Drug Antibody Ratios Made Easy by CE

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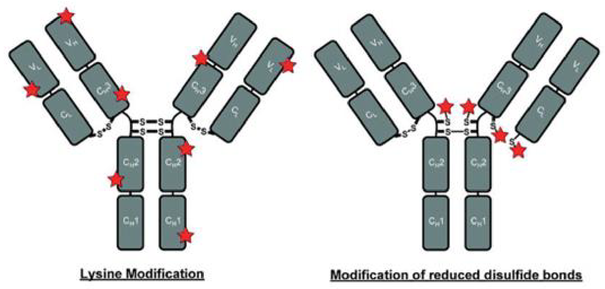
Introduction

Antibodies can be used as a delivery system to deliver a targeted payload. The targets can be fluorophores (for target cell labelling), peptides (targeted activation or deactivation of cells) or small molecules where the small molecule, often a cytotoxic compound, is used as a target treatment (ADCs). These payloads are usually attached through Lysine or Cysteine conjugation with the antibody. When antibodies are used in this way the amount of payload attached to the antibody is an important physical characteristic of the complex and payload analysis, referred to as drug antibody ratio (DAR) is achieved through various techniques cIEF, UV-Vis, HIC, MS etc.

Antibody peptide conjugate analysis is possible using Hydrophobic Interaction Chromatography (HIC), Mass Spectrometry (MS) and UV-vis spectrometry. In this study we show an alternative method using CE-SDS on the PA 800 Plus Pharmaceutical Analysis System to obtain Drug-to-Antibody-Ratio (DAR) values.



Nunes JP et al. Functional native disulfide bridging enables delivery of a potent, stable and targeted antibody-drug conjugate (ADC). Chem Commun (Camb). 2015;51(53):10624-7.

Figure 1. Location of conjugation of payloads on antibodies. The red stars highlight the potential conjugation points e.g. cysteine residues.

Materials and Equipment

Samples were analyzed by CE using a PA 800 Plus Pharmaceutical Analysis System with UV/VIS optical detection. Separations were performed using the EZ-CE cartridge (SCIEX, Part No. A55625). Samples were prepared using the SDS-MW assay Kit/Purity/Sizing (SCIEX, Part No. 390953).

Figure 2. PA 800 Plus Pharmaceutical Analysis System.

Methods

To calibrate the analysis a 10 -100 KDa sizing standard was first analysed to allow estimation of the ADC molecular weight. An example of a typical electropherogram of this standard is shown in Figure 3. The standard range from 10-100 KDa provided a fit >0.999 for molecular weight.

The antibody reduced and non-reduced samples were then analyzed using a EZ-CE cartridge which had an effective separation length of 20cm. Samples and buffer and method used following the standard CE-SDS protocol which comes with SDS-MW assay kit which was used in collaborative ring trial [1].

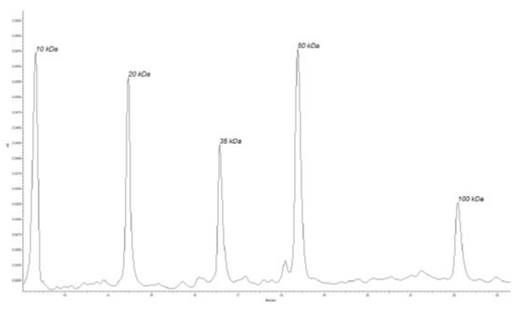


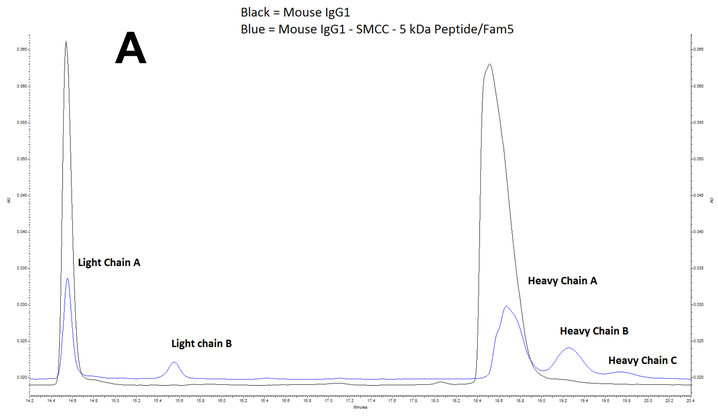
Figure 3. Electropherogram for 10-225 KDa sizing standard analyzed on a PA 800 Plus Pharmaceutical Analysis System.

Results

Lysine Antibody Conjugate Analysis

In this study a Mouse IgG1 antibody was conjugated in a 2-step protocol. Initially a SMCC linker was conjugated to lysine residues then the 5 kDa Peptide/Fam5 is attached to antibody bound SMCC linker so that when a full SMCC-5 kDa Peptide/Fam5 was attached it would result in ~5.225 kDa MW increase. The Mouse IgG 1 - SMCC - 5 kDa Peptide/Fam5 was then analyzed in it's non reduced form. The results from this analyses were inconclusive as the resolution observed was not sufficient to resolve unconjugated from a conjugated species which were separated by a 0.39m min corrected migration time shift. The antibody and lysine conjugate were therefore reduced and reanalyzed as shown in Figure 4.

A



B

Chain	Corrected Migration Time (mins)	Molecular Weight (kDa)
Light chain A	14.829 (± 0.006)	25.251 (± 0.000)
Light chain B	15.834 (± 0.012)	33.110 (± 0.050)
Heavy chain A	18.955 (± 0.018)	67.167 (± 0.153)
Heavy chain B	19.5415 (± 0.023)	75.003 (± 0.240)
Heavy chain C	20.017 (± 0.023)	81.571 (± 0.248)

C

	MW difference (kDa)	Divide by Payload	Payload
Light chain A	0.059	0.011	0
Light chain B	7.918	1.515	1
Heavy chain A	2.103	0.402	0
Heavy chain B	9.939	1.902	1
Heavy chain C	16.507	3.159	3

D

	load	Average Area %	Weighted Area %%	Weighted Area % x Load
Light chain A	0	17.034	84.389	0.000
Light chain B	1	3.151	15.611	15.611
Heavy chain A	0	34.611	65.568	0.000
Heavy chain B	1	15.4775	29.321	29.321
Heavy chain C	3	2.698	5.111	15.333
		Weighted Average DAR		1.205 DAR

Figure 4. CE-SDS Analysis of Mouse IgG and Mouse IgG ADC like analyte. 'A' Shows the overlaid electropherogram of mouse IgG and Mouse IgG ADC. Table B sows the MW Identification of peaks observed for the IgG ADC like analyte. Table C highlights the payload on each species it is calculated by subtracting the molecular weight of native chains from conjugated chains (a free linker conjugated to antibody without peptide was causing minor mass increases in addition to linker-peptide attachment). Table D shows the calculated DAR ratio for the Mouse IgG ADC like material. The DAR ratio was calculated by using the below calculation [2]

DAR=2x(Σ(weighted peak area of light chain)+Σ(weighted peak area of heavy chain)) / 100

In Figure 3 the DAR ratio was calculated by using the above calculation [2]. The Mouse IgG ADC like material was also analysed by UV-VIS to calculate a comparative DAR ratio. The UV/VIS was 1.21 and very close to the DAR ratio calculated by the new CE-SDS method. To verify that this this complementary nature of CE-SDS to UV-VIS analysis was not just related to one sample a series of different species were analysed by both techniques and the DAR ratios compared. Figure 5 highlights that the two techniques provide similar results.

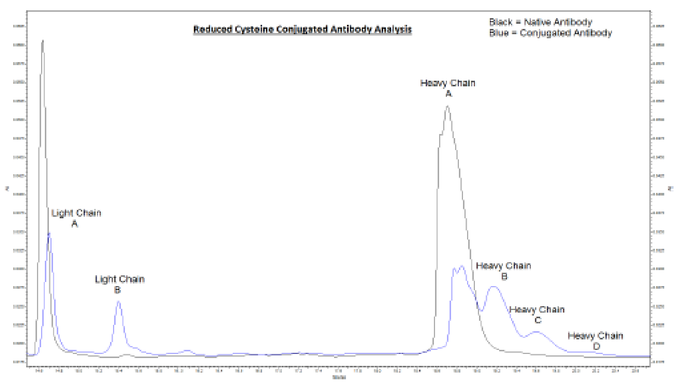
Antibody	Conjugation methodology	Linker	Payload	DAR calculations	
				UV/Vis Spectrometry	PA 800 Plus
Mouse IgG 1	Lysine	smPEG2	5kDa Peptide-Fam5	1.15	1.01
Mouse IgG 1	Lysine	smcc	5kDa Peptide-Fam5	1.21	1.205
Mouse IgG 1	Lysine	smcc	3.5kDa Peptide-Fam5	1	0.87

Figure 5. Comparison of UV/VIS spectrometry with CE (PA800 Plus) using a variety of peptide ADC samples.

Cysteine Antibody Conjugate Analysis

In this study a Mouse IgG1 antibody was reduced and conjugated via its cysteine residues. In this case a single peptide addition to the antibody resulted in an approximate 3.89 KDa mass increase. The cysteine conjugated IgG was first analyzed in it's non reduced form but it was found that the SDS broke non covalent bond opened up the antibody structure which made this ADC like material prone to fragmentation so again the cysteine antibody was analyzed in it's reduced form. The results from this analyses are shown in Figure 6.

A



B

Chain	Corrected Migration Time (mins)	Molecular Weight (kDa)
Light chain A	15.121 (±0.006)	21.848 (±0.034)
Light chain B	15.817 (±0.000)	26.228 (±0.000)
Heavy chain A	19.271 (±0.006)	59.687 (±0.076)
Heavy chain B	19.588 (±0.006)	63.907 (±0.080)
Heavy chain C	20.021 (±0.006)	70.033 (±0.086)
Heavy chain D	20.496 (±0.018)	77.228 (±0.277)

C

	MW difference (kDa)	Divide by Payload	Payload
Light chain A	0.409	0.105	0
Light chain B	4.790	1.231	1
Heavy chain A	1.818	0.467	0
Heavy chain B	6.038	1.552	1
Heavy chain C	12.164	3.127	3
Heavy Chain D	19.359	4.977	5

D

	load	Average Area %	Weighted Area %%	Weighted Area % x Load
Light chain A	0	19.921	75.015	0.000
Light chain B	1	6.635	24.985	24.985
Heavy chain A	0	26.379	46.626	0.000
Heavy chain B	1	22.841	40.371	40.371
Heavy chain C	3	6.728	11.892	35.676
Heavy Chain D	4	0.629	1.111	4.444
		Weighted Average DAR	2.132	DAR

Figure 6. CE-SDS Analysis of reduced cysteine linked Mouse IgG and Mouse IgG ADC like material. 'A' Shows the overlaid electropherogram of mouse IgG and Mouse IgG ADC like material. Table B sows the MW Identification of peaks observed in Mouse IgG ADC lie material. Table C highlights the payload on each species it is calculated by subtracting the molecular weight of native chains from conjugated chains (a free linker conjugated to antibody without peptide was causing minor mass increases in addition to linker-peptide attachment). Table D shows the calculated DAR ratio for the Mouse IgG conjugated material.

In Figure 5 the DAR ratio was calculated by using the same calculation as in Figure 3 [2]. The Mouse IgG ADC was also analysed by Hydrophobic Interaction Chromatography (HIC) to calculate a comparative DAR ratio. The HIC value measured at the time of conjugation was 2.23 again very close to the measured value of 2.132 observed by CE.

Conclusions

- Molecular weight (kDa) shifts in antibody light and heavy chains created through linker-peptide attachment via lysine and cysteine conjugation were successfully calculated by CE-SDS analysis on the PA 800 Plus
- Calculated molecular weight differences identify peptide payload attachment and DAR calculation is possible
- DAR values calculated using CE-SDS appear comparable to UV-vis spectrometer and Hydrophobic Interaction Chromatography calculated DAR

References

1.Nunally, B *et.al.* A series of collaborations between various pharmaceutical companies and regulatory authorities concerning the analysis of biomolecules using CE. *Chromatographia, 2006* , 64, P359-368.
2.Ouyan, J, Drug-to-Antibody Ratio (DAR) and Drug Load Distribution by Hydrophobic Interaction Chromatography and Reversed Phase Hugh-Performance Liquid Chromatography. *Antibody Drug conjugates Springer Protocols* 2013. P275-284

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