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

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**APPLICATION 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

**WATERS SOLUTIONS**
- ACQUITY UPLC H-Class Bio System
- BioSuite™ SEC Column
- ACQUITY UPLC Protein BEH SEC Column
- BEH200 SEC protein standard mix
- Empower® 3 Chromatography Data Software

**KEY WORDS**
Size exclusion chromatography, SEC, quality control, QC, AutoBlend Plus, automated mobile phase delivery, automated buffer management

**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 by demonstrating the benefits of Auto•Blend Plus Technology – which is included with all ACQUITY 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.
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 H₂O
Conventional mobile phase: 20 mM phosphate, 200 mM NaCl, pH 6.8

**Auto•Blend Plus**

Mobile phase A: A: 100 mM NaH₂PO₄
Mobile phase B: 100 mM Na₂HPO₄
Mobile phase C: 1 M NaCl
Mobile phase D: H₂O
Detection wavelength: 214 nm
Syringe purge: H₂O
Syringe wash: H₂O

**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
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.

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$_2$PO$_4$ buffer, 100 mM Na$_2$HPO$_4$ buffer, 1 M NaCl, and pure H$_2$O 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.

![Conventional SEC Method and Auto•Blend Plus SEC Method](image_url)
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.
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.

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. 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).

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.
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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.

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.
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.

Reference