A COMBINED WORKFLOW FOR IN-DEPTH CHARACTERIZATION OF CYSTEINE-CONJUGATED ANTIBODY DRUG CONJUGATES

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OVERVIEW

Intact Analysis	• HIC/UV • SEC-LC/MS • 2D-LC/MS (HIC/RPLC)
Subunit Analysis	• RP-LC/MS
Peptide Mapping	• LC/MS^E • LC/DDA

INTRODUCTION

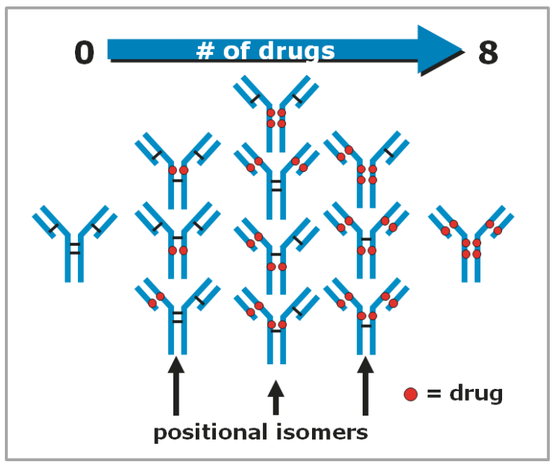


Fig. 1. Positional isoforms of cysteine-conjugated ADCs.

METHODS

- **HIC/UV, SEC-LC/MS, RP-LC/MS**

Instrumentation
LC: Waters ACQUITY H-Class Bio
MS: Waters Xevo G2-S QTof
Columns
Waters ProteinPak Hi Res HIC
ACQUITY UPLC Protein BEH C4 Column, 300Å, 1.7 µm, 2.1 mm X 50 mm
ACQUITY UPLC Protein BEH SEC Column, 200Å, 1.7 µm, 4.6 mm X 150 mm

- **2D LC/MS (HIC/RPLC)**

Instrumentation
LC: Waters ACQUITY H-Class Bio with 2D Technology
MS: Xevo G2 QTof
Columns
Waters Protein Pak Hi Res HIC
ACQUITY UPLC Protein BEH C4, 300Å, 1.7 µm, 2.1 mm X 50 mm

- **Peptide mapping**

Instrumentation
LC: Waters ACQUITY H-Class Bio
MS: Xevo G2-XS QTof
Columns
Waters ACQUITY UPLC CSH C18 Column, 130Å 1.7 µm, 2.1 mm X 100 mm

Intact Analysis

HIC/UV

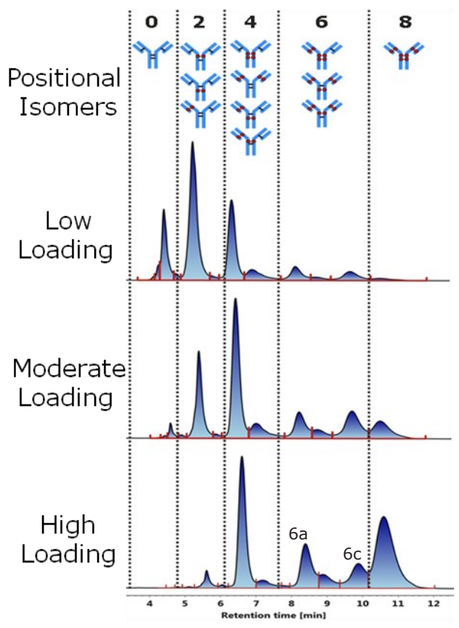


Fig. 2. Cysteine-conjugated ADC analysis using HIC. Drug distribution was determined for three different samples with increasing drug load.

SEC-LC/MS

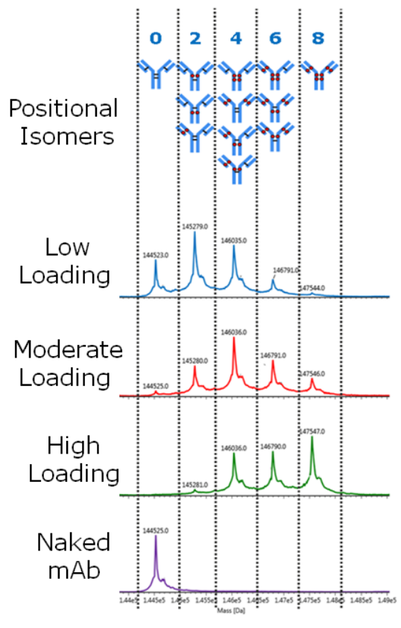


Fig. 3. Deconvoluted intact mass spectra for cysteine-conjugated ADCs from native SEC-LC/MS after deglycosylation.

Drug loading distribution and DAR						
	Low		Mod		High	
	HIC	LC/MS	HIC	LC/MS	HIC	LC/MS
ADC 2	0.81	0.74	0.38	0.41	0.07	0.09
ADC 4	1.14	1.17	1.67	1.57	1.23	1.11
ADC 6	0.75	0.60	1.61	1.45	1.72	1.72
ADC 8	0.12	0.21	0.78	0.97	2.95	3.05
DAR	2.83	2.72	4.44	4.40	5.97	5.97

Table 1. DARs comparison between HIC and native SEC/LC-MS experiments, which shows excellent agreement between the two methods for all three drug loading levels.

2D-LC/MS (HIC/RPLC) - Positional Isomers Determination

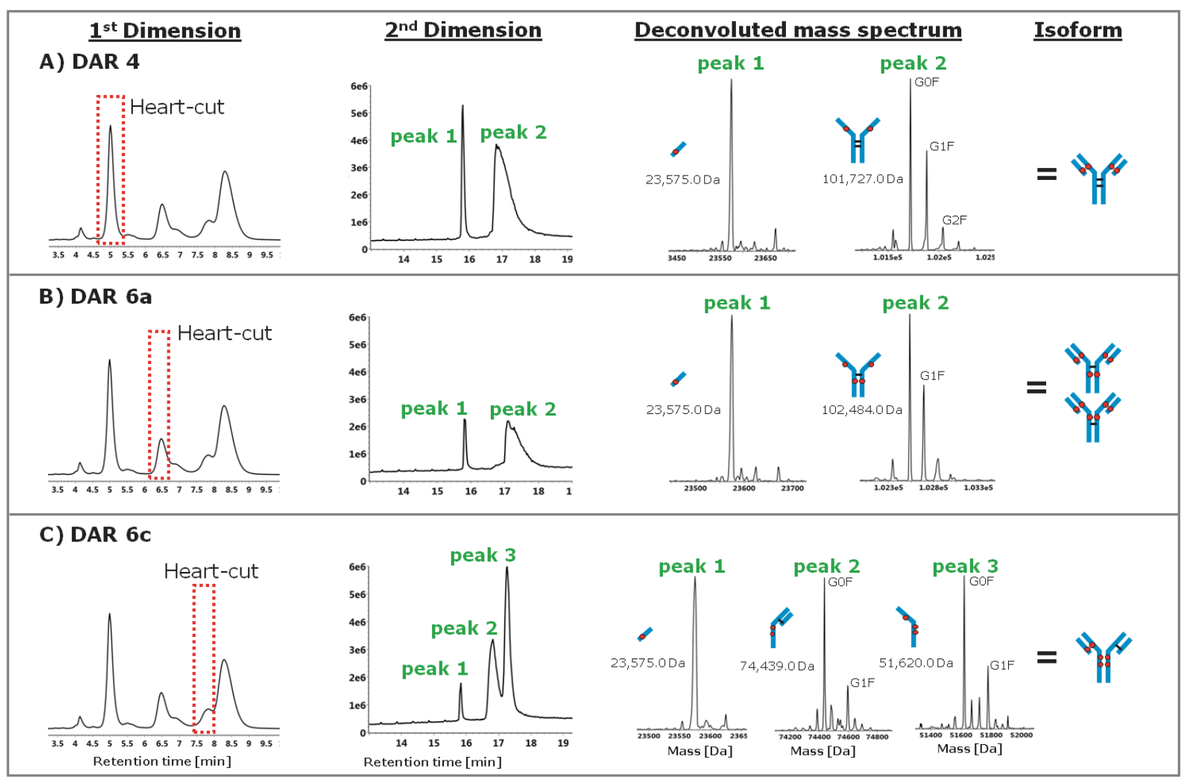
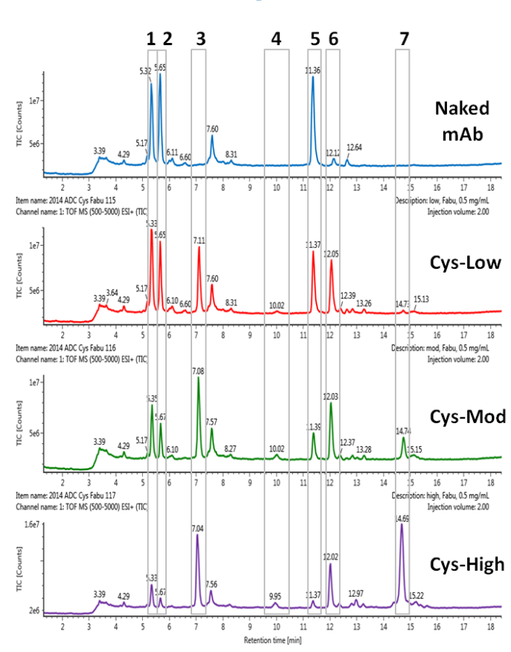


Fig. 4. Heart-cut fractions of A) DAR 4, B) DAR 6a, and C) DAR 6c were performed from individual HIC separations of cysteine-conjugated ADCs. A reversed phase gradient of each cut produced up to 3 peaks representing subunits of the positional isomers. Deconvolution of each peak resulted in unambiguous identification of the isoform for each fraction.

Subunit Analysis

RP-LC/MS



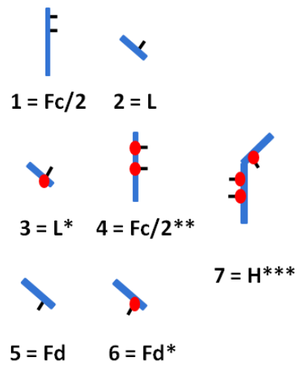


Fig. 5. Total ion chromatogram of reverse phase separated sub units. The subunit structures for peaks 1-7 were shown above. Cys-ADCs samples were treated by FabULOUS and then reduced by DTT.

- Steric hindrance inhibits cleavage, resulting in 7=H*** (+3 drugs) peak.

Peptide Mapping

LC/MS^E

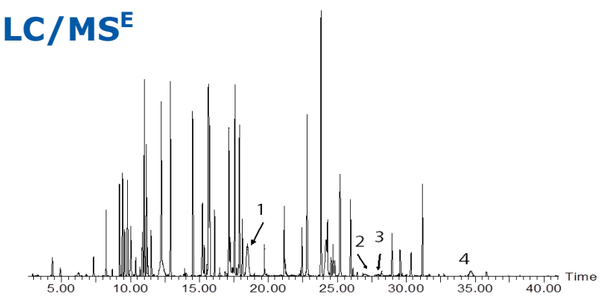


Fig. 6. Tryptic peptide mapping MS^E chromatogram of cys-conjugated ADC (Moderate). Heavy chain T21 peptides with two conjugation sites are shown as an example. Unconjugated T21 (1), T21 with 1 conjugation site (2 and 3), and T21 with 2 conjugation sites (4) are indicated on the chromatogram.

Chain	Pep#	Peptide sequence	Modifier	Drug Occupancy ratio
Light	1:T2	VTITCR	ADC_cys	7.3%
Light	1:T11	SGTASVVCLLNNFYPR	ADC_cys	1.2%
Light	1:T18	VYACEVTHQGLSSPVTK	ADC_cys	2.1%
Light	1:T20	GEC	ADC_cys	100.0%
Heavy	2:T11	AEDTAVYYCAR	ADC_cys	1.9%
Heavy	2:T15	STSGGTAALGCLVK	ADC_cys	1.9%
Heavy	2:T20	SCDK	ADC_cys	100.0%
Heavy	2:T21	THTCPPCPAPEAAGAPSVFLFPPKPK	ADC_cys, CAM	5.9%
Heavy	2:T21	THTCPPCPAPEAAGAPSVFLFPPKPK	ADC_cys, CAM	4.8%
Heavy	2:T21	THTCPPCPAPEAAGAPSVFLFPPKPK	ADC_cys x2	24.6%
Heavy	2:T23	TPEVTCVVVDVSHEDPEVK	ADC_cys	1.5%
Heavy	2:T37	NQVSLTCLVK	ADC_cys	3.5%
Heavy	2:T42	WQQGNVFSCSVMHEALHNHYTQK	ADC_cys	1.4%

Table 2. List of cys-conjugated peptides observed in the moderate loading sample. Drug occupancy ratio = MS intensity of conjugated/(MS intensity of unconjugated +conjugated peptides)

CONCLUSION

- DAR values and drug loading distributions for cysteine-conjugated ADCs are automatically acquired from HIC-LC analysis and from native SEC-LC/MS analysis, and the results show excellent agreement.
- 2D-LC/MS provides unambiguous identification of positional isomers in cysteine-conjugated ADCs.
- LC/MS^E indentifies 13 conjugation sites with drug occupancy ratio calculated.

References:

1. Details on the HIC-UV, SEC-LC/MS and RP-LC/MS analysis: 61st ASMS conference, poster number TP236
2. TP236 Details on 2D LC/MS analysis: 61st ASMS conference, poster number T2265

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