analytical chemistry

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Simultaneous Analysis of the Drug-to-Antibody Ratio, Free-Drug-Related Impurities, and Purity of Antibody−Drug Conjugates Based on Size Exclusion Chromatography

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Cite This: https://doi.org/10.1021/acs.analchem.4c06661 Read Online

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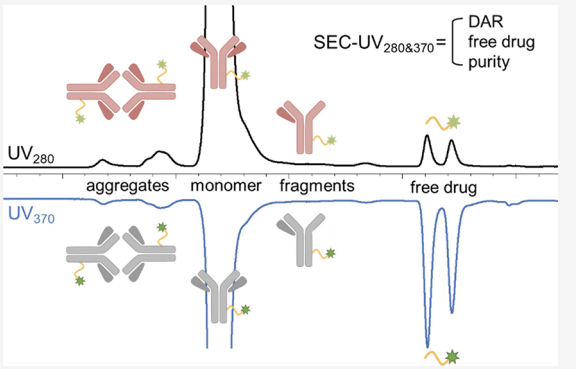
ABSTRACT: Drug-to-antibody ratio (DAR), free-drug-related impurities (FDRI) content, and purity are critical quality attributes of antibody−drug conjugates (ADCs), which substantially impact product safety and efficacy. However, growing efforts in developing ADCs with higher complexity in a faster timeline impose a great challenge to their analytical support. Herein, for the ADC carries an antibody and linker-payload with distinct UV/vis absorption maxima, we propose a high-throughput and multiattribute ADC analysis strategy based on size exclusion chromatography coupled with UV detection (SEC-UV). Briefly, in addition to the quantitation of aggregates and fragments, SEC excludes FDRI from ADC-related peaks and improves the accuracy of DAR determination via dual-wavelength UV detection. Relative FDRI content can be determined subsequently by comparing the free-drug-related and ADC-related peaks at a wavelength where UV absorbance is exclusively attributed to the linker-payload. Afterward, a quantitative consistency in DAR and FDRI analysis was established between SEC-UV and orthogonal methodologies widely adopted by the pharmaceutical industry. The applicability of the developed SEC-UV method was further extended through feasiblity studies on ADC process-intermediates, bispecific ADC, and photodegraded ADC. Despite lacking characterization of drug load distribution and profiling of individual FDRI species, its efficiency, simplicity, and high throughput make SEC-UV a phase-appropriate strategy for supporting in-process monitoring and early-stage process development of diverse ADC projects.



INTRODUCTION

The concept of antibody−drug conjugates (ADCs) was first introduced by Paul Ehrlich in 1913 as a "magic bullet" combining the advantages of chemotherapy and immunotherapy.[1,2] By conjugating a cytotoxic payload to an antibody through a linker, toxins can be specifically delivered to tumor tissues, which favors reducing systemic toxicity.[3] Since the approval of Mylotarg in 2000, 13 ADC products have been approved by the FDA and more than 100 ADC pipelines are under clinical trials,[4,5] indicating ADCs to be a promising class of antitumor therapy.[6,7] However, the inherent structural complexity and heterogeneity of ADCs present considerable technical challenges from an analytical and quality control perspective. From the standpoint of the pharmaceutical industry, development of a phase-appropriate analytical toolbox to support the process development of diverse ADC candidates has been a long-standing challenge.

Among the critical quality attributes of ADCs, drug-to-antibody ratio (DAR), which represents the average number of drugs conjugated to an antibody, is closely related to product efficacy. A lack of drug loading can depress potency, while elevated DAR might pose risks in safety, stability, and pharmacokinetics.[8−10] Meanwhile, residual unconjugated free-drug and related impurities, referred to as free-drug-related impurities (FDRI), may remain in the ADC product and lack the specificity to reach a desired target. The cytotoxic nature of the free drug necessitates control of the FDRI content throughout the lifetime of ADC products from in-process monitoring to release and stability tests.

State-of-the-art analytical techniques have been evolving to determine DAR, and most of them fall into three representative categories: UV/vis spectroscopy, liquid chromatography coupled with UV detection (LC-UV), and liquid chromatography coupled with mass spectrometry (LC-MS).[11−13] For a majority of ADC products in commercial and clinical stages, their linker-payload (LP) motif frequently

Received: December 9, 2024
Revised: March 16, 2025
Accepted: April 16, 2025

exhibits a unique UV/vis absorbance behavior compared to the antibody. When light-absorbing properties of the antibody and drug are not altered radically by the conjugation reaction, the absorbance of the ADC can be considered as the sum of absorbances contributed by the antibody and LP motif.[14] Therefore, antibody and drug concentrations together with the DAR value can be calculated by measuring the UV/vis absorbance of the ADC at two distinct wavelengths.[15,16] So far, the UV/vis method has been accepted broadly as a process analytical technology (PAT), but it typically lacks accuracy because FDRI can impact DAR determination.[17,18]

Through conjugation with LP, an alternation in hydrophobicity is commonly anticipated for the antibody; hence, different DAR species can be resolved with reversed-phase chromatography (RP) and hydrophobic interaction chromatography (HIC).[19,20] Peak assignment of HIC- or RP-separated species can be accomplished by comparing the UV signal at the absorbance maxima of the antibody and LP, respectively. Alternatively, the RP can be interfaced with an MS detector for direct peak identification, whereas multidimensional LC-MS strategies have been devised for online structural identification of HIC-separated peaks.[21,22] Furthermore, by preserving the native confirmation of ADC, SEC-MS techniques enable the determination of drug-load distribution (DLD), unconjugated antibody, and DAR simultaneously,[23−25] and the resulting DAR demonstrates quantitative equivalency with the HIC method.[26]

On the other hand, disparate studies concerning the characterization and quantification of FDRI have been performed broadly, among which RP-LC is predominantly employed by pharmaceuticals.[16] Absolute quantitation of RP-separated FDRI species with an external calibration curve and calculating a relative FDRI content by comparing the peak areas of FDRI and ADC are two leading routes of FDRI analysis.[27] In addition, multiple researchers have demonstrated the quantitation and characterization of FDRI with 2D-LC techniques, including SEC-RP[28] and SPE-RP,[29] where the removal of the ADC is achieved online through the first dimension of separation with an SEC or SPE column, and FDRI is diverted to the second dimension to profile its components with RP-LC, followed by quantification and characterization with UV and MS detectors. Recently, quantitation of FDRI has been further simplified to 1D-LC, where FDRI resolved in the SEC column is quantified directly with an external calibration curve, and a promising assay performance is established.[30]

Here, for an ADC composed of an antibody and LP exhibiting unique UV absorbing maxima, we extended the capability of SEC beyond the analysis of FDRI and enabled a simultaneous determination of the DAR value with high throughput. In addition to online ADC removal for FDRI quantitation, SEC can also eliminate the interference from FDRI on DAR determination with UV/vis. The aggregates, monomer, and fragments of the ADC were monitored at the UV absorbing maxima of the antibody and LP, and the total peak areas of ADC-related components were integrated to determine the average DAR value. Subsequently, instead of calculating an absolute FDRI content with an external calibration curve, the relative content of FDRI was determined by comparing the peak areas of ADC and FDRI, which can reduce the risk of exposure to OEB5 (occupational exposure band-5) materials.

In this article, the proposed SEC-UV-based ADC analysis strategy was evaluated in terms of quantitative consistency with orthogonal methodologies. The applicability of the method was subsequently evaluated through the analysis of process intermediates and force-degraded materials. Though lacking the characterization of DLD, unconjugated antibody, and individual FDRI components, the developed method can support diverse ADC projects with minimal cost in method development prior to the readiness of project-specific ADC analysis methods. Other than a phase-appropriate support of early-stage process development, the simplicity and throughput of SEC-UV enable in-process monitoring of process intermediates and alleviate the workload in supporting cutting-edge ADC projects.

EXPERIMENTAL SECTION

Materials and Reagents. The following materials were used: ammonium sulfate, DL-dithiothreitol, dimethyl sulfoxide, and tris(2-carboxyethyl) phosphine (TCEP) were purchased from Sigma-Aldrich. Formic acid and trifluoroacetic acid were purchased from Fisher Scientific. The antibody and LP were generated in-house by BeiGene. We used the following instruments and columns: Acquity Premier UPLC system, ARC Premier UHPLC system, Acquity Premier Protein SEC, 250 Å, 1.7 μm, 4.6 × 150 mm column, BioResolve RP mAb Polyphenyl, 450 Å, 2.7 μm, 2.1 × 150 mm column, BioResolve SEC mAb, 200 Å, 2.5 μm, 7.8 × 300 mm column from Waters; BioCore HIC-Phenyl, 5 μm, 4.6 × 100 mm column from NanoChrom, DAWN and OptiLab detector from Wyatt.

Preparation of the ADC. Cysteine-conjugated ADCs were produced in-house. Refer to the Supporting Information for details.

Determination of UV Extinction Coefficients. The LP standard was diluted with 20 mM histidine buffer (pH 5.5) to six concentration levels. Each diluted standard was measured by SoloVPE at 280 and 370 nm in fixed mode. The UV extinction coefficient of LP at 280 and 370 nm was obtained by calculating the slope of the standard curve. The theoretical UV extinction coefficients of antibodies were calculated with the ProtParam tool of the Expasy database.

SEC-UV Analysis to Determine DAR, FDRI Content, and Purity. Antibody and ADC samples were diluted to 2 mg/mL with the SEC mobile phase (50 mM sodium phosphate, 200 mM arginine-HCl, 10% ACN, pH 6.8) prior to analysis. SEC-UV was performed with an Acquity Premier Protein SEC column with an isocratic elution of 0.25 mL/min. Data was collected by UV detection at 280 and 370 nm.

SEC-MALS Analysis. ADC and antibody materials were analyzed with SEC-MALS with a Wyatt DAWN and Optilab detector. Refer to the Supporting Information for experimental details.

RP-UV Analysis to Determine DAR. A 100 μg aliquot of the ADC sample was dissolved in 10 mM Tris-HCl (pH 8.0) and 25 mM DTT to reach a final concentration of 1 mg/mL. Reduction reaction was allowed to proceed at 37 °C for 30 min, and the resulting samples were separated with a BioResolve RP mAb Polyphenyl column. A mobile phase of 0.05% TFA in H_2O/ACN was used for elution. UV detection was performed at 280 nm, and peak assignment was accomplished by a parallel LC-MS experiment. The DAR values of light and heavy chains were calculated separately by incorporating integrated peak areas and the assigned drug load

https://doi.org/10.1021/acs.analchem.4c06661
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for each peak. Subsequently, a weighted average DAR was determined by summing the DAR of light and heavy chains.

RP-MS Analysis to Determine DAR. Reduction of ADC was performed according to the condition used for the RP-UV experiment. Samples were separated via a BioResolve RP mAb Polyphenyl column with 0.1% FA in H_2O/ACN as mobile phases. MS data were collected with an online Xevo G3 QTOF detector and processed with UNIFI software. The method for determining DAR was consistent with RP-UV, but the area of detected ions was utilized for calculation.

HIC-UV Analysis to Determine DAR. ADC samples were diluted in PBS buffer to 2 mg/mL, followed by separation on a BioCore HIC-Phenyl column. Elution was performed with 1.5 M ammonium sulfate in 30 mM phosphate buffer (pH 7.0) as mobile phase A and 20% ACN in 30 mM phosphate buffer (pH 7.0) as mobile phase B. Different DAR species were separated and quantified by UV detection at 220 nm. Percentage peak area combined with the corresponding drug load number of each peak was used to calculate the weighted average DAR.

RP-UV Analysis to Determine FDRI Content. A calibration curve was prepared with a serial dilution of LP. The RP-UV experiment was performed on a UHPLC system via a BioResolve RP mAb Polyphenyl column. ADC samples were directly injected, and elution was conducted with 0.1% TFA in H_2O/ACN. Detection and quantitation were achieved by UV detection of FDRI at 370 nm.

RESULTS AND DISCUSSION

In this article, we demonstrate the development of a high-throughput ADC analysis method, enabling the measurement of the DAR value, FDRI content, and purity simultaneously. The developed SEC-UV-based ADC analysis strategy shows to be feasible for supporting in-process testing and early-stage process development of ADC projects.

Principle of Multiattribute ADC Analysis by SEC-UV. UV/vis has been employed broadly for DAR analysis, but its accuracy is often limited by unconjugated free-drug within ADC materials. Herein, we propose a SEC-based DAR analysis method, where FDRI can be eluted around the total inclusion volume of the SEC column and remains a minor impact on the UV signal of ADC components. Here, an in-house-prepared cysteine-conjugated ADC material, ADC-A, was used to demonstrate the feasibility of the proposed SEC-UV method. As shown in Figure S1, free antibody and LP showed maximum UV absorption at 280 and 370 nm, respectively. No apparent alternation in light-absorbing properties can be observed post conjugation reaction. Afterward, the UV extinction coefficient of LP at 280 and 370 nm was determined by SoloVPE analysis, while the calculated theoretical UV extinction coefficient of the antibody was utilized. As shown in eq 1, both antibody and LP contribute to the UV peak at 280 nm (A_{280}), whereas LP contributes exclusively to the peak area at 370 nm (A_{370}), since the antibody exhibits undetectable UV absorbance at 370 nm:

$$A_{280} = \varepsilon_{\mathrm{mAb}-280}\cdot C_{\mathrm{mAb}}\cdot L + \varepsilon_{\mathrm{LP}-280}\cdot C_{\mathrm{LP}} L$$
$$A_{370} = \varepsilon_{\mathrm{LP}-370}\cdot C_{\mathrm{LP}}\cdot L \quad (1)$$

where A is the UV peak area, ε is the extinction coefficient, C is the mass concentration, L is the light path, mAb represents the antibody, and LP represents the linker-payload.

Therefore, the ratio between A_{280} and A_{370} follows eq 2:

$$\frac{A_{280}}{A_{370}} = \frac{\varepsilon_{\mathrm{mAb}-280}}{\varepsilon_{\mathrm{LP}-370}}\cdot\frac{C_{\mathrm{mAb}}}{C_{\mathrm{LP}}} + \frac{\varepsilon_{\mathrm{LP}-280}}{\varepsilon_{\mathrm{LP}-370}} = \frac{\varepsilon_{\mathrm{mAb}-280}}{\varepsilon_{\mathrm{LP}-370}}\cdot\frac{M_{\mathrm{mAb}}\cdot W_{\mathrm{mAb}}}{M_{\mathrm{LP}}\cdot W_{\mathrm{LP}}} + \frac{\varepsilon_{\mathrm{LP}-280}}{\varepsilon_{\mathrm{LP}-370}} \quad (2)$$

where M is the molar concentration and W is the molecular weight. The DAR value, which equals the ratio of molar concentration between LP and antibody, can be applied to obtain eq 3:

$$\frac{1}{DAR} = \frac{M_{mAb}}{M_{LP}} = \left(\frac{A_{280}}{A_{370}} - \frac{\varepsilon_{LP-280}}{\varepsilon_{LP-370}}\right)\cdot\frac{\varepsilon_{LP-370}\cdot W_{LP}}{\varepsilon_{mAb-280}\cdot W_{mAb}} \quad (3)$$

Additionally, when DAR analysis is performed with SEC-UV, FDRI can be separated from ADC (Figure 1). Therefore,

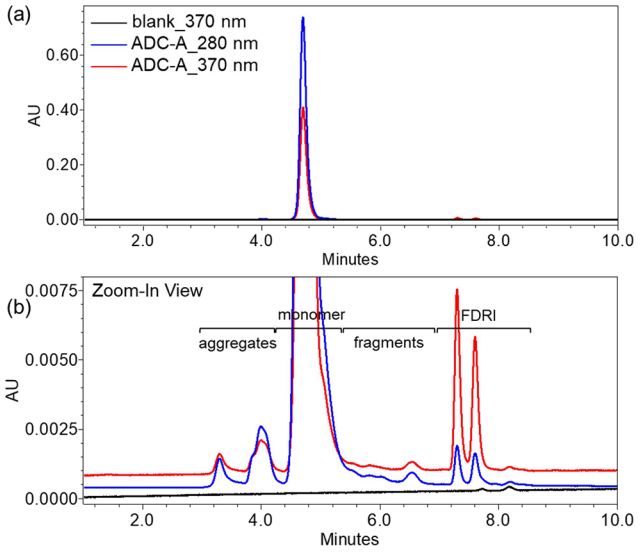

Figure 1. (a) SEC chromatograms of ADC-A with UV detection at 280 and 370 nm. A chromatogram of the blank detected at 370 nm is included to illustrate system peaks. (b) Zoom-in view to focus on aggregates, fragments, and FDRI-related peaks.

it is feasible to quantify the FDRI content together with the DAR value. For UV detection at 370 nm, mAb-A shows negligible absorbance, and only minimal system peaks are observed in the chromatogram of the blank. Therefore, the peak areas of FDRI and conjugated LP are proportional to their molar concentration, which can be implemented directly to quantify the relative FDRI content with eq 4:

$$\text{FDRI in } \frac{\text{mol}}{\text{mol}}\% = \frac{A_{370_{\mathrm{FDRI}}}}{A_{370_{\mathrm{FDRI}}} + A_{370_{\mathrm{ADC}}}} \times 100\% \quad (4)$$

where $A_{370_{\mathrm{FDRI}}}$ represents the total peak area of FDRI peaks at 370 nm and $A_{370_{\mathrm{ADC}}}$ represents the total peak area of the conjugated LP.

In the end, purity information, including aggregates and fragments, can be readily available with SEC separation. For confirmation, SEC-MALS analysis of mAb-A and ADC-A was performed, and aggregates in both samples were potentially dominated by dimers (Figure S2). Subsequently, multiple ADC-A and ADC-B materials were characterized with SEC-UV. Adequate separation among aggregates, monomer, and fragments was observed (Table S1). Considering the

https://doi.org/10.1021/acs.analchem.4c06661
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heterogeneity of mAb and resulting ADC, the peak areas of aggregates, monomer, and fragments were summed and used for the following calculation of DAR and FDRI.

Evaluation of Assay Performance. The performance of the SEC-UV method was first evaluated for the measurement of DAR (Table 1). In terms of loading amount, precision, and solution stability, ADC-A materials with relatively low, middle, and high DAR were analyzed to evaluate the suitability of the developed method. As shown in Table 1, adequate precision and solution stability in DAR determination are established. Moreover, considering that UV detection at two wavelengths is involved, UV detection with Waters TUV and PDA detectors was compared, and no apparent instrumental impact on DAR is observed (Table S2).

Table 1. Evaluation of Assay Performance on DAR Analysis

loading		precision		solution stability	
injection	DAR	prep	DAR	hold time	DAR
10 μg	4.49	1	6.31	0 h	7.99
14 μg	4.49	2	6.29	27 h	7.99
20 μg	4.48	3	6.28	74 h	7.99

Subsequently, process intermediates with various levels of FDRI were utilized for the evaluation of the assay performance on FDRI content (Table 2). For the range of loadings demonstrated for DAR analysis, appropriate robustness is observed. Considering a simultaneous measurement of DAR value and FDRI content, diluted ADC materials can be held in an autosampler (2−8 °C) for up to 74 h prior to injection.

Table 2. Evaluation of Assay Performance on FDRI Content Analysis

loading		precision		solution stability	
injection	FDRI	prep	FDRI	hold time	FDRI
10 μg	0.09%	1	2.35%	0 h	7.18%
14 μg	0.08%	2	2.37%	23 h	7.15%
20 μg	0.09%	3	2.39%	89 h	7.16%

SEC-UV vs Conventional DAR Analysis Methods. The developed SEC-UV-based DAR method was compared with other well-established DAR characterization approaches, including HIC-UV, RP-UV, RP-MS, and UV/vis, to demonstrate its accuracy (Table S1). Over the past few decades, HIC has been adopted extensively for DAR measurement. The HIC method was developed for ADC-A material, and adequate separation is achieved for DAR0, DAR2, DAR4, DAR6, and DAR8 species (Figure 2a). Afterward, 18 ADC-A samples with DAR ranging from 3 to 8 were analyzed with HIC-UV and SEC-UV. SEC-UV succeeds in establishing a quantitative consistency with HIC-UV in DAR measurement (Figure 3a). Other than the DAR value, the DLD and content of the unconjugated antibody become unavailable by simply coupling SEC with a UV detector. Future studies on integrating SEC-UV with a MS detector may broaden the capability of this multiattribute analysis technique to DLD and unconjugated antibody analysis.

RP-UV was subsequently compared with SEC-UV for the DAR determination. Light and heavy chains with different amounts of LP conjugated were separated with moderate resolution via the developed RP-UV method (Figure 2b). Peak assignments of DAR profiles were achieved by online mass

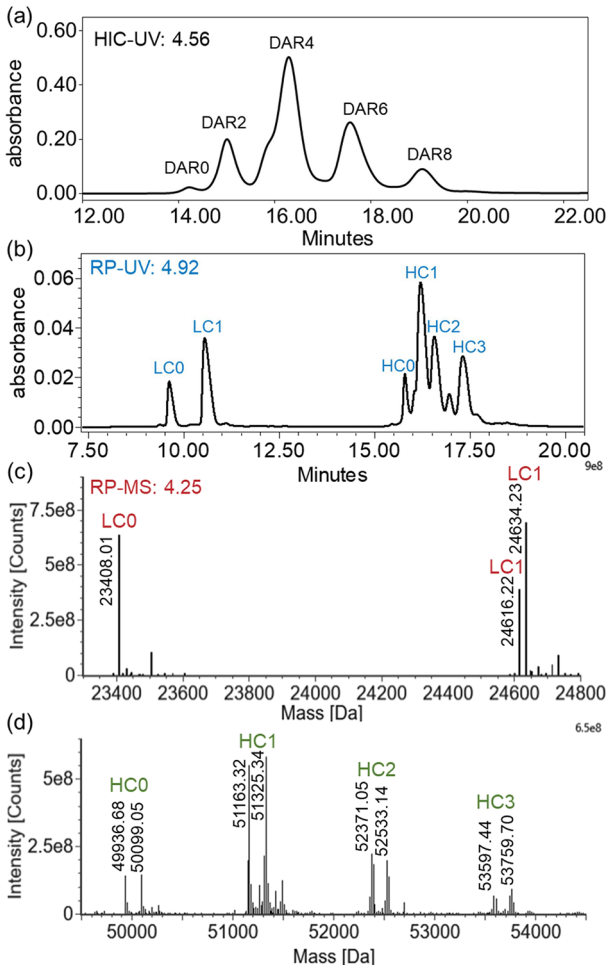

Figure 2. Chromatograms and mass spectra of ADC-A analyzed with (a) HIC-UV, (b) RP-UV, and (c, d) RP-MS. Deconvoluted mass spectra of light chains and heavy chains are presented in (c) and (d), respectively.

spectrometry detection (data not shown) and comparison of the UV signal collected from the absorbance maxima of the antibody and LP. Again, the quantitative consistency between SEC-UV and RP-UV was evaluated, and the results are shown in Figure 3b. Compared to the native condition used by SEC, the denaturing condition of RP might induce hydrolysis, fragmentation, and even loss of LP. These heterogeneity in LP may result in limited resolution among light and heavy chains with LP. Particularly, for cysteine-conjugated ADCs, samples with a moderate DAR value, DAR3−5 for ADC-A, typically exhibit higher heterogeneity and suffer significantly from factors impacting assay accuracy. Though ADCs with various degrees of conjugation are barely resolved in SEC, they are mostly free from degradation, and thus, an average DAR with generally higher accuracy can be determined with SEC-UV.

Mass spectrometry has been an incomparable technique in the identification of ADC products. RP-MS was subsequently compared with SEC-UV to calculate the DAR (Figure 2c,d). The incorporation of an MS detector enables the identification of various light- and heavy-chain species without ambiguity. Post conjugation with the antibody, maleimide-containing LP might undergo hydrolysis; hence, a peak with +18 Da is anticipated for each LP. For heavy chains, heterogeneity in N-glycosylation adds to the complexity of the mass spectrum. As expected, inconsistency in ionization efficiency of DAR

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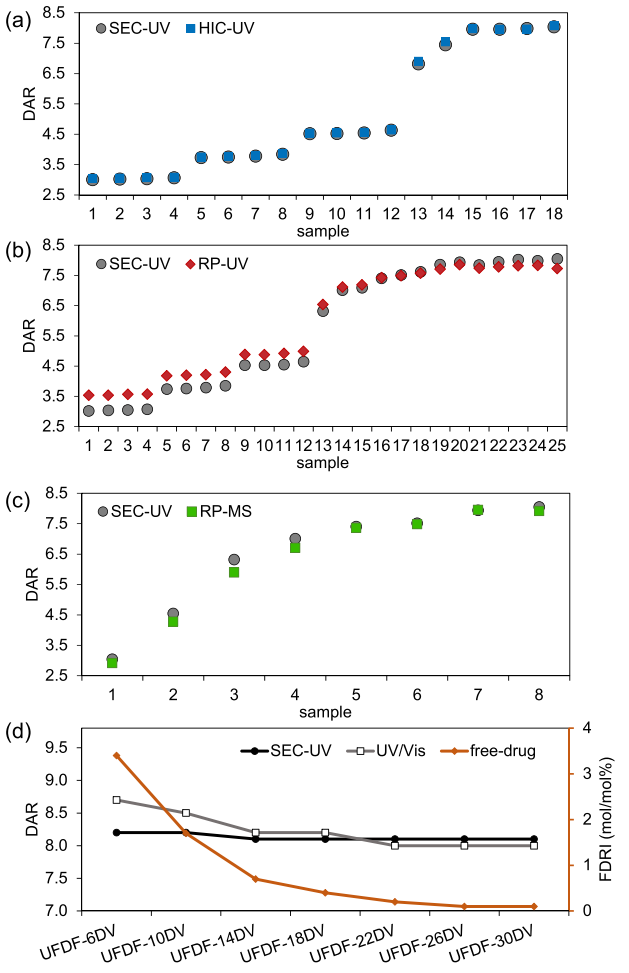

Figure 3. Comparison of the DAR value of ADC-A measured by SEC-UV with (a) HIC-UV, (b) RP-UV, (c) RP-MS, and (d) UV/vis.

variants in MS detection limits the implementation of the RP-MS method on DAR measurement.[31] For samples with relatively high drug load, higher homogeneity and good consistency between RP-MS and SEC-UV can be observed (Figure 3c). Meanwhile, a considerable discrepancy in DAR value (~0.3) is detected for samples with an average DAR around 4, as they exhibit higher heterogeneity in DLD and suffer significantly from inconsistency in ionization efficiency.

UV/vis has been predominantly accepted as a PAT support of ADC process development due to its advantages of throughput and operability. However, overestimation in DAR can be expected for ADC intermediates prior to a thorough ultrafiltration step to eliminate the impact from FDRI. In contrast, SEC enables separation of FDRI from the ADC, and an accurate estimation of DAR can be achieved with SEC-UV analysis. As shown in Figure 3d, with less than 14 diafiltration volumes (DV) of ultrafiltration/diafiltration (UFDF), a considerable amount of FDRI remains and results in overestimated DAR by UV/vis analysis. On the other hand, comparable DAR values are measured from ADC intermediates via SEC-UV, implying SEC-UV to be a promising in-process monitoring strategy to support the DAR analysis of both ADC and its process-intermediates.

Other than the monospecific ADC, an increasing effort to develop bispecific and trispecific ADCs has been observed in the field of therapeutic proteins.[32,33] Development of a DAR method with HIC-UV and RP-UV for such a complex ADC could be challenging because the assay accuracy will be limited by the resolution of separation. Meanwhile, the difficulty in method development can be significantly minimized for a SEC-UV-based DAR method. Here, LP is conjugated to a bispecific antibody with a knob-into-hole and common light chain structure. HIC-UV and RP-UV methods were developed for this bispecific ADC (Figure 4), which is referred to as ADC-B.

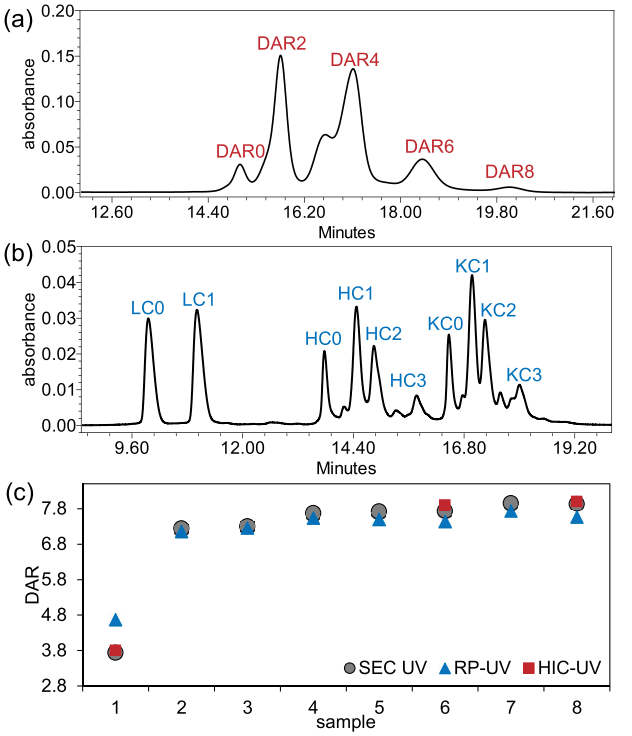

Figure 4. Chromatograms of a bispecific ADC, ADC-B, analyzed with (a) HIC and (b) RP-UV for the DAR value. (c) Comparison of the DAR values analyzed with SEC-UV, RP-UV, and HIC-UV.

Again, SEC-UV analysis was utilized to characterize the DAR value of ADC-B samples. As shown in Figure 4c and Table S1, good consistency can be observed between HIC-UV and SEC-UV, indicating that SEC-UV can be a feasible approach to characterize DAR values for bispecific ADCs. Consistent with ADC-A, RP-UV overestimates DAR for a ADC-B material with a moderate DAR value, of which the potential causes were discussed previously.

To conclude, the developed SEC-UV method enables high-throughput DAR analysis for monospecific ADCs, bispecific ADCs, and ADC process-intermediates, revealing its feasibility in supporting in-process tests and early-stage ADC process and formulation development. Additionally, SEC-UV can provide a comparable DAR value with HIC-UV, RP-UV, RP-MS, and UV/vis measurements, making the bridging from early-stage ADC support to release and stability method effortless.

SEC-UV vs RP-UV in FDRI Content Analysis. Incomplete conjugation and ADC degradation typically result in FDRI, which can significantly impact ADC product safety and requires close monitoring over process and formulation development. The feasibility of quantifying FDRI with SEC-UV is demonstrated with a series of ADC process-intermediates. As shown in Figure 5a, ADC-A prior to UFDF, referred to as UFDF-load, exhibits intense free-drug-

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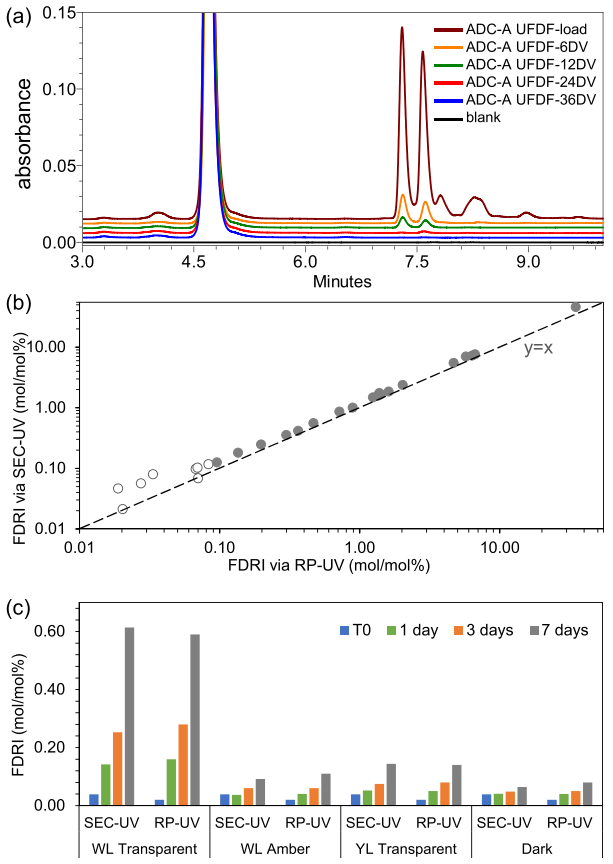

Figure 5. (a) SEC chromatograms of ADC-A process-intermediates. (b) Comparison between FDRI content measured with SEC-UV and RP-UV. Good consistency can be observed for samples with ≥0.10% free drug. (c) FDRI contents of ADC-A stability samples analyzed with SEC-UV and RP-UV. WL and YL represent containers exposed to white and yellow light, respectively. Transparent and amber represent samples stored in transparent and amber containers, respectively. A set of ADC-A stored in the dark was used as a negative control.

related peaks from 7.4 to 9.0 min. Subsequently, with the progression of UFDF, a decreasing amount of FDRI can be observed and significant clearance of FDRI can be achieved at 36 DV.

Subsequently, SEC-UV was compared with RP-UV for the determination of FDRI content (Figure S3 and Table S3). Multiple peaks were assigned to free-drug-related components in both assays, which might originate from the manufacture and storage of the linker, payload, LP, and ADC.[34] Comparable FDRI contents were detected (Figure 5b), and discrepancy was solely present in ADC with low FDRI content (<0.10% in mol/mol). Consequently, the limit of quantitation (LOQ) of SEC-UV-based FDRI content assay is set to 0.10% (mol/mol), which can meet the requirements of the ICH Q3A guideline and most ADC process and formulation development. Compared to RP-UV, the developed SEC-UV method can report FDRI content with a 10 min UPLC run consuming barely 15 μg of ADC. Risk of exposure to OEB5 material is mitigated in the SEC-UV method by substituting the external calibration curve with relative FDRI content analysis. Compared to RP-UV, SEC-UV is devoid of separation, characterization, and quantitation of individual FDRI components. Concerning the fact that most FDRI species generally share similar structure and extinction coefficient, reporting a total FDRI content can significantly enhance the efficiency of in-process monitoring. As for a path forward to characterize individual FDRI species, MS can be coupled with the current SEC-UV setup for further control of impurities.

In the end, the developed SEC-UV method is adopted to investigate the photostability of ADC-A. As shown in Figure 5c, with a set of ADC-A kept in the dark as a negative control, a substantial increase in FDRI is observed for samples exposed directly to white light in a transparent container. Yellow light or amber container can protect ADC-A from photoinduced degradation. Samples were analyzed with RP-UV in parallel, and a comparable trend is observed, indicating SEC-UV as a feasible approach to monitor the stability of ADC materials.

CONCLUSIONS

In terms of early process and formulation development, analytical support for ADC materials with high complexity, diversity, and quantity has been a long-standing challenge. The development of delicate DAR and FDRI analysis methods is mostly labor-intensive and time-consuming. Preceding the establishment of a comprehensive ADC analysis package, DAR, FDRI content, and purity are three key attributes impacting the optimization of the ADC process and formulation. Herein, for ADCs with LP possessing distinct UV absorption behavior from antibodies, we demonstrate an ADC analysis strategy to support early-stage process and formulation development with the following advantages: (1) High-throughput and multi-attribute analysis of ADC can maximize the efficiency of the analytical support. (2) Minimal method development is required to support diverse ADC molecules, enabling the support of process development from the very beginning. (3) Benefiting from the exclusion of complex matrix components, SEC-UV is applicable to support in-process monitoring of the ADC. (4) SEC-UV presents quantitative consistency with orthogonal methodologies, simplifying the bridging of the methods. Consequently, the developed multiattribute ADC analysis method can be a promising approach to support early-stage ADC process and formulation development, benefiting the exploration of challenging ADC products.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.4c06661.

Additional experimental details, chromatograms of ADC-A analyzed with SEC-UV and RP-UV, and tables summarizing the purity, DAR value, and FDRI content of ADC materials (PDF)

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https://doi.org/10.1021/acs.analchem.4c06661
Anal. Chem. XXXX, XXX, XXX−XXX

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The manuscript was written through contributions of all authors.

Notes

The authors declare no competing financial interest.

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