A SENSITITVE MULTIDIMENSIONAL LIQUID CHROMATOGRAPHY METHOD FOR THE CHARACTERIZATION OF FREE DRUG IMPURITIES IN ANTIBODY-DRUG CONJUGATES USING MASS SPECTRAL DETECTION

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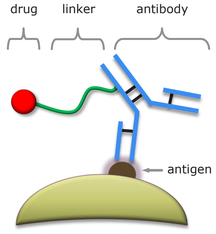
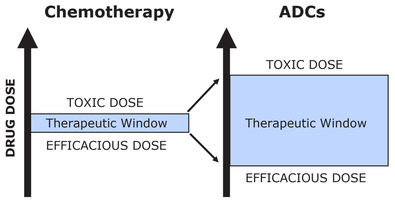
INTRODUCTION



Antibody-drug conjugates represent a growing class of immunoconjugate therapies for the treatment of cancer. Cytotoxic agents based on auristatin and maytansines are too potent to be used in traditional cancer treatment strategies such as chemotherapy. To overcome this challenge, highly potent drugs such as these are covalently attached to a linker molecule and conjugated to a monoclonal antibody (mAb).



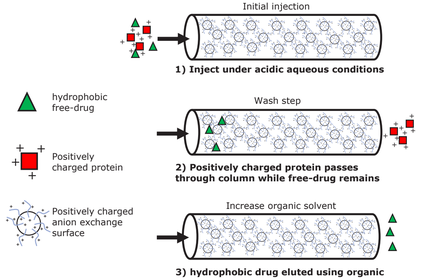
The conjugation of potent drugs to a mAb enables the targeted delivery of toxic payloads to tumor surfaces while minimizing systemic toxicity effects to healthy tissue, thus improving the therapeutic window for such modalities in the treatment of cancer.

Incomplete conjugation processes can result in free or non-conjugated drug, drug-linker, or drug-related impurities that co-exist with the ADC molecules in the samples.

As potentially more potent drug candidates for ADCs are identified, efforts to expand the therapeutic window will require assays with improved sensitivity for the assessment and characterization of residual free drug species to ensure product safety and efficacy.

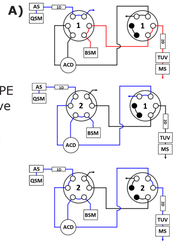
METHODS

The reduction of sample preparation steps through the incorporation of UV-based multidimensional techniques has afforded analysts more efficient methods in the assessment of trace drug species. However optical based methods have limited sensitivity and a narrow working range, a potential dilemma considering the diversity of biological substrates and drug candidates currently under investigation. The current study addresses these challenges through the development of an SPE-RPLC/MS approach that is specific and sensitive.



Extraction of analytes is accomplished through the exploitation of orthogonal physicochemical properties. Conceptually, molecules such as ADCs, will not be adsorbed on the SPE column (1st dimension) because both the ADC molecules and sorbent surface (a hydrophobic polymer chain interspersed with amine groups) bear the same net positive charge under acidic conditions. The free-drug species bearing a net neutral or basic (negative) charge are adsorbed to the sorbent surface and enriched for downstream analysis.

Figure 1. Instrument Configuration Schematic. **(A)** A column manager housing two 6-port 2-position valves was configured as illustrated by the schematic to facilitate transfer of retained drug species between the SPE (1st) and RPLC (2nd) dimensions. Valve position is denoted numerically as position 1 and 2. **(B)** Extracted drug species were transferred using at-column-dilution with both valves in position 2 to refocus eluting drug species at the head of the analytical column.



Abbreviations:
QSM: quaternary solvent manager
AS: auto sampler
TUV: tunable ultraviolet detector
BSM: binary solvent manager
MS: mass spectrometer
ACD: at-column-dilution

B) column manager: event table

Time (min)	Event	Action
Initial	Left Valve	Position 1
Initial	Right Valve	Position 1
12.00	Left Valve	Position 2
12.01	Right Valve	Position 2
17.50	Right Valve	Position 1
17.51	Left Valve	Position 1

Reference Standards Evaluation

To test the broadest applicability of the proposed method for free drug analysis in ADC samples, selected molecules should possesses the key structural features of a typical ADC (e.g., common conjugation methods and linker structures) and preferably exhibit low cytotoxicity for ease of use and handling.

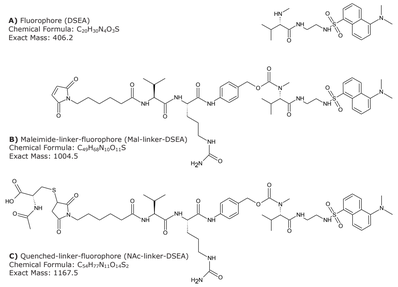


Figure 2. *AFC mimic drug components. Drug components used in the production of a non-toxic AFC to mimic chemistry and linker species of brentuximab vedotin were based on a* ***(A)*** *dansyl sulfonamide ethyl amine (DSEA) moiety attached to* ***(B)*** *a maleimidocaproyl valine-citrulline linker species (Mal-linker-DSEA). Residual reactive mal-linker-DSEA was quenched with N-acetyl-cysteine following the conjugation step, producing a* ***(C)*** *quenched-linker-fluorophore (NAc-linker-DSEA) adduct species.*

Reference standards composed of DSEA, linker-DSEA, and NAc-linker-DSEA were analyzed using an ACQUITY H-Class Bio with 2D technology in a 1DLC configuration to assess the suitability of the reference standards for the proposed method.

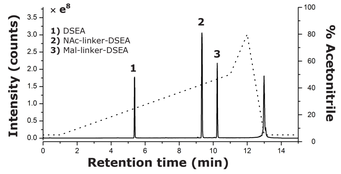
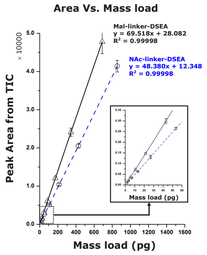


Figure 3. *Reference standard evaluation. Reference standards were separated over a 10 min gradient from 5 % - 50 % (dashed line) with acetonitrile containing 0.1 % FA v/v, as the organic mobile phase using a superficially porous 90 Å C18 2.7 µm column, 2.1x50 mm at 60 ℃. Combined spectrum from SIRs collected using the [M+1H]+1 and [M+2H]+2 charge state for each component using optimized MS settings is shown.*

RESULTS

Using MS to Extend Detection Limits of ADC Free-drug Impurities

Incorporation of MS detection increased the sensitivity of the current assay 125-fold for the mal-linker-DSEA and 250-fold for the NAc-linker-DSEA drug species with a nominal LOQ of 0.3 ng/mL (1.5 pg on-column) compared to UV detection. In addition to improved sensitivity, the ability to efficiently recover trace levels of drug species across a wide dynamic range makes the proposed method ideal for assessment of free drug species in formulation, stability studies, and clinical trials associated with the production of ADCs.



Mal-Linker-DSEA							
N=3			TUV			MS	
Ref. Sample	Conc. (ng/mL)	Mass load (pg)	Area	R.S.D. (%)	R.E. (%)	Area	R.S.D. (%)	R.E. (%)
1	4406.25	22031.25	196.67	2.35	99.80	1195858	18.32	78.08
2	2203.13	11015.65	99.35	2.13	100.55	667774	11.20	87.20
3	1101.56	5507.80	50.05	1.80	100.74	358237	9.78	93.55
4	550.78	2753.90	25.53	1.75	101.66	184526	8.42	96.37
5	275.39	1376.95	12.80	3.56	99.71	94516	7.52	98.71
6	137.70	688.50	6.64	4.04	99.25	47794	6.48	99.80
7	68.85	344.25	3.20	2.14	88.35	24108	4.49	100.62
8	34.42	172.10	1.67	2.73	80.16	12057	3.17	100.53
9	17.21	86.05				6089	4.47	101.29
10	8.61	43.05				2992	4.00	99.08
11	4.30	21.50				1476	2.95	96.89
12	2.15	10.75				728	6.81	93.78
13	1.08	5.40				373	0.61	92.79
14	0.54	2.70				191	2.61	88.67
15	0.27	1.35				103	2.74	85.11

NAc-Linker-DSEA							
N=3			TUV			MS	
Ref. Sample	Conc. (ng/mL)	Mass load (pg)	Area	R.S.D. (%)	R.E. (%)	Area	R.S.D. (%)	R.E. (%)
1	5468.75	27343.75	262.41	1.23	100.17	1081872	18.77	81.78
2	2734.38	13671.90	131.17	2.07	99.31	591076	14.83	89.36
3	1367.19	6835.95	66.52	2.35	99.08	311053	10.56	94.05
4	683.59	3417.95	35.87	3.50	103.49	161949	6.75	97.93
5	341.80	1709.00	18.59	4.21	100.90	82233	4.81	99.44
6	170.90	854.50	10.66	2.06	103.42	41406	3.66	100.13
7	85.45	427.25	5.61	2.62	89.68	20543	2.41	99.32
8	42.72	213.60				10409	2.77	100.60
9	21.36	106.80				5178	2.99	99.96
10	10.68	53.40				2646	2.15	101.93
11	5.34	26.70				1311	6.12	100.54
12	2.67	13.35				625	3.28	94.91
13	1.34	6.70				322	4.18	95.89
14	0.67	3.35				190	2.45	109.53
15	0.33	1.65				95	5.33	102.17

Current FDA guideline 1ug/mg (0.1%)

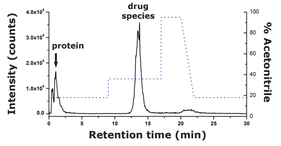
Formulated sample range (tested, 7.19 ng/mL; 3.82 ng/mL)

250-fold more sensitive (SNR = 10)

Figure 4. *Assay dynamic range. Analysis of reference standards were performed in triplicate using the same method shown in Figure 2. Calibration plots of the reference standards were generated using peak area from SIRs for the most abundant [M+2H]+2 charge state and fitted with an ordinary linear regression model. Using ICH guidelines the MS quadrupole dynamic range was determined to be 1.35 pg – 688.5 pg for the mal-linker-DSEA and 1.65 pg – 854.5 pg for the NAc-Linker-DSEA reference standards. MS settings: positive mode, SIRs for the [M+2H]+2 charge state were collected, Cone 2 V, Capillary 0.8 kV, Probe 400 °C.*

Method Evaluation with Spiked Samples

SPE Column (1D mode)



SPE Column to RPLC column (2D mode)

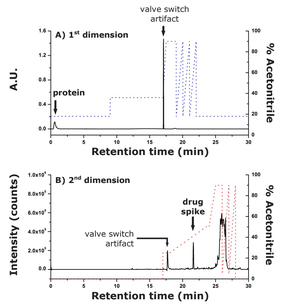
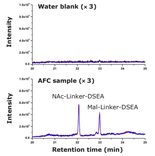


Figure 5. *Method evaluation of SPE with spiked sample. Mal-linker-DSEA and NAc-linker-DSEA was spiked into a dilute trastuzumab sample for SPE optimization. Optimal SPE loading conditions for the extraction of mal-linker-DSEA and NAc-linker-DSEA components from the spiked AFC sample were determined to be 18% acetonitrile containing 2% FA v/v. A step gradient to 36% acetonitrile containing 2% FA v/v was determined to be optimal conditions to elute bound drug components in a narrow peak centered around 13.5 min.*

Figure 6. *Evaluation of 2DLC configuration. NAc-linker-DSEA, spiked into a dilute trastuzumab sample, was successfully transferred from* ***(A)*** *the SPE column (1st dimension) to the* ***(B)*** *RP column (2nd dimension) using the 2DLC configuration illustrated in Figure 1 as proof-of-principle.*

SPE-RPLC/MS AFC Sample Analysis



Injection	NAc-Linker-DSEA			Mal-Linker-DSEA		
	Area	Experimental mass (pg)	Experimental concentration (ng/mL)	Area	Experimental mass (pg)	Experimental concentration (ng/mL)
1	3476	71.59	7.16	2716	38.67	3.87
2	3429	70.62	7.06	2746	39.10	3.91
3	3563	73.39	7.34	2599	36.98	3.70
Mean	3489	71.87	7.19	2687	38.25	3.82
S.D.	68	1.41	0.14	78	1.12	0.11
R.S.D.	1.95	1.96	1.96	2.89	2.92	2.92

Figure 7. *Reducing Sample Preparation. The increased sensitivity afforded by the incorporation of mass detection provides a means for analysts to bypass lengthy sample preparation steps for improved productivity in the biopharmaceutical production environment. The free-drug components were determined to be 7.19 ng/mL and 3.82 ng/mL for the NAc-linker-DSEA and mal-linker-DSEA components in a neat sample using the method shown in Figure 6. Detection of drug species at these levels, in a sample of modest concentration and injection volume (1.94 mg/mL, 10 uL), is not possible with optical detection alone, thus highlighting the utility of an MS detection in biopharmaceutical workflows.*

CONCLUSION

- **Increased Specificity and Sensitivity with MS Detection**
- **Extended Detection Limits for Increased Assay Robustness**
- **Reduced Sample Preparation for Increased Productivity**
- **Method Flexibility with Control of Both Dimensions**

Birdsall, R. E., et al. (2015). "A sensitive multidimensional method for the detection, characterization, and quantification of trace free drug species in antibody-drug conjugate samples using mass spectral detection." mAbs. DOI:10.1080/19420862.2015.1116659

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