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Application Note

Antibody Drug Conjugate (ADC) Separation using a Mixed Mode Cation Exchange Chromatography Step in a Monoclonal Antibody (mAb) Process

Introduction

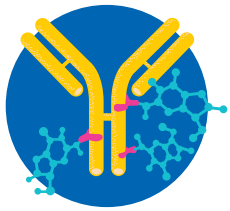
The aim of this technical note is to provide a description of the steps followed and considerations taken during the development of antibody drug conjugate separation using a mixed mode cation exchange chromatography step.

Antibody Drug Conjugates in mAb Process

Antibody drug conjugates (ADC) is a new class of high potency drugs dedicated to cancer treatment. Whereby the antibody molecule is dedicated for the specific recognition of cancer cells, the toxic payload carried by the antibody molecule destroys them (Adcetris®, Kadcyla®). These new drugs are manufactured by conjugating a toxic molecule (payload) on the monoclonal antibody (mAb) molecule and is achieved through controlled chemical conjugation with specific amino acid residues exposed on the surface of the mAb (for example: Lysine or Cysteine after cysteine bond reduction) that results into a heterogeneous mixture of ADC species with variable drug to antibody ratio (DAR).

To overcome the manufacturing challenges in the ADC heterogeneity, a mixed mode cation exchange chromatographic technology can be used to remove undesired low DAR and high DAR species. This technology enables to enhance the efficiency of the ADC drugs by separating individual antibody drug conjugate species and enabling the best pharmaco-kinetics, efficacy, half-life, and tolerability.

In the ADC downstream process, a mixed mode cation exchange step follows the antibody drug conjugation step to separate the previously mentioned antibody drug conjugates. The mixed mode cation exchange step is normally operated in a bind and elute mode at pH's between 4 to 5 and 150 to 250 mM NaCl. Under these conditions, most of antibody drug conjugates will bind to the mixed mode cation exchange resin and separation of low or too high DAR/antibody drug conjugate species will be achieved in selecting the optimal elution conditions. Elution conditions include the pH change and/or conductivity change, that increases the selectivity of the mixed mode cation exchange resin. This is achieved operating a gradient mode or in a step elution mode, where buffers having different pH and/or conductivity are applied.



Antibody Drug Conjugates (ADC)

- Separation of DAR Species

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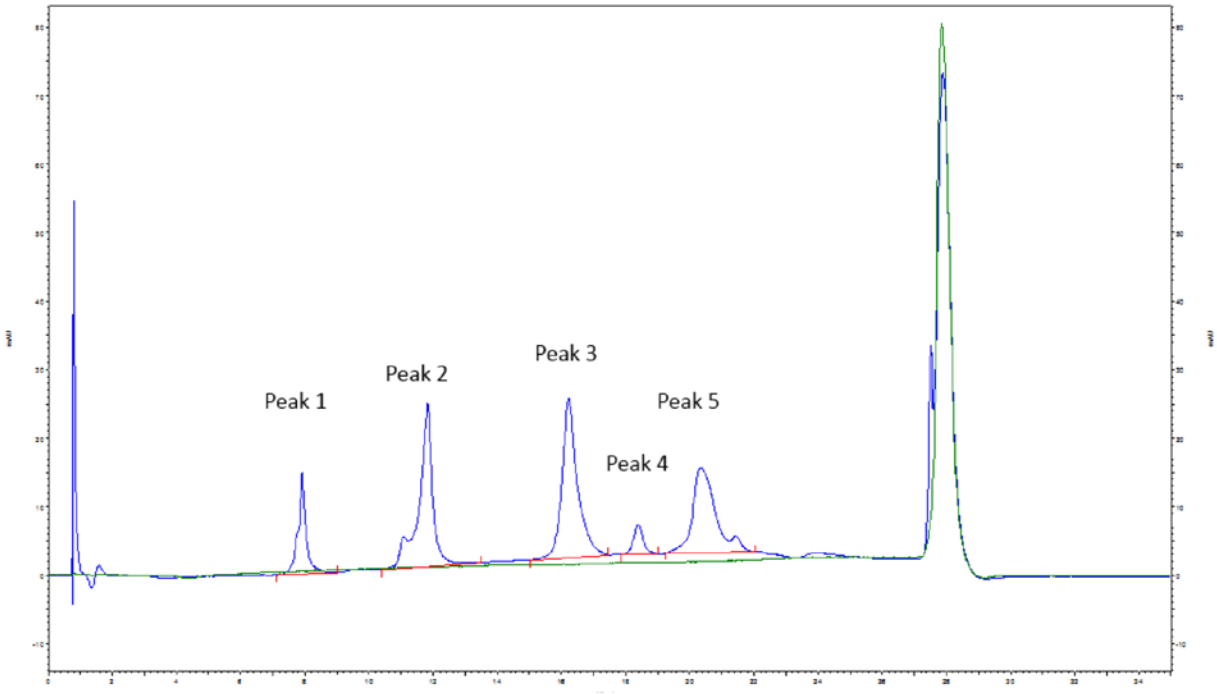

Figure 1. Qualitative example of antibody drug conjugate species elution using linear gradient of decreasing conductivity and increasing 2-propanol quantity on an analytical HPLC column (mAbPac HIC-Butyl 5 µm, 4.6 × 100 mm (P/N: 088558) Thermo Fisher Scientific). Buffer A consists of 1.5 M ammonium sulfate, 0,05 M Phosphate and 5% 2-propanol, whereby buffer B consists of 0,05 M Phosphate and 20% 2-propanol. HPLC column is operated in 1 mL/min flow rate and gradient from buffer A to buffer B is achieved in 20 minutes. ADC sample is diluted in buffer A at 5 mg/mL concentration and 10 µL is injected on the HPLC column prior gradient elution. Blue trace represents the UV adsorption for the antibody drug conjugate separation. Green trace represents the UV adsorption trace for a blank run.

Figure 1 illustrates the separation of antibody drug conjugate species during a linear elution gradient on an analytical HPLC column. Five consecutive elution pools are identified in the UV signal trace, representing five different antibody drug conjugate species: peak 1 represents antibody drug conjugate species without drug, peak 2 represents antibody drug conjugate species with two drug molecules conjugated to a single antibody molecule, peak 3 represents antibody drug conjugate species with four drug molecules conjugated to a single antibody molecule, peak 4 represents a mixture of antibody drug conjugate species, peak 5 represents antibody drug conjugate species with six drug molecules conjugated to a single antibody molecule. The identified antibody drug conjugate species differ by their average hydrophobicity, the one being more hydrophilic (e.g., left side) to hydrophobic (e.g., right side).

Considerations for Scale-up

Column bed height, flow rate, and system pressure should be considered during the resin evaluation process. Although these factors may not be as critical at the initial purification scale, they may become more important at commercial manufacturing scale. Selection of a resin that can be used throughout manufacturing — from clinical to commercial scale — simplifies process development and leads to a more robust final process. At this early stage in the development, end-users consider factors such as: the resin (ease of packing), previous experience with the vendor (security of supply) and commercial availability of the product (when resins in development are tested).

Initial Screening – Window of Operation for Binding (static)

The first evaluations of the mixed mode cation exchange resin involve binding capacity determinations under a range of pH and conductivity conditions. For antibody drug conjugate processes, the pH range is generally between pH 4.5 and 7.5. The salt concentrations (e.g., Na_2SO_4) tested are usually between 0–1 M at low buffer concentration (e.g., 50 mM acetate or 50 mM phosphate). Dilution and/or pH adjustment of the feed material might be needed.

At this stage, the binding capacity is generally determined under static conditions to maximize the number of conditions tested. The use of high throughput screening (HTS) tools, such as 96-well plates or micro-columns, can expedite the evaluation. Additionally, the small volumes required for these experiments allow the exploration of a wider experimental space. In cases where these HTS tools are not available, similar batch binding experiments can be performed manually using small resin volumes (e.g., in centrifuge tubes).

Although batch experiments can also be conducted to evaluate elution conditions, these evaluations are generally done in dynamic mode in a column format since linear gradients cannot be performed in batch mode and other parameters (e.g., protein loading) can also impact the resolution of antibody drug conjugate species. The results from these initial screenings should narrow down the number of resins and operating conditions to be tested in the next step of the development.

Figure 2 illustrates the window of operation for binding the antibody molecule under a range of pH and conductivity conditions.

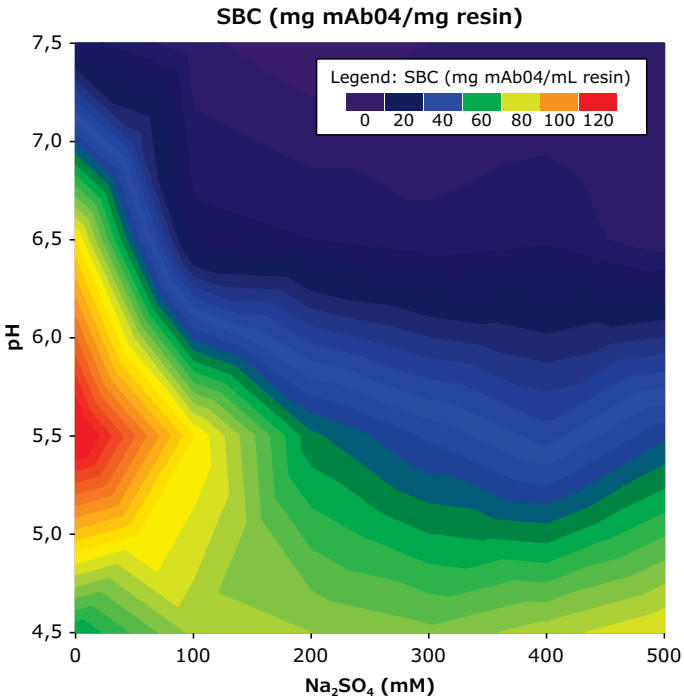

Figure 2. Quantitative example of antibody molecule binding under a range of pH and conductivity conditions. 50 mM acetate and 50 mM phosphate buffer system was used to achieve the pH with different Na_2SO_4 amounts. All values are average values from duplicates performed with 10 µL resin samples in the micro titer plates and using robotic Tecan system for the buffer and sample subjection and UV detection. Static incubation was done for 120 minutes in 0.2 mL sample suspension.

Considerations for Scale-up

Factors to consider before and during this step include buffer characteristics (e.g., pKa, type) and buffers used in the previous and subsequent steps. Buffers generally used for equilibration, binding and elution in a CEX step in a mAb process are acetate, citrate or phosphate. Other buffer related factors such as cost, ease of use and disposal (e.g., phosphate), particularly at large scale, should be evaluated as well.

Dynamic Binding Capacity

The static binding capacity studies narrow the binding conditions (pH and conductivity) and establishes the foundation for the optimization of capacity and antibody drug conjugate selectivity in a dynamic mode. However, it may be preferred to omit the static binding capacity studies and start the initial resin screening in a packed column format if time and/or ADC feed stock are limited, or if there are budgetary constraints.

The binding capacity of the mixed mode cation exchange resin is then evaluated in a dynamic mode in the narrowed range of pH and conductivity to establish the optimal binding conditions. A breakthrough curve of the ADCs is generated and the dynamic binding capacity (DBC) is determined at a percent breakthrough. A 5% breakthrough is more relevant to use in DBC measurements since the shape of the breakthrough curve is not as sharp as for some affinity resins and can also be influenced by impurity load. Generally, residence times in the order of 3 to 5 minutes for Eshmuno® CMX resin are good starting points for DBC evaluations. The mixed mode cation exchange step is normally operated in a bind and elute mode at pH's between 4 to 5 and 150 to 250 mM NaCl using pH change in step or gradient mode to elute the bound sample. The preferred column format for these studies is at a bed height that would be utilized upon scale-up (typically 15–25 cm). If feedstock is limited, a shorter bed height can be used for screening conditions and the DBC can be later confirmed at the desired bed height with the selected resin. In addition, the column diameter generally utilized is 1–2.5 cm whenever possible to minimize potential wall effects. Mixed mode cation exchange resins should be packed in storage buffer (150 mM NaCl in 20% Ethanol) using 11% compression rates. If there are constraints using ethanol, the storage solution can be exchanged with 0.1 M NaOH solution prior packing. Only 13% compression rates are used if 0.1 M NaOH is chosen. Please note to exchange the packing solution at least three times before recording the sediment amount necessary for the column. We strongly recommend following the supplier's recommendations to obtain the best assessment of a resin's capabilities.

Considerations for Scale-up: Systems and Hardware

At this point, an important consideration relates to the system and hardware capabilities at large scale, particularly pump capacity. Semi-rigid or rigid media, like Eshmuno® CMX media, have relatively high permeabilities (i.e., pressure drops below 2 bar at linear velocities higher than 400 cm/h for a 20 cm bed height). Therefore, the residence times chosen for DBC evaluations should meet those limitations. Finally, the system pressure as contributed by the piping, column hardware (screens, flow distributor, etc.), and head pressure due to tank height in large scale facilities must also be considered in addition to the resin bed pressure drop when designing large scale processes.

Antibody Drug Conjugate Separation Study Design

Pack a column with the desired bed height as can best be estimated from the final manufacturing conditions. The linear pH gradient study described should be tested at a variety of different salt concentrations ideally covering at least 150 mM and 300 mM NaCl. A linear increasing pH gradient is generally used for elution and fractions across the gradient are collected for antibody drug conjugate analysis. Load representative feedstock at the desired pH and conductivity followed by the column wash with the loading buffer until the absorbance reaches baseline (collect this wash fraction). Begin the elution by performing a gradient from 0 to 100% B buffer over 50 column volumes with the A buffer being the load buffer (e.g., 0.00796 M citric acid, 0.009068 M sodium di-hydrogen phosphate, 0.021543 M glycine, 0.010668 M TRIS, 0.007666 M

succinate, 0.0111 M NaOH and 0.2889 M NaCl of pH 4.5 and conductivity of ~24 mS/cm) and the B buffer (e.g., 0.00796 M citric acid, 0.009068 M sodium di-hydrogen phosphate, 0.021543 M glycine, 0.010668 M TRIS, 0.007666 M succinate, 0.046 M NaOH and 0.254 M NaCl of pH 8.5 and conductivity of ~24 mS/cm).

Table 1 illustrates an example of buffer components and the amounts thereof for the pH gradient.

Material	Art. Nr.	Mol	Amount in 1 L Buffer (g)
Buffer A			
Citric acid	1.00244	0,00796	1,6727
Natrium-di-hydrogen-phospate	1.06346	0,009068	1,2513
Glycine	1.04201	0,021543	1,6170
TRIS	1.08320	0,010668	1,2923
Succinate	8.18601	0,007666	1,2423
NaOH W=32%	1.05587	0,0111	1,3874
NaCl	1.06046	0,2889	16,8833
Buffer B			
Citric acid	1.00244	0,00796	1,6727
Natrium-di-hydrogen-phospate	1.06346	0,009068	1,2513
Glycine	1.04201	0,021543	1,6170
TRIS	1.08320	0,010668	1,2923
Succinate	8.18601	0,007666	1,2423
NaOH W=32%	1.05587	0,046	5,7496
NaCl	1.06046	0,254	14,8438

Table 1. Major components and amounts used for the 1 L buffer A and B. All components are obtained from Merck KGaA, Darmstadt, Germany.

Table 2 illustrates an example of method conditions for the pH gradient run. This example is covering a wide range of ADC physical properties and needs further optimization after the first gradient run is performed to reduce the duration of pH gradient elution or to change to step elution approach.

Starting Method

Steps	Duration	Solvent
Equilibration	5 CV	Buffer A
Load	10–60 mg/mL	Feed at pH 4.5 and 24 mS/cm
Wash	10 CV	Buffer A
Gradient Elution	120 CV	0–100% Buffer B
Strip	5 CV	Buffer B
CIP	7.5 CV	1 M NaOH
Reequilibration	10CV	Buffer A

Table 2. Sequential steps and duration for the pH gradient set-up on the liquid chromatography system including the column volumes for each step. Recommended velocity is four minutes residence time for 1 CV.

It is then generally sufficient to perform a final cleaning with buffer B to remove any remaining protein followed by 1 N NaOH and storage in 0.1 N NaOH. Analyze the fractions for drug distribution and HCP's. Based on the separation of the antibody drug conjugates and yield, further studies can be used to optimize the conditions.

The goals will be to obtain the desired purity targets for the mixed mode cation exchange step, optimize the antibody drug conjugate yield, develop manufacturing pooling conditions, and obtain manufacturing plant fit.

Figure 3 illustrates an example of the UV, pH and conductivity traces after applying the linear increasing pH gradient during an antibody drug conjugate separation.

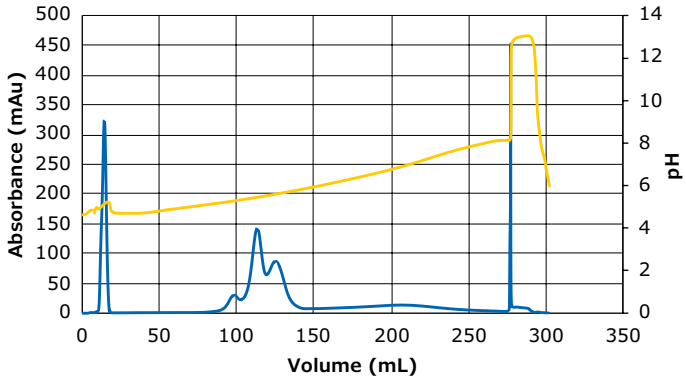

Figure 3. Example of pH (yellow) and UV (blue) traces detected using the chromatographic separation of Adcetris® sample loaded to 10 mg/mL on the mixed mode cation exchange resin, showing the elution of sample within the pH change in three main peaks (e.g., elution maximum of first peak at 98 mL, elution maximum of second peak at 104 mL, and elution maximum of third peak at 125 mL). The blue line represents protein concentration based on the UV adsorption. 150 cm/h velocity was chosen throughout all the chromatography steps.

Table 3 illustrates an example of the analytical evaluation using hydrophobic interaction chromatography of collected fractions, showing that the three obtained peaks are the separation of DAR species, namely the first peak is mainly the DAR 2 and DAR 6 species (e.g., elution maximum at 98 mL), second peak is mainly DAR 4 species (e.g., elution maximum at 104 mL), and the third one — DAR 6 species (e.g., elution maximum at 125 mL).

	Fraction	DAR 0 Species (%)	DAR 1 Species (%)	DAR 4 Species (%)	DAR 6 Species (%)
	Load	12.13	24.24	22.58	37.76
Peak 1	5A3	0.00	39.03	0.00	40.26
Peak 2	5A11	4.27	15.87	59.22	19.22
Peak 3	5B4	0.00	14.28	7.55	78.16

Table 3. Analytical results of sample fraction characterization performed with analytical hydrophobic interaction chromatography, where the results are displayed in % portion from the total ADC concentration.

Pooling Criteria

The parameters used to start and stop collecting the elution pool containing product are critical to achieving high yield, high purity and a successful scale-up to manufacturing. Due to the operational complexity and quality control work involved at manufacturing scale, collecting fractions for testing and pooling is rarely done. Instead easily measured output parameters such as absorbance and/or volume are usually used. A thorough understanding of the impurity profile across the elution peak is necessary to choose the appropriate starting and ending conditions.

Additional Approaches

If further purity optimization is needed, changing the salt or its concentration as well as buffer type can affect impurity separation from the product. Changing the pH or conductivity of either the wash buffer or elution buffer can also result in better antibody drug conjugate selectivity. If an undesired antibody drug conjugate species is present on the leading edge of the desired antibody drug conjugate species, increasing the absorbance when pooling is started will lower this undesired antibody drug conjugate species in the elution pool, although with slightly reduced yield. Conversely, increasing the absorbance of the pool end conditions will lower other antibody drug conjugate species on the trailing edge of the elution peak.

Process Optimization

Once the optimal conductivity for the separation has been established, the length and end of the increasing pH linear gradient can be reduced to reduce the total pool volume since shallow gradients result in larger pool volumes. This elution pool collection must meet tank volume limitations that can exist at pilot or large scale. In addition, the resolution of antibody drug conjugate species under these conditions must be confirmed since the slope of the gradient also affects the resolution of antibody drug conjugate species.

It should be noted that the loading and bed height can also affect the separation of antibody drug conjugate species. In the evaluations described above, a loading of approximately >30 mg/mL is generally used. A lower loading may result in an uneconomical process and, while a higher loading may provide the desired resolution, a safety factor is generally used. Longer bed heights can also improve the resolution and impurity clearance, but generally the condition optimizations are performed at the bed height that will be used at large scale, i.e., 15–20 cm. In some cases, up to 30–40 cm bed heights may be used. The desired bed height is based on the optimal height for desired resolution and the overall column volume (bed height x cross-sectional area) needed based on expected protein load and resin capacity (g/L).

In the evaluations described in this section, the wash step is usually conducted with equilibration buffer (e.g., 5–10 CV). In cases where a step elution is utilized, an intermediate wash with a conductivity between that of the equilibration and elution buffers can be utilized to remove loosely bound antibody drug conjugate species. In addition, 1 N NaOH can be used simultaneously for regeneration and cleaning. This approach can be efficient in processes where there is not a significant amount of protein remaining in the column after the elution step. In addition, one buffer can be removed from the process along with its potentially corrosive effect on equipment.

The effectiveness of the cleaning regime needs to be evaluated as part of the step optimization. A blank cycle, (all the step buffers except the load) is generally performed after a few cycles (e.g., 5, 10) and the elution is collected and analyzed for carryover of product or impurities. If impurities are found in this mock elution, it is necessary to identify them and assess the effect of the carryover with regards to performance (e.g., yield, purity) in the subsequent cycles. For mixed mode cation exchange chromatography in antibody drug conjugate species processes for non-capture steps, standard cleaning with 0.5 N NaOH is generally enough since the impurity loads are much lower compared to capture steps. Carryover of product or impurities generally necessitates an improved cleaning regime, for example a higher concentration of NaOH may be needed. In addition, if the carried-over impurities and their nature have been identified, more specific cleaning strategies can be evaluated, e.g., detergents for hydrophobic impurities. An ineffective cleaning regime can have an impact on chromatographic performance and/or ease of packing used resin.

The evaluation of resin lifetime is another important part of the development process. In the early stages, this goes hand in hand with the cleaning optimization described above since at least a few cycles are needed to ensure an acceptable cleaning regime. A truncated/short lifetime study can be conducted for products in early stage that would at least cover the expected number of cycles for a batch of clinical material. In later stages, once the process conditions have been finalized, the lifetime of the resin needs to be confirmed for the number of cycles that will be validated, generally >100 cycles.

Process Robustness

In addition, once the working parameters for pH, conductivity and product load have been established, the robustness of the process needs to be evaluated as well. Generally, an acceptable window of operation would be within ± 0.2 pH units, ± 1 mS/cm and loading between the minimum and maximum load expected at this step based on variability in cell culture expression. The pH and conductivity ranges tested should align with the manufacturing capability for these parameters. Impurity clearance and yield can be significantly impacted over a wide range of protein loading. Design of experiment (DOE) studies are valuable at this point to examine the ranges in which parameters (e.g., pH, conductivity, load) may interact with each other. The variability of the feed from the previous step and multiple lots of resin can also be evaluated at this stage. These studies will hopefully define a wide range of operating parameters or determine the “edge of failure” for a parameter about antibody drug conjugate separation. A robust process should perform comparably with regards to yield, purity, etc., within the before mentioned window of operation.

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