Waters™

Application Note

Future Proofing the Biopharmaceutical QC Lab: Benefits of Automating Mobile Phase Delivery to Improve pH Consistency in Size Exclusion Chromatography Methods

Eoin F.J. Cosgrave, Sean M. McCarthy

Waters Corporation

Abstract

This application note demonstrates the series on transitioning HPLC-based biopharmaceutical separations to UPLC-based methods focusses on the benefits of automating mobile phase delivery. The benefits of Auto▪Blend Plus Technology for consistent and reliable delivery of pH-dependent mobile phase for SEC-HPLC and SEC-UPLC and steps required to convert conventional SEC methods to Auto▪Blend Plus methods are described.

Benefits

- Using the ACQUITY UPLC H-Class Bio System to perform size exclusion chromatography (SEC) in biopharmaceutical quality control (QC)
- Experimental approach for converting conventional mobile phase delivery to Auto▪Blend Plus SEC assays

Introduction

Large molecule separations that require buffered mobile phases represent a challenge in analytical labs due to the potential sensitivity of analytes to changes in pH and salt concentration. One such large molecule assay includes size exclusion chromatography (SEC), which is typically used to measure the extent of aggregation in protein-based therapies.

Mobile phases for SEC separations have historically been prepared by combining individual components of the mobile phase followed by adjustment to the desired pH using an appropriate acid or base. In this scenario, calibration of the pH meter and the associated accuracy of pH measurements can directly influence the final pH of the mobile phase, affecting the quality of the final separation. As a result, subtle changes in the preparation of mobile phase can lead to differences in chromatography in situations where pH differs between mobile phase preparations.

In this application note, we continue our discussion of using the ACQUITY UPLC H-Class Bio System for size exclusion chromatography[1] by demonstrating the benefits of Auto▪Blend Plus Technology – which is included with all ACQUIT UPLC H-Class instruments – for consistent and reliable delivery of pH-dependent mobile phase for SEC-HPLC and SEC-UPLC.

Compared to manual approaches where mobile phase delivery is defined by percent composition of each solvent line, Auto▪Blend Plus allows the user to define individual method steps based on the desired pH and salt concentration. This enables the analyst to explore an extensive list of method parameters in a single set of buffer preparations.

Auto▪Blend Plus can be particularly advantageous in QC environments, where methods are expected to be accurate, precise, and robust. Variability in mobile phase preparation due to inconsistencies with pH can potentially lead to erroneous outcomes that can otherwise be controlled using automated chromatographic tools such as Auto▪Blend Plus.

In this application note, we demonstrate how a conventional SEC method is converted to an Auto▪Blend Plus-enabled method using the ACQUITY UPLC H-Class Bio System. There is no disruption to mobile phase composition when performing this conversion: Auto▪Blend Plus delivers identical chromatography to that obtained using mobile phase prepared and delivered in a conventional manner. The results presented in this application note show robust, precise, and reliable chromatography for both SEC-HPLC and SEC-UPLC, supporting the prospect of Auto▪Blend Plus as a technology that can be successfully deployed in large molecule QC environments.

Experimental

LC conditions

ACQUITY UPLC H-Class Bio System, comprised of:

- ACQUITY UPLC H-Class Bio Quaternary Solvent Manager (QSM)
- ACQUITY UPLC H-Class Bio Sample Manager (SM)
- ACQUITY UPLC Tunable UV Detector with Ti flow cell
- Extension loop: 100 µL (p/n 430002625)
- BioSuite SEC 10 µm, 250 Å Column, 7.5 mm x 300 mm (p/n 186002170)
- ACQUITY UPLC Protein BEH SEC Column, 200 Å, 1.7 µm, 4.6 x 150 mm (p/n 186005225)
- BEH200 SEC protein standard mix (p/n 186006518)

Column temp.: Ambient

Seal wash: 10% acetonitrile in H^2O

Conventional mobile phase: 20 mM phosphate, 200 mM NaCl, pH 6.8

Auto•Blend Plus

Auto•Blend Plus

Mobile phase A:	100 mM NaH_2PO_4
Mobile phase B:	100 mM Na_2HPO_4
Mobile phase C:	1 M NaCl
Mobile phase D:	H_2O
Detection wavelength:	214 nm
Syringe purge:	H_2O
Syringe wash:	H_2O

HPLC conditions

Injection vol.:	20 µL
Flow rate:	0.400 mL min^{-1}
Method length:	35 min

UPLC conditions

Injection vol.:	4 µL
Flow rate:	0.885 mL min^{-1}
Method length:	3 min

Results and Discussion

Experimental design of conventional and Auto•Blend Plus assisted SEC

Preparation of aqueous, pH dependent mobile phases can be a cumbersome aspect for both method development experiments as well as high-throughput assay environments where mobile phase is used in high volume. In the latter case, each preparation of new mobile phase can be susceptible to variability due to differences in pH meter calibration and accuracy, pH adjustment of the mobile phase, and general differences in the way analysts prepare mobile phase.

To get around this inconsistency, control of mobile phase preparation can instead be accomplished using Auto•Blend Plus Technology. Solutions of appropriate acid, base, salt, and water can be prepared separately as concentrated stocks and mixed together using Auto•Blend Plus, which combines the necessary proportions of each solvent required for delivering a specified pH and salt concentration. This strategy is made possible by the ACQUITY UPLC H-Class System's Quaternary Solvent Manager, which can combine four separate solvents to form a desired mobile phase composition.

To evaluate the similarity between conventional SEC-HPLC and Auto•Blend Plus assisted SEC-HPLC, we created two sets of mobile phase for each SEC assay. For conventional SEC, the mobile phase consisting of 20 mM phosphate with 200 mM NaCl adjusted to pH 6.8 was prepared at the bench. For Auto•Blend Plus assisted SEC, four separate stock solvents of 100 mM NaH_2PO_4 buffer, 100 mM Na_2HPO_4 buffer, 1 M NaCl, and pure H_2O were prepared.

In each case, a BioSuite SEC 10-µm 250 Å Column (7.5 x 300 mm) was used for comparison. Two separate protein samples were used to evaluate the HPLC approaches. The first protein sample was a Waters SEC200 protein standard mix consisting of five components intended for determining the total inclusion and exclusion volumes of SEC columns capable of separating proteins between approximately 10 kDa and 500 kDa. The second protein was the commercial monoclonal antibody, infliximab, previously shown to contain a minor amount of aggregate formation.[1]

For accurate delivery of a desired pH, an empirical table was generated that accounted for the effect of increasing salt concentration on mobile phase pH. Instrument methods for both conventional SEC-HPLC and Auto•Blend Plus SEC-HPLC were created using Empower 3 Software (Figure 1). For conventional SEC-HPLC, all relevant instrument details were outlined as depicted in Figure 1A. The Auto•Blend Plus SEC-HPLC method was created by selecting Auto•Blend Plus from the QSM option in the instrument method and itemizing the desired pH and salt concentration, as depicted in Figure 1B. Addition of empirical data was accessed by selecting Buffer System and then selecting the

Empirical Data option on the right side of the new window (Figure 2). It is recommended that labs generate their own Auto•Blend Plus tables as suppliers of raw chemicals and standard operating procedures may yield different pH values than those listed in the figure.

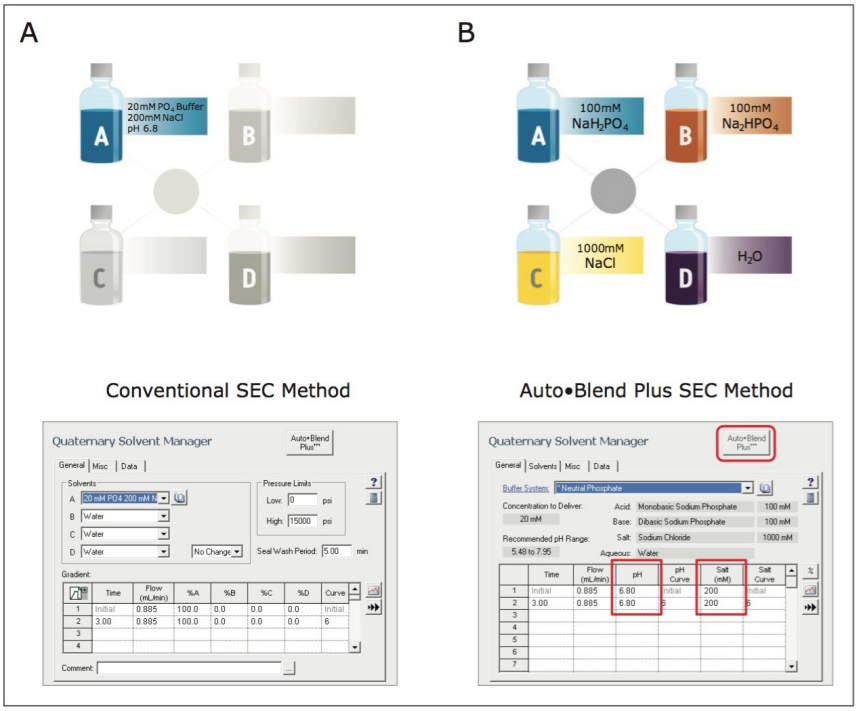

Figure 1. Conventional SEC and Auto•Blend Plus assisted SEC arrangements. Presented instrument method windows relate to the SEC-UPLC method. (A) Conventional SEC arrangement typically has a single prepared mobile phase on one solvent line, which is itemized in the instrument method as a 100% solvent A. (B) In Auto•Blend Plus SEC arrangements, mobile phases corresponding to acid (NaH_2PO_4), base (Na_2HPO_4), salt (NaCl), and water are configured on 4 solvent lines. The instrument method is modified to request the desired pH and salt composition rather than a percent mobile phase, as illustrated by the red boxed items. Similar windows exist for SEC-HPLC with appropriate changes to flow rate and method duration.

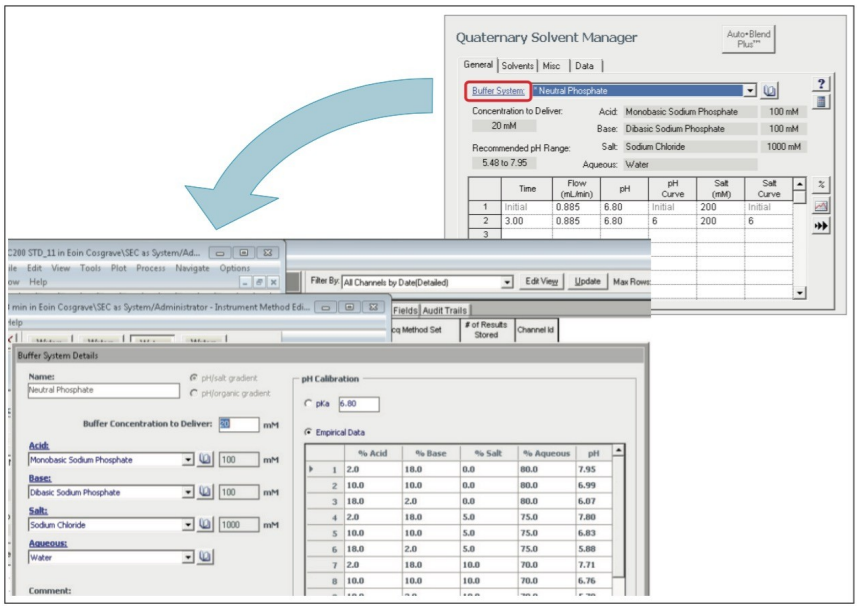

Figure 2. Recording empirical data in Auto•Blend Plus methods. Correcting pH due to salt concentration can be added to Auto•Blend Plus methods by first selecting the "Buffer System" in the QSM tab of the Empower instrument method window. In the new window, the option of "pKa" or "Empirical Data" is available. Selecting "Empirical Data" activates the table where pH values corresponding to the composition itemized in each row can be entered.

SEC-HPLC with Auto•Blend Plus generates identical results to conventional SEC-HPLC

To determine the comparability of Auto•Blend Plus for SEC HPLC, a benchmark separation was first established using the conventional SEC-HPLC method with the BioSuite SEC 10-µm column. In the first instance, the SEC200 protein standard mix was chromatographically separated and all peaks were shown to elute within the method run as expected (Figure 3A). All relevant chromatographic data is recorded in Table 1. With migration times established for each protein standard component, the ACQUITY UPLC H-Class Bio was configured to run Auto•Blend Plus methods by exchanging the conventional SEC mobile phase arrangement (Figure 1A) with the Auto•Blend Plus mobile phase arrangement (Figure 1B). The same column and SEC200 protein standard mix were used. Each component of the standard was shown to exhibit near identical migration times when compared to the conventional SEC-HPLC method results (Figure 3B and Table 1). Relative peak areas associated with each component were also shown to be highly comparable, indicating the ability of Auto•Blend Plus to generate identical chromatography when compared to conventional HPLC.

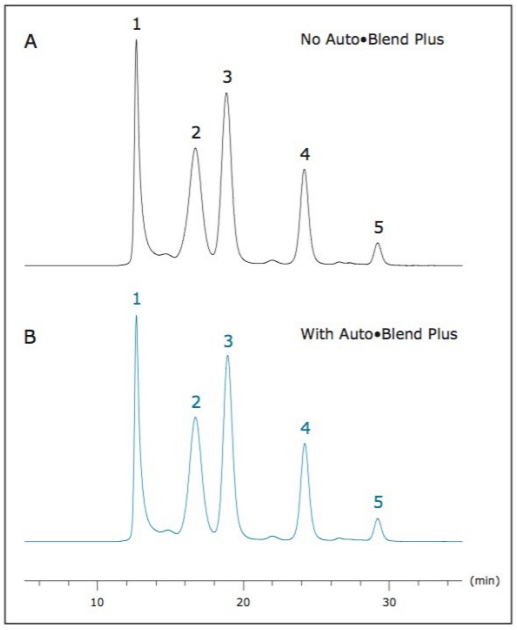

Figure 3. Auto•Blend Plus assisted SEC-HPLC generates equivalent chromatography to the conventional approach. In each chromatogram, 1 refers to thyroglobulin, 2 refers to IgG, 3 refers to BSA, 4 refers to myoglobin, and 5 refers to uracil. (A) SEC-HPLC using conventional mobile phase preparation; (B) SEC-HPLC using Auto•Blend Plus Technology for mobile phase delivery.

Peak	SEC component	Retention time (min)				Peak area (%)			
		Auto•Blend Plus				Auto•Blend Plus			
		-		+		-		+	
		$\bar{x}$	σ	$\bar{x}$	σ	$\bar{x}$	σ	$\bar{x}$	σ
1	Thyroglobulin	12.65	0.001	12.66	0.002	23.79	0.638	23.64	0.015
2	IgG	16.64	0.002	16.70	0.002	27.64	0.260	27.83	0.012
3	BSA	18.86	0.002	18.91	0.005	31.38	0.240	31.15	0.021
4	Myoglobin	24.07	0.004	24.19	0.003	14.45	0.107	14.60	0.015
5	Uracil	29.07	0.002	29.19	0.002	2.74	0.032	2.78	0.012
1	Mab dimer	14.19	0.022	14.29	0.003	0.47	0.000	0.53	0.010
2	Mab monomer	17.18	0.001	17.53	0.004	99.53	0.000	99.47	0.010
1	Thyroglobulin	1.03	0.001	1.03	0.001	25.19	0.151	25.34	0.200
2	IgG	1.27	0.001	1.27	0.001	27.14	0.217	27.17	0.209
3	BSA	1.42	0.000	1.43	0.001	30.52	0.482	30.19	0.511
4	Myoglobin	1.7	0.001	1.75	0.002	14.30	0.084	14.43	0.087
5	Uracil	2.23	0.001	2.23	0.001	2.85	0.032	2.87	0.021
1	Mab dimer	1.14	0.000	1.11	0.001	0.47	0.030	0.46	0.020
2	Mab monomer	1.32	0.001	1.30	0.000	99.53	0.040	99.54	0.020

Table 1. Quantitative comparison conventional SEC versus Auto•Blend Plus assisted SEC Retention time and peak area data represent the averaged data of triplicate analyses.

To investigate the comparison with a true commercial large molecule protein therapeutic, we used each SEC approach to measure the extent of aggregation in infliximab. As can be seen in Figure 4, the migration time for both the infliximab dimer and monomer were highly comparable, indicating Auto•Blend Plus as a suitable replacement for conventional mobile phase delivery.

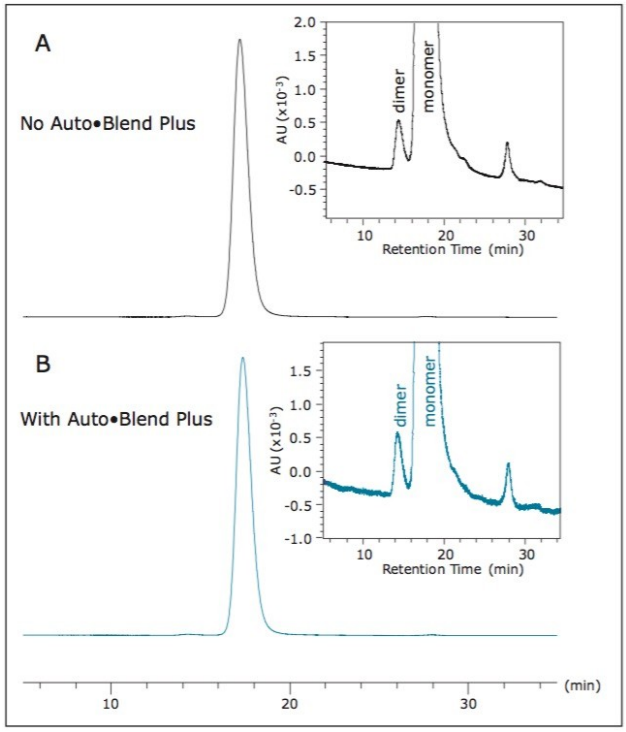

Figure 4. Auto•Blend Plus assisted SEC-HPLC of infliximab generates equivalent separation compared to conventional SECHPLC. (A) Infliximab separated using conventional SEC-HPLC; (B) Infliximab separated using Auto•Blend Plus assisted SEC-HPLC.

SEC-UPLC with Auto•Blend Plus generates identical results to conventional mobile phase preparation

Moving from SEC-HPLC to SEC-UPLC offers a number of improvements to chromatography previously described.[1] In addition to increasing chromatographic resolution and sensitivity by moving to SEC-UPLC, method robustness can also be improved by incorporating Auto•Blend Plus into the instrument method. Transferring the SEC-HPLC method to SEC-UPLC results in an increase in flow rate from 0.4 mL min^{-1} to 0.885 mL min^{-1} with a corresponding reduction in run time from 35 min to just 3 min.

To determine if Auto•Blend Plus could generate comparable results as those observed with SEC-HPLC, we ran both the SEC200 protein mix standard and infliximab using either conventional SEC-UPLC or Auto•Blend Plus-assisted SEC-UPLC. An ACQUITY UPLC Protein BEH SEC 200 Å Column (1.7-µm, 4.6 x 150 mm) was used with the ACQUITY UPLC H-Class Bio System for the assay. Benchmark SEC-UPLC using the SEC200 protein standard mix was generated as illustrated in Figure 5A. Auto•Blend Plus-assisted SEC-UPLC was then run and compared to the conventional SEC-UPLC, with results indicating no difference in individual component migration times (Figure 5B

and Table 1). The same comparison was performed using infliximab, where similar results were obtained (Figure 6 and Table 1).

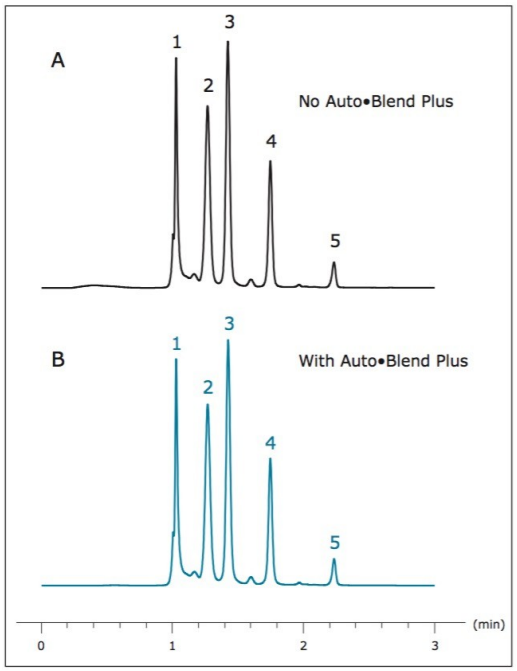

Figure 5. Auto•Blend Plus assisted SEC-UPLC generates equivalent chromatography to the conventional approach. In each chromatogram, 1 refers to thyroglobulin, 2 refers to IgG, 3 refers to BSA, 4 refers to myoglobin, and 5 refers to uracil. (A) SEC-UPLC using conventional mobile phase preparation; (B) SEC-UPLC using Auto•Blend Plus Technology for mobile phase delivery.

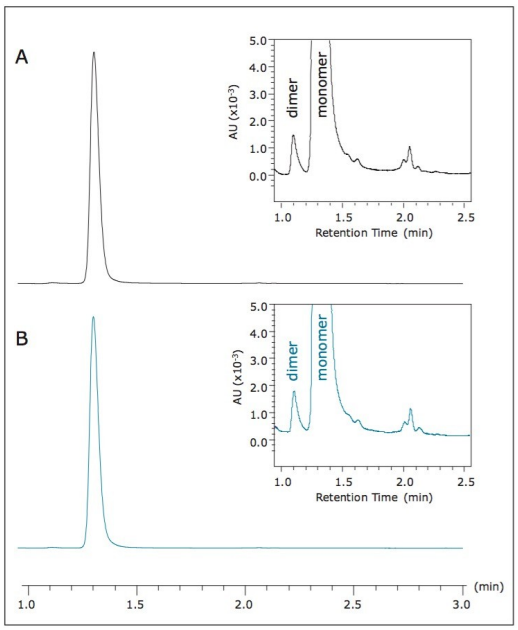

Figure 6. Auto•Blend Plus assisted SEC-UPLC of infliximab generates equivalent separation compared to conventional SEC-UPLC. (A) Infliximab separated using conventional SEC-UPLC; (B) Infliximab separated using Auto•Blend Plus assisted SEC-UPLC.

Results of SEC-UPLC unequivocally illustrate that using Auto•Blend Plus Technology for SEC-UPLC can replace conventional SEC-UPLC with no impact on component migration time or relative peak area.

Conclusion

Conventional SEC relies on the accurate preparation of pH dependent mobile phases where subtle variation in pH can lead to significant changes in chromatographic retention times. As a means for reducing variability in the preparation of buffered mobile phase, Auto▪Blend Plus Technology available through the Waters ACQUITY UPLC H-Class Bio System can prepare buffered mobile phase across a range of pH and NaCl concentrations from 4 standard stock solvents. In this application note, we have demonstrated the steps required to convert conventional SEC methods to Auto▪Blend Plus methods. The benefits of Auto▪Blend Plus span both SEC-HPLC and SEC-UPLC, where equivalent chromatography can be achieved with a more robust and reproducible solvent delivery system for pH dependent mobile phases.

References

1. Future-proofing the Biopharmaceutical QC Laboratory: Using the ACQUITY UPLC H-Class Bio to Run SEC HPLC and SEC UPLC. Waters Application Note. 2014: 720005057en.

Featured Products

ACQUITY UPLC H-Class PLUS Bio System

ACQUITY UPLC Tunable UV Detector

Auto•Blend Plus

Empower 3 Chromatography Data Software

720005264, January 2015