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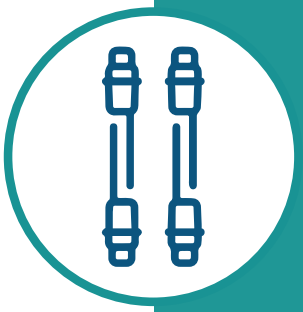

High-Resolution DAR Profiling with HIC-ADC Butyl

Your Challenge

- You deal with poor separation of high DAR species.
- You experience limited reproducibility in DAR quantification.

Our Solution

TSKgel® HIC-ADC Butyl column

- Provides high resolution separation of DAR species

What was done?

- Six ADCs were analyzed on the TSKgel HIC-ADC Butyl column and a reference column in terms of resolution and reproducibility.

What was the result?

- High DAR species separated better on the TSKgel HIC-ADC Butyl column, improving DAR calculation robustness and reproducibility.

The TSKgel HIC-ADC Butyl column outperforms conventional columns in ADC characterization, especially for high DAR species, with enhanced resolution, reproducibility, and QC compatibility.

Your Benefit

Generate reliable DAR data for regulatory compliance and enjoy the confidence in future DAR determinations.

Analysis of ADCs

TOSOH BIOSCIENCE

SEPARATION & PURIFICATION

CONNECTING MINDS.
TOUCHING LIVES.

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Application Note

In-Depth Characterization of Canonical ADC Platforms by Hydrophobic Interaction Chromatography

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Antibody-drug conjugates (ADCs) represent a rapidly growing class of biopharmaceuticals that combine the specificity of monoclonal antibodies with the potent therapeutic effects of cytotoxic drugs. The drug-to-antibody ratio (DAR) is a critical quality attribute of ADCs, as it directly impacts their efficacy, stability, and safety.

Cysteine-linked ADCs are traditionally analyzed by hydrophobic interaction chromatography (HIC) despite struggling with separation of high DAR species. In this application note, we explore the performance of a newly developed chromatographic column specifically designed for ADC analysis. The study demonstrates the column's ability to achieve superior separation for various cys-linked ADCs, including those with high DAR values—a critical advancement for the characterization and quality control of these therapeutics.

Experimental Conditions

Columns:	TSKgel® HIC-ADC-Butyl (5 µm, non-porous, 4.6 mm ID × 3.5 cm L) Reference HIC column (2.5 µm, non-porous, 4.6 mm ID × 3.5 cm L)
Mobile phase:	A: 1.5 mol/L ammonium sulfate / 25 mmol/L potassium phosphate pH 7.0 B: 25 % isopropanol / 25 mmol/L potassium phosphate pH 7.0
Gradient:	0-0.5 min 10 % B 0.5-12.5 min linear gradient from 10 % B–100 % B
Flow rate:	0.8 mL/min
Detection:	Absorbance at 280 nm
Samples:	5 ADCs approved by FDA & 1 ADC in clinical development - *Table 1* (injection amount 20 µg), non-conjugated mAbs (injection amount 5 µg), diluted 1:1 in mobile phase A

Results and Discussion

Characterization of Auristatin and PBD-Conjugated ADCs

First, the versatility of the HIC method was tested by analyzing the DAR of a panel of ADCs with heterogeneous payloads and average DARs between 2 and 4. As a control for identification of D0, the unconjugated mAb was run in parallel (data not shown). *Figure 1* shows the chromatograms for the ADC separations on the TSKgel HIC-ADC Butyl column and the comparison to a conventional (reference) column.

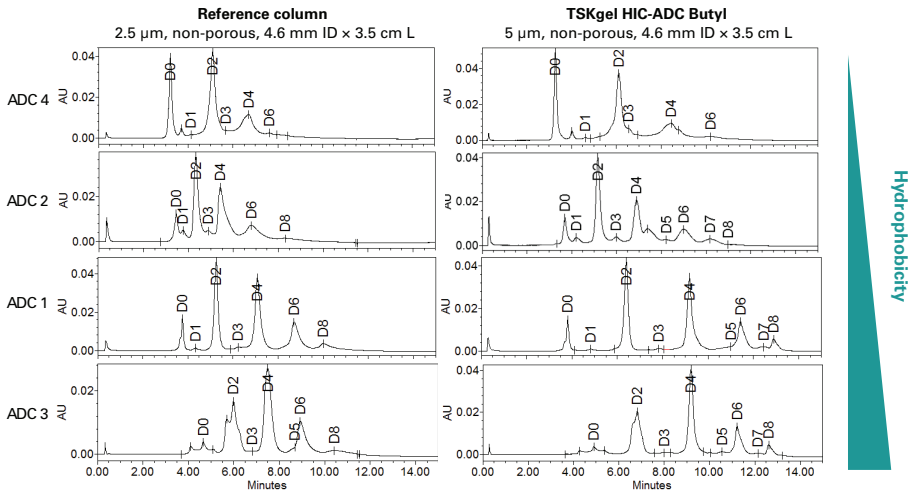
Retention and elution were achieved for all ADCs on both columns. Differences are visible in the elution time and peak shape of late eluting species such as DAR6, DAR7 and DAR8. These species elute later and show a clear peak on the TSKgel HIC-ADC Butyl column if compared to the reference column (e.g. ADC1, ADC2, ADC3 – *Figure 1*).

Comparison of the different ADCs reveals differences in retention time and accordingly in overall hydrophobicity. The elution times are in correlation with the AlogP values (octanol-water partition coefficient) of the linker+payload complexes that indicate their hydrophobicity *(Table 1)*. ADC4 with the lowest value elutes the fastest while ADC1 and ADC3 take the longest time to elute off the column.

Table 1. Overview of analyzed ADC

	ADC	Drug (mechanism of action)	DAR	AlogP (octanol-water partition coefficient of linker-payload)
Cys-linked ADC	ADC1: Brentuximab Vedotin	**Auristatin Derivatives** (Microtubule disruption)	4	4.79
	ADC 2		4	3.24
	ADC 3		4	4.79
	ADC 4	**PBD** (DNA cleavage)	2	2.15
	ADC 5	**SN-38 or DXd** (Topoisomerase inhibitor)	8	1.95
	ADC 6		8	0.29

Figure 1. **Analysis of a panel of ADCs by HIC.** Peaks with are assigned with the according DAR species.



For further comparison, performance parameters such as theoretical plate (TP) number, asymmetry (As) on DAR2 peak and resolution (between DAR2 and DAR4) were analyzed for ADC1 *(Table 2)*. The new HIC-ADC column outperforms the reference column by higher TP numbers hinting to better separation performance as well as by a better resolution. The improved performance is achieved despite a larger particle size and can be explained by particle size homogeneity as well as optimized particle chemistry and ligand density. In terms of asymmetry, both columns were comparable with the reference column showing an As factor of 1.1 (slight tailing) whereas the HIC-ADC column with an As factor of 0.9 indicates a slight fronting.

Table 2. Performance parameters of the separation of ADC1.

	Reference column			TSKgel HIC-ADC Butyl		
Performance parameter	**Average**	**SD**	**SD(%)**	**Average**	**SD**	**SD(%)**
Theoretical plates (DAR2)	4085	422	10.3	5963	187	3.1
Asymmetry (DAR2)	1.1	0.04	3.9	0.9	0.01	0.9
Resolution (DAR4-DAR2)	4.3	0.07	1.6	7.0	0.11	1.6

Characterization of High DAR Topoisomerase-Conjugated ADC Platforms

In contrast to ADC 1-4, ADC5 and ADC 6 out of the panel of tested ADCs have a homogenous payload distribution of 8 drugs/mAb and are considerably less hydrophobic, as indicated by the lower AlogP values of their linker-payload construct. For comparison, the unconjugated mAb is overlaid with the ADC chromatogram *(Figure 2)*. The ADC traces show a prominent peak which elutes approximately 0.5 min (ADC 5) or > 2 min (ADC6) after the unconjugated mAb *(Figure 2)*. Notably, for ADC 6 a shoulder accounting for DAR6 elutes before the main peak which is better separated on the HIC-ADC Butyl as compared to the reference column. Accordingly, the subsequent DAR calculation results in a DAR of 7.9 *(Figure 2)*.

Low variability in DAR determination with TSKgel HIC-ADC-Butyl column

A second aspect evaluated on the TSKgel HIC-ADC Butyl column was average DAR calculation. To this end, the analysis was repeated on 3 different days with changing analysts and repeated sample preparations. A comparison of the calculated DARs and their variability is shown in *Table 3*. For most of the analyzed ADCs, DAR determinations were more reproducible on the TSKgel HIC-ADC Butyl column as compared to the reference column.

Figure 2. **Analysis of ADC with homogenous payload distribution of DAR8.** DAR species are assigned.

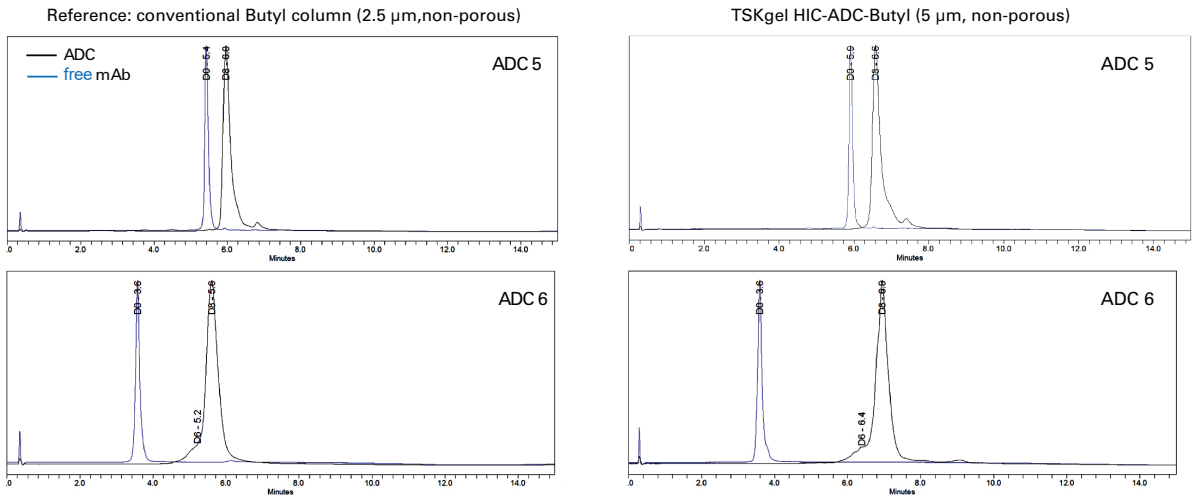


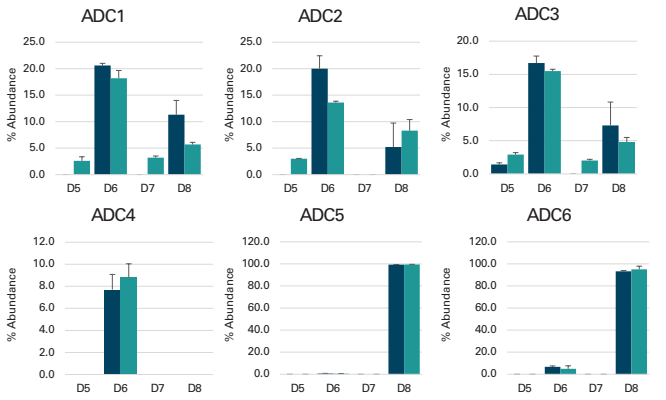
Table 3. **Stability of DAR determination.**

		ADC1	ADC2	ADC3	ADC4	ADC5	ADC6
Reference column	Average DAR (%SD)	4.0 (3.3 %)	3.6 (5.3 %)	3.7 (4.0 %)	2.4 (4.2 %)	8 (0 %)	7.9 (0.3 %)
TSKgel HIC-ADC Butyl	Average DAR (%SD)	3.8 (1.6 %)	3.6 (3.3 %)	3.7 (0.5 %)	2.5 (2.4 %)	8 (0 %)	7.9 (3.8 %)

Superior separation of high DAR species in ADCs with heterogeneous payload distribution

To elucidate robustness of average DAR calculation on the two columns, quantification of each DAR species was investigated. Two findings were made: first, intermediate DARs were better separated and thus quantifiable on separations done with the TSKgel HIC-ADC Butyl column while not being identified on the reference column (e.g. DAR5 in ADC1 and ADC2, DAR7 in ADC1 and ADC3). Second, for high DAR species (e.g. DAR6 and DAR8), the area determination, in percent, has higher variation when analyzed on the reference column compared to the TSKgel HIC-ADC Butyl column. Please note that the high DAR areas have a higher impact on the overall DAR calculation, a more reliable quantification of the high DAR areas hence results in highly accurate and robust average DAR calculations.

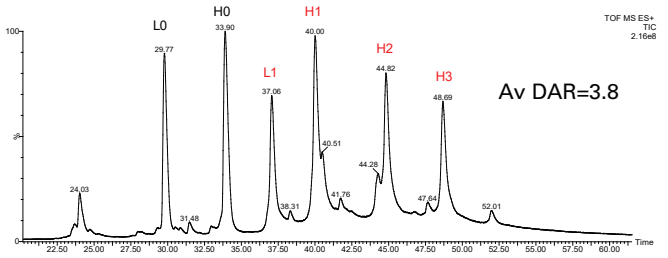
Figure 3. **Robustness of high DAR species quantitation.** Quantification of relative peak areas of all high DAR species (5-8). Experiments were repeated three times on different days with different experimenters. Blue bars refer to the reference column, teal bars refer to TSKgel HIC-ADC Butyl column. Error bars display standard deviation.



Comparison of DAR determination with LC-MS

For verification of the DAR calculation after HIC-separation, ADC 1 was additionally analyzed by RP-LC-MS in denaturing conditions. The ADC was reduced resulting in dissociation into light and heavy chains. Mass determination by ESI-TOF (Synapt G2Si Waters instrument) reveals presence of non-conjugated light chain (L0) and light chain conjugated with a single drug (L1). Heavy chain was either non-conjugated (H0) or carries up to three drugs (H1-3). Quantification of the MS data results in an average DAR of 3.8 *(Figure 4)* which is consistent with the DAR calculation based on HIC using the TSKgel HIC-ADC Butyl column *(Table 3)*.

Figure 4. **DAR determination of ADC1 by LC-MS.** DAR was determined in a reduced ADC sample by verifying conjugation state of light chain and heavy chain based on the mass determination (deconvolved) and peak quantitation in total ion chromatogram (TIC).



Conclusions

This application note benchmarks a novel HIC column featuring innovative particle technology against a reference column with conventional chemistry. Evaluated using a panel of ADCs with varying payloads and average DARs, the new TSKgel HIC-ADC Butyl column demonstrated suitability across a broad range of molecules. Notably, the enhanced separation performance resulted in high robustness particularly of high DAR (>5) quantification and visibility of DARs not separated on a reference column (e.g. DAR5, DAR7) leading to accurate average DAR calculations. Comparing average DAR determination by non-denaturing HIC on a TSKgel HIC-ADC Butyl column with RP-MS gave identical results, suggesting the use of HIC as a convenient and more QC-friendly method for average DAR determinations. By addressing the limitations of traditional HIC columns, particularly regarding challenges associated with achieving high-quality peak shapes for high DAR variants, the TSKgel HIC-ADC Butyl column offers a reliable and robust tool for ADC characterization.

Featured Product

Part #	Description
0023538	TSKgel HIC-ADC-Butyl (5 µm, 4.6 mm ID × 3.5 cm L)