# High-Resolution Separation of Cysteine-Linked Antibody-Drug Conjugate Mimics Using Hydrophobic Interaction Chromatography

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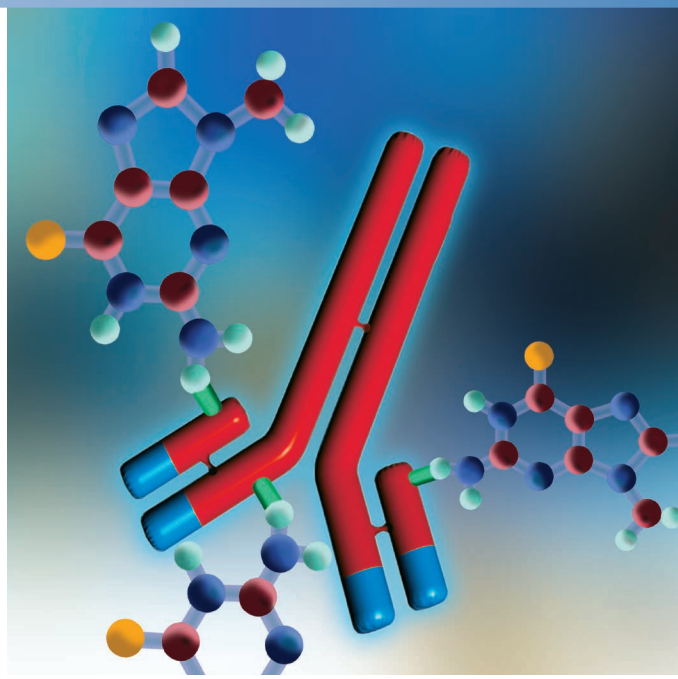
Application Note 21079

**Key Words**
Hydrophobic interaction chromatography, HIC, monoclonal antibody, mAb, antibody-drug conjugate, ADC, drug-to-antibody ratio, DAR, MAbPac HIC-Butyl

## Goal

To demonstrate the analysis of antibody-drug conjugate (ADC) mimics using a Thermo Scientific™ MAbPac™ HIC-Butyl column.



## Introduction

Monoclonal antibody drugs are the fastest growing class of biotherapeutics and have become a major part of the biopharmaceutical market. Monoclonal antibodies have demonstrated their effectiveness against autoimmune disorders, cardiovascular diseases, infectious diseases, and cancer.[1] Monoclonal antibodies selectively recognize antigens that are present on the tumor cells and exert their cytotoxic effect by receptor agonist activity, immune response, or vascular and stromal cell ablation. However, most of these mAbs are used in combination with chemotherapy, and many others have shown a lack of clinical efficacy. The success and the limitation of monoclonal antibodies have fueled the development of another form of antibody based therapeutics–antibody-drug conjugates (ADCs).[2] Antibody-drug conjugates utilize the exquisite selectivity of the antibody to achieve targeted delivery of cytotoxic drugs. ADCs have gained tremendous interest among pharmaceutical companies due to their significantly improved clinical efficacy over native monoclonal antibodies.[3]

The conjugation of drugs often results in an ADC molecule that is heterogeneous with respect to both the distribution and loading of cytotoxic drugs on the mAb. The number of drugs attached to the mAb has been shown to directly affect the safety and the efficacy of the drug. Unconjugated mAbs have significantly lower potency, and the ADCs with high drug load are subject to rapid renal clearance. Therefore, it is critical to fully characterize and monitor the heterogeneity of ADCs during development and production.[4]

Hydrophobic interaction chromatography (HIC) is a technique for separation of proteins, including monoclonal antibodies, by hydrophobicity. The HIC mobile phase usually consists of a salting-out agent, which at high concentration retains the protein by increasing hydrophobic interaction between the protein and the stationary phase. Bound proteins are eluted by decreasing the salt concentration.

HIC has been widely used as an orthogonal method to size-exclusion chromatography and ion-exchange chromatography for the characterization of mAb heterogeneity. Analysis of succinimides, antibody fragments, oxidated mAbs, and C-terminal lysine modifications were successfully carried out to monitor the stability and, in some cases, the potency of the drug.[5,6] HIC is also suitable for the separation of ADCs since attachment of cytotoxin alters the hydrophobicity of the antibody. The least hydrophobic unconjugated antibody

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elutes first and as the number of drugs attached increases the elution time increases. Therefore, HIC is considered to be the method of choice to characterize the distribution of ADC molecules with different drug-to-antibody ratios (DARs).[7]

The MAbPac HIC-Butyl column is a polymer-based HIC column designed for separation of mAbs and variants, including ADCs. The hydrophilic nature of polymer particles and the optimal density of butyl functional groups lead to excellent biocompatibility, low carryover, and high resolution. Here we describe the analysis of ADC samples that were conjugated via interchain disulfide bonds on the MAbPac HIC-Butyl column.

## Experimental

### Chemicals and Reagents

- Deionized (DI) water, 18.2 MΩ-cm resistivity
- 2-Propanol (Fisher Scientific P/N A461-4)
- Sodium phosphate monobasic monohydrate ($NaH_2PO_4 \cdot H_2O$, ≥98.0%)
- Ammonium sulfate [$(NH_4)_2SO_4$, ≥99.0%]

### Sample Handling Equipment

Polypropylene, 0.3 mL vials (P/N 055428)

### Sample Preparation

Cysteine-conjugated ADC mimic samples were provided by a customer. 10 mg/mL ADC mimic samples were diluted in half with 2 M $(NH_4)_2SO_4$, 100 mM $NaH_2PO_4$, pH 7.0 solution. 25 mg/mL control mAb was diluted five-fold with 2 M $(NH_4)_2SO_4$, 100 mM $NaH_2PO_4$, pH 7.0 solution.

### LC Separation

The LC separation conditions were as follows:

| | | | |
|---|---|---|---|
| Instrumentation | Thermo Scientific™ Dionex™ UltiMate™ 3000 BioRSLC system equipped with: | | |
| | SR-3000 Solvent Rack (without degasser) (P/N 5035.9200) | | |
| | LPG-3400RS Biocompatible Quaternary Rapid Separation Pump (P/N 5040.0036) | | |
| | WPS-3000TBRS Biocompatible Rapid Separation Thermostatted Autosampler (P/N 5841.0020) | | |
| | TCC-3000RS Rapid Separation Thermostatted Column Compartment (P/N 5730.0000) | | |
| | VWD-3400RS Rapid Separation Variable Wavelength Detector) equipped with a micro flow cell (P/N 5074.0010 | | |
| Column | MAbPac HIC-Butyl, 4.6 × 100 mm (P/N 088558) | | |
| Mobile phase A | 1.5 M ammonium sulfate, 50 mM sodium phosphate, pH 7.0 / 2-propanol (95:5 v/v)<br>Dissolve 6.55 g of $NaH_2PO_4 \cdot H_2O$ and 188.30 g of $(NH_4)_2SO_4$ in 750 mL DI water, adjust the pH to 7.0 using 50% sodium hydroxide (NaOH) solution. Bring the volume to 950 mL with DI water. Then bring the volume to 1000 mL with 2-propanol. | | |
| Mobile phase B | 50 mM sodium phosphate, pH 7.0 / 2-propanol (80:20 v/v)<br>Dissolve 5.52 g of $NaH_2PO_4 \cdot H_2O$ in 750 mL DI water, adjust the pH to 7.0 using 50% NaOH solution. Bring the volume to 800 mL with DI water. Then bring the volume to 1000 mL with 2-propanol. | | |
| Gradient | Time (min) | %A | %B |
| | -5.0 | 100 | 0 |
| | 0.0 | 100 | 0 |
| | 1.0 | 100 | 0 |
| | 15.0 | 0 | 100 |
| | 20.0 | 0 | 100 |
| Flow rate | 1.0 mL/min | | |
| Run time | 20 min | | |
| Injection volume | 5 µL | | |
| Temperature | 25 °C | | |
| UV detector wavelength | 280 nm | | |

### Data Processing

Thermo Scientific™ Dionex™ Chromeleon™ 6.8 Chromatography Data System

## Results and Discussion

The ADC mimics used in this work were conjugates between a drug mimic and mAb via the sulfhydryl group of interchain cysteine residues (Figure 1a). This procedure results in a mixture of drug-loaded antibody species with 0 to 8 drugs (Figure 1b). The stoichiometry of the drug mimic was varied in the conjugation reaction. Samples are labeled low, moderate, and high based on the average number of the drug mimic attached to the mAb. The typical separation of cysteine-conjugated ADC mimic on a MAbPac HIC-Butyl column is demonstrated in Figure 2. HIC peak identities were assigned by examining the UV spectra of each peak using a diode array detector using a literature method.[7]

Concentration of organic solvent in mobile phases and temperature are critical factors for the resolution and peak shape when analyzing protein samples in HIC. The best peak shape and separation was achieved using 5% 2-propanol in mobile phase A and 20% 2-propanol in mobile phase B at 25 °C. Without organic solvent, DAR species were not well resolved, while addition of more 2-propanol in mobile phase A or mobile phase B resulted in a very broad peak for the 8-drug form, probably due to denaturing of the heavy and light chain. All the ADC species with 0 to 8 drugs were observed with three additional peaks for the 6-drug species. This indicates there are at least four hydrophobic variants of the 6-drug species.

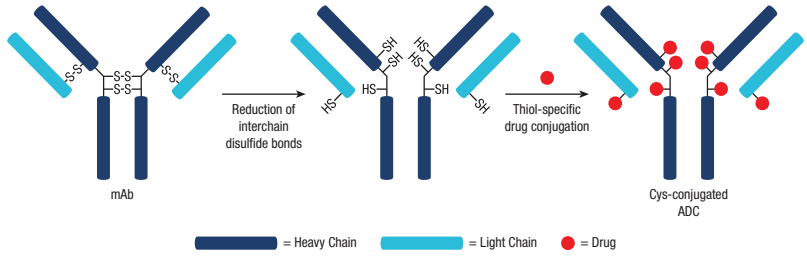


Figure 1a. Schematic representation of conjugation of drug mimic via interchain cysteine residues.

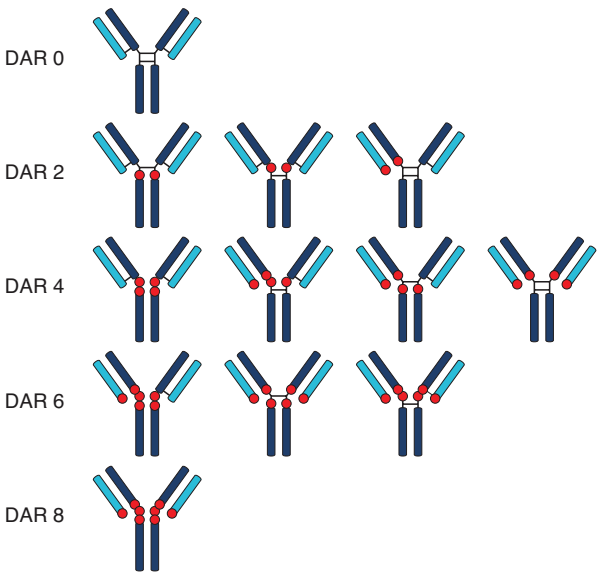


Figure 1b. Heterogeneity of cysteine-conjugated ADCs.

| | | | |
|---|---|---|---|
| Column: | **MAbPac HIC-Butyl**, 5 µm | | |
| Format: | 4.6 × 100 mm | | |
| Mobile Phase A: | 1.5 M ammonium sulfate, 50 mM sodium phosphate, pH 7.0 / 2-propanol (95:5 v/v) | | |
| Mobile Phase B: | 50 mM sodium phosphate, pH 7.0 / 2-propanol (80:20 v/v) | | |
| Gradient: | Time (min) | %A | %B |
| | -5.0 | 100 | 0 |
| | 0.0 | 100 | 0 |
| | 1.0 | 100 | 0 |
| | 15.0 | 0 | 100 |
| | 20.0 | 0 | 100 |
| Flow Rate: | 1.0 mL/min | | |
| Inj. Volume: | 5 µL | | |
| Temp.: | 25 °C | | |
| Detection: | UV (280 nm) | | |
| Sample: | Cys-conjugated ADC mimic (5 mg/mL) | | |

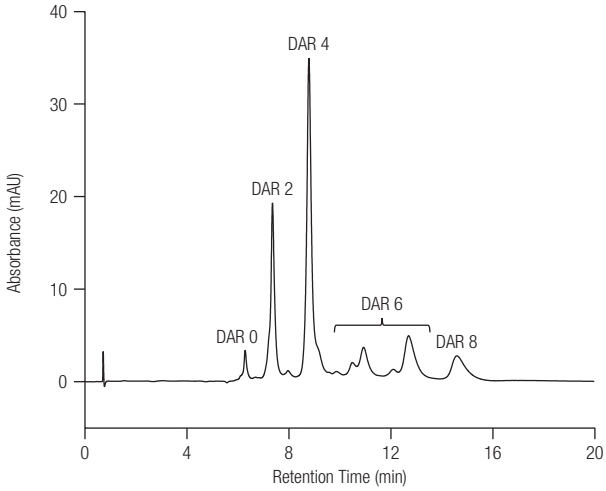


Figure 2. Separation of cysteine-conjugated ADC mimic.

Figure 3 compares the analysis of cysteine-conjugated ADC mimics with different drug loads assuming the UV absorption of the drug mimic is minimal at 280 nm. The mimic with high drug load shows no unconjugated mAb and a higher intensity of the 8-drug form while the low drug load sample contains a significant amount of unconjugated mAb, 2-drug form, and 4-drug form.

| | | | |
|---|---|---|---|
| Column | **MAbPac HIC-Butyl**, 5 µm | | |
| Dimension: | 4.6 × 100 mm | | |
| Mobile Phase A: | 1.5 M ammonium sulfate, 50 mM sodium phosphate, pH 7.0 / 2-propanol (95:5 v/v) | | |
| Mobile Phase B: | 50 mM sodium phosphate, pH 7.0 / 2-propanol (80:20 v/v) | | |
| Gradient: | Time (min) | %A | %B |
| | -5.0 | 100 | 0 |
| | 0.0 | 100 | 0 |
| | 1.0 | 100 | 0 |
| | 15.0 | 0 | 100 |
| | 20.0 | 0 | 100 |
| Flow Rate: | 1.0 mL/min | | |
| Inj. Volume: | 5 µL | | |
| Temp.: | 25 °C | | |
| Detection: | UV (280 nm) | | |
| Sample: | a. Unconjugated mAb (5 mg/mL)<br>b. Cys-conjugated ADC mimic (low load; 5 mg/mL)<br>c. Cys-conjugated ADC mimic (moderate load; 5 mg/mL)<br>d. Cys-conjugated ADC mimic (high load; 5 mg/mL) | | |

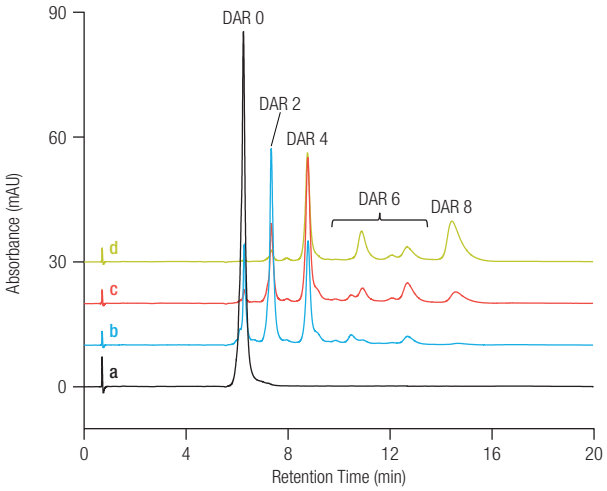


Figure 3. Comparison of cysteine-conjugated ADC mimics with different drug loads

## Conclusion

- The MAbPac HIC-Butyl column provides high resolution for unconjugated mAbs and cysteine-linked ADCs with different drug-to-antibody ratios (DARs).
- The drug load distribution can be monitored using the MAbPac HIC-Butyl column.

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