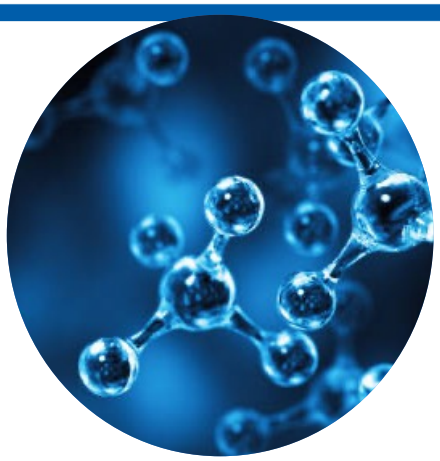

TN-1371

Impact of Mobile Phase Conditions and Temperature on Antibody Drug Conjugates Separation using Biozen Native RP-5 Column

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Introduction

Antibody drug conjugates (ADCs) represent a novel biotherapeutic approach that utilizes the specificity of an antibody to target an antigen, delivering a cytotoxic compound directly to the desired location in the body. ADCs are created by attaching small molecule cytotoxic drugs to an antibody through either a permanent or labile linker. The cytotoxic drug is typically hydrophobic which, when combined with the effect of the linker molecule, significantly affects the hydrophobicity and chromatographic behavior of the ADC. Consequently, innovative analytical solutions that offer faster and more comprehensive characterization of ADCs are essential for advancing this therapeutic technology.

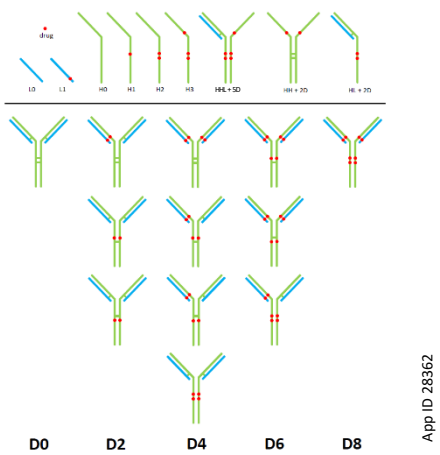
This technical note explores the impact of column temperature, mobile phase ionic strength, and pH on the LC-HRMS analysis of an intact cysteine-linked ADC using the Biozen Native RP-5 HPLC column, a non-porous, hydrophilic particle designed to preserve the native forms of intact biomolecules under reversed phase conditions. Observations on retention, peak shape, selectivity, and MS sensitivity of DAR species are discussed, and recommendations for optimal analysis conditions are provided.

Sample Preparation

The commercial cysteine linked ADC drug Polivy® (polatuzumab vedotin-piiq from Genentech Inc) was used as model test compound.

The ADC was buffer exchanged into 20 mM Ammonium Acetate (pH 5.2) using Vivaspin® 500 30 kDa centrifugal concentrators. Buffer exchange was performed to reduce fouling of the MS source by components of the ADC formulation and to improve sensitivity for the DAR0 species which elutes close to the void time of the column. The relatively high concentration of salts and other formulation components will likely cause MS signal suppression for any components eluting near the void time.

Figure 1. Antibody Drug Conjugate Subunits and Intact DAR Species with Positional Isomers



LC Conditions

Column: Biozen Native-RP-5
Dimensions: 50 x 2.1 mm
Part No.: 00B-4800-AN
Mobile Phase: See Table 1
Gradient: See Table 2
Flow Rate: 0.2 mL/min
Injection Volume: 20 µg ADC on-column
Temperature: 30-40 °C
LC System: Agilent 1290 Infinity II UHPLC
Detection: HRMS, UV (280 nm)
Detector: SCIEX X500B HRMS, Agilent DAD (UV)

Note: The Agilent 1290 Infinity II UHPLC system contained the following components: G7120A high speed binary pump with JetWeaver V35, G7167B multisampler, G7116B multicolumn thermostat and G7117B diode array detector (10mm pathlength). Also, plastic mobile phase reservoirs were used to reduce any metallic impurities (especially sodium and potassium) that may leach from standard borosilicate glass reservoirs. Finally, to ensure ADCs remained in their native, intact state the mobile phase organic composition was limited to a maximum of 25 v/v% IPA except for a brief column clean-up step (50 v/v% IPA) at the end of the gradient.

HRMS Conditions

Ionization Mode: ESI
Polarity: Positive
Source Temperature: 450 °C
Declustering Potential: 200 V (50 V spread)
CE ± CE Spread: 5 ± 0V
Ion Spray Voltage: 5000 V
Scan Range: 1000-8000 m/z

Table 1. Mobile Phases

Mobile Phase	A	B
High Ionic Strength	50 mM Ammonium Acetate in Water (no pH adjustment, pH ~6.8)	Isopropanol/50 mM Ammonium Acetate in Water (50/50, v/v)
Low Ionic Strength	20 mM Ammonium Acetate in Water (no pH adjustment, pH ~6.8)	Isopropanol/40 mM Ammonium Acetate in Water (72/25, v/v)
Low Ionic Strength/ Low pH MP	20 mM Ammonium Acetate in Water, pH 5.2	Isopropanol/40 mM Ammonium Acetate in Water, pH 5.2 (72/25, v/v)

Table 2 . Gradient Profiles

Gradient Profile	Time (min)	%B
High Ionic Strength	0	0
	2	0
	5	15
	20	50
	24	50
	26	100
	27	100
	27.1	0
	30	0
Low Ionic Strength (including low pH)	0	0
	2	0
	5	7.5
	20	25
	24	25
	26	75
	27	75
	27.1	0
	30	0

Results and Discussion

The illustration in Figure 1 shows the general arrangement, nomenclature, and structure of the different DAR species of cystine linked ADCs. Extracted ion current (XIC) chromatograms of the DAR species in the commercial drug Polivy were used to assess the differences in performance between the 3 different mobile phase systems: high ionic strength, low ionic strength, and low ionic strength with low pH. XIC chromatograms were extracted for the G0F/G0F glycoform using the Sciex software and the theoretical masses for the different DAR species given in Table 3. For DAR species where more than one peak elute, the letter that follows the DAR value denotes the elution order.

The effect of mobile phase ionic strength on chromatographic performance is shown by comparing the XIC chromatograms using the high and low ionic strength mobile phases shown in Figure 2 and Figure 3, respectively. These chromatograms along with the data in Table 4, show that reducing the ionic strength of the mobile phase increases retention for all DAR species. Also, MS signal intensity significantly increased with the lower ionic strength mobile phase (data not shown). The difference in retention time between the two mobile phase systems is the least pronounced with the DAR0 peak and this is likely due to the unconjugated antibody (DAR0) being effectively unretained with this column. The largest increases in retention time were for the earlier eluting DAR species (except for DAR0) and the effect decreases with increasing retention. These retention time increases may indicate slight changes in conformation of the protein with changes in ionic strength or the presence of limited ion exchange interaction with the sorbent.

The peak widths for the DAR species with high and low ionic strength mobile phases at pH 6.8 (unadjusted) are listed in Table 5. The low ionic strength mobile phase had a minor impact on peak widths except for the DAR4b species whose width decreased significantly. These results are more consistent with conformational changes in the protein than with slightly increased ion exchange interaction. Table 6 shows the resolution of the different pairs of DAR species. The low ionic strength mobile phase gave slightly increased resolution between DAR2 and DAR4a but slightly lower resolution between DAR4b and DAR6a and DAR6a and DAR8 compared to the high ionic strength mobile phase. The moderate changes in resolution with ionic strength are consistent with the observations for peak width and retention times. Reduced ionic strength increased the retention of earlier eluting DAR species more than later eluting ones while peak widths generally showed a slight decrease.

The combined effect of low ionic strength and low pH (pH 5.2) mobile phase is illustrated in the XIC in Figure 4. As also shown in Table 4, the lower pH gave reduced retention for all DAR species except DAR8. Reducing the pH while holding the ionic strength low essentially negated the retention increase observed with lower ionic strength at pH 6.8. As shown in Table 5, an increase in peak widths was also observed at lower pH. The general changes in chromatographic behavior with the change in pH likely indicate a change in configuration of the protein at the lower pH. The low pH may also affect selectivity since 4 peaks are observed for the DAR4 species and 2 peaks for the DAR6. These additional peaks may be other positional isomers or different conformations of the protein. Additional work is required to elucidate the identity of these additional peaks. As shown in Table 6, the resolution of DAR species with lower pH mobile phase is slightly lower at reduced pH, in line with changes in retention times and peak widths.

The effect of column temperature was investigated using a prototype column which is too hydrophobic to elute the DAR8 species; however, the results are representative of the general trend observed for temperature with other reverse phase sorbents. Figures 5 and 6 show the 280 nm UV chromatograms of Polivy on the prototype column using the high ionic strength mobile phase at 30°C and 40°C, respectively. The range of temperatures used was narrow to ensure the ADC remains in its native, intact state. As shown in Table 7, the retention times of all DAR species were reduced at elevated temperature. Also, additional peaks are resolved for the DAR4 and DAR6 species at 40°C. An XIC chromatogram at 40°C is shown in Figure 7. The orange and green traces for DAR4 and DAR6, respectively, clearly show that the additional peaks are DAR4 and DAR6 species indicating that temperature may be a useful parameter for exploring configuration changes and positional isomers of different DAR species.

Figure 2 . XIC with High Ionic Strength Mobile Phase

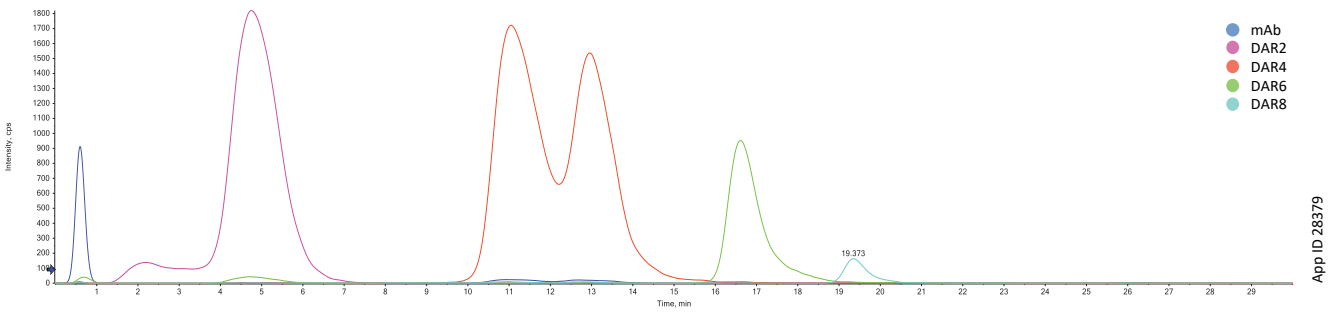


Figure 3. XIC with Low Ionic Strength Mobile Phase

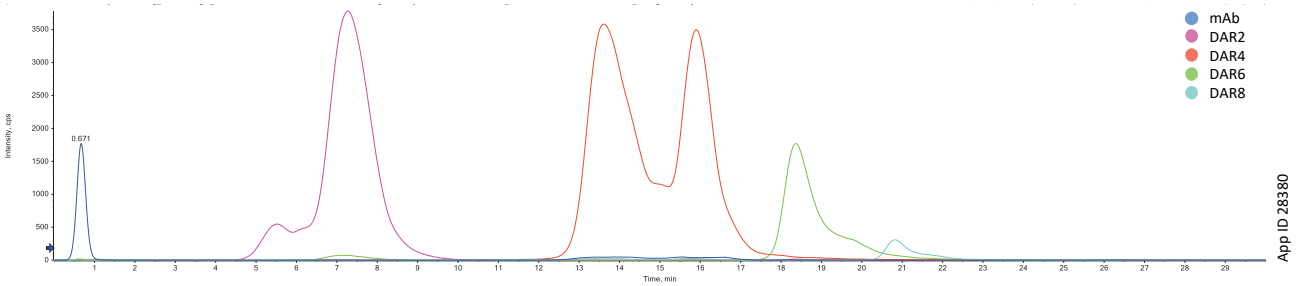


Figure 4. XIC Using Low pH and Low Ionic Strength Mobile Phase

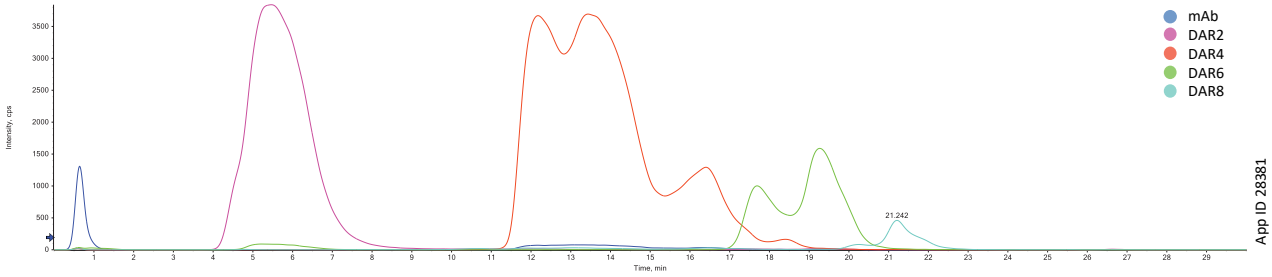


Figure 5. UV Chromatogram of Polivy® at 30°C with High Ionic Strength Mobile Phase

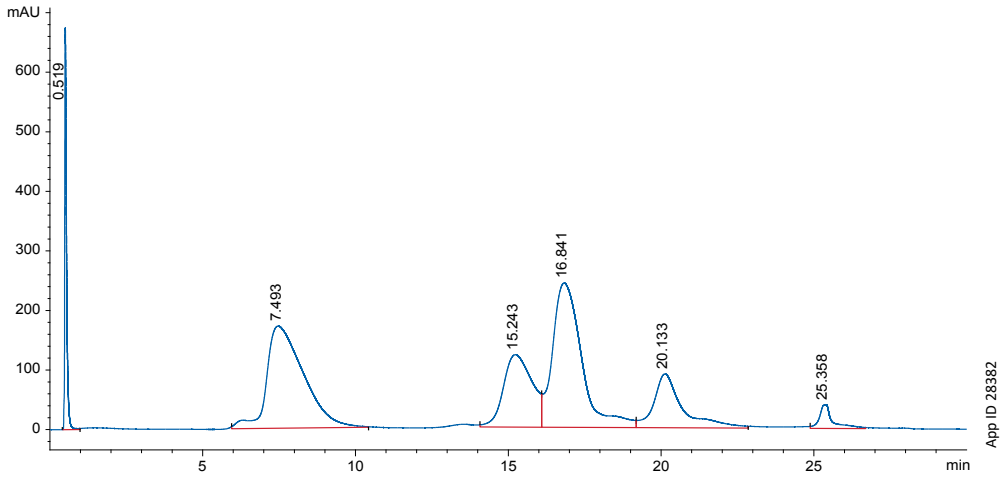


Figure 6. UV Chromatogram of Polivy at 40 °C with High Ionic Strength Mobile Phase

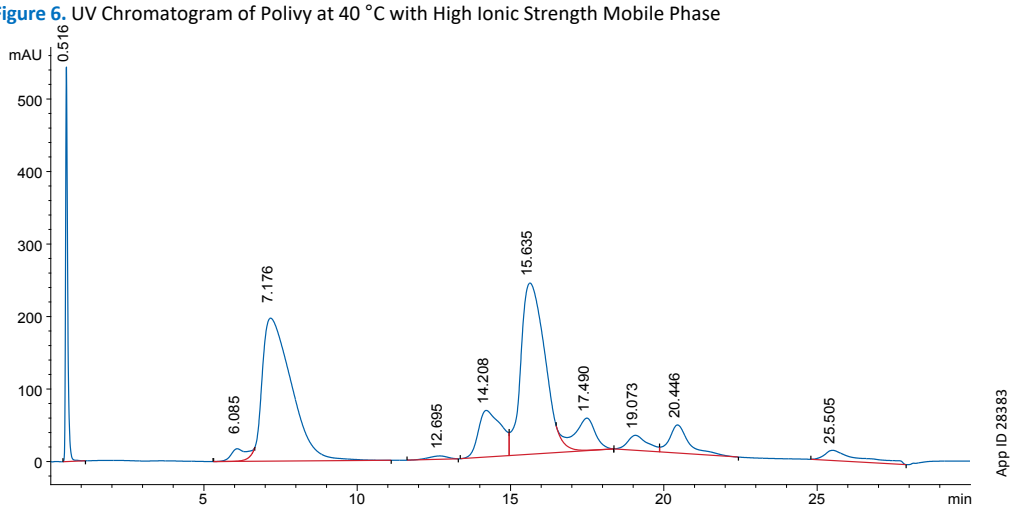


Figure 7. XIC of Polivy at 40 °C with High Ionic Strength Mobile Phase

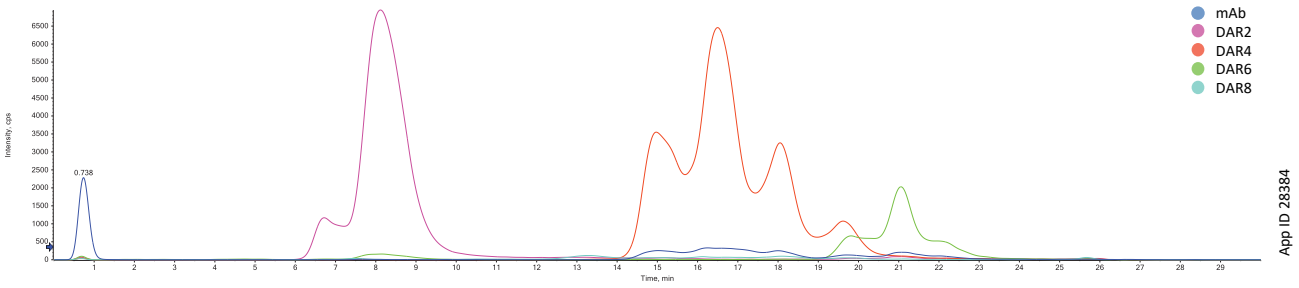


Table 3 . Table for XIC G0F/G0F +26

Expected Mass	Mass Window	Species Name	Trace Color
5689.05	1.15	mAb G0F-2	Blue
5790.41	1.15	DAR2 G0F-2	Pink
5891.77	1.15	DAR4 G0F-2	Orange
5993.13	1.15	DAR6 G0F-2	Green
6094.48	1.15	DAR8 G0F-2	Light Blue
Gaussian Smoothing Width: 10 Points			

Table 4. Retention Times (RT) of DAR species

Mobile Phase	DAR0 RT (min)	DAR2 RT (min)	DAR4a RT (min)	DAR4b RT (min)	DAR4c RT (min)	DAR6a RT (min)	DAR6b RT (min)	DAR8 RT (min)
High Ionic Strength	0.60	4.8	11.2	13.0	N/A	16.6	N/A	19.4
Low Ionic Strength	0.67	7.3	13.7	15.9	N/A	18.4	N/A	20.8
Low Ionic Strength/ Low pH MP	0.64	5.6	12.3	13.6	16.4	17.8	19.3	21.2

Table 5. Peak Width at Half Height (PW 50%) of DAR species

Mobile Phase	DAR0 PW 50% (min)	DAR2 PW 50% (min)	DAR4a PW 50% (min)	DAR4b PW 50% (min)	DAR4c PW 50% (min)	DAR6a PW 50% (min)	DAR6b PW 50% (min)	DAR8 PW 50% (min)
High Ionic Strength	0.25	1.2	1.3	1.2	N/A	0.80	N/A	0.58
Low Ionic Strength	0.25	1.1	1.2	0.73	N/A	0.78	N/A	0.61
Low Ionic Strength/ Low pH MP	0.26	1.7	1.1	1.8	1.1	1.2	1.1	0.68

Table 6. Resolution (Rs) of DAR Species

Mobile Phase	DAR2/DAR4a Rs	DAR4b/DAR6a Rs	DAR6/DAR8 Rs
High Ionic Strength	3.0	2.2	2.3
Low Ionic Strength	3.3	1.9	2.1
Low Ionic Strength/ Low pH MP	2.9	1.7	2.2

Table 7. Retention Times of Polivy® with Biozen Native RP-5 Column at Different Temperatures

Column Temperature (°C)	DAR 0 RT (min)	DAR2 RT (min)	DAR4a RT (min)	DAR4b RT (min)	DAR4c RT (min)	DAR6a RT (min)	DAR6b RT (min)
30	0.52	7.5	15.2	16.8	N/A	20.1	N/A
40	0.53	7.2	14.2	15.7	17.5	19.1	20.5

Conclusion

Factors affecting the chromatographic performance of a new HPLC column, Biozen™ Native RP-5, for the rapid characterization of native, intact cysteine linked ADCs with online mass spectrometry and UV detection were demonstrated. Reduced ionic strength generally increased retention while slightly reducing peak widths of the DAR species. Reduced pH had mixed effects generally showing reduced retention but increased peak widths. Reduced mobile pH also allowed resolution of additional peaks for several DAR species, possibly additional positional isomers or different conformations of the protein. Increasing column temperature gave reduced retention of all DAR species but allowed resolution of additional peaks of several DAR species. Again, the additional peaks may be positional isomers or different configurations of the protein. Ultimately, the recommended chromatographic conditions will correspond with the analytical objectives of the analyst using this column and whether they are interested in resolving these configurational or positional isomers. This new approach to ADC analysis allows coupling native, intact reversed phase analysis online with high resolution mass spectrometry and greatly reduces the time required for more comprehensive characterization of ADCs.

Biozen Ordering Information

Biozen Columns (mm)		
	50 x 2.1	50 x 4.6
Biozen Native-RP-5	00B-4800-AN	00B-4800-E0
Biozen Native-RP-1	00B-4799-AN	00B-4799-E0

Need a different column size or sample preparation format?

No problem! We have a majority of our available dimensions up on www.phenomenex.com, but if you can't find what you need right away, our super helpful Technical Specialists can guide you to the solution via our online chat portal www.phenomenex.com/Chat.

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