Using design of experiments to optimise SEC method conditions for proteins

Size exclusion chromatography (SEC) is routinely used in the pharmaceutical industry to characterise the higher and lower molecular weight species proteins. Here, Gregory Webster, Senior Principal Research Scientist in Analytical Research and Development at AbbVie, explores the challenges, opportunities and optimisations needed to execute design of experiment studies to project SEC method conditions for antibody-drug conjugate (ADC) formulations.

PROTEINS PROVIDE a unique challenge in robustness testing and liquid chromatographic methods development, as the profile is typically less resolved than with small molecules. As with their small molecule counterparts, traditional elements of validation are required for regulatory approval. Due to the complexity of the chromatographic profile, the elements of robustness can be particularly challenging to evaluate for methods involving antibodies.

Robustness evaluation of the size exclusion chromatography (SEC) and hydrophobic interaction chromatography (HIC) methods can be efficiently evaluated using a design of experiments (DOE) platform. Ganorkar et al. remind us that DOE is a series of experimental designs using statistical techniques for planning, conducting, analysing and interpreting data from experiments.[1] Accordingly, quality by design (QbD) is a systematic approach to method development based on statistical knowledge that once required "complex statistical expertise and a significant amount of time".[2] However, this notion of complexity has diminished with the arrival of several user-friendly software platforms to the market. With this availability, QbD implementation can be practical for use by non-statisticians. As a result, regulatory agencies such as the US Food and Drug Association (FDA) and the European Medicines Agency (EMA) now advocate for QbD method development designs and are developing review criteria for evaluation of QbD-based analytical methods.[3]

Chromatography for large molecules, such as proteins, is distinct from traditional small molecule applications conventionally seen in pharma. These large molecules do not often play by those rules embraced by chromatographers since Snyder and Kirkland.[4] Highly resolved separations with sharp peaks as the gold standard is replaced with poorly resolved groups of like isomers, conformations, etc. Oftentimes, the application of ultra-high-performance liquid chromatography (UHPLC) does not resolve this, but only serves to provide slightly sharper details within the group since pore size, and not particle size, plays a more important role in large molecule chromatography.[5,6] Additionally, many laboratories still rely on manual integration for protein chromatography due to the complexity of the profile. One must note that the eye is an important detector in analytical chemistry, particularly when spotting outliers; the eye can readily depict a resolution factor of 0.8 under manual conditions for large molecule groupings.

Even with these limitations, chromatography still plays an important role with proteins. It is used for intact protein reversed-phase liquid chromatography (RPLC),[8] reduced protein RPLC,[9] SEC,[10] HIC,[11] free drug,[12] as well as with traditional residual solvents (GC/HPLC) investigations.[13,14] Thus, DOE method development and optimisation can still play a significant role in large molecule chromatography. Typical chromatographic parameters to be optimised are column temperature, mobile phase modifiers, salt strength, gradient slope and flow rate.[15]

Liquid chromatography (LC) methods to characterise antibody-drug conjugate (ADC) formulations typically involve SEC and HIC. SEC methods are used to characterise the higher and lower molecular weight of the ADC. The effect of column temperature, flow rate, and need for a salt or organic modifier is typically addressed in method robustness studies.

Table 1

LC columns used for SEC optimisation

Column	Vendor	Dimension	Particle size
C1	A	300x4.6mm	1.7µm
C2	B	300x4.6mm	1.9µm
C3	C	300x4.6mm	1.9µm
C4	D	150x4.6mm	3.0µm
C5	D	150x4.6mm	1.8µm
C6	B	150x4.6mm	2.7µm

Experimental

Instrumentation

The LC data reported was generated using Thermo Scientific™ – UltiMate™ 3000 LC Method Development Systems (Bannokburn, IL, USA) configured with multiple solvent and column capability.

The system was operated using Thermo Scientific™ Dionex™ Chromeleon™ 7 Chromatography Data System, v7.2.10 and 7.3 (Thermo Scientific, Waltham, MA USA) with sequences generated using Fusion QbD Professional, v9.9.1 (S-Matrix Corp., Eureka, CA USA). The DOE investigations were developed, executed and analysed using the Fusion QbD Professional software.

SEC columns

Per journal guidelines, specific column vendors are not disclosed. The SEC column used in the original method was a standard 30cm × 7.8mm column packed with 7µm particles. The method re-optimisation focused on columns that fit more universally into LC column compartments and evaluation of small particle size packings. The columns used for this re-optimisation DOE are listed in **Table 1**.

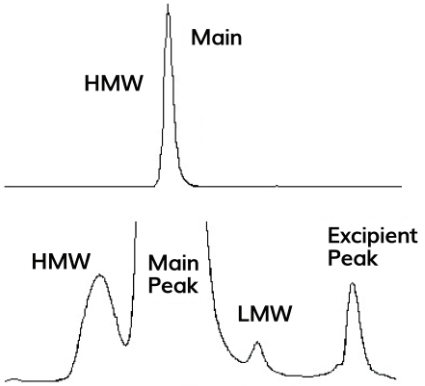
DOE conditions

The DOE probe analyte was a representative and proprietary ADC. The DOE platform for the original column robustness optimisation and the lower flow optimisation are listed in **Tables 2 and 3**, respectively. The DOE system conditions for the method column re-optimisation study are listed in **Table 4**. All SEC runs were isocratic.

Results and discussion

In **Figure 1**, the representative protein profile presents a challenge as to where to consistently draw the line between the main peak and the low molecular weight peak in the SEC profile. Initial versions of DOE and chromatography software did not have this issue since they were optimised for small molecule separations. When applying the typical algorithms of method development software to protein separations, the results often underpredicted the robustness of the method. Manual integration was more representative of the quality control applications of the method. While time consuming, manual integration resulted in a stronger predictive power of the DOE output. More recent updates to these software packages allow for 'peak-to-valley' software evaluation of the chromatographic response. This not only simulates the data evaluation in quality investigations but also such automation eliminates analyst bias in its integrations as well.

Figure 1



Representative profile of an ADC separation. The primary goal in a protein SEC separation is resolving the HMW species. LMW species are better characterised with techniques such as capillary electrophoresis.

In our laboratory, we were experiencing column robustness issues with an industry standard SEC column. Method suitability performance would diminish with time. While this is less of an issue in a research setting, better control of this performance is vital in a quality control laboratory. Lost time and repeating runs are both regulatory and cost prohibitive issues. A DOE investigation was initiated to test the robustness of the method to small changes in mobile phase salt concentrations, pH and injection volume.

Using DOE software coupled to a multi-column and flow valve HPLC, these changes could all be directly implemented into the sequence for an uninterrupted and unattended fashion, thus making the investigation quite efficient. The robustness issues our laboratory experienced were demonstrated within a few injections of the DOE conditions (see **Table 2**). The SEC profile deteriorated rapidly, and this result was reproducible. After reviewing the conditions it was noted that, unlike antibodies alone, ADCs typically run with isopropyl alcohol in the mobile phase. The alcohol modifier is used to sharpen the peaks and help the later retaining peaks to elute. This addition of isopropyl alcohol also increases the viscosity of the mobile phase. Thus, SEC conditions commonly developed for antibodies tend to run at a lower system pressure than seen with their corresponding ADC method. Thus, the upper robustness flow rate of 1.2 ml/min in the DOE study quickly pushed the system over the manufacturer recommended upper limit for column pressure. To run the DOE, the system parameters were adjusted to use a median flow rate of 0.75 ml/min and an upper flow rate of 1.0 ml/min. (see **Table 2**)

The adjusted DOE predicted conditions where the column would meet resolution criteria. In the representative performance »

Table 2

DOE platform for original column robustness investigation

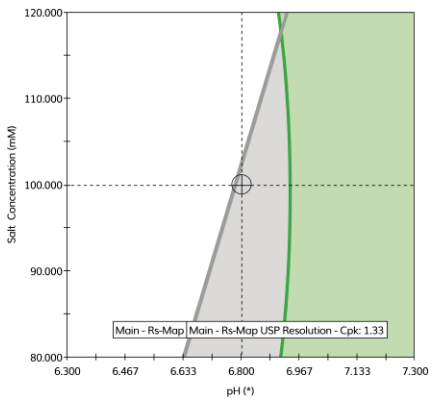
Variable	DOE range
Pump flow rate	0.8 – 1.2ml/min
Mobile phase salt modifier concentration	80 – 120mM Na_2SO_4 (in 100mM PO_4 buffer)
Mobile phase buffer pH	6.3 – 7.3
Mobile phase modifier (IPA)	0 – 11 percent
Column oven temperature	18° – 30°C
Injection volume	7.0 – 8.0µl

Table 3

DOE platform for lower flow optimisation

Variable	DOE range
Pump flow rate	0.5 – 1.0 ml/min
Mobile phase salt modifier concentration	80 – 120mM Na_2SO_4 (in 100mM PO_4 buffer)
Mobile phase buffer pH	6.3 – 7.3
Mobile phase modifier (IPA)	0 – 11 percent
Column oven temperature	18 – 30°C
Injection volume	7.0 – 8.0µl

Figure 2



Representative optimal performance region for the 30cm × 7.8mm column.

region plot illustrated in **Figure 2**, the white area is the region where desired limits for the high-molecular-weight (HMW) and low-molecular-weight (LMW) peaks should be achieved. It should be noted that all DOE projections should be experimentally confirmed prior to implementation. Optimum QbD ranges for each of the chromatographic variables were derived for an updated method to meet suitability while operating in a more robust manner.

Modern DOE of SEC ADC separations

This preliminary DOE work set the foundation for continued optimisation of the SEC method. The current method was much more robust. However, the use of 30cm × 7.8mm, 7µm particle columns seemed 'dated' in our current small particle UHPLC era. Additionally, these larger SEC columns are bigger than the default column departments used in some new LC systems currently marketed. Switching the method to a smaller column would allow the column to fit inside the column compartments to enable temperature control. The system was configured with three columns from different manufacturers using UHPLC particles (C1-C3). The 30cm length of the column was maintained as it was suspected that length was an important factor for resolving the HMW peak from the main peak in the SEC separation. However, our laboratory had some success with a 15cm column when using smaller particle size stationary phases. Column C4 was chosen to be a 15cm column based on a 3µm particle size. Columns C5 and C6 were chosen to be a 15cm column using both a UHPLC particle and a superficially porous particle from a separate vendor.

Because 'ambient' has different meanings throughout the world, our laboratory routinely confirms ambient method robustness for 18 – 30°C. The goal for the optimised method would be to update from 'ambient' to a more desired column temperature controlled at X° ± 2°C.

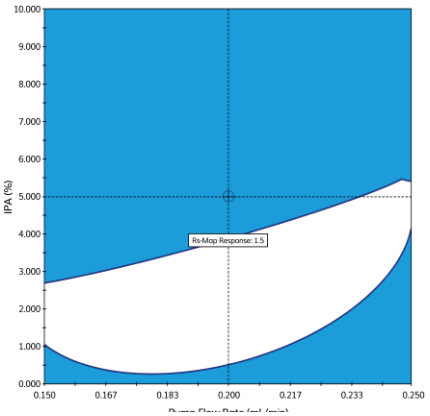
Optimisation of injection volume was originally thought to be an issue in the method using the original 30cm × 7.8mm, 7µm particle column. The DOE investigations did not support this concern and evaluation of this parameter was dropped from the modernising DOE study. An injection volume of 7.5µl was used for all runs.

The issue of short lifetimes for SEC columns when running commonly proposed parameters is a common challenge in the pharmaceutical industry,[16-18] even with columns from different commercial vendors. Our work has noted the issue of using isopropanol in the mobile phase to improve peak shape and selectivity. As a viscous solvent, the viscosity of isopropanol increases column pressure. It is likely that the resulting pressure, even when under column manufacturer recommendations, shortens the effective lifetime performance of the column for ADC separations. Our new SEC platform must operate in regions where this pressure issue is not suspect and well under manufacturer column pressure recommendations.

Finally, it was noted that the literature is inconsistent in the choice of salt modifiers for the SEC of proteins. Thus, the DOE optimisation used three common SEC mobile phase modifier salts to investigate the optimal performance regions of commercial SEC columns at lower flow rates than traditionally used for ADC separations. The DOE platform conditions for the column optimisation study are listed in **Table 3** – these ranges will be used as a basis for further method development specific to each ADC in development.

For the antibody profile in SEC, the primary goal in a protein SEC separation is resolving the HMW species. LMW species are better characterised with techniques such as capillary electrophoresis (CE). The DOE must focus on optimisation of the peak-to-valley resolution between the HMW peak response to the main peak seen in **Figure 1** and the main peak's peak-to-valley resolution with the first low molecular peak response in the profile. Use of these ratios provides excellent metrics for a protein separation, does not require baseline separation of the components and assures peak shapes, which support repeatable quantitation.

Figure 3



KCl study: representative optimal performance region for column 1 (Rs Map Response = 1.5).

Table 4

DOE platform for SEC method re-optimisation

Variable	DOE range
Pump flow rate	0.15 – 0.25 ml/min
Mobile phase salt modifier concentration (in 100mM PO_4 buffer)	50 – 250mM KCl
	50 – 150mM NaCl
	50 – 150mM Na_2SO_4
Mobile phase buffer pH	6.3 – 7.3
Mobile phase modifier (IPA)	0 – 10 percent
Column oven temperature	18 – 30°C

KCl DOE

The use of potassium chloride (KCl) was evaluated in a salt range consistent with what was seen in the literature: 50 – 250mM KCl. The pH was controlled with the additional use of 50 – 100mM PO4 in the modifier. The DOE results were project optimum performance regions for the separation where the resolution response of 1.5 resulted for columns C1, C2, C3 and C6. Resolution response is defined for this study as the minimum peak-pair-pair resolution achieved at conditions that maximise "Start p/v" and "End p/v" results for HMW and LMW peaks. Columns C4 and C5 could only project a resolution response of <1.0. Thus, for columns C1, C2, C3 and C4, the DOE projected suitable method conditions for flow rate, KCl modifier concentration, pH, IPA mobile phase concentration and column temperature. For C1, these acceptable performance regions, which can be further optimised, were a flow rate range of 0.17-0.23 ml/min, an IPA mobile phase content of 1.0 – 2.7 percent, pH 6.3 – 7.3, a column temperature of 20 – 30° C and a mobile phase KCl salt content of 75 – 250mM.

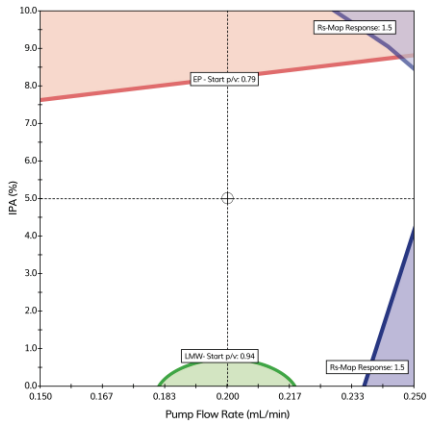
As illustrated in **Figure 3**, the DOE could also project the critical parameters that produced an immediate region of failure for resolution response (represented by the coloured regions).

NaCl DOE

The use of NaCl was evaluated in a salt range consistent with that seen in the literature: 50 – 150mM NaCl. The pH was controlled with the addition of 50 – 100mM PO4 in the modifier. With this salt, the DOE results projected an optimum performance region for the separation where the resolution response of 1.5 resulted for columns C1, C3 and C4. Column C2 could project a resolution response of <1.25. Columns C4 and C5 could only project a resolution response of <1.0. Thus, for column C1, C3 and C4, the DOE projected suitable method conditions for flow rate, NaCl modifier concentration, pH, IPA mobile phase concentration and column temperature. For C1, these acceptable performance regions, which can be further optimised, were a flow rate range of 0.15 – 0.21 ml/min, an IPA mobile phase content of 0.0 – 7.5 percent, pH 6.3 – 7.3, a column temperature of 18 – 30°C and a mobile phase NaCl salt content of 50 – 150mM.

The DOE projected that the excipient peak start peak-to-valley (p/v) ratio and the LMW p/v ratio were critical parameters to monitor in the region of failure for resolution response (see **Figure 4**).

Figure 4

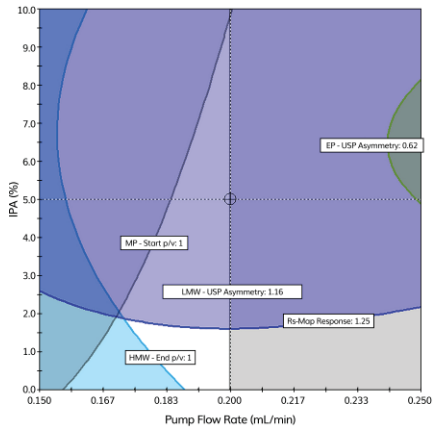


NaCl study: representative optimal performance region for column 1 (Rs Map Response = 1.5).

Na_2SO_4 DOE

The use of Na_2SO_4 acted noticeably differently to that seen with the chloride salts. This salt modifier was more sensitive to pressure issues. Na_2SO_4 was evaluated in a salt range of 50 – 150mM. The pH was controlled with the addition of 50 – 100mM PO4 to the modifier. With this salt, the DOE »

Figure 5



Na_2SO_4 study: representative optimal performance region for column 1 (Rs Map Response = 1.5).

results projected an optimum performance region for the separation where the resolution response of 1.5 only resulted for column C1. Columns C2, C3 and C6 could project a resolution response of <1.25. Columns C4 and C5 still could only project a resolution response of <1.0. Thus, for column C1 only, the DOE projected suitable method conditions for flow rate, Na_2SO_4 modifier concentration, pH, IPA mobile phase concentration and column temperature. For C1, these acceptable performance regions, which can be further optimised, were a flow rate range of 0.16 – 0.20 ml/min, an IPA mobile phase content of 0.0 – 1.5 percent, pH 6.3 – 7.0, a column temperature of 24 – 30°C and a mobile phase Na_2SO_4 salt content of 50 – 150mM.

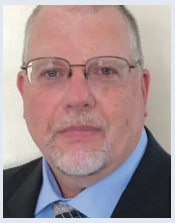
As illustrated in **Figure 5**, the DOE projected that the USP asymmetry for the excipient and low molecular weight peak, main peak start p/v ratio and the HMW p/v ratio were critical parameters to monitor in the region of failure for resolution response.

Conclusion

The DOE approach efficiently enabled an unbiased evaluation of the optimal performance ranges for operating commercial SEC columns at lower flow rates than typically assumed for antibodies. This work assured that the method performance would ensure longer column lifetimes with use of isopropanol as the mobile phase organic modifier, as it not only modelled the parameters themselves, but also considered the interactions among parameters. All DOE projects must be confirmed experimentally along with mass spectrometric confirmation of the peak assignments for this final optimised method.

The DOE approach found not only the optimal performance ranges for three common SEC mobile phase salt modifiers (KCl, NaCl, Na_2SO_4) but that the representative ADC could be modelled most effectively using KCl. The other modifier can be dropped from initial SEC method development platforms. Because our laboratory experiences robustness issues from column pressures, methods using Na_2SO_4 as the mobile phase salt modifier should be specifically re-evaluated with KCl as the alternative. As a platform, the DOE projected ranges should be used to further optimise future SEC methods for ADCs within these settings.

As expected, the longer SEC columns exhibited better selectivity performance for the representative ADC. The use of shorter SEC columns needs to be eliminated until further DOE optimisation studies can evaluate and optimise the conditions for the short 15cm column lengths.



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Disclosure

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