TOSOH

Mobile phases for hydrophobic interaction chromatography (HIC)

Your Challenge

- You need to develop a HIC method.
- You need to know which parameters affect your separation and how.

Our Solution

TSKgel® HIC-ADC Butyl column

- Versatile HIC column for a large range of hydrophobic molecules

What was done?

- By systemically changing the mobile phase, its influence on mAb and ADC separation was studied.

What was the result?

- Effective HIC separation and elution of molecules with varying hydrophobicities is possible by fine-tuning the mobile phase.

pH, organic solvent, salt concentration, and gradient mixing rates influence HIC separations. Understanding these factors allows for effective separations of mAbs and ADCs on the TSKgel HIC-ADC Butyl column.

Analysis of mAbs and ADCs

Your Benefit

One column for a wide range of molecules – understand how to optimize HIC separation

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TOUCHING LIVES.

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Application Note

Impact of Mobile Phase Modification in HIC Chromatography for IgG and ADC analysis

Introduction

In the analysis of biomolecules, particularly immunoglobulins (IgG) and antibody-drug conjugates (ADCs), maintaining the native state of these complex structures is crucial. Hydrophobic Interaction Chromatography (HIC) addresses this challenge by separating biomolecules under conditions that preserve their functional integrity. HIC uses a mobile phase with high salt concentrations to bind biomolecules, and elution is achieved by gradually decreasing the salt concentration. This process results in the early elution of more hydrophilic molecules and the later elution of more hydrophobic ones.

This application note describes how modifications to the mobile phase – specifically pH, organic solvent addition, salt concentration, and gradient mixing rates - can significantly enhance the efficacy of HIC separations using the TSKgel HIC-ADC Butyl column. Understanding the impact of each variable provides a foundation for fine-tuning separations of complex biomolecules such as ADCs and monoclonal antibodies.

Experimental Conditions

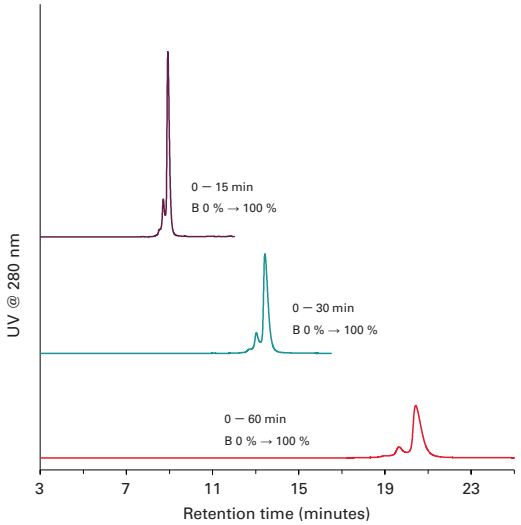
Column:	TSKgel® HIC-ADC Butyl (4.6 mm ID × 10 cm L)
Mobile phase:	pH, organic solvents, salt concentrations, and gradient slopes were systematically varied to explore their impact on the elution profiles of the monoclonal antibody and ADC mimic. The specifics of each gradient elution are detailed in the corresponding figure legends.
Gradient:	10 - 35 % B linear in 20 min, 100 % B for 5 min, 10 % B for 5 min
Flow rate:	0.5 mL/min (Sample A), 0.8 mL/min (Sample B)
Temperature:	25 °C
Detection:	UV @ 280 nm (Sample A); UV @ 215 nm (Sample B)
Injection vol.:	10 µL
Samples:	A: Human IgG1 (1 mg/mL) B: ADC mimic (1 mg/mL)

Results and Discussion

Modifying Gradient Slope

Different linear mixing gradients were tested on an IgG1 antibody (Sample A) by lowering the salt concentration of 1 mol/L Ammonium Sulfate to 0 mol/L over 15, 30, and 60 minutes. Under these conditions, the elution time shifts later with shallower gradients because the elution salt concentration is reached at later run times (*Figure 1*). However, the resolution between the shoulder and main peak improves with longer gradients due to a larger time-lapse to achieve the critical salt concentration needed to elute each peak. Though separation is increased, the tradeoff is a loss of sensitivity, as band broadening is observed with flatter gradients. An optimal gradient slope is, therefore, a compromise between run time, resolution, and sensitivity.

Figure 1. Impact of gradient slope on HIC separation of a mAb

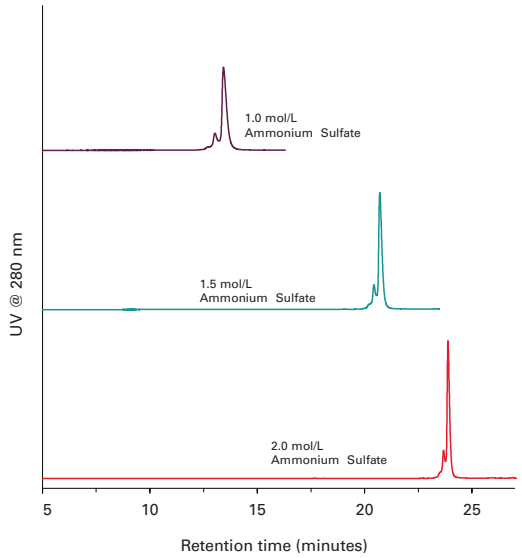


Buffer A: 0.1 mol/L sodium phosphate pH 7 + 1.0 mol/L ammonium sulfate; Buffer B: 0.1 mol/L sodium phosphate pH 7; Gradient: B 0 – 100% (linear, time as indicated in the graph)

Influence of Salt Concentration

Mobile phases starting with 1 mol/L, 1.5 mol/L, and 2 mol/L ammonium sulfate were tested on the IgG1 antibody (sample A) to investigate the effect of relative retention and separation (*Figure 2*). The ammonium sulfate concentration decreases linearly to 0 mol/L over 30 minutes. In effect, the gradient is steeper when starting with a higher salt concentration.

Figure 2. Impact of salt concentration on HIC separation of a mAb



Buffer A: 0.1 mol/L sodium phosphate pH 7 + ammonium sulfate as indicated in the graph; Buffer B: 0.1 mol/L sodium phosphate pH 7; Gradient: B 0 – 100% (0-30 min linear)

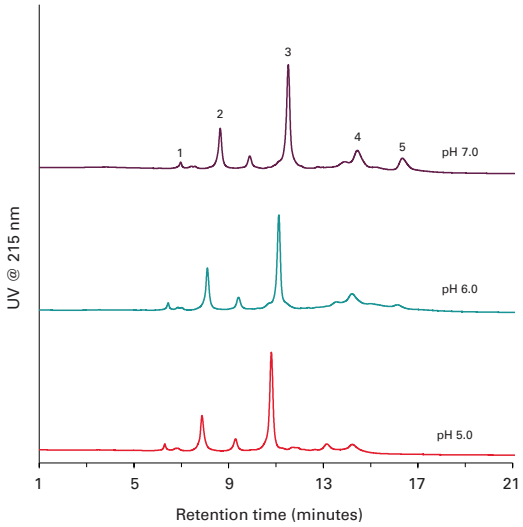
Separations that start with 1 mol/L ammonium sulfate elute at earlier retention times and more efficiently separate the shoulder from the mAbs' main peak than separations using higher initial salt concentrations. Although the separation improves at lower salt concentrations, peak heights increase as the salt concentration in the weak mobile phase increases. This results in increased sensitivity, mirroring the effect of a steeper mixing gradient, as described in *Figure 1*.

Modifying pH

Mobile phase pH critically affects the surface charge near the isoelectric point of proteins and antibodies, altering the distribution of charge variants present in solution. These effects can be further exacerbated by covalently linked drug-conjugates used for ADCs, as the local pKA of charged residues can be affected by non-native intramolecular interactions. As a result, the variably hydrophobic ADC variants can be separated using HIC.

The influence of pH on the separation of an ADC mimic (Sample B) was determined by analyzing mobile phases buffered at pH 5-7. At pH 5, the low DAR variants (DAR = 0, 2, and 4) of the ADC mimic elute earlier, and the peaks for high DARs are slightly less retained than at pH 6 and 7 (*Figure 3*). Peaks of high DAR species (DAR = 6 and 8) analyzed at low pH suffer from band broadening and decreased sensitivity. As the pH is increased, the chromatographic efficiency for high DAR species is significantly improved, as observed by the sharper peak shapes. As the molecule approaches the p*I* of the ADC mimic (close to 7), the molecule is less charged and more hydrophobic, leading to the observed higher retention.

Figure 3. – Impact of pH value on HIC separation of an ADC mimic



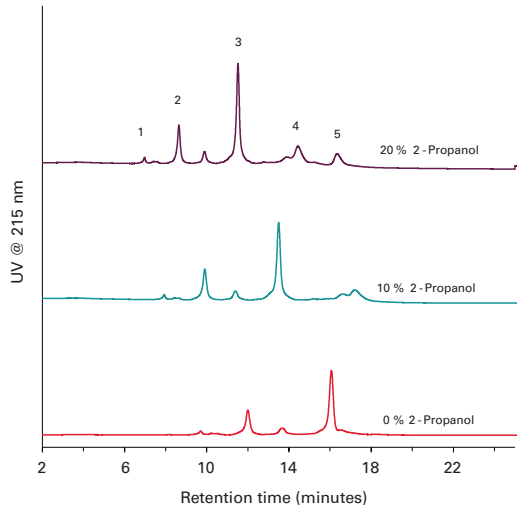
Buffer A: 0.05 mol/L sodium phosphate + 1.2 mol/L ammonium sulfate; Buffer B: 0.05 mol/L sodium phosphate + 20 % 2-propanol; Gradient: B 0 – 100% (0-15 min linear); pH is indicated in the graph. 1. DAR = 0; 2. DAR = 2; 3. DAR = 4; 4. DAR = 6; 5. DAR = 8

Utilizing Organic Solvent

The TSKgel HIC-ADC Butyl column features an increased ligand density tuned toward separating variably conjugated DAR isoforms. Adding organic solvents can modify hydrophobic interactions between analytes and the stationary phase, which are the main drivers of hydrophobic interaction separations. Thus, they can be used to optimize HIC analyses.

To test the effects of organic solvents on the separation of an ADC mimic, HPLC runs with different concentrations of isopropanol (IPA) (0 %, 10 %, 20 % v/v) in the strong mobile phase were performed (*Figure 4*). A comparison of separations with and without the organic modifier IPA shows a faster and complete elution when IPA is added to the run, while in the absence of IPA high DAR species (DAR=6, DAR=8) do not elute from the column. At higher isopropanol concentrations, high DAR variants elute as sharp peaks. In contrast, little or no addition of IPA to the elution buffer results in band broadening of hydrophilic variants and only partial elution of DAR species.

Figure 4. Impact of organic solvent addition on HIC separation of an ADC mimic



Buffer A: 0.05 mol/L sodium phosphate pH 7 + 1.2 mol/L ammonium sulfate pH 7; Buffer B: 0.05 mol/L sodium phosphate pH 7 + 2-propanol as indicated in the graph; Gradient: B 0 – 100% (0-15 min linear) 1. DAR = 0; 2. DAR = 2; 3. DAR = 4; 4. DAR = 6; 5. DAR = 8

Both early and complete elution in the presence of 20 % IPA can be explained by its ability to reduce hydrophobic interactions between the stationary phase and the DAR variants retained on the column. While hydrophobic interactions are the main mechanism for HIC retention, the combination of highly hydrophobic molecules with a stationary phase of high hydrophobicity leads to over-retentive behavior and non-elution of hydrophobic analytes. As shown here, one method to overcome inadequate elution is the addition of low organic solvent composition in the mobile phase.

Conclusion

We demonstrated effective separation and elution of molecules with varying hydrophobicities, such as ADCs and monoclonal antibodies, on TSKgel HIC-ADC Butyl. By adjusting the mobile phase conditions—both weak and strong—we were able to fine-tune the separation parameters to achieve optimal results.

In conclusion, pH, organic solvent composition, salt concentration, and gradient mixing rates all play crucial roles in Hydrophobic Interaction Chromatography (HIC) separations. A comprehensive understanding of how these factors interact allows for the systematic optimization of HIC methods, leading to more efficient and effective separations.

Featured Product

Part #	Description	Column dimensions
0023539	TSKgel HIC-ADC-Butyl	4.6 mm ID × 10 cm L