Biopharma

High-sensitive multi-attribute analysis of ADCs under native conditions by using an online multiple heart-cut 2D-LC-HRAM mass spectrometry system

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Abstract

Purpose: To develop a multi-attribute analysis for cysteine-linked antibody-drug conjugates (ADCs) using a two-dimensional liquid chromatography (2D-LC) system coupled with high-resolution accurate mass (HRAM) mass spectrometry (MS).

Methods: A Thermo Scientific™ Vanquish™ 2D UHPLC system, coupled with a Thermo Scientific™ Orbitrap Exploris™ 240 mass spectrometer with Biopharma option, were utilized to configure the multiple heart-cut 2D-LC-HRAM MS system. HIC-SEC and SCX-SEC methods were developed and optimized for the charge variants, drug-to-antibody ratio (DAR) and drug load distribution (DLD) characterization of ADCs under native conditions.

Results: The configured system successfully characterized multi-attributes, such as charge heterogeneity, DAR, and DLD of polatuzumab vedotin, a cysteine-linked ADC, under native conditions with high sensitivity.

Introduction

ADCs are a rapidly expanding class of biotherapeutics for cancer treatment. Critical quality attributes such as charge heterogeneity, DAR, and drug distribution are essential for ensuring the safety, stability, and efficacy of ADCs. Strong cation exchange (SCX) and hydrophobic interaction chromatography (HIC) are conventional methods employed for charge variant analysis and DAR determination of ADCs[1]. However, these methods frequently utilize MS-incompatible salts, limiting their use for MS characterization. Online 2D-LC addresses this by enabling desalting in the second dimension([2]D) and offering multi-attribute analysis in one method. Here, multiple heart-cut 2D-LC-HRAM MS methods were developed to achieve high sensitivity for the multi-attribute analysis of polatuzumab vedotin under native conditions.

Materials and methods

Sample Preparation

Commercial product polatuzumab vedotin, dissolved in deionized water with a concentration of 5.0 mg/mL, was provided by the customer and used directly in the experiment.

Test Methods

Figure 1 shows the configuration of the 2D-LC-HRAM MS system, Table 1 shows the UHPLC and MS conditions.

Figure 1. Overview of the experimental study design for polatuzumab vedotin multiple-attribute analysis using the online multiple heart-cut 2D UHPLC-HRAM MS system.

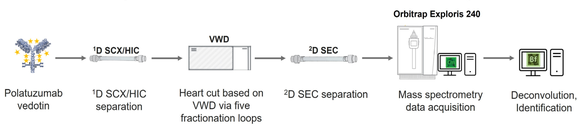


Table 1: UHPLC and MS conditions.

[1]D SCX and HIC chromatographic conditions:

Columns	SCX method: Thermo Scientific™ ProPac™ 3R SCX column, 100×4 mm, 3 µm (P/N 43103-104068)	
	HIC method: Thermo Scientific™ MAbPac™ HIC-Butyl, 100×4.6 mm, 5 µm (P/N 088558)	
Mobile Phase	SCX method	Eluent A: Thermo Scientific™ CX-1 pH gradient buffer A (10-fold diluted) Eluent B: Thermo Scientific™ CX-1 pH gradient buffer B (10-fold diluted)
	HIC method	Eluent A: 1.5 M ammonium sulfate in 50 mM sodium phosphate buffer, pH 7.0 Eluent B: 50 mM sodium phosphate buffer/IPA (80:20, v/v)
Column Temperature	40ºC for SCX method, 30ºC for HIC method	
Autosampler Temperature	4ºC	
Flow Rate	0.3 mL/min for SCX, 0.5 mL/min for HIC	
Autosampler Wash	10% MeOH in water	
Injection Volume	10 µL for method optimization; 20 µL for HRAM MS characterization	
Detector	280 nm, 0.2 Hz	

[2]D SEC chromatographic and MS conditions:

Column	Thermo Scientific™ MabPac™ SEC-1, 300×4 mm, 5 µm (P/N 074696)
Mobile Phase	100 mM ammonium acetate
Flow Rate	0.12 mL/min
Column Temperature	30ºC
Autosampler Temperature	4ºC
Autosampler Wash	10% MeOH in water
Injection Volume	[1]D fraction volume
Detector	280 nm, 0.2 Hz

Parameters	**Value/ settings**	**Parameters**	**Value/ settings**
Spray Voltage: Positive Ion (V)	3800	Microscans	10
Sheath Gas (Arb)	35	Normalized AGC Target	300%
Aux Gas (Arb)	10	RF Lens (%)	200
Ion Transfer Tube Temp (ºC)	250	Maximum Injection Time Mode	custom
Vaporizer Temp (ºC)	175	DataType	Profile
Orbitrap Resolution	60,000	Source Fragmentation	120 eV

Gradient and column switching valve position:

Fraction collection from SCX and HIC						
[1]D Gradient	SCX method:			HIC method:		
	Time(min)	A%	B%	Time(min)	A%	B%
	0	100	0	0	95	5
	2	100	0	2	95	5
	2.1	95	5	25	0	100
	32	50	50	30	0	100
	33	0	100	31	95	5
	35	0	100	40	95	5
	36	100	0			
	41	100	0			
[2]D Gradient	Isocratic elution, 41 minutes					
Column switching valve position	CutValve: 1-2; DivertValve: 1-6; ArrayValves: Triggered by the valve switching time set in the [1]D					
SEC separation and HRAM MS data acquisition						
[1]D Gradient	Isocratic elution with 100% A, 30 minutes					
[1]D flow rate	0.10 mL/min					
[2]D Gradient	Isocratic elution, 40 minutes					
[2]D flow rate	0.12 mL/min					
Column switching valve position	CutValve: 1-6; DivertValve: 0-10 min (1-6); 10-25.5 min (1-2); 25.5-30 min (1-6) ArrayValves: Corresponding to the fractionation loop that was eluted into the [2]D					

Data Analysis

Thermo Scientific™ Chromeleon™ Software Chromatography Data System (CDS) 5.3.2 was used for instrument control, data acquisition, and UHPLC data analysis. Thermo Scientific™ Biopharma Finder ™ Software version 5.3 was used for HRAM MS data deconvolution and analysis.

Results

Multiple heart-cut 2D-LC-MS system setup

Five 250 µL fractionation loops were used in the 2D-LC system to offer flexible [1]D fraction volumes, as shown in Figure 2. To minimize the fraction dispersion in the fractionation loops, the fluidic connections were set up to enable reverse flow directions in the loops for fraction transfer to the [2]D (backflush, shown in Figure 2). Three [1]D SCX peaks (main, acidic, and basic peaks) were utilized to demonstrate the benefit of the backflush fraction transfer; the results are listed in Figure 3.

Figure 2. Fluidic scheme of multiple heart-cut 2D-LC-HRAM MS setup.

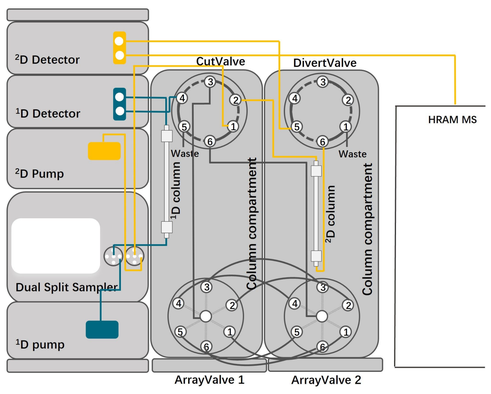
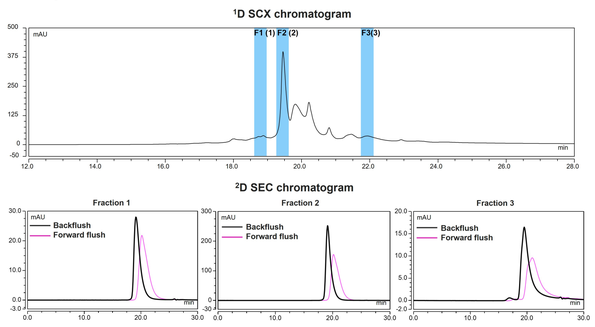


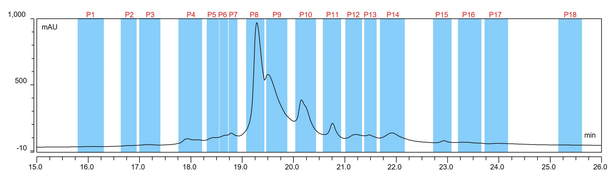
Figure 3. The [1]D SCX and [2]D SEC UV chromatograms for three fractions, with a fraction volume of 105 µL from [1]D, showed significant improvements in peak shape and sensitivity when fractions were transferred using the backflush system compared to forward flush.



SCX-SEC-HRAM MS analysis for polatuzumab vedotin

The SEC was used in the [2]D method to keep the ADCs under the native state. In the [1]D SCX UV chromatogram, 18 peaks were separated and transferred to the [2]D system for intact mass analysis, as shown in Figure 4.

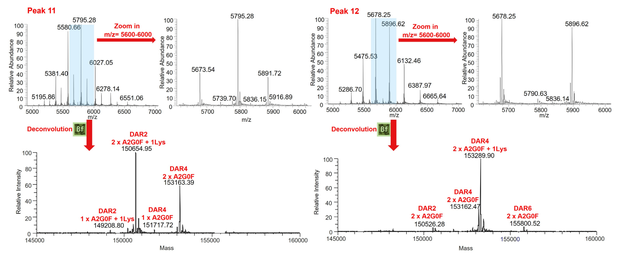
Figure 4. The [1]D SCX-UV chromatogram and the relative abundance of each peak. The relative abundance of these charge variants was calculated using their [1]D peak areas. The fraction volume was determined based on the peak width, ranging from 48 µL (0.16 min width) to 150 µL (0.5 min width).



Peak No.	Relative Abundance (%)	Fraction volume (µL)	Peak No.	Relative Abundance (%)	Fraction volume (µL)
1	0.24	150	10	11.26	120
2	0.47	90	11	5.10	105
3	0.91	120	12	4.41	99
4	4.15	135	13	2.76	69
5	2.45	72	14	6.54	135
6	1.53	48	15	1.80	105
7	2.53	51	16	1.67	135
8	27.90	105	17	1.72	135
9	24.14	120	18	0.43	135

After acquiring the MS data using Chromeleon CDS, the raw data were imported into the BioPharma Finder software for deconvolution and identification. Figure 5 shows the deconvolution results of peaks 11 and 12. The average mass of the main components detected in peaks 11 and 12 showed a mass shift of 128 Da, indicative of a lysine (Lys) truncation. This Lys truncation modification was also identified for the subsequent peaks 13, 15, 16,17, and 18. And a D_n + 1 Lys and D_{n+2} + 0 Lys pattern was found in these peaks, indicating a drug payload and Lys truncation induced charge heterogeneity.

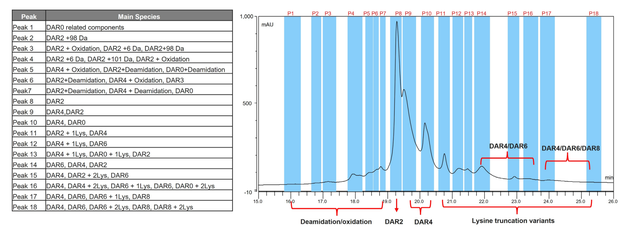
Figure 5. The full mass spectra (top) and deconvoluted spectra (bottom) of peak 11 and peak 12. The expanded view on the right of each spectra displays the details of the blue shaded area. The major payloads/glycoforms/PTMs were labelled in the deconvoluted spectra.



Besides the payload conjugation and Lys truncation, other modifications such as deamidation and oxidation can also affect the charge heterogeneity. The summary of the identified principal species for each [1]D SCX peak is listed in Figure 5. The DAR0, DAR2, DAR4, and DAR8 species were eluted sequentially, which suggests that the payload conjugation alters the surface charge of the mAbs, thereby increasing their retention on the SCX column. Additionally, deamidation and oxidation resulted in the formation of acidic variants, whereas Lys truncation leads to the formation of basic variants.

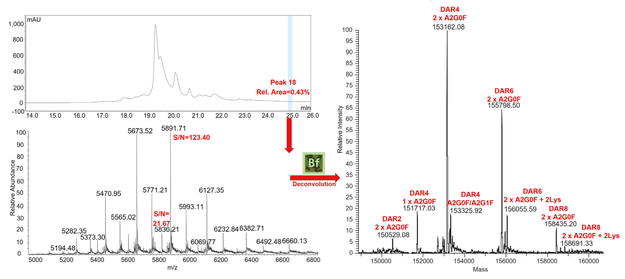
Figure 6: The summary of the main species for all charge variant peaks.

Peak	Main Species
Peak 1	DAR0 related components
Peak 2	DAR2 +98 Da
Peak 3	DAR2 + Oxidation, DAR2 +6 Da, DAR2+98 Da
Peak 4	DAR2 +6 Da, DAR2 +101 Da, DAR2 + Oxidation
Peak 5	DAR4 + Oxidation, DAR2+Deamidation, DAR0+Deamidation
Peak 6	DAR2+Deamidation, DAR4 + Oxidation, DAR3
Peak7	DAR2+Deamidation, DAR4 + Deamidation, DAR0
Peak 8	DAR2
Peak 9	DAR4,DAR2
Peak 10	DAR4, DAR0
Peak 11	DAR2 + 1Lys, DAR4
Peak 12	DAR4 + 1Lys, DAR6
Peak 13	DAR4 + 1Lys, DAR0 + 1Lys, DAR2
Peak 14	DAR6, DAR4, DAR2
Peak 15	DAR4, DAR2 + 2Lys, DAR6
Peak 16	DAR4, DAR4 + 2Lys, DAR6 + 1Lys, DAR6, DAR0 + 2Lys
Peak 17	DAR4, DAR6, DAR6 + 1Lys, DAR8
Peak 18	DAR4, DAR6, DAR6 + 2Lys, DAR8, DAR8 + 2Lys



Owing to the high sensitivity of the Orbitrap Exploris 240 MS, it was feasible to identify charge variants even at very low abundance levels. As illustrated in Figure 7, peak 18, which has an abundance of merely 0.43% as detected by the [1]D UV, still exhibits a high signal-to-noise (S/N) ratio in the raw mass spectra.

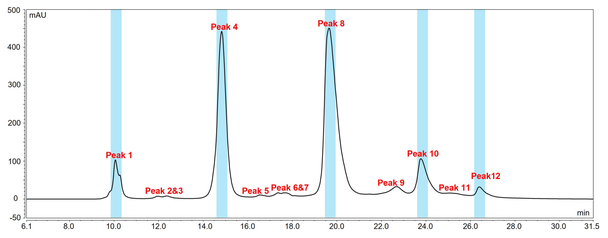
Figure 7. The [1]D SCX-UV chromatogram, full mass spectrum, and deconvoluted spectrum for peak 18. The identification results were labelled on the deconvoluted spectrum.



HIC-SEC-HRAM MS analysis for polatuzumab vedotin

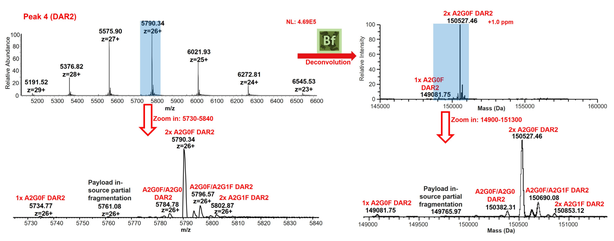
In the HIC-SEC-LC HRAM MS study, a total of 12 peaks were separated in the [1]D HIC chromatogram. The five main peaks separated on the HIC chromatogram were putatively assigned with DAR values from 0 to 8 based on retention time.

Figure 8: The [1]D HIC UV chromatogram showed a total of 12 separated peaks. The highlighted five main peaks were collected in the first cycle and transferred to SEC-HRAM MS for native MS characterization. The average DAR of polatuzumab vedotin was calculated to be 3.48 using the peak area in HIC chromatography, which closely matches its theoretical value (3.4 to 3.5) [2].



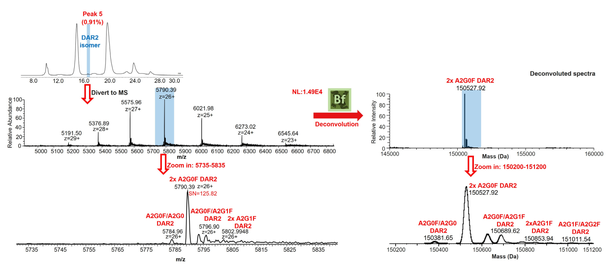
The deconvoluted mass analysis for these five main peaks revealed major species with DAR0 (147891.86 Da), DAR2 (150527.46 Da), DAR4 (153162.74 Da), DAR6 (155798.10 Da), and DAR8 (158433.65 Da), respectively. Different glycoforms of the polatuzumab vedotin were also identified in this study. The identification results in Figure 9 reveal the glycosylation pattern for peak 4.

Figure 9. The full mass spectra (left) and deconvoluted spectra (right) of peak 4. The expanded view below displays the details of the blue shaded area. The predominant glycoform for DAR2 was A2G0F+A2G0F, with a deconvoluted mass of 150527.46 Da, which is within 1 ppm deviation of the theoretical mass, demonstrating the high mass accuracy of the Orbitrap Exploris 240 HRAM MS.



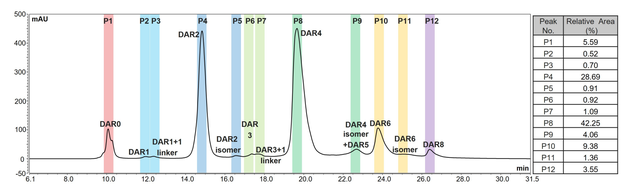
The high sensitivity of the Orbitrap Exploris 240 HRAM MS effectively facilitates the identification of all the minor peaks in the [1]D HIC, as shown in Figure 10.

Figure 10. The [1]D HIC chromatogram (top), raw MS spectra (middle left), and deconvoluted mass spectra (middle right) of peak 5, which has a relative area of only 0.91% in the [1]D HIC chromatogram, were successfully identified using the high-quality mass data. The results indicate that this peak is a DAR2 isomer.



In addition to the expected positional isomers, ADCs with odd-number payloads such as DAR1, DAR3, and DAR5 were also identified. The main species of each HIC peak in [1]D were summarized in Figure 11.

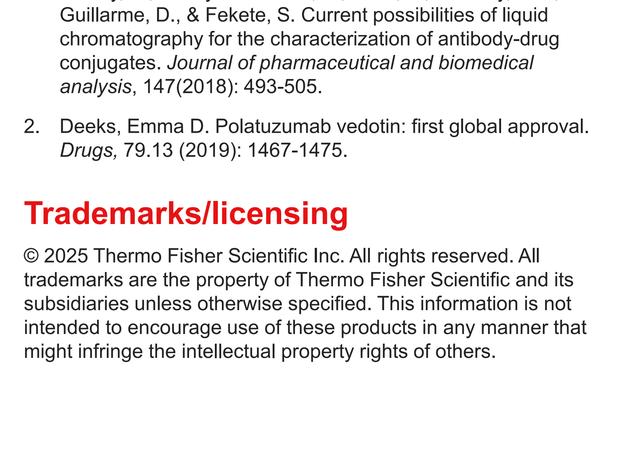
Figure 11. The summary of the main species for all the [1]D HIC peaks, the relative area was calculated based on the [1]D UV peak area.



Peak No.	Relative Area (%)
P1	5.59
P2	0.52
P3	0.70
P4	28.69
P5	0.91
P6	0.92
P7	1.09
P8	42.25
P9	4.06
P10	9.38
P11	1.36
P12	3.55

Notably, ADCs with linkers only, such as DAR1+1 linker (peak 3) and DAR3+1 linker (peak 7), were identified in our study. This indicates that the ADCs either lost one payload during production or storage, or that only one payload was linked to the free thiol after a disulfide bond was reduced. These are not the desired products in the ADC drug production process and can therefore be considered as impurities.

Figure 12: The identification results of peak 7, which was identified as DAR3+1 linker.



Conclusions

In this study, an online multiple heart-cut 2D-LC-HRAM MS system was configured and optimized for the multi-attribute analysis of polatuzumab vedotin. This approach provides detailed and comprehensive insights into the charge heterogeneity analysis, DAR, and DLD analysis of polatuzumab vedotin under native conditions.

- The online multiple heart-cut 2D-LC system enables the direct transfer of SCX and HIC fractions to mass spectrometry analysis by utilizing SEC as a desalting step, thereby overcoming the MS-incompatibility buffer in the LC separation.
- The backflush transfer of fractions was employed to enhance peak shape and sensitivity in the [2]D SEC separation.
- The [2]D SEC-MS analysis enables the characterization of ADCs under native conditions.
- The high sensitivity of the Orbitrap Exploris 240 MS allows for the identification of fractions with low abundance in the [1]D chromatography.

References:

1. Bobály, B., Fleury-Souverain, S., Beck, A., Veuthey, J. L., Guillarme, D., & Fekete, S. Current possibilities of liquid chromatography for the characterization of antibody-drug conjugates. *Journal of pharmaceutical and biomedical analysis*, 147(2018): 493-505.
2. Deeks, Emma D. Polatuzumab vedotin: first global approval. *Drugs,* 79.13 (2019): 1467-1475.

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