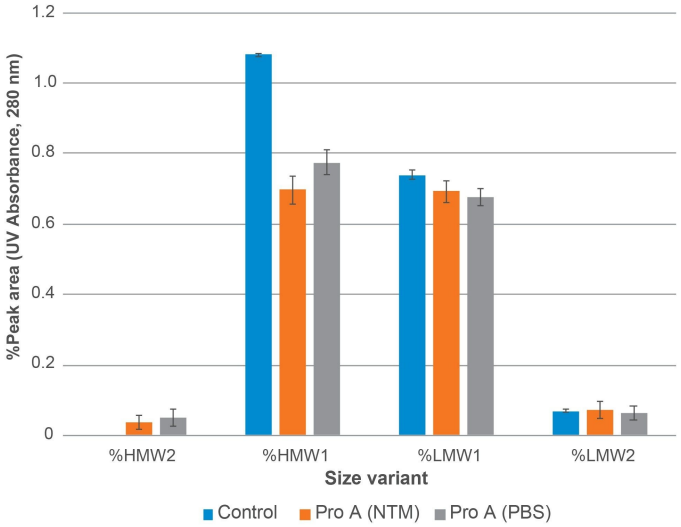

Figure 4. Shown is an evaluation the SEC-UV quantitative results for protein A purified trastuzumab-anns. Trastuzumab-anns spiked into PBS (n=8) and non-transfected CHO cell conditioned media (NTM, n=8) are compared to a spike control (n=3). Chromatographic conditions are provided in the text. Error bars represent the range of values obtained. These data were collected on an ACQUITY Premier BSM UPLC.

Although further investigation was outside the scope of this study, it is noted that the Protein A based mAb purification procedure used modestly biases the HMWS results for trastuzumab-anns. Trace level amounts (<0.05%) of HMW2 are artifactually generated while HMW1 is partially recovered. Absolute HMW1 size variant recoveries were estimated as 68% for the spiked NTM sample and 59% for the spiked PBS sample, assuming that additional HMW1 forms were not also generated by the Protein A purification process. Challenges with the quantitative recovery of HMW mAb variants when using Protein A affinity purification,

even when deploying a more precise LC-based methodology, have been previously reported.[6]

In total, these results demonstrate that the developed SEC-UV method can be effective for the determination of mAb size variants for a low range of sample concentrations (≥0.5 mg/mL) both in typical protein formulation buffers and in neutralized Protein A elution buffer. The finalized method has a 4 minute analysis time that is capable of recovering and resolving the HMW forms of the mAbs tested and can also provide separation of low molecular weight (LMW) size variants for several of the mAbs evaluated. It should be noted that while ESI-MS detection can be used for this separation, the high AMA concentration and flow rate resulted in low quality MS data.

Method Reliability

Two principal components of method reliability are column performance over time and column-to-column reproducibility. An unplanned assessment of column lifetime when using AMA mobile phases for more than 700 samples run over the course of more than 3 months (Figure 5) was obtained. Over this period the column was subjected to multiple stops and starts, long periods of inactivity, and was exposed to a variety of Protein A purified, partially purified, and even crude cell-culture samples. In comparing the earlier and later performance of the column, a slight overall retention shift was observed although much of the initial resolution was retained. It can also be seen that the abundance of multimeric HMW2 is lower. This can be primarily attributed to sample stability based on the comparable performance between this column and a new column using the same trastuzumab sample at nearly the

same time (Figure 6). Additionally, the new column used for comparison was also packed with particles manufactured at a later time. Taken together, these results indicate that these columns are be capable of sustained performance and can deliver reliable column-to-column performance.

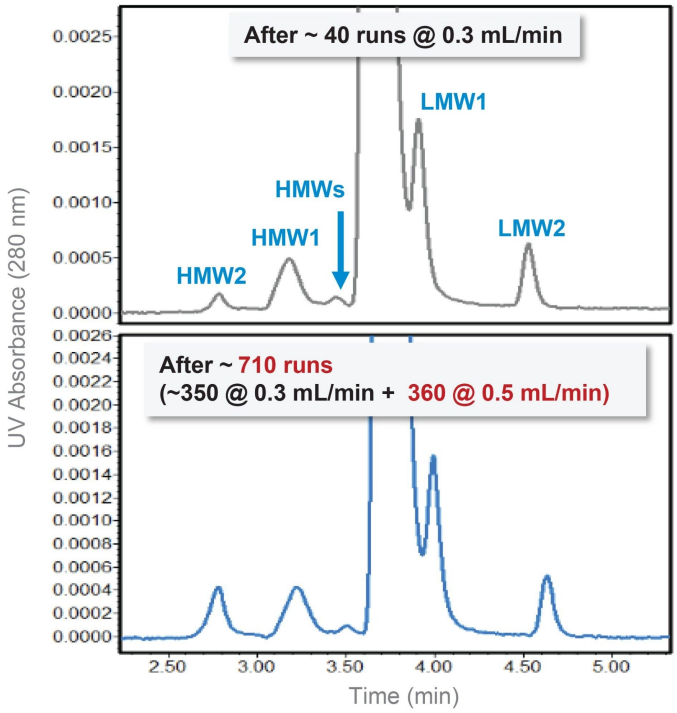

Figure 5. Real-use SEC column life-time when using a 200 mM ammonium acetate mobile phase at flow rates of 0.3 to 0.5 mL/min. A comparison of chromatograms spanning over more than 700 analyses and 3 months is presented for trastuzumab (1 mg/mL in PBS). Chromatographic conditions and further discussion are provided in the text. These data were collected on an ACQUITY Premier BSM UPLC at a flow rate of 0.3 mL/min.

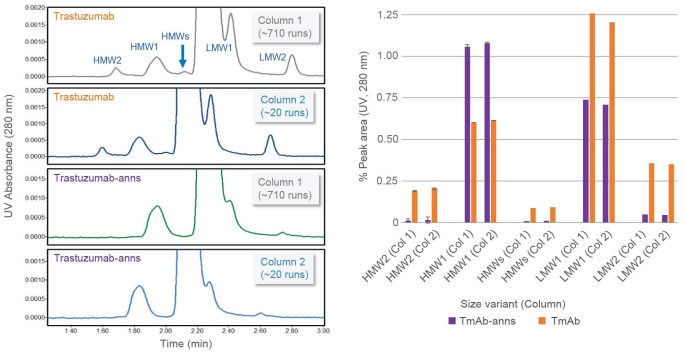

Figure 6. Column-to column reproducibility is demonstrated for two columns packed with different batches of SEC particles manufactured several months apart. Samples evaluated are trastuzumab-anns and trastuzumab @ 1 mg/mL (PBS). A mobile phase of 200 mM ammonium acetate was used at a flow rate 0.5 mL/min. Column 1 was used for more than 700 analyses and 3 months, while Column 2 data were generated after 20 runs. Chromatographic conditions and further discussion are provided in the text. Error bars represent the range of values obtained (n=2). These data were collected on an ACQUITY Premier BSM UPLC.

Conclusion

Rapid non-denaturing size exclusion chromatography (SEC) methods can be useful to support the development of biotherapeutic cell-culture and purification processes, and the development of drug substance and drug product formulations. In addition, a rapid SEC-UV method using an ammonium acetate mobile phase can also be more

efficiently deployed on an LC system or column dedicated for LC-MS analysis versus using a traditional SEC mobile phase comprised of non-volatile salts and buffers (NaCl, phosphate, *etc.*).

Specifically, for this application, we successfully optimized a high-throughput (4 minute run time) SEC-UV method with an ACQUITY Premier Protein SEC (250 Å, 1.7 µm, 4.6 x 150 mm) Column using a 200 mM ammonium acetate mobile phase at a flow rate of 0.50 mL/min and UV absorbance detection (280 nm) for size variant (aggregation and fragmentation) analysis of monoclonal antibodies. Reliable method performance was also evaluated on stand-alone ACQUITY Premier UPLC Systems in addition to a BioAccord LC-MS (ESI-Tof) System, all with low dispersion volumes.

In addition, column lifetime performance with more than 700 analyses over the course of 3 months, and column-to-column reproducibility was also demonstrated. For this evaluation, the mobile phase was 0.1 µm sterile filtered and the samples were 0.2 µm filtered separately or as part of the Protein A purification procedures used, which can greatly reduce the probability of column fouling. However, if development samples containing larger quantities of subvisible or larger particles are being analyzed it is recommended that a guard column (MaxPeak™ Premier Protein SEC Guard, 250 Å, p/n: 186009969 <https://www.waters.com/nextgen/global/shop/columns/186009969-maxpeak-premier-protein-sec-guard-250a-25--m-46-x-30-mm-1-pk.html>) or sample pretreatment such as centrifugation be used.

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