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Review

Recent Advances in Drug–Antibody Ratio Determination of Antibody–Drug Conjugates

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Antibody–drug conjugates (ADCs) are biopharmaceuticals produced by chemically linking small molecules (payloads) to antibodies that possess specific affinity for the target cell. The ADCs currently on the commercial market are the result of a stochastic conjugation of highly-potent payloads to multiple sites on the monoclonal antibody, resulting in a heterogeneous drug–antibody ratio (DAR) and drug distribution. The heterogeneity inherent to ADCs not produced site-specifically may not only be detrimental to the quality of the drug but also is less-desirable from the perspective of regulatory science. An ideal method or unified approach used to measure the DAR for ADCs, a critical aspect of their analysis and characterization, has not yet been established in the ADC field and remains an often-challenging issue for bioanalytical chemists. In this review we describe, compare, and evaluate the characteristics of various DAR determination methods for ADCs featuring recently reported technologies. The future landscape of bioconjugate DAR analysis is also discussed.

Key words antibody–drug conjugate; drug antibody ratio; site-specific conjugation; native analysis

1. Introduction

Antibody–drug conjugates (ADCs) are complex biopharmaceuticals produced by chemically attaching a highly-active small molecule drug (payload) *via* an appropriate linker to an antibody or antibody fragment^{1–4)} (Fig. 1). The antibodies used in ADCs typically possess high affinity and specificity for antigens preferentially expressed on tumor cells compared to regular tissues and direct the attached payloads to the tumor cells and tumor microenvironment. While attached to the antibody, cytotoxic payloads behave as inactive prodrugs while in circulation and display their activity when liberated from their antibody or linker. To date, nine ADCs have been approved for clinical use by the U.S. Food and Drug Administration (FDA), and more than 90 ADCs are in clinical development⁵⁾ (Table 1).

Traditionally, ADCs are synthesized through stochastic payload conjugation onto natural amino acid residues, usually lysine or reduced interchain cysteine residues, *via* chemically stable linkers. These conjugation approaches lead to ADCs with varying levels of heterogeneity that may be limited in their efficacy and therapeutic index due to this heterogeneity.⁶⁾

With the rapid growth of the ADC field in recent years, analytical characterization to measure drug–antibody ratio (DAR) continues to play an important role in the discovery, development, and manufacturing of ADCs.⁷⁾ Regulatory bodies such as FDA and European Medicines Agency require the biopharmaceutical industry to analyze antibody–small

molecule bioconjugates similarly to both large-molecule and small-molecule drugs, and the complex nature of ADCs require well-thought analytical strategies.^{8,9)} Although many analytical chemists have reported unique methodologies in the scientific literature that are geared toward the establishment of a gold standard technique for DAR characterization, challenges remain because of the structural complexity, diversity and heterogeneity of ADCs.^{7,10)} In addition to heterogeneity due to conjugation methodology as described above, there is antibody-related issue to be considered. Naked antibodies, starting material of ADCs, are usually produced as complex mixtures of Fc glycoforms. Due to the complexity and heterogeneity, traditional analytical methodology which is used for protein-characterization cannot be directly applied to DAR determination of ADCs. Therefore, specific approaches focusing on DAR determination are required. Herein, we describe an overview of DAR characterization methods, focusing on recent advances, with the aim of providing a guidance for the analytical community working on relevant biopharmaceutical development.

2. Analytical Approaches for DAR Determination

The reported strategies for DAR determination can be classified into three representative approaches: HPLC/UV, liquid chromatography–quadrupole time-of-flight mass spectrometry (LC-QTOF/MS), and UV-Vis¹¹⁾ (Fig. 2).

HPLC/UV methods are commonly used because of the changes to the antibody or heavy/light chain hydrophobicity due to conjugated payloads. The relative area under each peak in an HPLC chromatogram can be used to calculate an

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Fig. 1. Overview of ADCs

A: Requirement for each ADC component. B: Synthetic scheme and typical DAR distribution of lysine-based ADCs. C: Synthetic scheme and typical DAR distribution of cysteine-based ADCs. (Color figure can be accessed in the online version.)

Table 1. FDA-Approved ADCs

General name	ADC	Approval	Strategy	DAR (method)
Mylotag [®]	Gemtuzumab ozogamicin	2000, 2017	Stochastic conjugation	2.5 (UV-Vis)
Adcetris [®]	Brentuximab vedotin	2011	Stochastic conjugation	3.5 (HPLC/UV)
Kadcyla [®]	Trastuzumab emtansine	2013	Stochastic conjugation	3.5 (UV-Vis)
Besponsa [®]	Inotuzumab ozogamicin	2017	Stochastic conjugation	5–6 (UV-Vis)
Polivy [®]	Polatuzumab vedotin	2019	Stochastic conjugation	3.5 (HPLC/UV)
Padcev [®]	Enfortumab vedotin-ejfv	2019	Stochastic conjugation	3.8 (HPLC/UV)
Enhertu [®]	Fam-trastuzumab deruxtecan-nxki	2019	Site-specific conjugation	7.7 (HPLC/UV)
Trodelvy [®]	Sacituzumab govitecan-hziy	2020	Site-specific conjugation	7.6 (HPLC/UV)
Blenrep [®]	Belantamab mafodotin-blmf	2020	Site-specific conjugation	4.0 (HPLC/UV)

average DAR. This analysis is a well-known method due to accuracy and reproducibility and can also be used to compare biosimilar-type ADCs against the original ADCs.¹²⁾ There are two typical HPLC methods employed for DAR measurement: hydrophobic interaction chromatography (HIC) and reverse phase (RP)-HPLC. The DAR can be obtained from the ratio of the peak areas in the UV chromatogram measured at an absorption wavelength of 280nm in both methods. However, this analytical approach has a disadvantage in that it is not possible to identify the molecular weight of each different DAR species (Fig. 2A) and is often used in conjunction with another method that can provide peak identity information.

Another significant approach to determine the DAR is by LC-QTOF/MS. The combination of HPLC with QTOF/MS systems is frequently used methodology for both intact and

subunit ADC analysis (Fig. 2B). This technique can provide not only payload distribution, but also molecular weight of each DAR species. Therefore, this approach has great potential as an idealized DAR measurement method. However, this approach may not work well for payloads that are particularly hydrophilic or that impart properties that negatively affect ionization of the conjugated species.

The third approach to measuring DAR is based on UV-Vis. (Fig. 2C). This method is a simple and commonly used technique adapted from protein concentration measurement methods. Using absorbances of an ADC at appropriate wavelengths and the extinction coefficients of the antibody and payload, the average DAR can be determined. This technology is user-friendly because there is no need to establish HPLC or MS conditions or complex sample preparation; however, it requires

Fig. 2. Analytical Approaches to Determine the DAR

A: HPLC/UV approach. B: LC-QTOF/MS approach. C: UV-Vis approach.¹¹⁾

that the absorption maximum of the payload is sufficiently shifted from that of the unconjugated antibody in to determine the average DAR value. With this method, knowledge of the ratio of individual DAR species within the mixture is not obtainable.

3. HPLC/UV Approach

3.1. HIC/UV HIC-HPLC is a well-established technique used to separate ADCs based on their intrinsic hydrophobicity. HIC can be considered a standard approach for DAR determination because it can be performed on intact ADCs under native conditions^{13,14)} requiring minimal sample preparation. Conventional cysteine-based ADCs targeting a DAR of less than 7-8 commonly yield major five species (DAR=0, DAR=2, DAR=4, DAR=6, and DAR=8) and smaller amounts of odd-DAR species. HIC columns can separate these species in a mixture based on hydrophobicity shifts from varying amounts of payload per species and the resultant chromatogram can be used to calculate DAR (Fig. 3). Because HIC can separate DAR species under native conditions, it is used not only analytically for DAR determination but also for purification.^{13,15,16)} Furthermore, a combination of site-specific conjugation followed by preparative HIC purification can provide site-specific and homogeneous-DAR ADCs.¹⁶⁾

However, HIC separation has some disadvantages. This technique does not provide compatibility with MS analysis because HIC-HPLC requires a high initial concentration of low volatility salts in the mobile phase. Recently, Wei *et al.* successfully used HIC coupled with MS to analyze monoclonal antibodies using MS-compatible buffers, but no studies that apply this method to ADC analysis have yet been reported.¹⁷⁾ Lower recovery of ADC species is the other drawback of HIC

Fig. 3. HIC/UV Analysis of Cysteine-Based ADCs Prepared and Analyzed as Previously Reported²²⁾

A: naked mAb (Trastuzumab). B: Cysteine-based ADCs (Trastuzumab-MMAE). (Color figure can be accessed in the online version.)

separation. To overcome this issue, the reduction of strong lyotropic salts in the mobile phase¹⁸⁾ and the use of membrane chromatography¹⁹⁾ are being explored. A high concentration of salts in the mobile phase typically has negative effects on peak resolution, which may result in low reproducibility. Introduction of isopropyl alcohol (IPA)²⁰⁾ or acetonitrile (ACN)²¹⁾ as an organic modifier sometimes improves peak shape, resolution, and separation.

3.2. RP-HPLC/UV From a peak separation and resolution perspective, reduced RP-HPLC/UV analysis may be a high-utility method for DAR determination. Elevated column temperatures and mobile phases containing trifluoroacetic acid (TFA) can enhance peak separation and improve peak resolution. In the analysis of samples by reduced RP-HPLC/UV, the ADCs are fully fragmented by pretreatment with DL-dithio-

Fig. 4. RP-HPLC Analysis of Cysteine-Based ADCs

A: abbreviation and illustration of cysteine-based ADC. B: RP-HPLC analysis of cysteine-based ADCs. (Color figure can be accessed in the online version.)

threitol (DTT) reduction to break the covalent links between antibody chains prior to analysis. The ADC has four inter-chain disulfide bonds, which, when cleaved by the reductive pretreatment, generate antibody fragments derived from the light and heavy chains of the antibody. In native immunoglobulin Gs (IgGs), the light and heavy chains have large differences in molecular weight (usually about 25kDa for the light chain and 50kDa for the heavy chain) as well as hydrophobicity, which allows them to be separated by HPLC, another advantage of this technique. When the resulting antibody fragments are injected onto a RP-HPLC column, multiple peaks may be observed in the chromatogram. Cysteine-based ADCs, especially those with a highly hydrophobic maleimide-C6-valine-citrulline-p-aminobenzoxycarbonyl-monomethyl auristatin E (MMAE) payload, are often synthesized with the aim of achieving a DAR of 4 (instead of saturating the 8 possible interchain cysteine present in an IgG1 for example) as well as lysine-based ADCs to avoid decreasing the pharmaceutical properties of an ADC. Aggregation tends to increase and toxicity and pharmacokinetic profiles may shift undesirably with ADC conjugated with excessive payload.²²⁾ It is generally difficult to obtain homogeneous ADCs with a DAR of 4 by a traditional reductive synthetic approach. Incomplete reduction of the disulfide bonds in the antibodies results in uncomplete modification and produces a mixture of ADCs shown in Fig. 1C. This DAR mixture contains six peaks of antibody-related fragments (unconjugated light chain (L0), conjugated light chain by 1 MMAE (L1), unconjugated heavy chain (H0), heavy chain conjugated by 1 MMAE (H1), heavy chain conjugated by 2 MMAEs (H2), and heavy chain con-

jugated by 3 MMAEs (H3) in the RP-HPLC chromatogram (Fig. 4). These peaks may be well-separated, which allows the average DAR to be calculated.

The RP-HPLC/UV approach has another drawback in that the elevated temperatures required both for measurement (column temperature) and denaturing pretreatment using a combination of guanidine and DTT can potentially degrade the ADC species. This potential degradation may result in DARs calculated from RP-HPLC analysis that are artificially lower than those from HIC or other analyses.^{9,12,23)} The establishment of an ideal analytical procedure balancing mild conditions and sufficient separation is required.

Recently, several groups reported reverse phase HPLC analysis for intact ADCs without any sample pretreatment.^{23–26)} This HPLC system is compatible with MS analysis because of the salt-free buffer system that also promotes ionization efficacy. In 2019, Wirth and colleagues reported the application of intact RP-HPLC for cysteine-based ADCs.²⁵⁾ Furthermore, our research group recently demonstrated this analytical procedure for *in-situ* monitoring of payload conjugation.²⁶⁾ These results indicate that the intact RP-HPLC methodology has great potential application to a variety of ADC analyses.

3.3. Other HPLC/UV Approaches In addition to HIC/UV and RP-HPLC/UV approaches, some other chromatographic analyses have been reported for ADC analysis. The affinity capture approach is considered a promising method to evaluate an antibody's properties.^{27–30)} This analysis is conducted under native conditions and has already been applied to ADC analysis and can therefore be expected to be applied for DAR characterization.³¹⁾

Hydrophilic interaction chromatography (HILIC) can assess the glycosylation profile of an intact protein³²⁾ and may be applied to evaluate DAR distribution for ADCs based on appropriately glycosylated antibodies. Columns based on this type of stationary phase has been used for qualitative comparison of the original and biosimilar antibodies^{33,34)}; however, there are currently limited published reports of HILIC analysis applied to ADCs.³⁵⁾

4. LC-QTOF/MS Approach

4.1. RPLC-QTOF/MS The mobile phases of RP-HPLC (RPLC) can be directly injected into an MS through an appropriate interface, and if the instrument is constructed as an RPLC-QTOF/MS, theoretically, all the DAR species of an ADC may be analyzed (Fig. 5). Complete separation of the DAR species is not required for DAR determination, and relatively small amounts of ADC sample (typically approx. 5 μ g) are required for this analysis. These advantages suggest that RPLC-QTOF/MS can be used for initial reaction condition screening studies, such as a design-of-experiments (DOE) optimization.

RPLC-QTOF/MS systems face similar liabilities as the RP-HPLC/UV approach. The use of potentially denaturing conditions (elevated column temperatures and organic solvents in the mobile phases) can degrade ADCs on the column during the analysis. Because of this potential situation, the LC-QTOF/MS methodology may not be appropriate for sensitive antibodies, such as bispecific antibodies or some masked-antibodies.

4.2. SEC-QTOF/MS Size exclusion chromatography (SEC) is a widely compatible and powerful tool for user-

friendly analysis and purification of proteins. Columns based on SEC stationary phases are typically used for aggregation analysis, preparative HPLC, and buffer exchange. Additionally, its compatibility with favorable mass spectrometry conditions has also been confirmed.³⁶⁾ In 2012, Valliere-Douglass *et al.* reported the first application of a combination SEC couple with QTOF/MS for DAR determination.³⁷⁾ The use of an ammonium buffer, which is known to form highly volatile salts, enables the characterization of the DARs under native conditions. Native analysis using SEC-QTOF/MS has recently become one of the popular methods in antibody analysis as reported in the chemical literature.³⁶⁾ However, the ionization efficiency of ammonium cations is relatively weaker than that of formic acid (FA), which is typically used for RPLC-QTOF/MS as the acidic modifier. Because of this characteristic, SEC-QTOF/MS requires a purification step to remove interfering small molecules that have stronger ionization efficiencies than ADCs.²⁰⁾

From an ionization efficacy perspective, SEC-QTOF/MS using mobile phases containing FA is another useful approach for DAR determination despite that this methodology is denaturing analysis. As mentioned above, SEC can separate larger ADC compounds from smaller molecules, such as payloads and linkers, in the column without the need for any other pretreatments. This advantage of denaturing SEC-QTOF/MS enabled the development of *in-situ* monitoring of the conjugation reaction. However, the reliability of the DAR data from denaturing SEC/QTOF-MS has remained questionable. This issue was pointed out by Beck and Cianfèrari's group.³⁸⁾ They compared denaturing SEC-QTOF/MS with native SEC-QTOF/MS for the analysis of Kadcyla[®], a commercially available ADC, and found that native SEC-QTOF/MS provided a reliable DAR, while denaturing SEC-QTOF/MS gave a semi-accurate DAR.³⁸⁾ However, there is no sufficient comparative study or published discussions on the cause of the different DARs given by the two methods.

LC-QTOF/MS methods using denaturing conditions, such as denaturing SEC-QTOF/MS and RPLC-QTOF/MS, require a complicated composition of the mobile phase. For peak resolution, TFA is an essential acid because of the effectiveness as an ion-pairing agent that reduces secondary interactions in the HPLC column. However, this hydrophobic acid does not have strong ionization efficacy. To avoid ion suppression, mobile phases for denaturing LC-QTOF/MS need to have FA, which is a weak ion-pairing modifier and reduces peak separation in the HPLC column. Therefore, there is no commonly used optimal acid modifier, and sub-optimal combinations may have to be employed.

To overcome this issue, difluoroacetic acid (DFA) was recently reported by Nguyen *et al.*³⁹⁾ This acid modifier can increase MS sensitivity while producing higher chromatographic resolution than that of a combination of FA and TFA.

4.3. IEX-QTOF/MS Native-QTOF/MS-methods based on SEC are promising tools for complex analytical separations that does not lead to the denaturation of the ADCs. However, SEC columns provide inadequate peak separation; therefore, when using LC-MS as the detection method, it may be difficult to calculate the DAR distribution from the HPLC chromatogram (Table 2).

Another promising approach for DAR analysis under native conditions is utilizing ion-exchange chromatography

Fig. 5. RPLC/QTOF-MS of Site-Specific ADC

A) total ion chromatogram. B) UV chromatogram (280nm). C) mass spectrum. D) deconvoluted spectrum. (Color figure can be accessed in the online version.)

Table 2. Summary of Four Different LC Modes for ADC Analysis

Characteristics	RPLC	HIC	SEC	IEX
Column Temperature	70–80 °C	RT	RT	RT
MS Compatibility	Yes	Typically, No	Yes	Yes
Separation of Fc and F(ab') ₂	Yes	Unknown	No	Yes
Compatibility for Native Intact Analysis	No	No	Yes	Yes
Compatibility for Native Subunit Analysis	No	No	Limited	Yes
Application to ADC	Known	Known	Known	Limited

(IEX).⁴⁰⁾ IEX can provide an informative chromatogram, including information about the charge variation, glycoforms, and structural alternations.^{41–44)} IEX is often used for protein analysis; however, there are a limited number of reports of its application to ADC analyses published in the literature. Recently, IEX coupled with MS under native conditions was applied for antibody characterization by several groups.^{42,45–48)} These results strongly suggest that this unique native-MS has great potential for use in DAR characterization. In 2020, our research group successfully conducted native IEX-QTOF/MS analysis of a site-specific ADC that resulted in both DAR determination and charge variant characterization in a single analysis.⁴⁹⁾ Furthermore, subunit analysis after enzymatic digestion of the ADC was also performed. These results suggest that the native IEX-QTOF/MS for ADC characterization has great potential.

4.4. Other LC-QTOF/MS Approaches Even though several LC-QTOF/MS methods have been reported, DAR characterization of ADCs remains non-trivial due to several factors, among them structural complexity and heterogeneity. Therefore, exploration of novel mass spectrometric-based methodologies for DAR characterization are needed. Källsten and Bergquist's group reported an analytical methodology for DAR determination using triple quadrupole MS⁵⁰⁾ or matrix-assisted laser desorption ionization (MALDI)-TOF/MS.⁵¹⁾ Very recently, they also reported the impact of sample preparation on MS-based DAR determination.⁵²⁾ In addition to their study, HILIC-MS,³⁵⁾ capillary electrophoresis (CE)-MS,^{53,54)} and affinity chromatography (AC)-MS^{55–58)} are also valuable analytical techniques that can be considered as promising approaches for ADC analysis.

The evolution of LC-MS methods can deliver not only intact protein analysis but also conjugation site analysis, such as peptide mapping, which is an essential tool for drug regulation.^{59–61)} To overcome the issues inherent to conventional QTOF/MS technology, mass spectrometry approaches based on Orbitrap instruments may be promising alternatives due to improvements in resolution.⁶²⁾ MS techniques based on this instrument have already been applied to the analysis of ADC catabolites⁶³⁾ and conjugation-site determination of ADCs.⁶⁴⁾

5. UV/Vis Approach

As described above, LC-QTOF/MS and HPLC/UV approaches are well-established as DAR determination methodologies; however, these techniques can be low-throughput due to time-demands, especially when the samples and sample preparations are handled manually. If the analytical chemists need to measure only the average DAR value for an ADC sample without knowledge of either the DAR distribution or the molecular weight of each DAR species, a simple method relying on UV/Vis absorption measurements can be consid-

ered a useful option.⁶⁵⁾ This analysis requires the payload to possess a different local maximum absorption band from that of the naked antibodies (typically centered at $\lambda = 280$ nm). Based on the absorption at both the maximum value and the respective value of extinction coefficients, the molar concentrations of the naked antibody and payload compounds can be calculated. This technique has been applied for several ADCs based on different ADCs, including maytansinoid payload (DM1)⁶⁶⁾ and MC-VC-MMAE.¹⁵⁾ Recently, Hubbuck and colleagues established an analytical procedure based on the UV-Vis methodology for conjugation reaction monitoring.⁶⁷⁾

Ellman's assay is known as a thiol titration method based on the calculation of the inhibition rate by measuring the absorbance at 412 nm by UV-Vis.⁶⁸⁾ When Ellman's reagent (5,5'-dithiobis (2-nitrobenzoic acid)) reacts with a free sulfhydryl, 2-nitro-5-mercaptobenzoic acid, which has an absorption maximum at 412 nm is formed. This method has been applied to cysteine-based ADCs because the residual thiol group can be measured after conjugation; therefore the DAR can be calculated indirectly.⁶⁹⁾

6. Summary and Future Landscape

This review provides a guidance and describes representative analytical techniques used for DAR characterization with an emphasis on recent advances. HPLC/UV, LC-QTOF/MS, and UV-Vis methods are well-established approaches and are often implemented as in-process control (IPC) in ADC manufacturing processes or for the characterization of ADC samples; however, the heterogeneity and structural complexity of ADCs remain obstacles for establishment of ideal DAR determination method which can be applied for a wide variety of ADCs.

To reduce the heterogeneity seen in ADCs conjugated stochastically and the potentially clinically limiting characteristics that may result, site-specific ADCs will likely continue to have an increased presence in the clinic and literature. DAR characterization of site-specific ADCs is simpler because of their less-complicated composition.⁷⁰⁾ Recently, several non-engineered chemically site-specific conjugation methods, such as using an Fc affinity compound have been reported.⁶⁴⁾ One of the advantages of this technology is that it generates homogeneous DAR by a standard chemical reaction to native antibodies, which results in streamlined reaction sequence, which simplifies IPC.⁸⁾ This approach has already been applied to large-scale ADC synthesis showing its great potential to be applied to a wide variety of next generation ADCs.⁷¹⁾

The ADC analytical field has room for further development and optimization and are needed to keep the pace as new complex large-molecule constructs and formats are developed for clinical therapeutics. As described in this review, intact MS analysis for DAR characterization has been implemented

over the last few years to complement the existing analytical toolbox. However, some challenges have remained, such as sensitivity enhancement and the optimization of the resolution of the chromatogram. To overcome these issues, on-line comprehensive two-dimensional liquid chromatography (LC $\times$ LC or 2D) emerged as an attractive technique.^{72–74} 2D LC combines two different chromatography columns, and thereby enables a rapid and efficient analysis to provide greater resolution for the resulting chromatogram and obtain more information within a single injection. Furthermore, this combination system can be coupled with MS (2D LC/MS) to provide a wide variety of options for ADC characterization.⁷⁴ For example, the combination of HIC $\times$ SEC may provide ideal synergy for DAR determination with non-volatile salts in the first dimension (HIC) and the use of a second dimension (SEC) to desalt and remove the non-volatile salts prior to arrival at the MS chamber.^{75,76} This complex but attractive LC/MS approach could address the analyses of complicated structures of new modalities such as complicated bioconjugates derived from novel drug delivery system,^{77,78} which we expect will continue to diversify in the future.

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