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REPORT

Native size-exclusion chromatography-mass spectrometry: suitability for antibody–drug conjugate drug-to-antibody ratio quantitation across a range of chemotypes and drug-loading levels

Jay Jones[a], Laura Pack [b], Joshua H. Hunter[c], and John F. Valliere-Douglass[a]

[a]Analytical Sciences, Seattle Genetics Inc., Bothell, WA, USA; [b]Quality, Seattle Genetics Inc., Bothell, WA, USA; [c]Conjugation Process Development, Seattle Genetics Inc., Bothell, WA, USA

ABSTRACT

Native size-exclusion chromatography-mass spectrometry (nSEC-MS) is an analytical methodology that is appropriate for accurately quantitating the drug-to-antibody ratio (DAR) on a wide variety of interchain cysteine-linked antibody-drug conjugates (ADCs), irrespective of chemotype. In the current preclinical environment, novel ADCs conjugated with unique drug-linkers need to progress toward the clinic as quickly as possible. Platform analytical approaches can reduce time-to-clinic because key process development and optimization activities can be decoupled from the development of bespoke, molecule-specific analytical methods. In this work, we assessed the potential of nSEC-MS as a platformable, quantitative DAR method. The nSEC-MS method was evaluated according to performance characteristics and parameters described in the ICH guideline Validation of Analytical Procedures: Text and Methodology Q2(R1). In order to comprehensively assess the accuracy and bias of nSEC-MS DAR quantitation, ADCs were generated using three different drug-linker chemotypes with DARs ranging from 2 to 8. These molecules were tested by hydrophobic interaction chromatography (HIC) and nSEC-MS, and DARs obtained from both methods were compared to assess the degree to which nSEC-MS quantitation aligned with the HIC release assay. Our results indicated that there is no bias introduced by nSEC-MS quantitation of DAR and that SEC-MS data can be bridged to HIC data without the need for a correction factor or offset. nSEC-MS was also found to be suitable for unbiased DAR quantitation in the other ADC chemotypes that were evaluated. Based on the totality of our work, we conclude that, used as intended, nSEC-MS is well suited for quantitating DAR on a variety of interchain cysteine-linked ADCs in an accurate, unbiased manner.

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Introduction

Antibody-drug conjugates (ADCs) are an increasingly important class of cancer therapeutics. ADCs are composed of a monoclonal antibody (mAb) that is attached to a potent cytotoxic drug via a cleavable linker. The specificity of the mAb component of the ADC to cancer antigens expressed on the cell surface ensures that the cytotoxic drug is delivered to the site of the tumor in a target-specific manner.[1-3] There are currently five ADCs approved for the treatment of cancer in the US: Adcetris, Bestponsa, Kadcyla, Mylotarg, and Polivy (Table 1). Additionally, sacituzumab govitecan and enfortumab vedotin have been granted breakthrough designation (BTD) by the US Food and Drug Administration (FDA) and are currently under FDA review, while trastuzumab deruxtecan received BTD and fast track designation and belantamab mafadotin received BTD.[4] While these ADCs are broadly similar in that they are composed of a mAb, cytotoxic drug, and a cleavable linker, there is diversity in the types of linkers, drugs and mAb attachment sites. All of the ADCs mentioned above are heterogeneous with respect to drug load and distribution. Thus, a given ADC may have on average ~4 drug per mAb, but the average is composed of a distribution of individual molecules typically ranging from 0 to 8 or more drugs per mAb, depending on the IgG subclass and conjugation strategy employed. While the ADCs listed in Table 1 all include IgG1 molecules, IgG2 and IgG4 molecules have been evaluated in preclinical and clinical settings.[5-7] Lysine conjugate ADCs have many more residues in the primary sequence that are available for conjugation than interchain cysteine conjugate ADCs, and this results in a more heterogenous, covalent ADC than an interchain cysteine conjugate.[8,9] On the other hand, interchain cysteine-linked ADCs are composed of antibodies with drugs conjugated to interchain cysteine residues.[10,11] The implication of this is that the heavy-heavy and heavy-light chain associations in the ADC monomer are a composite of covalent and non-covalent associations because some of the interchain disulfides have been reduced to accommodate covalent attachment of the drug-linker. The ADC modalities described above share a commonality in that endogenous cysteine or lysine amino acids on the IgG backbone are used as the sites of conjugation. Other conjugation strategies involve homogeneous site-specific attachment of drug-linkers by strategically modifying the primary sequence by inserting unpaired cysteine residues or enzymatic motifs into the primary sequence.[12-15]

CONTACT John F. Valliere-Douglass jdouglass@seagen.com Seattle Genetics Inc., Bothell, WA 98021 USA

Table 1. ADCs that are approved in the US or have completed pivotal clinical trials and received breakthrough/fast track designation by the FDA.

Molecule	Approval status	Antibody	Drug	Attachment site
Adcetris® (brentuximab vedotin)	Approved (2011)	IgG1	vcMMAE (Auristatin)	Interchain cysteine
Besponsa® (inotuzumab ozogamicin)	Approved (2017)	IgG1	Ozogamicin (Calicheamicin)	Lysine
Kadcyla® (ado-trastuzumab emtansine)	Approved (2013)	IgG1	DM-1 (Maytansine)	Lysine
Mylotarg® (gemtuzumab ozogamicin)	Approved (2017)	IgG4	Ozogamicin (Calicheamicin)	Lysine
Polivy® (polatuzumab vedotin-piiq)	Approved (2019)	IgG1	vcMMAE (Auristatin)	Interchain cysteine
Enfortumab vedotin	BTD, priority review	IgG1	vcMMAE (Auristatin)	Interchain cysteine
Sacituzumab govitecan	BTD, priority review, complete response letter issued	IgG1	SN-38 (Camptothecin)	Interchain cysteine
Trastuzumab deruxtecan	BTD, fast track	IgG1	Dxd (Camptothecin)	Interchain cysteine
Belantamab mafadotin	BTD	IgG1	mcMMAF (Auristatin)	Interchain cysteine

The potency of an ADC is determined in large part by the average number of drugs attached to the mAb, i.e., the drug-to-antibody ratio (DAR).[16] Consequently, key assays for measuring DAR should be developed and optimized as soon as possible in preclinical development. For the commercial and pre-commercial molecules listed in Table 1, hydrophobic interaction chromatography (HIC), reversed-phase chromatography (RPC) and UV absorbance have been used to quantitate DAR.[17] HIC provides a holistic view of the drug distribution of an ADC, but the assay does not provide insight into the drug attachment site and the analytical separation cannot be directly characterized by mass spectrometry (MS).[18] Reduced reversed-phase analysis of ADCs provides a readout on the heavy and light chain drug distribution, and this result can be used to recapitulate the DAR on the intact molecule.[19] Additionally, drug-positional isomers can also be inferred on the basis of the relative chromatographic retention of drug-linked heavy and light chains. However, while the assay provides an accurate quantitation of DAR for a sample, the drug-distribution on the intact ADC cannot be determined from the separation. UV absorbance-based quantitation of DAR is the most operationally simple method to execute and, like HIC and reduced RPC, provides an accurate quantitation of DAR.[20] However, UV absorbance-based quantitation methods provide no information about ADC drug-distribution and require that the drug-linker component of the ADC has a unique absorbance relative to the mAb. Finally, all of the approaches discussed above require some (potentially substantial) development when applied to a new ADC modality or drug-linker chemotype.

MS-based approaches have also been implemented to assess ADC DAR. There are several examples in the literature demonstrating that MS can be used for quantitative or qualitative assessments of DAR and drug-distribution for the types of molecules described in Table 1.[21-24] All of these MS-based approaches have commonalities: the multiply charged mass spectrum of the ADC is deconvolved into a zero charge mass spectrum, the species observed in the mass spectrum are identified on the basis of agreement between theoretical and observed mass and, finally, the relative levels of each of the individual drug-loaded species are inferred based on the apparent-relative height or area of that species. Compared to the conventional methods for quantitating ADC DAR, MS-based approaches have inherent advantages because DAR quantitation does not necessarily depend on liquid chromatography (LC)-based separation of the individual drug-loaded species. As an example, some RPC- and SEC-based MS methods described in the literature use LC separation only for the purpose of exchanging the ADC into a mobile phase that is amenable for MS detection.[25] In these approaches, the distinct ADC drug-loaded species are not separated, rather, the entire sample bolus is monitored and the DAR is quantitated by deconvolution of the MS from a single time-averaged scan.[25-27] It should be noted, however, that LC-MS-based approaches for monitoring DAR of lysine conjugate ADCs may not be appropriate for interchain cysteine conjugate ADCs because certain LC conditions may cause the analyte to dissociate into its constituent non-covalently associated, drug-linked heavy and light-chain subunits. This issue can be overcome by carrying out the LC separation in native conditions, e.g., in the presence of a neutral pH, volatile salt buffer.

Novel ADC molecules may not be amenable to a typical platform method such as HIC, RPC or UV absorbance without considerable development and optimization. Consequently, we saw a need to deploy a universal analytical methodology for measuring DAR that is agnostic to ADC modality and chemotype. The purpose of this work was to systematically evaluate our hypothesis that native LCMS methodologies run at microscale flow rates (e.g. 100 µL/min) may fill that need by providing reliable, accurate quantitation of DAR for interchain cysteine-linked ADCs. For this evaluation, we chose an SEC-MS-based methodology that we found to be rugged and reliable and had previously described in the literature.[25] However, the choice of LC separation conditions is somewhat ancillary to the larger questions we are seeking to answer: Is native MS actually a viable alternative/as good as existing methodologies such as HIC for quantitating DAR of interchain cysteine-linked ADCs? If so, can a native MS approach potentially be used as a standard DAR method for interchain cysteine-linked ADCs irrespective of the target DAR range or chemotype? To address the questions posed above, we adopted a two-part investigational strategy. The first part consisted of assessing the suitability of the method for the determination of ADC DAR in a routine laboratory setting. Put simply, this step determined whether the method is fit for purpose. The second part of the inquiry focused on carrying out a rigorous assessment of the accuracy of the method for determining the DAR of interchain cysteine-linked ADCs. To that end, we purposefully created a set of ADC samples with DAR values from ~2.5 to 8 that were conjugated to a variety of drug-linker chemotypes and compared the results obtained from the analysis of those samples by native SEC-MS and HIC in a head-to-head fashion.

Results

nSEC-MS method performance

The experiments described here were conducted over a 3-month period. During that time, ADC-A, which is an

interchain cysteine-linked maleimidocaproyl-valine-citrulline -*p*-aminobenzyloxycarbonyl MMAE (vcMMAE) ADC, was injected no less than 3 times during every sample set, twice at the beginning and once at the end of the sample set. The purpose of gathering this data was to determine how much the DAR reported by the method varied over time and, ultimately, to set up system suitability criteria for the assay. Across the 43 control injections that were carried out, the average DAR recorded was 4.195, the standard deviation was 0.015 and the range was 4.170 to 4.230.

nSEC-MS qualification

We envisioned implementing nSEC-MS for routine, high-throughput testing of a variety of ADCs. To ensure that the nSEC-MS method was fit for purpose, it was qualified according to the analytical method validation criteria outlined in ICH Q2(R1) (https://www.ema.europa.eu/en/ich-q2-r1-validation-analytical-procedures-text-methodology). Since the intended use of the method is to quantitate DAR in a chemotype agnostic manner, any given method characteristic was assessed on the basis of impact to DAR. For example, assay linearity was assessed over a range of 20–120% of a nominal injection of ADC-A on the column, and the results were evaluated on the basis of the extent to which DAR varied over that range. The qualification encompassed the following method characteristics: accuracy, precision, specificity, quantitation limit, linearity, range, and robustness. The results from the ADC-A qualification are summarized in Table 2. Across the entire DAR range tested (2.8–5.7), HIC and nSEC-MS profiles were qualitatively similar and the nSEC-MS DAR estimation differed from HIC by an average of 0.02 DAR (±1%) (Figure 1a, b). Additionally, a plot of nSEC-MS DAR vs HIC DAR for this experiment demonstrated a linear relationship with a slope of 1.016 and R^2 of 0.9997 (Figure 1c). The qualification and robustness assessment were executed on one MS instrument, a Bruker MaXis II, which is a high-resolution quadrupole time-of-flight (Q-TOF)-type mass spectrometer. MS instrument comparisons are beyond the scope of the current work. Table 3 contains a summary of all accuracy data obtained for the ADCs discussed above.

Table 2. Summary of qualification results for nSEC-MS.

Method characteristic	Experiment	Result
Accuracy	Test ADC-A over a DAR range of 2.8 to 5.7, compare to orthogonal result from HIC	nSEC-MS measured DAR was 0.02 higher than HIC (avg across all measurements).
Repeatability	Evaluate consistency of DAR measurement over 6 nominal injections of ADC-A	●Avg DAR: 4.189 ●Std Dev: 0.006 ●%RSD: 0.138
Intermediate Precision	Evaluate consistency of ADC-A DAR measurement while varying: ● Analysts (2) ● Columns (2) ● Mobile phase preps (2)	ADC-A measurements were collected over 4 days ● Avg DAR: 4.181 ● Std Dev: 0.006 ● %RSD: 0.133
Specificity	Assess carryover from injection of ADC-A and interference from ADC-A matrix	No carryover observed from previous sample in the deconvoluted MS and no interferences from ADC-A matrix
Recovery	Assess observed chromatographic UV area at 280 nm, compare to theoretical	Observed UV area at 280 nm was 104% of expected UV area
Quantitation Limit	Confirm practical QL with triplicate injections of ADC-A sample containing DAR 0 at a 0.5 to 2.5% weight ratio	ADC-A containing 0.5% DAR 0 was accurately quantitated ● DAR 0: 0.54% (108% recovery) ● %RSD: 4.74
Linearity	Evaluate the consistency of DAR measurement of ADC-A over a range of 20–120% nominal load injection	Slight bias observed at 20% nominal load; DAR measured 4.194, high degree of linearity from 40–120%. UV area-based recovery was 99–103% for linearity samples in the range of 40–120%. ● Avg DAR: 4.212 ● Std Dev: 0.014 ● %RSD: 0.339%
Range	Determined from linearity experiment	Slight bias (lower DAR) observed with the 20% nominal injection. Recommended range is 40–120% nominal injection (20–60 µg)
Robustness	Evaluate the consistency of DAR measurement of ADC-A while varying the following method characteristics: ● Ammonium acetate concentration in mobile phase ● Deglycosylated vs glycosylated ADC-A	● Varying the ammonium acetate concentration in the mobile phase from 150 mM to 250 mM had no significant impact on DAR measurement ● DAR is significantly biased if the ADC is not deglycosylated. DAR is underestimated by 12% on avg across DAR range of 2.8 to 5.7

nSEC-MS accuracy assessment with other ADC chemotypes

Similar accuracy experiments were performed using a DAR series of ADC-B, which was conjugated with maleimidocaproyl MMAF (mcMMAF), and ADC-C, which was conjugated with a novel second-generation MMAE-derived drug-linker (2gMMAE) consisting of a self-stabilizing maleimidyl PEG12 glucuronide MMAE.[28] ADC-B has a target DAR of 4, while ADC-C has a target DAR of 8; thus, the accuracy experiments were carried out on a series of samples where the DAR was purposefully varied from 2.7 to 5.7 for ADC-B and 2.2–7.9 for ADC-C. In the case of ADC-B, the HIC assay separated some drug-positional isomers and the resolution was generally poorer than that observed for ADC-A, which is a vcMMAE ADC (Figure 2, panel A). Nevertheless, nSEC-MS determination of ADC-B DAR differed from HIC by 0.03 DAR on average, and across the range of 2.7–5.7, nSEC-MS DAR was ±2% of HIC DAR. Similar to ADC-A Figure 1, panel C, a comparison of DAR determined by the two methods across the ADC-B drug-load series was plotted, and a least-squares regression line yielded an equation with a slope of 1.05 and an R^2 of 0.9999 (data not shown). HIC and nSEC-MS runs from low, medium, and high DAR ADC-B samples are shown in Figure 2, panels A and B, respectively.

Like ADC-B, the HIC profile for ADC-C is also quite complicated (Figure 3, panel A). The nSEC-MS determination of ADC-C DAR differed from HIC 0.01 DAR on average; however, it should be noted that the nSEC-MS values obtained for the lowest DAR ADC-C sample varied by 0.21

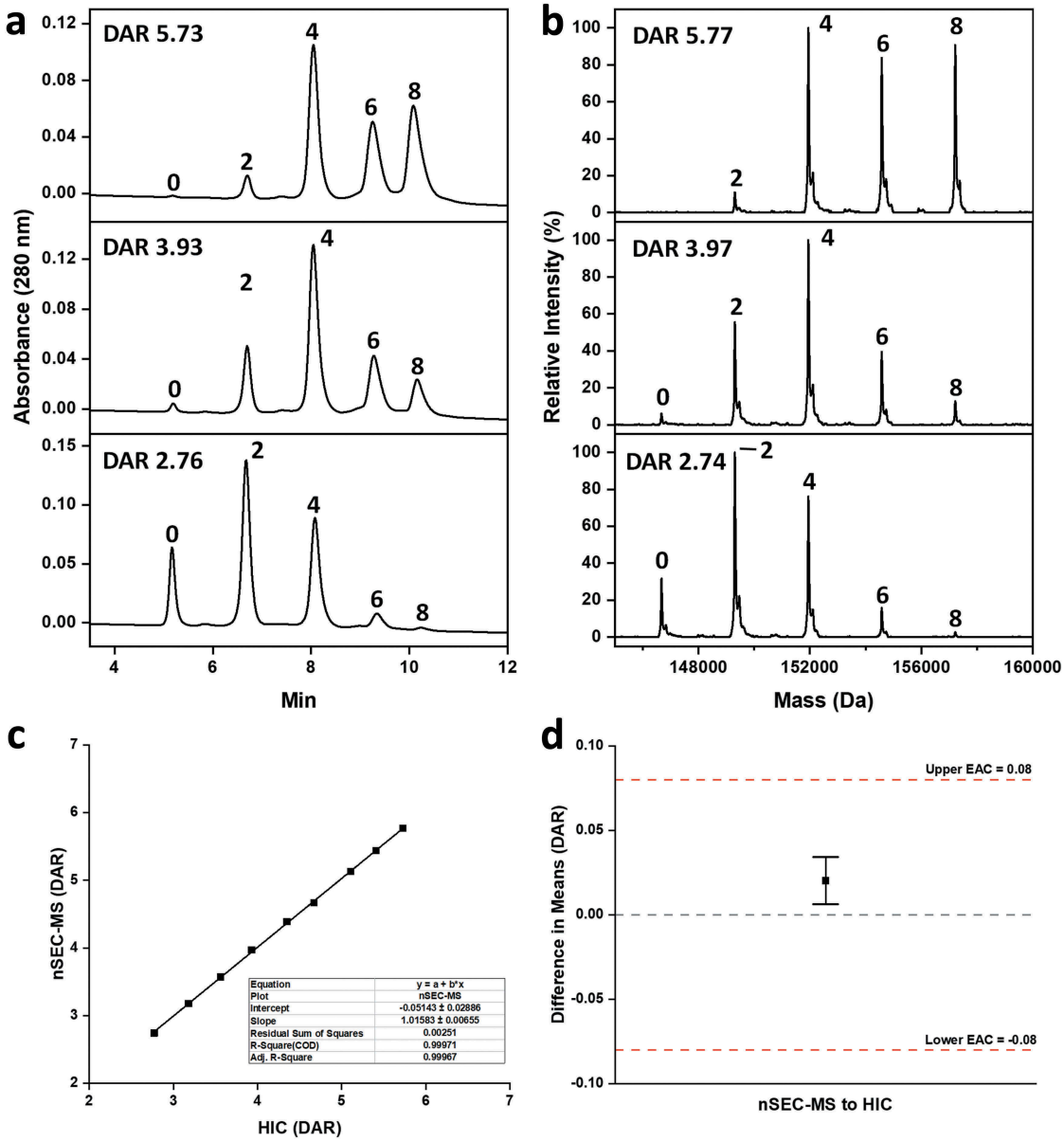

Figure 1. Comparison of HIC (panel A) and nSEC-MS (panel B) profiles of ADC-A samples at three different DAR levels. Linear correlation of ADC-A DAR determination by both methods is shown in panel C and a graphical representation of nSEC-MS equivalence to HIC for ADC-A DAR given previously established EAC is shown in panel D.

Table 3. Summary of nSEC-MS and HIC quantitation of ADCs A-C across the DAR range used for accuracy experiments.

ADC	Method	ADC DAR range								
ADC-A	HIC	2.76	3.18	3.56	3.93	4.35	4.67	5.11	5.41	5.73
	nSEC-MS	2.74	3.07	3.57	3.97	4.39	4.68	5.12	5.43	5.77
	nSEC-MS – HIC	−0.02	−0.10	0.01	0.04	0.04	0.01	0.01	0.02	0.04
ADC-B	HIC	2.69	3.06	3.37	3.81	4.19	4.59	4.97	5.30	5.66
	nSEC-MS	2.63	3.03	3.36	3.83	4.21	4.63	5.03	5.37	5.78
	nSEC-MS – HIC	−0.06	−0.03	−0.01	0.02	0.02	0.04	0.06	0.07	0.12
ADC-C	HIC	2.23	3.26	4.41	5.42	5.96	7.07	7.90		
	nSEC-MS	2.44	3.29	4.28	5.36	6.15	6.79	7.88		
	nSEC-MS – HIC	0.21	0.03	−0.13	−0.06	0.19	−0.28	−0.02		

DAR (9%) from the HIC value. For all other ADC-C samples, nSEC-MS differed from HIC by ±4% or less. The linear plot of DAR determined by the two methods had a slope of 0.96 and an R^2 of 0.994. HIC and nSEC-MS runs from low, medium, and high DAR ADC-C samples are shown in Figure 3, panels A and B, respectively.

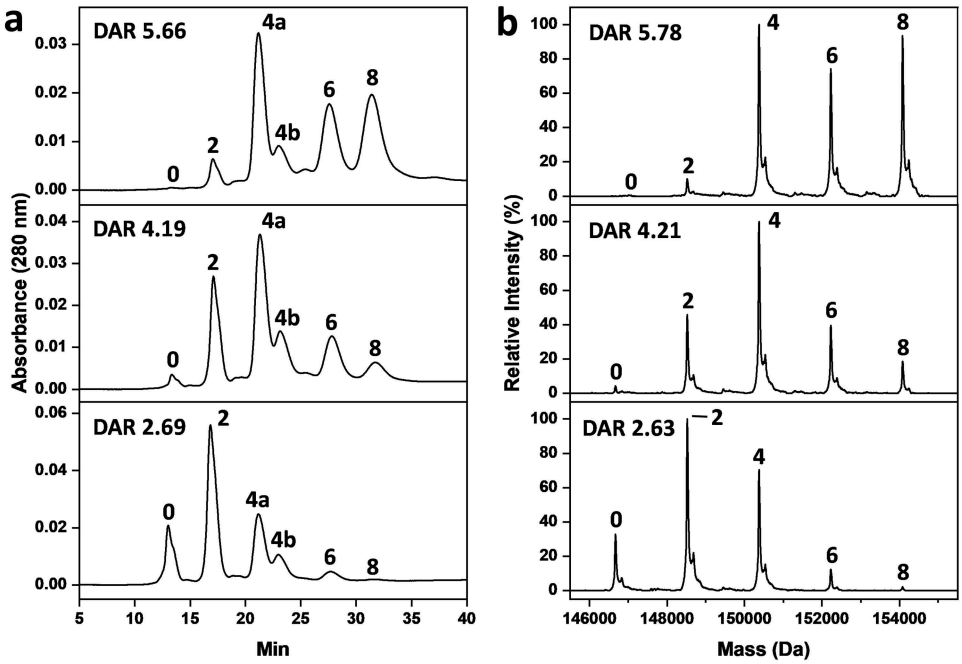

Figure 2. Comparison of HIC (panel A) and nSEC-MS (panel B) profiles of ADC-B samples at three different DAR levels.

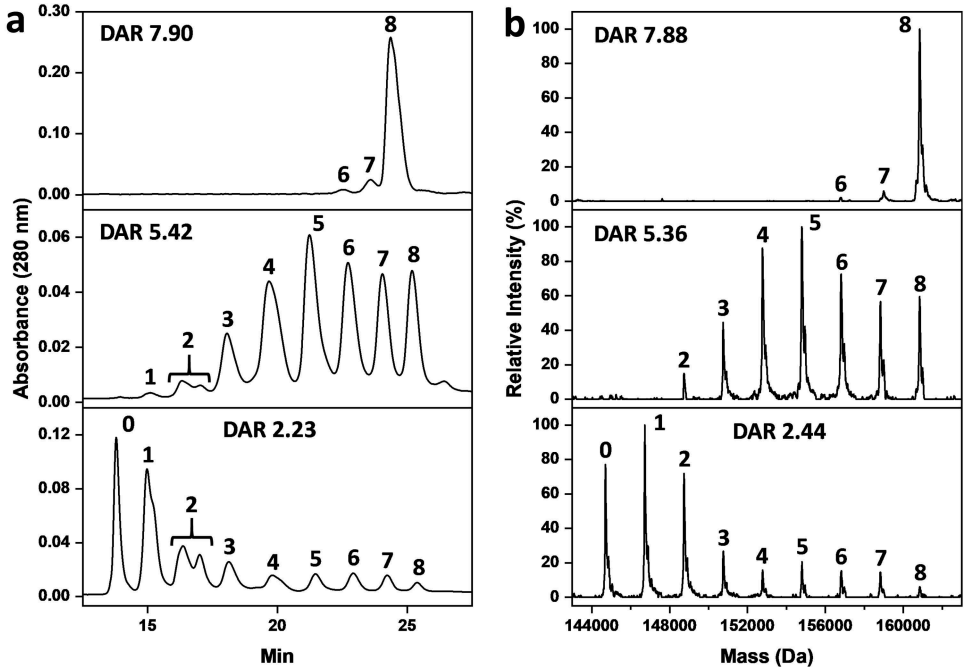

Figure 3. Comparison of HIC (panel A) and nSEC-MS (panel B) profiles of ADC-C samples at three different DAR levels.

Determination of nSEC-MS quantitation limit

An empirical quantitation limit (QL) for the nSEC-MS method was determined by spiking unconjugated mAb into the ADC-A sample that had an nSEC-MS determined DAR of 5.77. The highest DAR sample was chosen for this evaluation because it did not contain a detectable level of unconjugated antibody. ADC-A samples containing unconjugated mAb at levels ranging from 0.5% to 2.5% by mass were analyzed by nSEC-MS in triplicate and the relative intensity of the unconjugated mAb was divided by the summed intensity of all forms of ADC-A. The result (in percent) was compared to the theoretical (spiked) level of unconjugated mAb. The quantitated level of unconjugated mAb was reproducibly recovered across all spike levels, thus supporting the establishment of a practical QL of 0.5% (Table 2). A zoomed MS of unconjugated mAb found in the QL panel is shown in Figure 4a and a plot of the relative signal intensity (%) versus amount spiked (shown in Figure 4b) demonstrated that the MS signal response is linear for low abundance species.

Statistical equivalence of nsec-ms to HIC for DAR quantitation

The results from the ADC-A accuracy experiment were statistically assessed to determine if nSEC-MS was equivalent to HIC for the measurement of DAR. The approach taken was to perform a method bridging evaluation that uses a statistically derived equivalence acceptance criteria (EAC) to define the magnitude of difference between the two methods that is practically important.[29] System suitability data for HIC were used to define mathematically based EACs because these data are expected to most accurately reflect expected method variability. Based on the HIC system suitability data, the EAC was ±0.08. As indicated above, the average difference in the mean estimation of DAR for ADC-A by nSEC-MS and HIC was 0.02 and the 90% confidence interval (CI) around the average is 0.-0063–0.0341. Since the 90% CI around the average difference between the two methods falls within the EAC, it was determined that nSEC-MS and HIC are equivalent (Figure 1d).

Discussion

Since the initial publication of our work on the intact mass determination of interchain cysteine-linked ADCs,[25] additional publications have emerged describing similar nSEC-MS methods that have been deployed for unique and interesting purposes. In these studies, researchers have used nSEC-MS for assessing the composition of ADCs recovered from in vivo studies in rats,[30] to gain a better understanding of the gas-phase conformation of ADCs in ion-mobility separations,[31,32] and as a component of a suite of MS-based assays that can be used for comprehensive ADC characterization.[33] While the particulars of the nSEC-MS methods described above vary, they all share fundamental key attributes: a microbore SEC column is used to exchange the ADC into ammonium acetate, thus facilitating online native electrospray ionization (ESI) analysis. While this approach has been adopted with the understanding that it is appropriate for enabling direct determination of ADC mass and provides a qualitative assessment of DAR and drug-distribution, it was not clear whether the DAR and drug-distribution information derived from nSEC-MS was quantitatively accurate and valid. With this work, we answer that question.

Prevailing wisdom would suggest that the nSEC-MS method would not be quantitatively accurate because of the potential for bias arising from: (1) differential ionization efficiency of various drug-loaded species,[34] and (2) harsh, conventional ESI source conditions leading to ADC dissociation that affects higher loaded species preferentially. Indeed, at the time of our first publication on this method, we did not envision that the assay would be suitable for DAR quantitation. As we and others have implemented the assay more broadly, we began to question our initial assessment that nSEC-MS was not suitable for highly accurate DAR quantitation. In order to evaluate this question in a definitive empirical manner, we designed the accuracy study described in the results. In setting up the nSEC-MS accuracy panel, the HIC result was treated as the 'true' value for DAR. In this context (method qualification), the accuracy of the nSEC-MS assay was adequately demonstrated. However, a method may be shown by qualification to be accurate, but might not be able to be bridged to an existing assay due to differences in response across the product quality attribute range tested, or because there is a consistent offset between the two methods. Conceptually, in a graph of nSEC-MS DAR vs HIC DAR across the range of ADC DAR tested, this would manifest as a significant deviation from a slope of 1 or a y-intercept that is significantly different than 0 (respectively). The accuracy results obtained for ADC-A, B and C were sufficient to support the use of nSEC-MS as a quantitative DAR method for all three ADCs. However, in order to fully assess method bridging, the difference between the estimation of DAR by nSEC-MS and HIC needs to be contextualized to provide an understanding of whether the observed difference is acceptable. System suitability data from HIC were used to define the EAC, which is the range within which the difference between HIC and nSEC-MS needs to fall in order for the two methods to be assessed as being equivalent. As indicated in the results of the accuracy assessment, the 90% CI around the difference between the methods falls well within the EAC, indicating that the methods are equivalent and can be used interchangeably for DAR without the need for a correction factor or concern for bias. From the accuracy experiments, we

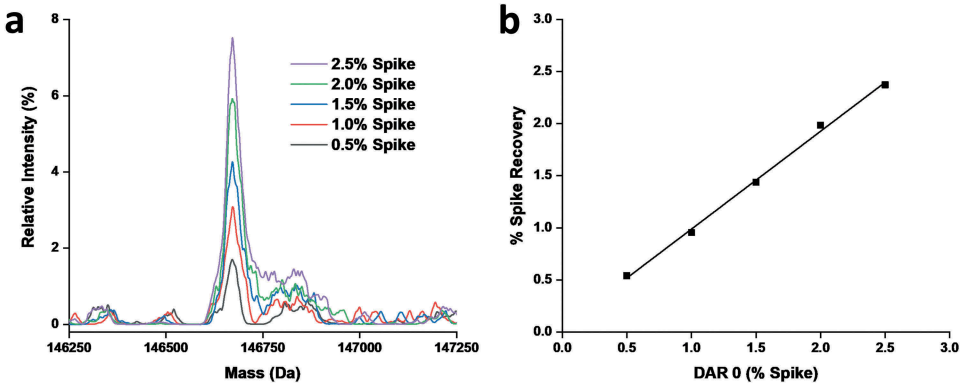

Figure 4. Mass spectra of ADC-A spiked with levels of unconjugated mAb ranging from 0.5% to 2.5% (panel A) and linear plot of % spike vs observed unconjugated mAb (panel B).

conclude that nSEC-MS provides quantitatively accurate measurements of the DAR of interchain cysteine-linked ADCs across a wide range of drug-loading.

Limited robustness assessment was carried out on the nSEC-MS method to gain a better understanding of method parameters that would have an effect on DAR quantitation. Changes in the concentration of the nSEC-MS mobile phase were found to have no impact on DAR quantitation, but the glycosylation status of the ADC had a significant impact on DAR assessment of ADCs conjugated with vcMMAE. As an example, ADC-A was assessed to have a DAR of 3.93 by HIC, and DARs of 3.97 and 3.58 were determined by nSEC-MS on the deglycosylated and glycosylated molecules, respectively. In order to eliminate bias in DAR determination by nSEC-MS, we recommend deglycosylation prior to analysis as a standard practice.

The qualification exercise described in the results section was useful for gaining a thorough understanding of the sensitivity, reproducibility, and ruggedness of the nSEC-MS method and to better understand if it is suitable for implementation in a high-throughput environment. The method performed well across all characteristics assessed, which indicated that this approach can be implemented for routine, high-throughput analysis of cysteine-linked ADCs. However, while accuracy experiments were carried out on ADCs with other chemotypes, the method was not formally qualified for those chemotypes. Our general conclusion was that nSEC-MS has performed well for all interchain cysteine-linked ADCs discussed here and for the analysis of others that have not been communicated in this work. However, due diligence in the form of a method assessment should be carried out to determine that the method is fit for purpose prior to application to new ADC chemotypes or modality. The extent and rigor of the method assessment would depend on how radically the ADC chemotype diverges from standard auristatin-based drug-linkers. Some drug-linker chemistries may be labile to sample preparation or ionization conditions. We, therefore, suggest that a minimal set of experiments should be executed to establish that deglycosylation conditions and source ionization settings do not cause conjugated drug-linker decomposition. Additionally, it is crucial to establish that the method is fit for the purpose of a new ADC chemotype and can be used for accurate DAR quantitation. This may be difficult to comprehensively assess for a novel ADC chemotype, but a simple spiking experiment involving titration of unconjugated mAb into an ADC sample should provide sufficient insight to either move forward with nSEC-MS or consider an alternative method.

We have deliberately emphasized the quantitative strengths of nSEC-MS for tracking ADC DAR and drug distribution, but the approach has the added benefit of providing an experimental mass for all species that are quantitated. Obviously, mass verification is important for guaranteeing that ADC forms are quantitated correctly, but the intact mass can also provide additional insight. For example, Figure 5 demonstrates that nSEC-MS and HIC quantitation of even-load vcMMAE ADCs agree quite well across the DAR range over which method accuracy was assessed. However, the odd-load vcMMAE ADCs do not line up nearly as well. While these species are at low levels to begin with, nSEC-MS results indicate that DAR1 and DAR3 species are, on average, observed at considerably lower levels than they are by HIC (Figure 4). Conversely, DAR5 and DAR7 species were not detected at all by HIC, but were detected by nSEC-MS at levels higher than the QL and were definitively identified on the basis of experimental mass. Previous work on the characterization of ADC separations by HIC provides some insight into why nSEC-MS and HIC do not align on detection and quantitation of odd-loaded species.[35] Gilroy et al. used two-dimensional chromatography to show that putative DAR3 species isolated from HIC separation actually consisted predominantly of DAR4 ADC with 1 aglycosylated HC and/or a Man-5 N-glycan and that DAR3 was likely a minor component. This observation suggests that HIC over-represents DAR3, which aligns with our finding that true DAR3 is not as abundant as HIC results would indicate. Regarding the higher odd-loaded ADCs, the HIC chromatogram is not as well resolving in the post 4-load region and the peaks are relatively broad, which suggests that DAR5 and DAR7 are not sufficiently resolved to allow detection and quantitation of these species. In principle, HIC is a hydrophobicity-based separation, but molecule attributes such as glycosylation status and composition may have an impact on hydrophobicity, and therefore HIC retention,[36] potentially leading to the conflation of these species with drug-load variants. It is important to note that our findings may depend on the specifics of the HIC method and the glycosylation status of the ADCs used in this study. Nevertheless, our findings highlight a key point, which is that off-line chromatographic methods used for quantitation of DAR may be influenced by unexpected coelution of odd-loaded species with even-loaded ADC post-translational variants. While HIC is well suited for measuring DAR and is stability-indicating, MS approaches may offer a key advantage, namely: the quantitated species are unambiguously identified by mass.

In summary, the totality of the results presented here demonstrates that nSEC-MS can be used to quantitatively determine the DAR on cysteine-linked ADCs. We have addressed the uncertainty around using the approach for DAR quantitation by empirically demonstrating that the nSEC-MS is comparable to HIC. Finally, we conclusively established that the method is validation amenable for at least one ADC chemotype and sufficiently rugged to be deployed in research or early preclinical development as a chemotype agnostic DAR method.

Materials and methods

Materials

IgG1 recombinant mAbs were expressed in Chinese hamster ovary cells and purified according to standard platform procedures. All ADCs were conjugated to interchain cysteine residues. ADC-A was conjugated with vcMMAE, ADC-B was conjugated with mcMMAF and ADC-C was conjugated to 2gMMAE according to established procedures.[10,37] Variably loaded ADCs were generated by modulating the molar equivalents of tris(2-carboxyethyl)phosphine (TCEP) reductant added to the antibody prior to the addition of the maleimide containing drug-linker.

nSEC-MS

Prior to mass spectrometric analysis, antibodies were deglycosylated by adding 3 µL of PNGase F (New England Biolabs, Ipswich, MA) per 100 µg of antibody or ADC and incubated at 37°C for at least 2 h. ADCs were separated on a polyhydroxyethyl-A (PHEA) column (PolyLC, Columbia,

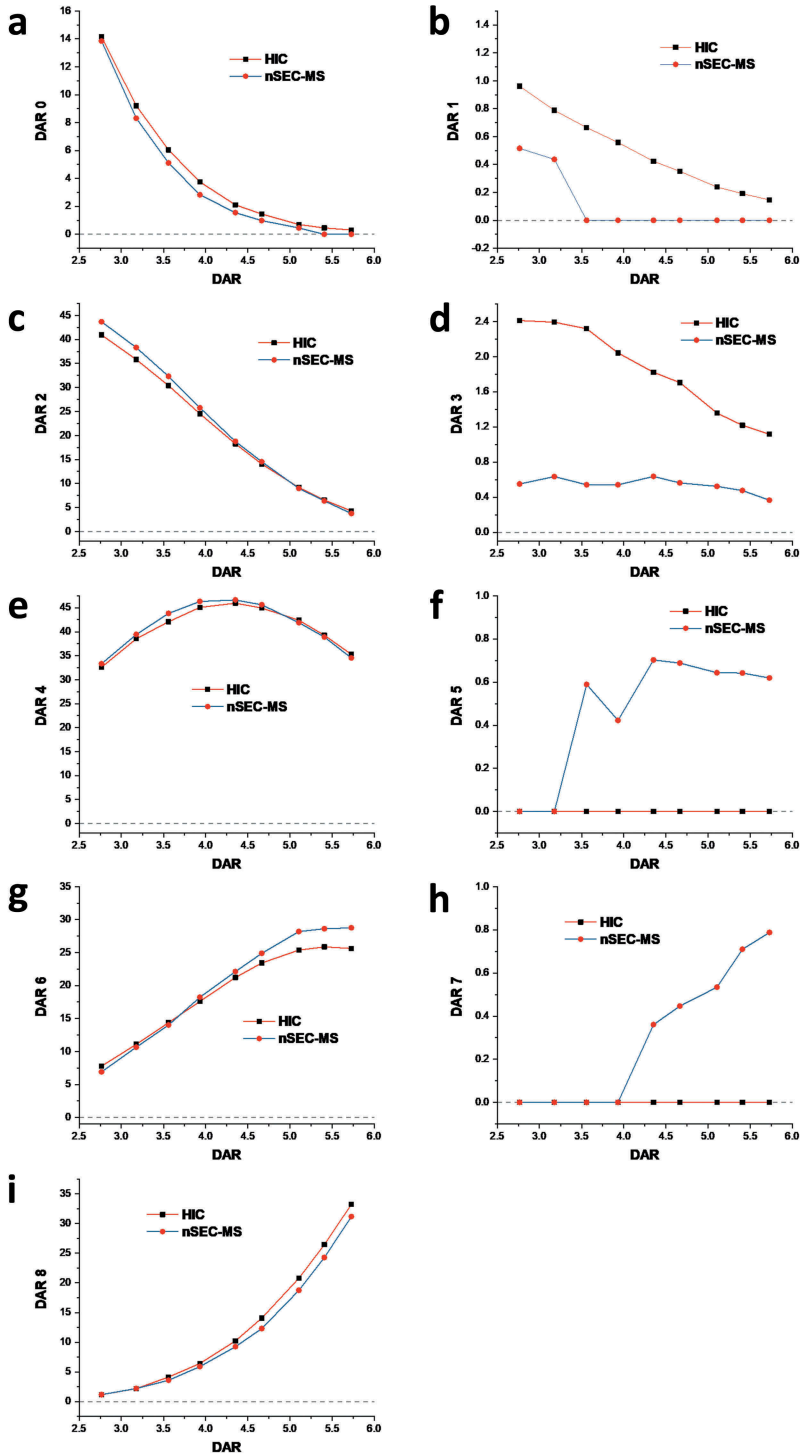

Figure 5. Linear comparison of nSEC-MS and HIC ADC-A assessment of individual drug-load variants across the entire DAR range that was assessed during accuracy experiments. Each panel represents a drug-load variant, e.g., A = DAR0, B = DAR1. The x-axis for each chart represents the DAR of particular samples that were assessed during accuracy experiments and the y-axis represents the relative percent of that species in a given sample.

MD) with dimensions of 2.1 mm × 200 mm and containing 5 µm particles with 300 Å pores. The column was equilibrated in 200 mM ammonium acetate, pH 7. The flow rate was maintained at 0.1 mL/min during the run, and the ADC was typically eluted between 3.5 and 4.5 min. The flow and buffer composition were maintained following elution of the mAb or ADC, and the total cycle time was 10 min per run. The column eluent was directed into a Bruker MaXis II Q-TOF mass spectrometer (Bruker, Billerica, MA) starting at approximately 2.75 min and diverted to waste at 4.6 min. The source capillary voltage and endplate offset were 4500 and 500 V, respectively. The source drying gas and nebulizing gas were set at 8.0 L/min and 1.8 bar, respectively, and the source funnel pressure was maintained at ~3.5 mbar. The source drying temperature was set at 150°C. ADC spectra were obtained over a range of 800–8000 m/z with a rolling average

of two spectra and a scan rate of 0.5 Hz. ADC Mass spectra were batch deconvoluted using Protein Metrics Intact Mass™ software (Protein Metrics, Cupertino, CA) and the DAR was computed using workflows within the Intact Mass deconvolution software according to the following algorithm.

$$DAR = \sum_{n=0}^{8} (ion\ intensity)_n\ xn$$

where:DAR = average drug-to-antibody ratio
Ion intensity = the height of the nth species
n = drug-to-antibody ration of the nth species

The nSEC-MS recovery of ADCs was calculated by integrating the chromatographic UV area of the nSEC-MS protein elution peak at 280 nm and comparing the experimental UV area to the theoretical area expected based on Beer's law.

Hydrophobic interaction chromatography

ADCs were separated on the basis of the molar ratio of antibody to the drug (MR) by HIC. A 4.6 mm x 35 mm Butyl-NPR column (Tosoh, JPN) was used to separate ADC-A drug-load variants and the same column with dimensions of 4.6 mm × 100 mm was used for separation of ADC-B and C drug-load variants. The ADC-A mobile phase A consisted of 50 mM sodium phosphate, 1.5 M ammonium sulfate, pH 7.0, and mobile phase B consisted of a mixture of 50 mM sodium phosphate, pH 7.0 and isopropanol in a 3:1 ratio. Mobile phases for the separation of ADC-B were the same except the concentration of sodium phosphate was lowered to 25 mM. Separation of the ADC-A drug-load variants was obtained with a linear gradient of 0 – 100% B over 12 min at a flow rate of 0.8 mL/min. Separation of the ADC-B drug-load variants necessitated a longer gradient of 15 – 85% B over 50 min at a flow rate of 0.4 mL/min and ADC-C drug-load variants were separated with a linear gradient of 0–100% B over 28.6 min at a flow rate of 0.7 mL/min. Quantitation of drug-loaded species was obtained by integrating the UV area of each separated species at 280 nm and the DAR of each sample was calculated using the following equation:

$$DAR = \sum_{n=0}^{8} (\%\ Area)_n\ xn$$

where: DAR = average drug-to-antibody ratio
% $Area_n$ = % area of the nth species
n = drug-to-antibody ration of the nth species

Statistical assessment of nsec-ms and HIC comparability

Statistical analysis was conducted to assess whether SEC-MS and HIC were comparable in both mean and variance. To compare the means, the equivalence approach was used such that the magnitude of a practically important difference was defined based on acceptable method variability. The EAC was calculated as 2 times the 80% upper confidence bound on the total variance in historical HIC system suitability data (n = 45).[29] The difference in SEC-MS and HIC means, along with a 90% CI on the difference, was calculated using data from an accuracy experiment where each sample was tested with both methods (n = 9 per method). Two one-sided t-tests were used to assess equivalence such that the 90% CI was compared to the EAC range. The means of the two methods were deemed equivalent because the 90% CI on the difference (0.0063–0.0341 DAR) in means fell completely within the EAC interval of ± 0.08 DAR. The variance of the two methods was compared by calculating a ratio of variances

$$\left(\frac{\widehat{\sigma}^2_{SEC-MS}}{\widehat{\sigma}^2_{HIC}}\right)$$

from two different lots tested at different times, where SEC-MS data were generated on one lot (n = 43) and HIC data were generated on a different lot (n = 45). The 90% CI on the ratio of variance was calculated using equation 2.66 in Burdick *et al.*[29] The estimated ratio of variances is 0.2946. The upper 90% confidence bound on the ratio of variances is 0.5667, indicating that the variance of the SEC-MS method is approximately 57% of the total variance present in the HIC method.

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Declaration of Interest Statement

The authors report no conflict of interest.

ORCID

Laura Pack http://orcid.org/0000-0002-3439-3610

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