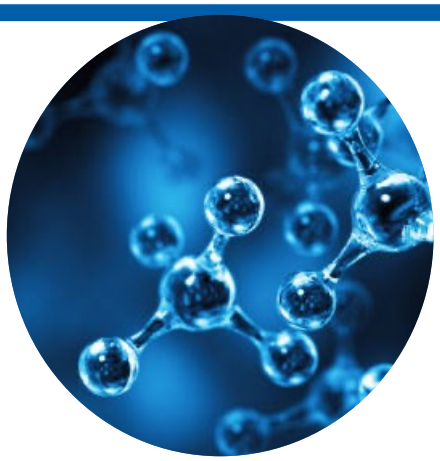


TN-1370

Utilizing Biozen™ Native RP-5 Column for Rapid RPLC-HRMS Separation and Identification of Native, Intact Antibody Drug Conjugate Species

Carl Sanchez and Juan M. Ferefetti
Phenomenex Inc., 411 Madrid Ave., Torrance, CA 90501, USA

Introduction

Antibody-Drug Conjugates (ADCs) are a proven, highly effective drug modality for cancer treatments, maximizing drug efficacy while minimizing systemic exposure and associated side effects. Padcev® and Polivy®, to name a few, are approved ADCs used to treat urothelial cancer and large b-cell lymphoma, respectively.

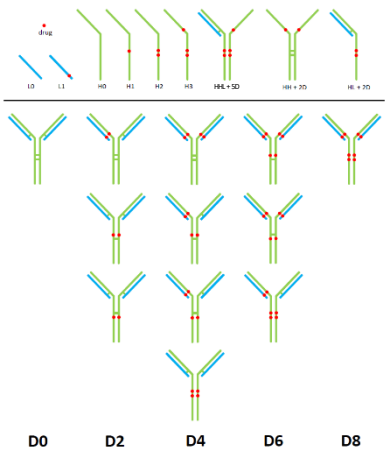
Determining the average drug-to-antibody ratio (DAR) is a key quality attribute for commercial ADCs. Conventional techniques for DAR analysis, such as Hydrophobic Interaction Chromatography (HIC), are commonly used to fractionate the individual DAR species prior to offline MS characterization. Also, this approach is time consuming and impacts reliability and reproducibility due to the need for buffer exchanges prior to MS analysis. Comprehensive characterization of the individual DAR species is a critical part of the ADC development process. With more than 100 ADCs currently in different stages of clinical development, efficient and robust characterization methods are critical to making timely drug development decisions.

This technical note demonstrates the performance of Biozen Native RP-5, a non-porous, hydrophilic reversed phase sorbent specifically designed for the chromatographic separation and online MS identification of native, intact ADCs. Chromatographic separation and MS identification of all DAR species, including positional isomers, of three commercially available cysteine linked ADCs are demonstrated.

Sample Preparation

ADCs were 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 as it 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. Three commercial cysteine linked ADC drugs were tested: 1) Padcev (enfortumab vedotin from Astellas Pharma Inc.), 2) Polivy (polartuzumab vedotin-piiq from Genentech Inc.), 3) Tivdak® (tisotumab vedotin-tftv, from Seagen Inc.).

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: A: 50 mM Ammonium Acetate in Water
B: Isopropyl alcohol/50 mM Ammonium acetate in Water (50/50, v/v)

Gradient:

Time (min)	% B
0	0
2	0
5	15
20	50
24	50
26	100
27	100
27.1	0
30	0

Flow Rate: 0.2 mL/min
Injection Volume: 20 µg ADC on-column
Temperature: 25 °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 (10 mm 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

Results and Discussion

An illustration of the general structure, nomenclature and arrangement of drug conjugation sites of cysteine linked ADCs are shown in **Figure 1**. The three commercial cysteine linked ADCs used in this study were Padcev®, Polivy® and Tivdak®. The primary difference between these ADCs is the antibody. For Padcev, the antibody is enfortumab while, for Polivy, the antibody is polatuzumab and for Tivdak the antibody is tisotumab. The antibody provides the specificity of drug delivery by targeting an antigen specific to the cancer cells. The same drug molecule, monomethyl auristatin E, a mitotic inhibitor, is conjugated to the antibody with the same enzyme cleavable linker in all three ADCs.

An extracted ion chromatogram (XIC) for the ADC Padcev is shown in **Figure 2a**. The XIC mass window is limited to the native region of the mass spectrum for this compound (m/z 5000-8000) to reduce noise and enhance visualization of the different DAR species. This view of the data is superior to the total ion current (TIC) and base peak current (BPC) chromatograms due to the selected mass window (see Figures 2B and 2C) since all DAR species are clearly evident. Additionally, the XIC chromatogram closely matches the UV chromatogram (recorded at 280 nm) shown in **Figure 2d**. The slight offset in retention times between the UV and MS signals are due to the detectors being connected in series with the UV detector first in line. The calculated average DAR value for the MS and UV chromatograms was 3.6 for both, in good agreement with the literature value for the average DAR of 3.8 for the Padcev ADC.[1]

The full-range raw HRMS spectrum for the DAR0 species of Padcev is shown in **Figure 3a**. As seen in the spectrum, the majority of the signal is in the native region of the spectrum, m/z 5000-8000, corresponding to charge states of +19 to +28. There is no evidence of adducts, as demonstrated in **Figure 3b**. The reconstructed neutral mass spectrum of the DAR0 species calculated using the Bio Tool Kit software and data from the native MS region is shown in **Figure 3c**. As seen in the figure, the primary species in the spectrum corresponds to the G0F/G0F glycoform with both C-terminal lysines clipped (-2K) giving an average mass of 146669.3 Da and a mass error of 35 ppm.

The full range raw HRMS spectrum for the DAR2 species of Padcev is shown in **Figure 4a**. In the spectrum of these species, peaks corresponding to in-source dissociation of the light chain with one conjugated drug molecule are prominent in the m/z 1700-3100 range. Again, a significant portion of the overall signal is in the native region of the spectrum, m/z 5000-8000, as seen in **Figure 4b**. The reconstructed neutral mass spectrum for the DAR2 species calculated as described previously is shown in **Figure 4c**. As seen in the figure, the primary species in the spectrum corresponds to the G0F/G0F glycoform with both C-terminal lysines clipped (-2K) and two conjugated drug molecules giving an average mass of 149303.6 Da and a mass error of 28 ppm.

The DAR4 species of all cysteine linked ADCs studied here elute as two chromatographically resolved peaks. The native region of the raw spectrum for the first eluting DAR4 species of Padcev (DAR4A) is shown in **Figure 5a**. The reconstructed neutral mass spectrum for this species using data from the native MS region is shown in **Figure 5b**. The native region of the spectrum for the second eluting DAR4 positional isomer of Padcev, DAR4B, is shown in **Figure 5c**. The reconstructed neutral mass spectrum for this DAR4B isomer using data from the native MS region is shown in **Figure 5d**. The identical raw mass spectra for these two chromatographically resolved peaks provide strong evidence that they are different positional isomers of the DAR4 species. Chromatographic resolution of these species suggests two pairs of isomers with different hydrophobicity. Further work is underway to identify the species eluting at the two different retention times. The general approach described above was applied to the remaining peaks and a full summary of results is shown in **Table 3**.

Similar analyses of the MS data for the other two cysteine linked ADCs (Polivy, Tivdak) were performed and XIC chromatograms are shown in **Figures 6** and **7**. Interestingly, the chromatographic profile is similar for all commercial ADCs tested. A summary of masses and mass errors for these ADCs is shown in **Tables 4** and **5**. All DAR species in all ADCs were positively identified with MS mass errors <50 ppm.

Figure 2a. XIC Chromatogram of Padcev ADC Native MS Region m/z 5000-8000

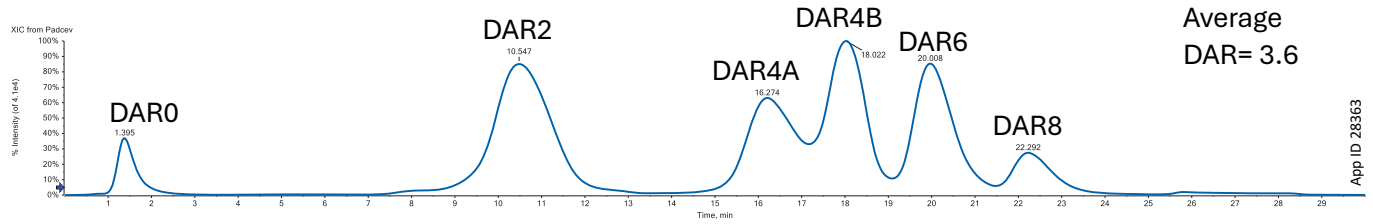


Figure 2b. TIC Chromatogram of Padcev ADC (full acquisition range, m/z 1000-8000)

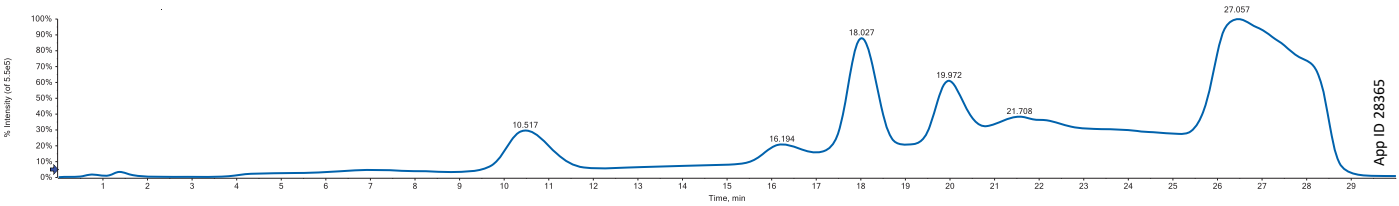


Figure 2c. BP Chromatogram of Padcev ADC (full acquisition range, m/z 1000-8000)

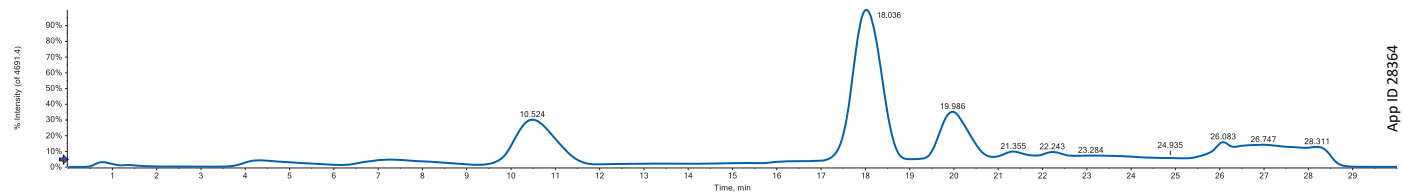


Figure 2 d . UV Chromatogram of Padcev® ADC (280 nm)

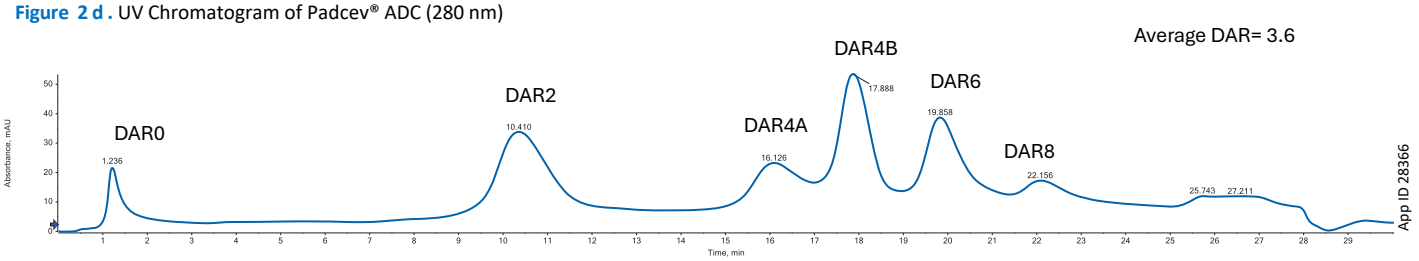


Figure 3 a . Full Raw HRMS Spectrum of Padcev DAR0 m/z 1000-8000

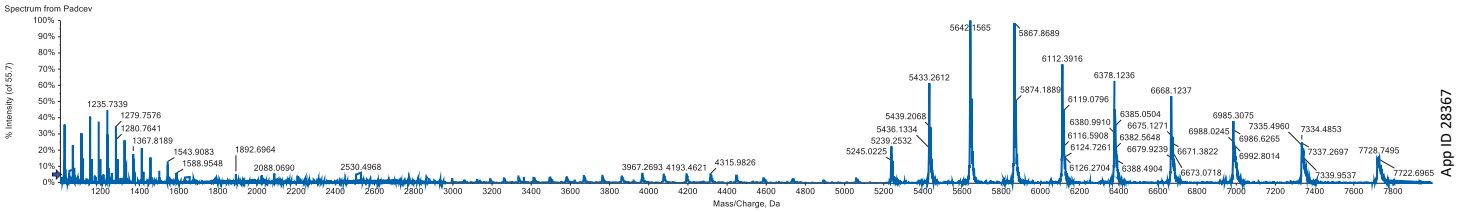


Figure 3 b . Native Region of Raw HRMS Spectrum of Padcev DAR0 m/z 5000-8000

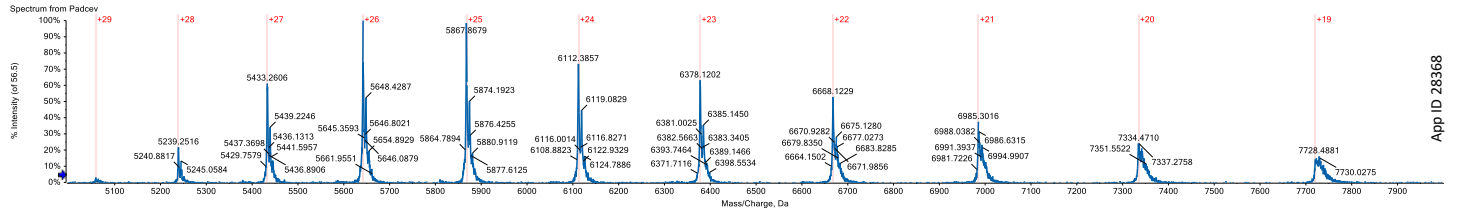


Figure 3 c . Reconstructed Neutral Mass Spectrum of Padcev DAR0 – G0F/G0F, -2K, mass error = 35ppm

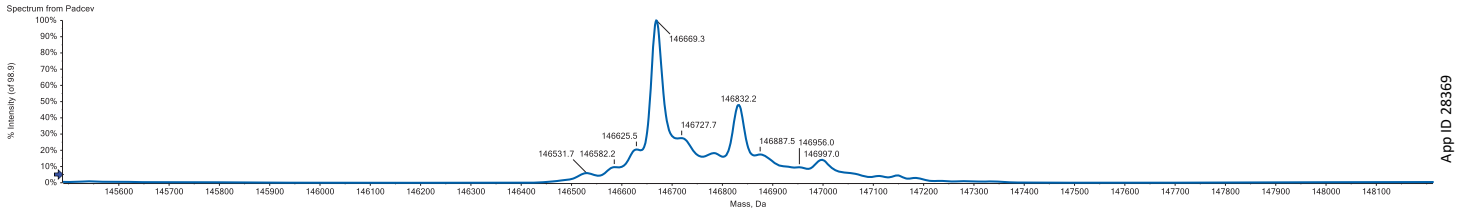


Figure 4a. Full Raw HRMS Spectrum of Padcev DAR2 m/z 1000-8000

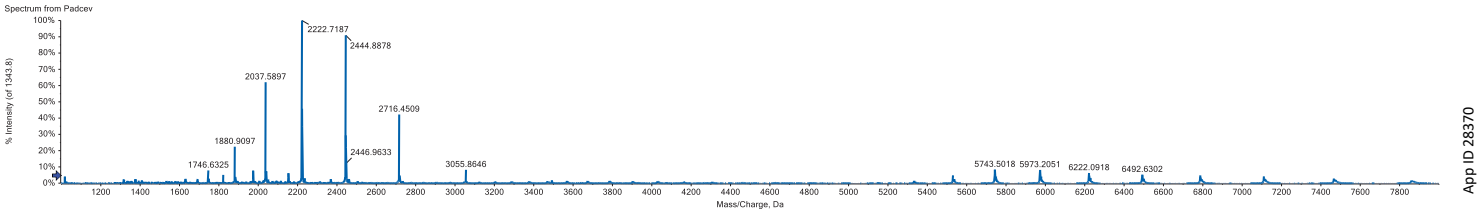


Figure 4b. Native Region of Raw HRMS Spectrum of Padcev DAR2 m/z 5000-8000

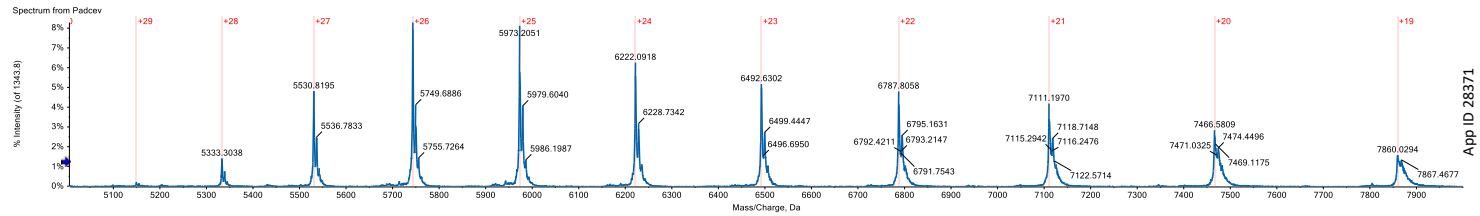


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Figure 4 c . Reconstructed Neutral Mass Spectrum of Padcev® DAR2 – G0F/G0F, -2K, mass error = 28ppm

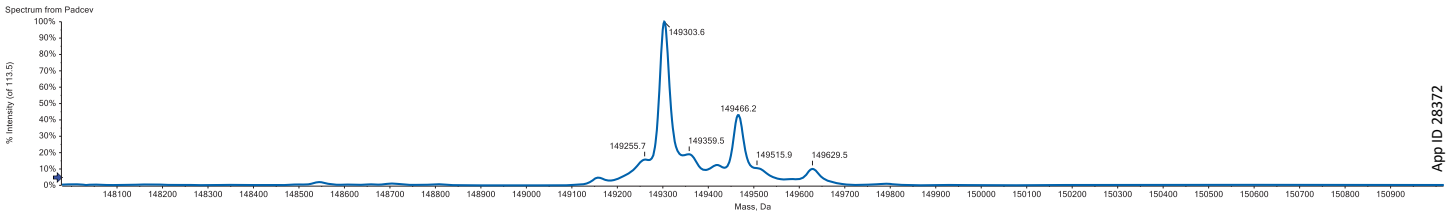

Figure 5a. Native Region of Raw HRMS Spectrum of Padcev DAR4 isomer 1 (DAR4A) m/z 5000-8000

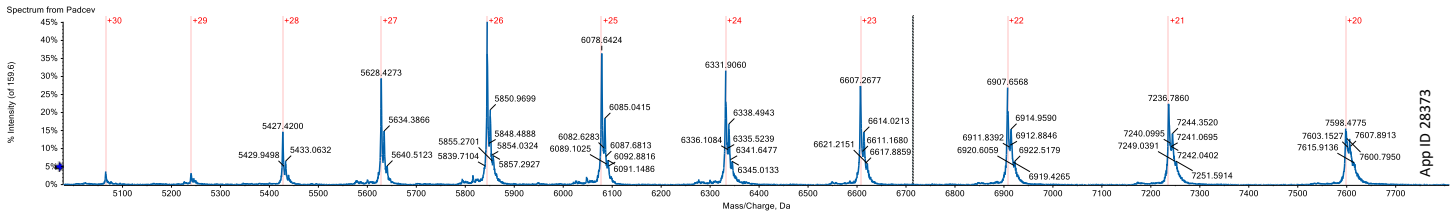

Figure 5 b . Reconstructed Neutral Mass Spectrum of Padcev DAR4 isomer 1 (DAR4A) – G0F/G0F, -2K, mass error = 27ppm

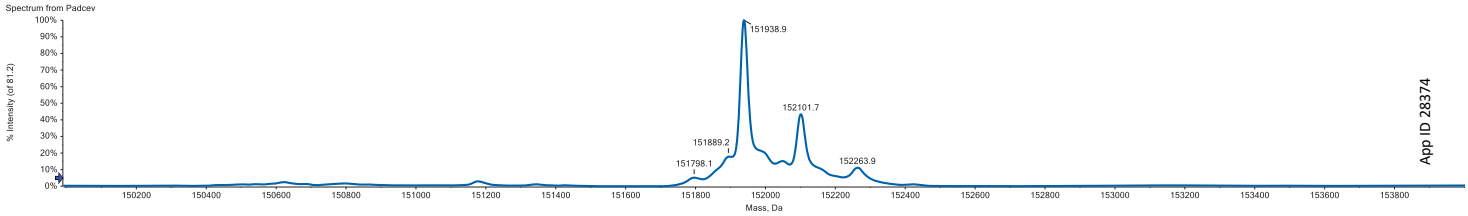

Figure 5 c . Native Region of Raw HRMS Spectrum of Padcev DAR4 positional isomer 2 (DAR4B) m/z 5000-8000

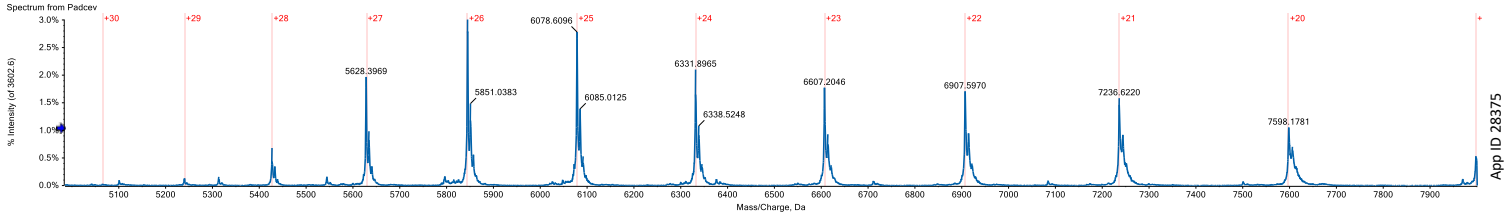

Figure 5d. Reconstructed Neutral Mass Spectrum of Padcev DAR4 positional isomer 2 (DAR4B) – G0F/G0F, -2K, mass error = 26ppm

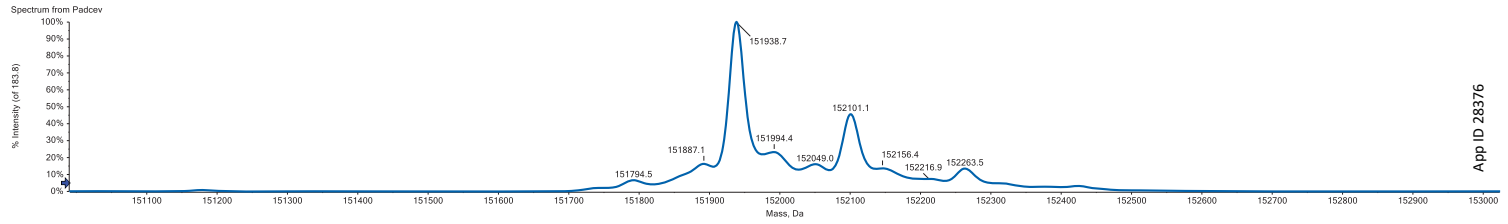

Figure 6. XIC Chromatogram of Polivy® using Native MS Region m/z 5000-8000

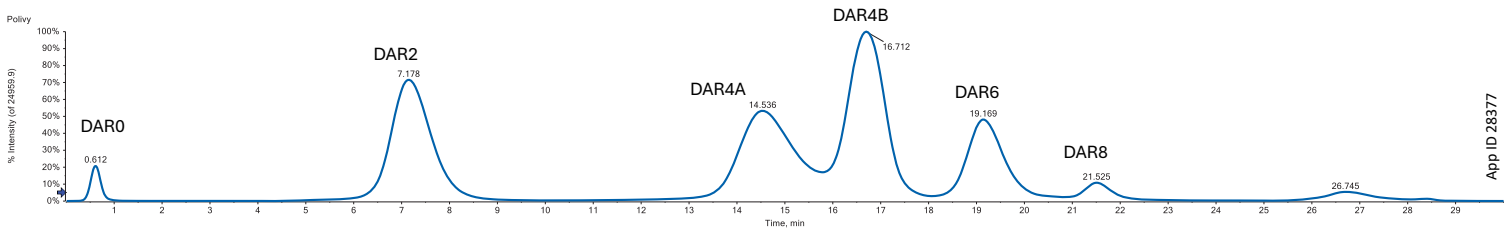

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Figure 7. XIC Chromatogram of Tivdak® using Native MS Region m/z 5000-8000

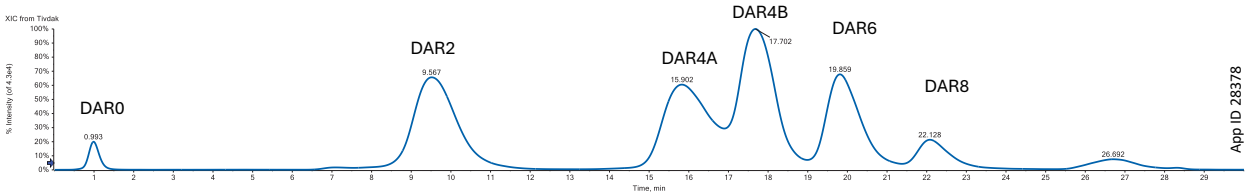

Table 3. Summary of Masses and Mass Errors for Padcev® DAR Species.

DAR Species	Expected Mass, Da	Experimental Mass, Da	Mass Error, ppm
DAR0	146664.16	146669.3	35
DAR2	149299.47	149303.6	28
DAR4 isomer1 (DAR4A)	151934.79	151938.9	27
DAR4 isomer2 (DAR4B)	151934.79	151938.7	26
DAR6	154570.10	154574.1	26
DAR8	157205.42	157210.3	31

Table 4. Summary of Masses and Mass Errors for Polivy® DAR Species

DAR Species	Expected Mass, Da	Experimental Mass, Da	Mass Error, ppm
DAR0	147889.3	147895.2	40
DAR2	150524.7	150529.4	32
DAR4 isomer 1 (DAR4A)	153160.0	153165.7	37
DAR4 isomer 2 (DAR4B)	153160.0	153165.3	35
DAR6	155795.3	155799.6	28
DAR8	158430.6	158435.8	33

Table 5. Summary of Masses and Mass Errors for Tivdak DAR Species

DAR Species	Expected Mass, Da	Experimental Mass, Da	Mass Error, ppm
DAR0	147416.83	147424.0	49
DAR2	150052.15	150058.6	43
DAR4 isomer1 (DAR4A)	152687.46	152693.6	40
DAR4 isomer2 (DAR4B)	152687.46	152693.5	40
DAR6	155322.78	155329.3	42
DAR8	157958.10	157964.4	40

Conclusion

The Biozen™ Native RP-5 column provides robust chromatographic separation of all DAR species (DAR0-DAR8), including positional isomers, in their native, intact state with the cysteine linked ADCs tested. The high-resolution MS spectra demonstrate elution of all major DAR species in their native, intact state with good sensitivity. The HRMS spectra provide positive identification (<50 ppm mass error) of all major DAR species in each of the ADCs that were tested.

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

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[1] Tong, J.T.W.; Harris, P.W.R.; Brimble, M.A.; Kavianinia, I. An Insight into FDA Approved Antibody-Drug Conjugates for Cancer Therapy. Molecules 2021, 26, 5847. https://doi.org/10.3390/ molecules26195847

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t: +61 (0)2-9428-6444
auinfo@phenomenex.com

Austria
t: +43 (0)1-319-1301
anfrage@phenomenex.com

Belgium
t: +32 (0)2 503 4015 (French)
t: +32 (0)2 511 8666 (Dutch)
beinfo@phenomenex.com

Canada
t: +1 (800) 543-3681
info@phenomenex.com

China
t: +86 400-606-8099
cninfo@phenomenex.com

Czech Republic
t: +420 272 017 077
cz-info@phenomenex.com

Denmark
t: +45 4824 8048
nordicinfo@phenomenex.com

Finland
t: +358 (0)9 4789 0063
nordicinfo@phenomenex.com

France
t: +33 (0)1 30 09 21 10
franceinfo@phenomenex.com

Germany
t: +49 (0)6021-58830-0
anfrage@phenomenex.com

Hong Kong
t: +852 6012 8162
hkinfo@phenomenex.com

India
t: +91 (0)40-3012 2400
indiainfo@phenomenex.com

Indonesia
t: +62 21 3952 5747
indoinfo@phenomenex.com

Ireland
t: +353 (0)1 247 5405
eireinfo@phenomenex.com

Italy
t: +39 051 6327511
italiainfo@phenomenex.com

Japan
t: +81 (0) 120-149-262
jpinfo@phenomenex.com

Luxembourg
t: +31 (0)30-2418700
nlinfo@phenomenex.com

Mexico
t: 01-800-844-5226
tecnicomx@phenomenex.com

The Netherlands
t: +31 (0)30-2418700
nlinfo@phenomenex.com

New Zealand
t: +64 (0)9-4780951
nzinfo@phenomenex.com

Norway
t: +47 810 02 005
nordicinfo@phenomenex.com

Poland
t: +48 22 51 02 180
pl-info@phenomenex.com

Portugal
t: +351 221 450 488
ptinfo@phenomenex.com

Singapore
t: 800-852-3944
sginfo@phenomenex.com

Slovakia
t: +420 272 017 077
sk-info@phenomenex.com

Spain
t: +34 91-413-8613
espinfo@phenomenex.com

Sweden
t: +46 (0)8 611 6950
nordicinfo@phenomenex.com

Switzerland
t: +41 (0)61 692 20 20
swissinfo@phenomenex.com

Taiwan
t: +886 (0) 0801-49-1246
twinfo@phenomenex.com

Thailand
t: +66 (0) 2 566 0287
thaiinfo@phenomenex.com

United Kingdom
t: +44 (0)1625-501367
ukinfo@phenomenex.com

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t: +1 (310) 212-0555
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