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Application News

Optimal Separation Conditions for Antibody-Drug Conjugates by Hydrophobic Interaction Chromatography

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User Benefits

- The average drug-to-antibody ratio (DAR) of antibody-drug conjugates (ADCs) can be analyzed by hydrophobic interaction chromatography on Shim-pack™ Bio HIC Butyl column.
- It ensures high reproducibility in the mobile phase analysis with high salt concentration.

■ Introduction

Antibody-drug conjugates (ADCs) are biopharmaceuticals produced by covalently linking cytotoxic drugs to monoclonal antibodies. Because the average drug-to-antibody ratio (DAR) and DAR distribution influence the stability and efficacy of ADCs, proper evaluation, including separation, detection, and analysis, is required. Hydrophobic interaction chromatography (HIC) is considered one of the useful techniques for DAR analysis, as it allows the separation of ADC species with different DAR values under native conditions based on differences in hydrophobicity. This article presents an example of optimization of HIC separation conditions for ADCs using a model ADC.

■ Analytical Condition

A 10 mg/mL solution of brentuximab vedotin diluted with ultrapure water was used as the sample. Brentuximab vedotin is a cysteine-conjugated ADC. As shown in Fig. 1, cysteine-conjugated ADCs contain isomers that differ in the number and positions of drug molecules conjugated to the antibody. Therefore, hydrophobic interaction chromatography may enable the separation of ADC species with different DAR values. The type of salt added to the mobile phase, the salt concentration, and the percentage of 2-propanol were investigated as parameters affecting retention and separation. First, sodium chloride, ammonium sulfate, and sodium sulfate were selected as the salts to be added. For each salt, the mobile phase consisted of 100 mmol/L sodium phosphate buffer (pH 7.0) containing the salt at a concentration of 1 mol/L. For the condition that provided the best result, the salt concentration was further varied in 0.5 mol/L increments to investigate the separation behavior. Under the most favorable condition, the 2-propanol concentration was then varied to 10, 15, 20, and 30% to evaluate the effect of organic solvent addition on separation behavior. The other analytical conditions are shown in Table 1.

Table 1 Analytical condition

System	: Nexera XS inert
Mobile Phase	: Pump A : 100 mmol/L phosphate(Na) buffer (pH 7.0) + 1mol/L of salt
	: Pump B :100 mmol/L phosphate(Na) buffer (pH 7.0) + 15% 2-propanol(standard)
Column	: Shim-pack Bio HIC Butyl (100 mm ×4.6 mm I.D., 4 µm)*1
Sample	: Brentuximab vedotin, 10 mg/mL in water
Vial	: Shim-vial H glass*2
Injection Volume	: 10 µL
Time Program	: B Conc. 0%(0 min)→100%(20 min) →100%(20.01-25 min) →0%(25.1-35 min)
Column Temp.	: 25℃
Detection (PDA)	: 280 nm (SPD-M40, UHPLC inert cell)

*1 P/N: 227-31174-01
*2 P/N: 227-34500-01

■ Salt Screening and Selection for ADC Separation

Three salts, sodium chloride, sodium sulfate, and ammonium sulfate, were selected as additives for mobile phase A, and each salt was initially added to mobile phase A at a concentration of 1 mol/L. The results are shown in Fig. 2. When mobile phase A containing 1 mol/L sodium chloride was used, retention of the ADC was insufficient; however, at 4 mol/L, separation of ADC species with different drug-to-antibody ratios (DARs) was achieved. In contrast, mobile phase A containing 1 mol/L sodium sulfate or ammonium sulfate provided better peak shape and separation than mobile phase A containing 4 mol/L sodium chloride. In addition, because ammonium sulfate has higher solubility in water than sodium sulfate and is therefore more readily applicable to high-salt-concentration mobile phases, ammonium sulfate was used in the subsequent studies.

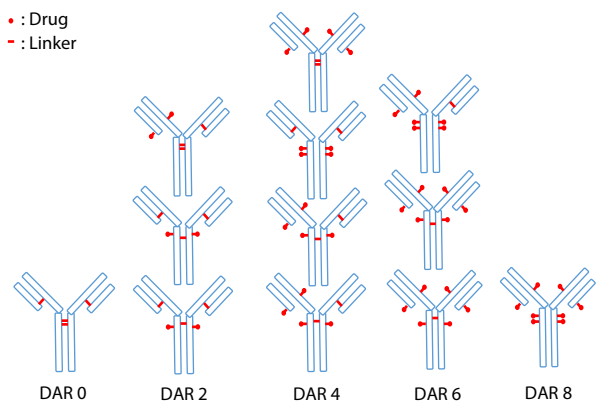

Fig. 1 Isomer distribution of cysteine-conjugated ADCs

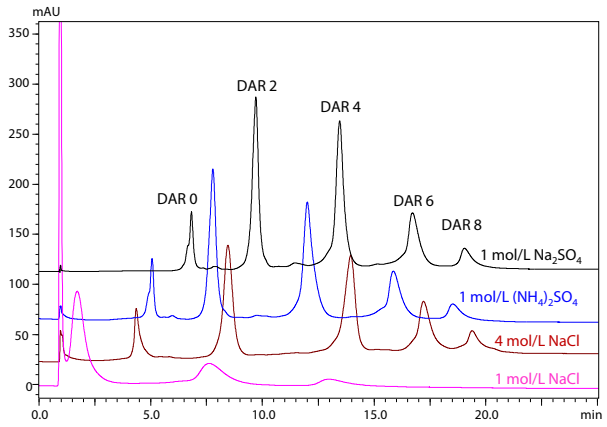

Fig. 2 Differences in separation behavior by salt type

■ Effect of Salt Concentration on ADC Separation

The separation behavior of the ADC was investigated using mobile phase A containing ammonium sulfate at concentrations of 1.0, 1.5, and 2.0 mol/L. The results are shown in Fig. 3. As the ammonium sulfate concentration increased, the peaks of the respective ADC species became sharper, and both retention and peak height increased. Based on these results, mobile phase A containing 2.0 mol/L ammonium sulfate was selected for the subsequent studies.

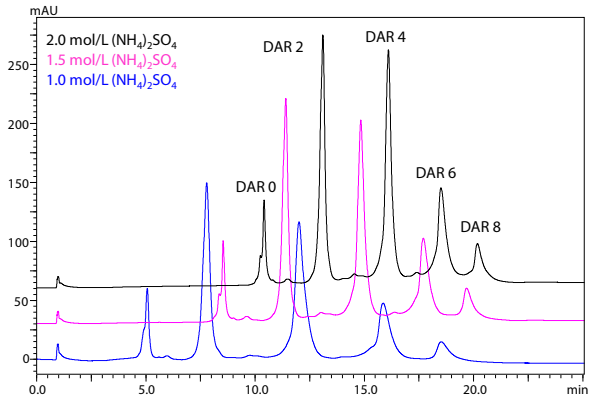

Fig. 3 Effect of Ammonium sulfate concentration on separation of ADC

■ Evaluation of 2-propanol Optimal Concentration

The effect of the 2-propanol concentration in mobile phase B, set at 10, 15, 20, and 30%, on the separation behavior of the ADC was evaluated. The chromatograms of the respective ADC species are shown in Fig. 4, and the peak area values are summarized in Table 2. When the 2-propanol concentration was 15%, the peak area values for DAR 6 and DAR 8, which have higher drug loading, reached their maximum levels. At 20%, the peak area values decreased, and at 30%, the peak area values further decreased and the overall peak shape deteriorated. Based on these results, the 2-propanol concentration was set at 15%.

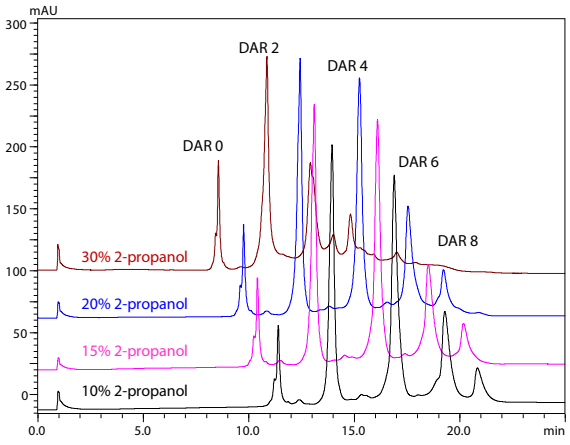

Fig. 4 Effect of 2-propanol concentration on separation of ADC

Table 2 Differences in the area values of each according to the concentration of 2-propanol

Conc. of 2-propanol	Peak area				
	DAR 0	DAR 2	DAR 4	DAR 6	DAR 8
10%	961,490	3,746,447	4,181,866	2,281,302	765,566
15%	990,998	3,695,578	4,158,322	2,392,434	885,265
20%	781,211	3,403,276	3,712,114	1,896,431	507,986
30%	929,603	3,222,159	1,787,304	-	-

■ Confirmation of Intra-day Repeatability and DAR

The chromatograms obtained from six repeated analyses of brentuximab vedotin under the optimized conditions and the repeatability (%RSD) of the peak area values for each peak are shown in Fig. 5 and Table 3, respectively. The repeatability of all peak area values was less than 5%. In addition, when the DAR was calculated using Equation (1) below, the result was approximately 4.04, which was consistent with the value reported in the literature.*1

$$DAR = \sum_{n=0}^{8} \frac{\text{Peak area} \times \text{Drug binding number}}{\text{Total value of peak area}} \quad \cdots (1)$$

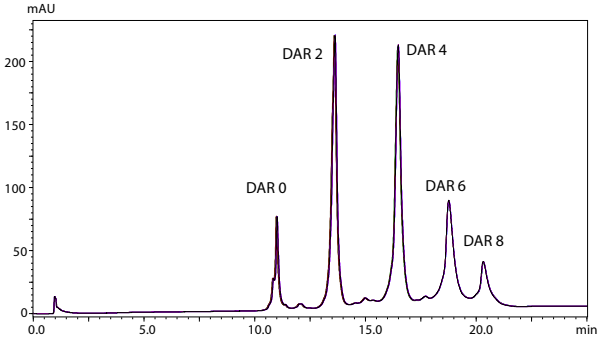

Fig. 5 Repeatability(%RSD) in six replicate analyses

Table 3 Reproducibility of area values in six repeated analyses (%RSD)

	%RSD
DAR 0	1.09
DAR 2	0.99
DAR 4	0.26
DAR 6	4.36
DAR 8	0.93

■ Conclusion

In the calculation of the average drug-to-antibody ratio (DAR) of antibody-drug conjugates, variations in separation behavior and peak intensity were observed depending on the type and concentration of salt in the mobile phase, as well as the amount of organic solvent.

In this study, the combination of the Nexera XS inert, which offers high durability against salt-induced corrosion, and the Shim-pack Bio HIC Butyl column enabled the analysis of ADC species with different DAR values, achieving both high separation performance and excellent repeatability.

01-01287-EN First Edition: Sep. 2026

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