

## Separation of Antibody Drug Conjugate Payload Drugs Using MaxPeak™ Premier Columns

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### Abstract

Antibody drug conjugates (ADCs) are a form of biotherapeutic which combines a monoclonal antibody and a small molecule therapeutic drug, which is designed to combat certain cancers. These drugs differ from traditional chemotherapy, as they target the cancerous cells while sparing the healthy cells. In creating these compounds, the challenge comes from not only dealing with the monoclonal antibody and ensuring proper attachment of the small molecule drug, but also the manufacture of both components. Characterization of the small molecule drug is a critical part of the process, and to accurately characterize the compound, an analytical method must be created.

This application note focuses on developing a method for the analysis of six ADC payload drugs which can be used to characterize the cytotoxic chemical compound. A final method was created using an ACQUITY™ Premier CSH Phenyl-Hexyl Column, low pH-modified mobile phases, and methanol as the strong solvent.

### Benefits

- Fast method development using the systematic screening protocol

- Baseline resolution of six antibody drug conjugate (ADC) compounds
  - Separation of compounds using a phenyl-hexyl stationary phase to promote secondary retention mechanisms in the presence of methanol mobile phases
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## Introduction

Antibody drug conjugates (ADCs) are complex biotherapeutics that combines a monoclonal antibody (mAb) and a cytotoxic small molecule drug that targets cancer and tumor cells.<sup>1,2</sup> By attaching the small molecule therapy to the mAb, it can be delivered directly to the cancerous site, making ADCs a targeted approach to cancer treatments. While these therapies offer effective treatment, their manufacture poses several challenges. First, the mAb portion of the therapy needs to be synthesized and quality controlled. Next, the small molecule drug needs to be created, and the purity assessed and proven. Finally, the ADC itself needs to be created, ensuring that the small molecule has been attached to the correct part of the mAb and that when introduced to a biological system it is readily cleaved.<sup>1</sup> All these steps are intricate and need to be tightly monitored to ensure the quality of the final ADC.

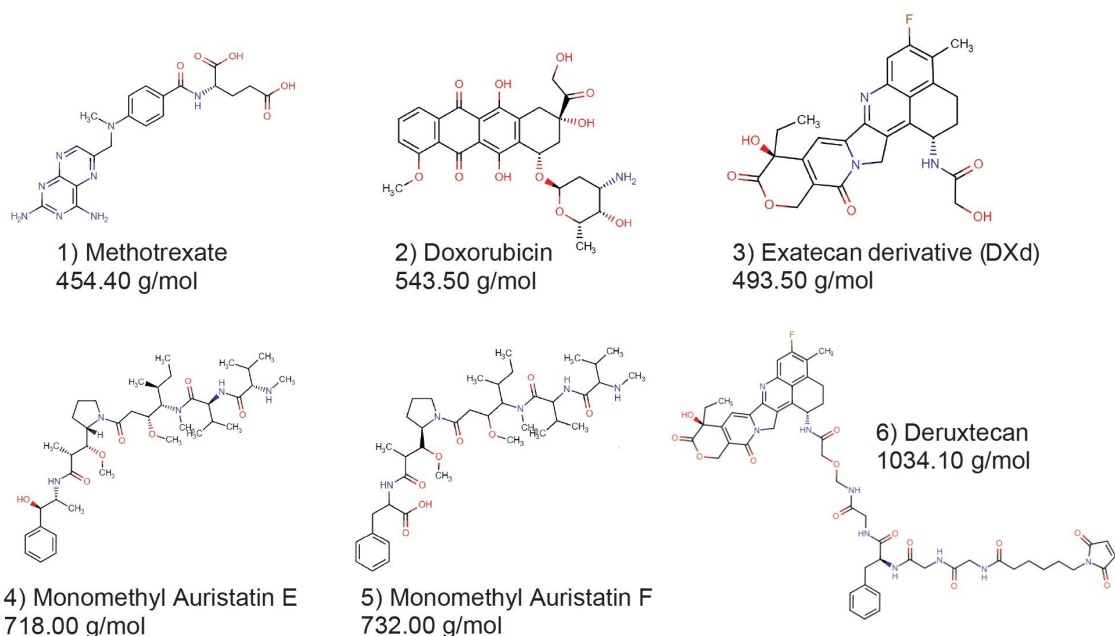
To ensure quality at each step, analytical methods are needed to properly characterize the samples. Characterization of the mAb component is usually performed using Size Exclusion Chromatography (SEC) to ensure no aggregates or dimers are formed. This technique is also viable for monitoring attachment of the small molecule to the mAb as the final ADC is created. However, for manufacturing and characterization of just the small molecule, reversed-phase liquid chromatography (RPLC) is better suited. Creating a single analytical method for the detection of the small molecule compounds, often called payload drugs, allows for faster characterization. However, creating such a method provides its own intrinsic challenges, which can be overcome easily by implementing structured method development workflows.

The systematic screening approach to method development is a structured, tiered approach to method development which can be used not only by expert chromatographers, but also novice users.<sup>3</sup> Previously, the systematic screening approach has been used to develop methods for a variety of samples including pharmaceutical impurities testing, antibiotics, and forced degradation samples, to name a few.<sup>4-6</sup> Coupling the systematic screening approach with MaxPeak Premier Columns, which use MaxPeak Premier High Performance Surfaces (HPS) Technology, improves not only the speed at which methods can be developed but ensures the data collected is accurate and reproducible. MaxPeak Premier Columns improve separation quality by

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eliminating secondary interactions between analytes and the metal surfaces of the column.<sup>6-9</sup> This ensures the only interactions are those between the analyte and stationary phase, increasing both analyte recovery but also improving separation reproducibility.

The systematic screening approach and MaxPeak Premier Columns were used to create a method for the separation of six ADC payload drugs, shown in Figure 1. These compounds range in use and chemical structure, providing a unique challenge for method development. While in real-world applications, these compounds would be analyzed in the presence of mAb's, the work shown in this application note is only applicable for the payload drugs. Analysis of mAb's or fully conjugate ADCs may require wider pore particle columns in order to elute the larger components of the formulation.



*Figure 1. Chemical structures of six ADC payload drugs including monoisotopic masses which were used for peak tracking during method development. Numerical identifiers used in later figures.*

## Experimental

## Sample Description

1 mg/mL stock solutions of each small molecule payload drug were created. From those solutions, a mixture containing 0.1 mg/mL of each was created with a final sample composition of 23% acetonitrile in water.

## LC Conditions

LC system:	ACQUITY UPLC H-Class Plus System with Quaternary Solvent Manager (QSM) with optional solvent select valve, Sample Manager Flow Through Needle (SM-FTN), Column Manager, Two Column Manager Auxs, and QDa mass detector
Detection:	MS full scan (ESI+)
Columns:	ACQUITY™ Premier BEH™ C <sub>18</sub> , 2.1 x 50 mm, 1.7 µm (p/n: 186009452)  ACQUITY Premier CSH™ Phenyl-Hexyl, 2.1 x 50 mm, 1.7 µm (p/n: 186009474)  ACQUITY Premier BEH Shield RP18, 2.1 x 50 mm, 1.7 µm (p/n: 186009497)  ACQUITY Premier HSS T3, 2.1 x 50 mm, 1.7 µm (p/n: 186009467)  ACQUITY Premier HSS PFP, 2.1 x 50 mm, 1.7 µm (p/n: 186010036)
Column temperature:	30 °C
Sample temperature:	10 °C
Injection volume:	1.0 µL

Flow rate:	0.50 mL/min
Mobile phase A:	Milli-Q Water
Mobile phase B:	Acetonitrile
Mobile phase C:	Methanol
Mobile phase D1:	2% Formic acid in water
Mobile phase D6:	200 mM Ammonium hydroxide in water
Screening gradient conditions:	Constant 5% Dx maintained throughout gradient. Linear gradient of 5–95% B/C in 6.86 minutes, hold for 1.14 minutes. Return to 5% B/C and hold for 2.3 minutes. Total run time: 10.30 minutes.

## Data Management

Chromatography software:	Empower™ 3 Feature Release 4
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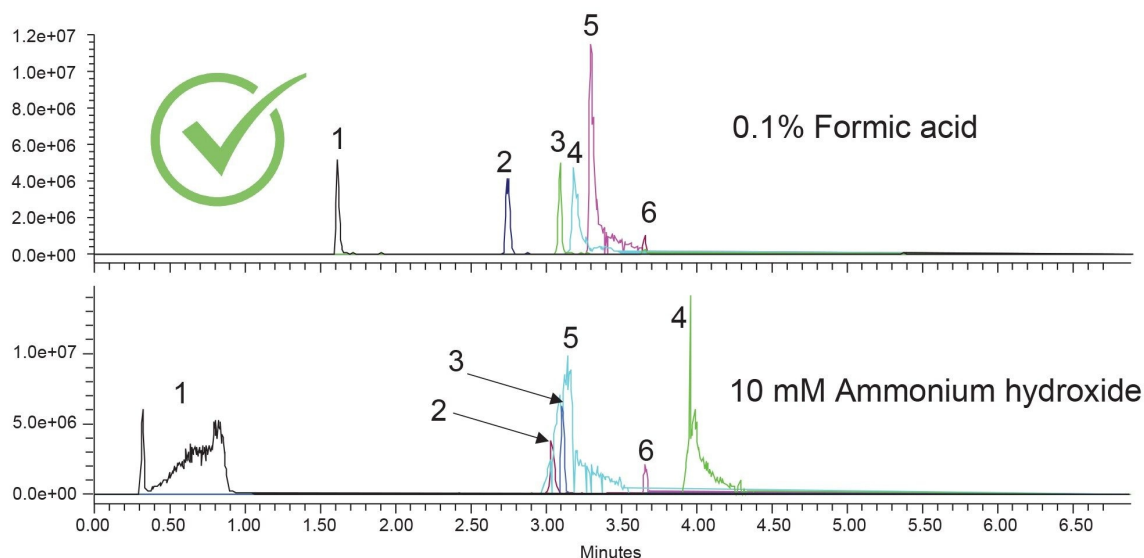
## Results and Discussion

As outlined in the systematic screening protocol, the first step is to define the method objectives and system parameters. The system being used is outlined in the experimental section of this application note. Method objectives can be set at the discretion of the analyst and should be mindful of the intended end use of the method. For instance, if the method is moving to a QC lab, then perhaps the use of a fully scalable column technology would be important to reduce potential issues with method migration to the other site. Conversely, if the method is being created to eventually be used in a Prep LC environment, not only is scalability important, but also ensuring the highest resolution between peaks to better facilitate purification. For this application note, the

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method objectives are simple: full resolution of all analytes with USP resolutions greater than 1.5. Once method objectives have been set, the experiments can begin.

For the systematic screening protocol, the first step is to assess mobile phase pH to determine which modifier provides the best retention for the analytes. In some cases, if the analyte structure and chemical properties are known, this can be predicted by looking at pKa and logP values, however, for unknowns or complex mixtures it is often best to run the experiments. This step calls for the use of a low pH mobile phase modifier, formic acid, and a high pH mobile phase modifier, ammonium hydroxide. Then, using a high pH stable column, such as the ACQUITY Premier BEH C<sub>18</sub> Column, the sample is tested under both low and high pH conditions. This step is referred to as rapid scouting. Figure 2 shows the separation of the ADC payload drugs at both low and high pH.



*Figure 2. Chromatograms of pH scouting using the ACQUITY Premier BEH C<sub>18</sub> Column. Full Scan ESI+ mass spectrometry detection used for peak identification. 1) methotrexate, 2) doxorubicin, 3) exatecan derivative, 4) monomethyl auristatin E, 5) monomethyl auristatin F, 6) deruxtecan.*

Under high pH conditions, methotrexate is poorly retained with part of the analyte eluting in the void of the column. Additionally, while not a factor in the rapid scouting step, peak shapes are overall worse at high pH compared to low pH. Focusing primarily on retention, and considering methotrexate's early elution, low pH mobile phases will be used in all subsequent testing. These conditions allow the use of a wide range of column selectivities which would be otherwise unavailable due to column stability.

The next step of the systematic screening protocol is to perform a more traditional column and solvent screen. This includes using acetonitrile and methanol on a panel of columns with diverse properties. Selecting the columns to test can be a challenge. For this application, the first column selected is the ACQUITY Premier BEH C<sub>18</sub> Column, which was used in the rapid scouting step. This column has a full coverage C<sub>18</sub> ligand and has been well documented in a variety of workflows. The next column selected is the ACQUITY Premier CSH Phenyl-Hexyl Column. This column employs a charged-surface hybrid (CSH) particle which improves the peak shape for basic analytes at low pH by having a slight positive charge, which repels the positively charged basic probes. Additionally, this column uses a phenyl-hexyl ligand, which can retain analytes not only by the traditional reversed-phase mechanism but also through  $\pi$ - $\pi$  interactions, especially when methanol mobile phases are used. Third, the ACQUITY Premier HSS PFP Column was selected. Unlike the previous two columns, this one employs a silica base particle, providing a unique set of conditions to retain the analyte. The higher amount of surface silanols improves retention of analytes, and the pentafluorophenyl (PFP) ligand provides additional retention mechanisms including dipole interactions and hydrogen bonding potential. Next, the ACQUITY Premier HSS T3 Column was selected. This column uses the same base particle as the PFP column, but instead employs a mid-coverage C<sub>18</sub> ligand. This means that analytes have more interaction with the base particle. This column is well known for retaining polar analytes and is commonly used in bioanalytical and metabolomics workflows. Lastly, the ACQUITY Premier BEH Shield RP18 Column was selected. This column, using the BEH particle, contains a straight chain ligand with an embedded polar group. The polar group attracts more water in the mobile phase to the surface of the column, reducing interactions between basic analytes and the base particle. This, in effect, improves the peak shape for basic probes while offering slightly different selectivity compared to a C<sub>18</sub> column.

The above-mentioned five columns were selected in order to have a diverse panel to screen. Additionally, all columns use MaxPeak HPS Hardware, as that has shown to reduce unwanted interactions between analytes and metal surfaces, such as column hardware. Lastly, some of these columns have been evaluated by My Green Lab for the product environmental impact and the columns have been given ACT Eco Labels. Waters is the first column manufacturer to have columns with ACT Eco Labels, which is beneficial for labs trying to reduce their environmental impact.<sup>10</sup> Figures 3 and 4 show the chromatograms of column screening using acetonitrile and methanol, respectively.

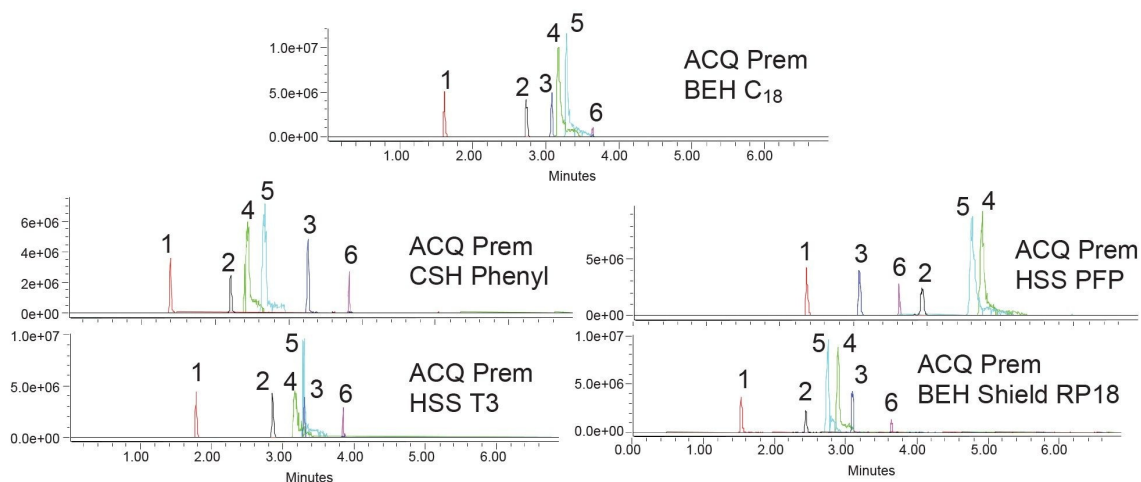


Figure 3. Separation of six ADC payload drugs using five columns and acetonitrile mobile phases. Peak identification outlined in Figure 1. ACQUITY Premier abbreviated to ACQ Prem to save space in the figure. SIRs used to track peaks during column screening.

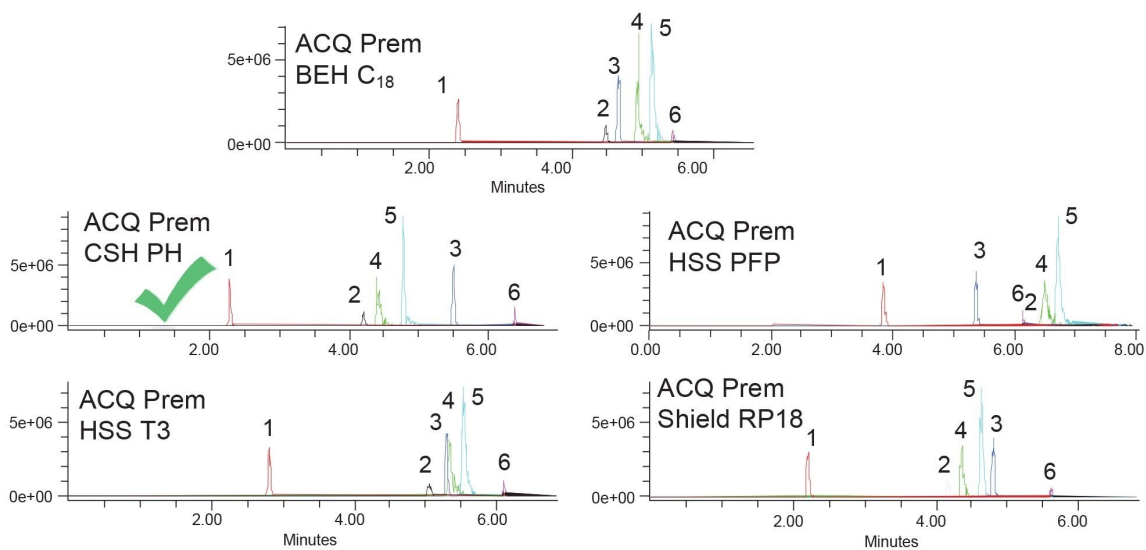
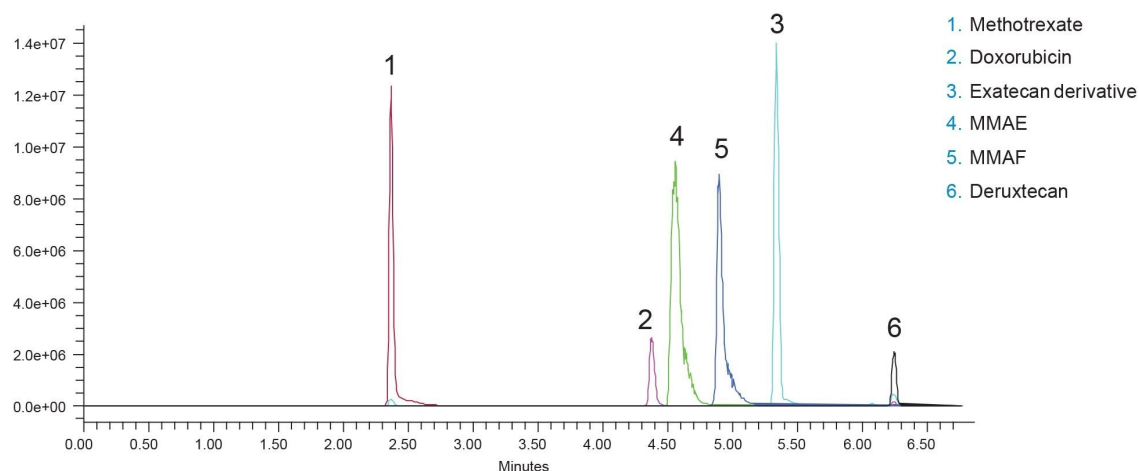


Figure 4. Separation of six ADC payload drugs using five columns and methanol mobile phases. Peak identification outlined in Figure 1. ACQUITY Premier abbreviated to ACQ Prem to save space in the figure. SIRs used to track peaks during column screening.

When acetonitrile is used, none of the columns achieve the method objective of full resolution. The ACQUITY Premier CSH Phenyl-Hexyl Column is close; however, due to peak shape disturbances of both analytes 4 and 5, the resolution is not quite baseline. Those two analytes are co-eluting on the other columns as well. However, some notable selectivity differences are seen across the five columns used. For instance, analytes 4 and 5 elute after all of the other compounds using the ACQUITY Premier HSS PFP Column. The other columns show these two eluting in the middle of the run. Additionally, while the ACQUITY Premier BEH C<sub>18</sub> and BEH Shield RP18 columns both use the same particle, the ligand produces slightly different selectivity for peak 3, the exatecan derivative. The C<sub>18</sub> ligand shows that the analyte is eluted before the monomethyl auristatin E (4) peak, while the Shield RP18 causes the exatecan derivative to elute after the monomethyl auristatin E. Additionally, the elution order of the monomethyl auristatin E and F peaks change depending on the column used. The BEH C<sub>18</sub>, CSH Phenyl-Hexyl, and HSS T3 columns all show monomethyl auristatin E eluting first, while the HSS PFP and BEH Shield RP18 columns have monomethyl auristatin F eluting first. These differences highlight why selecting diverse columns for screening is so important.

Methanol mobile phases produce a single chromatogram that meets the method objectives, which is achieved using the ACQUITY Premier CSH Phenyl-Hexyl Column. Using that column with methanol mobile phases promotes any  $\pi$ - $\pi$  interactions that could take place, providing a different selectivity than the same column with acetonitrile mobile phases. The BEH Shield RP18 column could have also been used but would have required additional optimization to get a better separation between peaks 3 and 5. The different selectivity and retentivity achieved using methanol mobile phases highlights why screening more than just one strong solvent is important during method development.

Final method conditions using the ACQUITY Premier CSH Phenyl-Hexyl Column with methanol mobile phases modified with formic acid are shown in Figure 5. By employing the systematic screening protocol and MaxPeak Premier Columns, the separation of six ADC payload drugs was achieved.



*Figure 5. Final method conditions for the separation of six ADC payload drugs. ACQUITY Premier CSH Phenyl-Hexyl Column using formic acid-modified methanol mobile phases on an ACQUITY UPLC H-Class Plus System with QDa detection.*

## Conclusion

Antibody drug conjugates (ADCs) are complex biotherapeutics that contain not only a monoclonal antibody (mAb) but also a small molecule “payload” drug. Characterizing the small molecule is critical to the success of these biotherapeutics, and having a single analytical method to detect several of these compounds can be an important aspect of that characterization.

This application note focused on creating a method to separate six ADC payload drugs using the systematic screening protocol and MaxPeak Premier Columns. The implementation of the structured method development protocol, coupled with the advanced hardware of MaxPeak Premier Columns, provided a final method using a methanol mobile phase and formic acid modifier. Baseline separation was achieved for all six compounds, and the method could now be used in a variety of different workflows including quantification or even bioanalytical testing. It should be noted that this application and the final method conditions developed are appropriate only for the small molecule payload drugs, and may not be suitable for ADCs as a combined final product. Analysis of mAbs or conjugated ADCs may require a specialized, wide pore particle column in order to elute the larger mAb

under reversed-phase conditions.

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