Hydrophobic Interaction Chromatography- Native Mode Separation of Bio-Therapeutic Workflows

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HPLC 2018
P-M-0324

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Introduction

Hydrophobic interaction chromatography (HIC) is a powerful technique used in protein purification applications. HIC separates and purifies protein molecules based on their hydrophobicity, but unlike traditional reversed phase chromatography, it uses non-denaturing mobile phase solutions to preserve the biological activity of the intact proteins. HIC is a useful characterization tool for separating Monoclonal Antibodies (mAb), mAb variants, and is widely adapted for analyzing Antibody Drug Conjugates (ADCs).[1]

The Agilent AdvanceBio HIC column has been designed for the optimum separation of intact mAb and ADCs. A 3.5 µm fully porous particle was chosen to have maximum column efficiency while keeping pressure within HPLC operating ranges. Using a larger pore size of 450Å allows for the effective mass transfer of larger molecules. The bonding density and the surface area of the column has been optimized to increase the hydrophobicity of the stationary phase, which allows for the use of low salt gradients. In this poster, we describe use of this novel column to separate mAb oxidized variants and characterize oxidized mAb variant coupling with 2D-LC/MS approach. AdvanceBio HIC column was also applied for analysis of cysteine linked ADCs in their intact state to determine overall Drug to Antibody Ratio (DAR) value.

Furthermore, when performing HIC workflows extreme high salt concentrations are used, the Agilent 1260 Infinity II Bio-Inert system is an excellent fit to prevent corrosion and can be applied for routine analysis of Bio-therapeutics.

Experimental

Sample Preparation

All samples were diluted with mobile phase B at a 1:1 volume ratio to avoid injection viscosity differences. Baseline subtraction was performed for all HIC chromatograms.

Oxidation Conditions

2 % (v/v) of 70 % tert- Butyl hydroperoxide (*t*-BHP) solution was added to 1 mL sample of NIST mAb A (1 mg/mL), and the reaction mixture was injected onto the HIC column. The sample vial was held at 7 °C and multiple injections from the same vial were carried out.

mAb B used in 2D-LC/MS application was subject to forced oxidation using 1% *t*-BHP for 72 hours at 37 °C.

Mobile Phase Preparation

Mobile phases were prepared by diluting a stock salt solution (as indicated) with water (for Mobile Phase A and B). Both solutions were adjusted with 50% sodium hydroxide solution to pH 7.

Instrumentation

All Samples were analyzed using an Agilent 1260 Infinity II Bio-Inert LC system. Project 1, 2D-LC was performed using Agilent 1290 Infinity 2D-LC solution with an Agilent 6224 TOF MS system.

LC Conditions – mAb oxidized variant Analysis				
Column	Agilent AdvanceBio HIC, 4.6 x 100 mm (p/n 685975-908)			
Column Temp	25 °C			
Sample Conc.	1 mg/mL			
Inj. Vol.	5 µL			
Mobile Phase	**UV Detection: 220 nm** A = 50mM Sodium Phosphate pH 7.0 B = 2M ammonium Sulfate in 50mM Sodium Phosphate pH 7.0			
Flow Rate	0.3 mL/min			
Gradient Program	**Time**	**% A**	**%B**	
	0	40	60	
	40	90	10	
	45	90	10	
	50	40	60	
	60	40	60	

Experimental

LC Conditions – ADC DAR Analysis				
Column	Agilent AdvanceBio HIC, 4.6 x 100 mm (p/n 685975-908) Agilent AdvanceBio HIC, 4.6 x 30 mm (p/n 681975-908)			
Column Temp	25 °C			
Mobile Phase	**UV Detection: 220 nm** A = 50mM Sodium Phosphate pH 7.0 B = 2M ammonium Sulfate in 50mM Sodium Phosphate pH 7.0 C = propan-2-ol			
Inj. Vol.	5 µL			
Flow Rate	0.5 mL/min			
Gradient Program	**Time**	**% A**	**%B**	**%C**
	0	45	50	5
	15	75	0	25
	20	75	0	25
	21	45	50	5
	31	45	50	5

Results and Discussion

Project 1: Characterization of mAb oxidized variants

In this study, *t*-BHP used as chemical oxidant to promote oxidation of mAb samples. In Figure 1, oxidation reaction of NIST mAb A was monitored at various time points using shallow gradient, represented by an overlay of chromatograms. Multiple peaks eluted earlier than the mAb main peak, suggesting these oxidized species might be more polar and heavier resulting from surface accessible oxidation of Methionine (Met) residues.

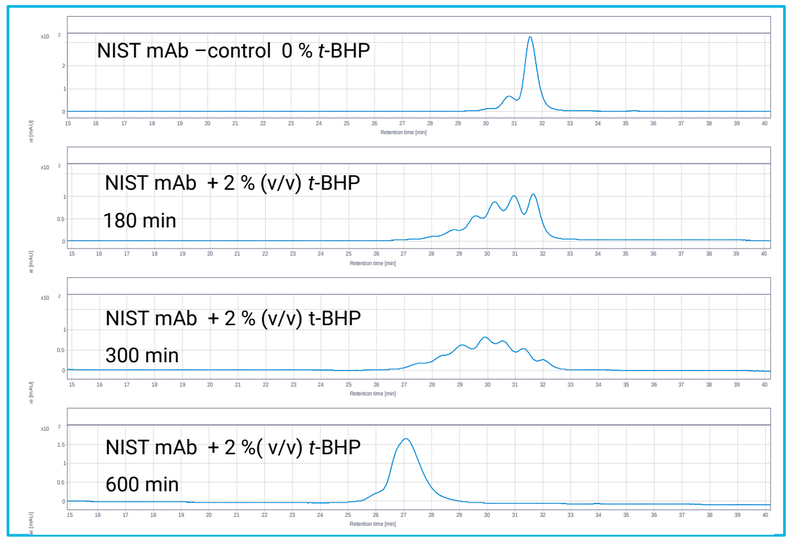

Figure 1. Monitoring the *t*-BHP oxidized mAb reaction

mAb B treatment with *t*-BHP produced a peak that eluted 0.5 minutes earlier than the main mAb peak at 12.5 minutes, as shown in Figure 2, A. Each of the two peaks was selected for mass measurement using the MHC 2D-LC/MS approach. As expected, the peak resultant from TBHP treatment was 64 Da higher in mass than the main mAb peak, as shown in Figure 2, B and C, which is presumably due to oxidation of four Met residues.[2]

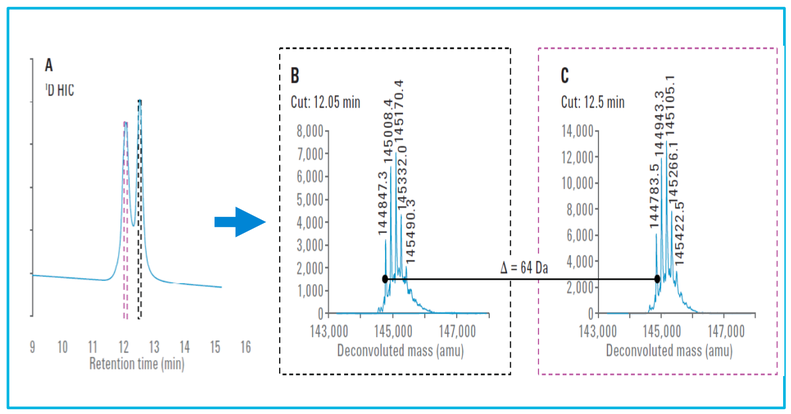

Figure 2. Analysis of oxidized mAb B using the HIC/RP 2D-LC/MS

Results and Discussion

Project 2. DAR Analysis of Antibody Drug Conjugates (ADCs)

Here we demonstrate the use of new AdvanceBio HIC column for analysis of the DAR value of ADCs with both speed and accuracy.[3]

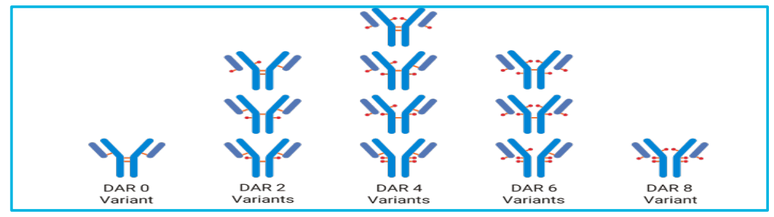

Figure 3. Drug distribution in cysteine linked ADCs

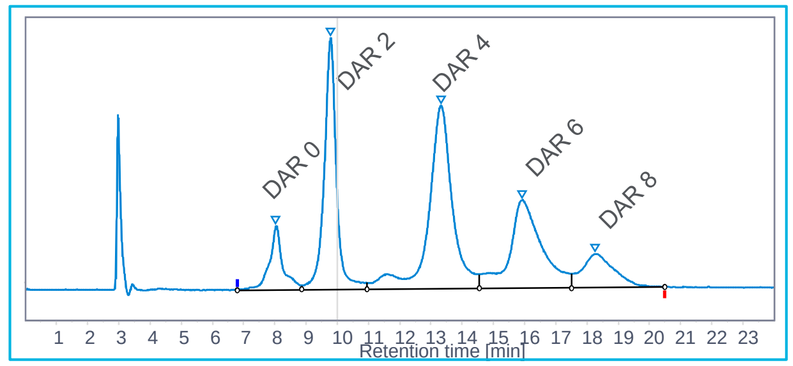

Figure 4. HIC separation of brentuximab vedotin (Adcetris)

Peak area used to calculate DAR results using equation to the right

No.	RT (min)	Area	%Area	DAR	Weighted Average
1	8.03	763	6.9	0	0
2	9.77	2759	25.1	2	0.5
3	13.31	3936	35.8	4	1.43
4	15.91	2565	23.3	6	1.4
5	18.26	978	8.9	8	0.71
				DAR	4.04

$$DAR = \sum_{n=0}^{8} \frac{\text{LC peak area} \times n_{drug}}{\text{Total LC peak area}}$$

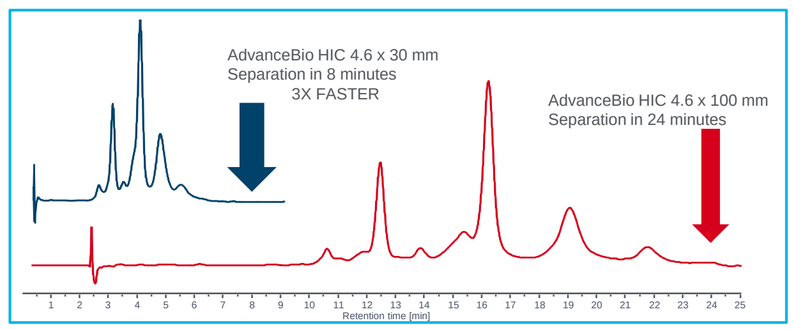

Figure 5. Comparison of Sigma mimic ADC faster separation

Conclusions

- The New AdvanceBio HIC column demonstrated optimal performance in analysis of Intact mAbs , oxidized mAb variants and ADCs.
- Use of high salt concentration mobile phases are required for HIC separations, this limits its use in characterization with Mass spec. AdvanceBio HIC column coupled with Agilent Infinity II Bio-Inert LC and 2D-LC workflows presented here help resolve these issues and provide an easy solution for routine characterization of large bio-molecules.

References

[1]Fekete, S.; Veuthey, J. L.; Beck, A.; Guillarme, D. Hydrophobic interaction chromatography for the characterization of monoclonal antibodies and related products, *J. Pharm. Biomed. Anal.* 2016, *130*, 3–18.

[2]Staples, G. Analysis of monoclonal antibodies using MHC 2D-LC/MS.. Agilent Technologies, Application Note. 2014. (5991-6376EN)

[3]Coffey A, Kondaveeti, S. An AdvanceBio HIC Column for Drug-to-Antibody Ratio (DAR) Analysis of Antibody Drug Conjugates (ADCs) . Agilent Technologies, Application Note. 2018. (5994-0149EN)