**In-situ** Reverse Phased HPLC Analysis of Intact

Antibody-Drug Conjugates

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ABSTRACT

The field of oncology has recently seen an exponential growth in antibody-drug conjugates (ADCs) as a biopharmaceutical class with seven ADCs being launched onto the market in the last ten years. Despite the increase in the industrial research and development of these compounds, their structural complexity and heterogeneity continue to present various challenges regarding their analysis including reaction monitoring. Robust and simple reaction monitoring analysis are in demand in the view of at-line in-process monitoring, and can instill control, confidence and reliability in the ADC manufacturing process. Aiming at providing chromatographic methods for conjugation monitoring, we evaluated herein the potential of utilizing reverse phase HPLC analysis, without sample pretreatment, for characterization of traditional cysteine-based ADCs. This analysis can be used for estimation of drug antibody ratio (DAR), which has shown the same trends and results as other well-established HPLC techniques. This methodology was also applied to three ADCs derived from three different antibodies. Additionally, we analyzed unpurified ADC samples existing in a complex reaction matrix and separated ADC species and payload compounds. This investigation was conducted using three different ADCs based on different payloads. The results described herein indicate the potential application of this RP-HPLC methodology in reaction monitoring studies.

KEYWORDS: Antibody-Drug Conjugate, Cysteine Conjugation, Reverse Phase HPLC, in-situ analysis, In Process Control
Introduction

Antibody-drug conjugates (ADCs) have rapidly become one of the major approaches for cancer therapy in recent years. To date, nine ADCs are on the market and more than 85 ADCs are in clinical stage trials. 1-3

ADCs consist of a recombinant monoclonal antibody, which has specificity to bind to the target cell, conjugated to a highly potent drug through covalent bond. Various methods have been developed to construct this linkage between an antibody and a payload. The well-established synthetic approach is based on nonspecific drug conjugation using reduced interchain cysteines.4-6 While this conjugation technology provided four commercially available ADCs, analysis for these cysteine-based ADCs remains a challenge due to their heterogeneity.7 Recently, the importance of analytical strategy was discussed in several literatures. 8-10 Due to the structural complexity of ADCs, multiple analytical methods are required for analysis depending on the project type and reaction stage (SI, Table S1). In the reaction screening stage, data accuracy is not a strict requirement due to limitations in the quantity of the ADC samples. The objective for this stage is simply to obtain a “green light” or “red light”. High throughput analysis is of ultimate importance in this stage, therefore crude analysis, which requires no sample preparation and/or pretreatment, is preferred. In the case of later stages, the analytical objective is different. For reaction monitoring like an in-process control (IPC), data accuracy is not as crucial since the purpose is to see the status of the reaction. On the other hand, the final ADC product requires thorough and accurate analysis through the utilization of several different analytical methods. For the IPC in ADC manufacturing, real-time DAR analysis for conjugation reaction is important, however, there are very limited reports explaining ADC monitoring, to the best of our knowledge.9,11-13 Furthermore, no examinations have shown the monitoring of conjugation reaction by direct HPLC technology requiring no sample treatment and/or preparation before injection. To minimize the workload of the manufacturing operators, more user friendly and rapid monitoring methods are in demand.
HPLC analysis is commonly used for DAR characterization because of the hydrophobicity of the payloads. Hydrophobic interaction chromatography HPLC (HIC-HPLC) and reduced reverse phase HPLC (RP-HPLC) are routinely used for DAR analysis of cysteine-based ADCs, but previous comparison studies have reported DAR discrepancies between the two techniques.\textsuperscript{8-10,14-16} Additionally, these HPLC methods are not suitable for reaction monitoring without pretreatment.\textsuperscript{17} HIC-HPLC requires a purification step to remove hydrophobic molecules such as payloads that have the potential to clog the column, while reduced RP-HPLC requires a pretreatment step to reduce the ADC samples.

With the goal of providing a reliable HPLC technique for in-situ monitoring of ADC conjugation, we decided to demonstrate reverse phase HPLC analysis for intact cysteine-based ADCs without any sample pretreatment. Limited reports in the chemical literature examine RP-HPLC for intact ADC characterization\textsuperscript{18,20} and no investigation of application for unpurified cysteine-based ADC analysis without any pretreatment has been reported in peer-reviewed literature to our knowledge.

When we began this investigation, the intact RP-HPLC technique was used for only site-specific ADCs produced by antibody engineering methods,\textsuperscript{18,19} which are the preferred models to demonstrate new analytical techniques due to their simple composition. However, the most well-established approach to synthesize ADCs in the market is through cysteine-based conjugation technology, therefore it was required to apply this HPLC approach for the characterization of cysteine-based ADCs. In 2019, Wirth and co-workers reported excellent methodology to apply RP-HPLC analysis for intact and purified cysteine-based ADCs.\textsuperscript{20} To the best of our knowledge, this is the first example of RP-HPLC for intact cysteine-based ADC characterization. Furthermore, Wirth’s group succeeded in the application of a RP-HPLC method for native MS analysis. 50 mM ammonium acetate, which is a well-known mass spectrometry (MS) compatible buffer, was used for mobile phases in order to be applied for native RP-HPLC-MS. This method allowed DAR characterization and distribution in a single analysis; however, Wirth’s group evaluated only one ADC with this RP-HPLC technique and did not investigate unpurified ADC analysis. Typically, payload related compounds have higher hydrophobicity which increases the risk of clogging the column, therefore, careful
mobile phase investigation is considered to be essential for crude ADC analysis. These limitations and the potential demand for in-situ conjugation monitoring prompted us to investigate RP-HPLC analysis that can be applicable to crude ADCs.

Here we reported the feasibility study of RP-HPLC analysis for intact cysteine-based ADCs. The reliability of this methodology was confirmed by three different ADCs produced by three different reduction conditions. The peak distributions obtained from the resulting ADCs provided reasonable trends that aligned with previous DAR information generated by reduced RP-HPLC analysis. This methodology was also applied to three different ADC syntheses, derived from three antibodies having different isoelectric points (PIs). Furthermore, a feasibility study using this methodology for crude ADC analysis was also performed. This crude analysis was applied to three different ADCs derived from three different payloads to demonstrate that this strategy has the potential for IPC use in ADC manufacturing.

Materials and Methods

Materials

IgG1 trastuzumab (commercial name: Herceptin®) and rituximab (commercial name: Rituxan®) were purchased from Roche Pharmaceutical Company (Switzerland). Human IgG1 infliximab (commercial name: Humira®) was purchased from Sigma-Aldrich (USA). MC-VC-MMAE (CAS#: 646502-53-6) was purchased from NJ Biopharmaceuticals LLC (USA). SMCC-DM1 (CAS#: 1228105-51-8) and MC-MMAF (CAS#: 1228105-51-8) were purchased from Abzena (USA). T-DM1 (commercial name: Kadcyla®) was acquired from Genentech (USA) and reconstituted to 5 mg/mL formulation buffer (20 mM histidine containing 5% trehalose, pH 5.2) by gel-filtration. All other chemical reagents were acquired from Sigma-Aldrich (USA).

General synthetic procedure for cysteine-based ADC
The IgG1 antibody (1.0 mg) was dissolved in water and then buffer exchanged into conjugation buffer (0.25 mL, pH 7.5, 50 mM PBS, 10 mM EDTA) to prepare for the conjugation process. The reduction reaction began with the addition of a defined molar ratio of tris-(2-carboxyethyl)-phosphine hydrochloride (TCEP-HCl) to antibody which was stirred mildly for 2.5 h at 20 °C. Dimethylacetamide (DMA) (8% v/v) and 7 eq. of drug-linker were sequentially added to the resulting reaction mixture and stirred mildly for 1 h at 20 °C. Unreacted drug linker was quenched with the addition of 25 eq of N-Acetyl-L-cysteine (NAC) and mixed for 25 min at 20 °C. The final mixture was purified using NAP-10 desalting columns (purchased from GE Healthcare Life Sciences, USA) and eluted with pH 5.2, 20 mM histidine, 5% trehalose.

**Protein concentration**

Protein concentration was determined by the Slope Spectroscopy® method with a Solo-VPE system as previously reported.²¹

**RP-HPLC analysis for reduced ADCs (Condition A)**

RP-HPLC analysis was performed based on previously reported literature.²² Each sample was prepared as follows: 1.0 mg/mL of ADCs in 500 mM tris buffer, pH 7.5, was diluted to 0.6 mg/mL in 8 M guanidine HCl, and reduced by addition of 1 M DL-Dithiothreitol (DTT). The mixture was incubated at 80 °C for 10 min and was analyzed using AdvanceBio RP-mAb Diphenyl, 2.1 × 100 mm, 3.5 μm column (Agilent), connected to an Agilent 1260 HPLC system containing a binary gradient pump, temperature-controlled column compartment, autosampler, and a diode array detector. The system conditions were as follows: flow rate = 0.4 mL/min at 70 °C; mobile phase A (MPA) = 0.1% trifluoroacetic acid (TFA) and 2% acetonitrile in water; mobile phase B (MPB) = 0.1% TFA in acetonitrile. The absorbance was monitored at 280 nm (reference wavelength at 450 nm). Each ADC (20 μL) was injected into the system and eluted over a 35 min run consisting of a 2 min isocratic hold at 30% MPB, a 22 min linear gradient from 30% to 48% MPB, a 3 min wash using 95% MPB, and an 8 min re-equilibration at 3 0% MPB.
RP-HPLC analysis for reduced ADCs (Condition B)

Each sample was prepared as follows: 1.0 mg/mL of ADCs in 500 mM tris buffer, pH 7.5, was diluted to 0.6 mg/mL in 8 M guanidine HCl and reduced by addition of 1 M DL-Dithiothreitol (DTT). The mixture was incubated at 80 °C for 10 min and was analyzed using Sepax Proteomix RP-1000 5 µm 2.1 × 50 mm column (Sepax Technologies, Inc., USA), connected to an Agilent 1260 HPLC system containing a binary gradient pump, temperature-controlled column compartment, autosampler, and a diode array detector. The system conditions were as follows: flow rate = 0.5 mL/min at 80 °C; MPA = 0.1% TFA and 2% acetonitrile in water; MPB = 0.1% TFA in acetonitrile. The absorbance was monitored at 280 nm (reference wavelength at 450 nm). Each ADC (20 μL) was injected into the system and eluted over a 22 min run consisting of a 1 min isocratic hold at 30% MPB, a 15 min linear gradient from 30% to 45% MPB, a 3 min wash using 95% MPB, and a 3 min re-equilibration at 30% MPB.

RP-HPLC analysis for intact ADCs (Condition C)

RP-HPLC analysis was performed on a Sepax Proteomix RP-1000 5 µm 2.1 × 50 mm column (Sepax Technologies, Inc., USA), connected to an Agilent 1260 HPLC system containing a binary gradient pump, temperature-controlled column compartment, autosampler, and a diode array detector. The system conditions were as follows: flow rate = 0.5 mL/min at 80 °C; MPA = 0.1% TFA and 2% acetonitrile in water; MPB = 0.1% TFA in acetonitrile. The absorbance was monitored at 280 nm (reference wavelength at 450 nm). Each ADC (20 μL) was injected into the system and eluted over a 22 min run consisting of a 1 min isocratic hold at 30% MPB, a 15 min linear gradient from 30% to 45% MPB, a 3 min wash using 95% MPB, and a 3 min re-equilibration at 30% MPB.
Results and Discussion

Peak distribution obtained from RP-HPLC for intact ADCs

For feasibility study of RP-HPLC analysis, trastuzumab-MMAE was prepared by traditional cysteine-based conjugation (Figure 1),\textsuperscript{23,24} this method includes a partial reduction of the interchain disulfide bonds of the antibody by TCEP, followed by thiol maleimide coupling with commercially available MC-VC-MMAE.

To apply RP-HPLC to intact cysteine-based ADCs, measurement conditions were modified based on previous literature.\textsuperscript{18} To improve peak separation, a slow flow rate (0.5 mL/min) and slightly longer gradient (14 min) were required (condition C in materials and methods section). Since cysteine-based ADCs have a more complicated composition than site-specific ADCs, a wider peak distribution will be observed. Especially with the most hydrophobic species (H3: heavy chain modified with 3 MMAEs), as they will be eluted at a later retention time. Due to this widened distribution, a slow flow rate and longer gradient was needed. Several commonly used RP-HPLC columns were evaluated including an Agilent AdvanceBio RP-mAb Diphenyl (resin matrix: diphenyl) and a Zorbax RRHD EclipsePlus C18 (resin matrix: C18), but a Proteomix RP-1000 column (resin matrix: cross-linked polystyrene and divinylbenzene) provided the most sufficient peak separation. In 2016, Yin and co-workers reported that RP-HPLC using Proteomix RP-1000 columns gave the best performance for site-specific ADC characterization in their investigation.\textsuperscript{18} Our present comparison study supported Yin's report and indicated that the cross-linked polystyrene and divinylbenzene can be the most suitable resin matrix even for use with more complicated ADCs. In the chromatograms for blank samples after ADC injection, no evidence of carryover was observed, indicating that this condition can be used for high throughput analysis with no requirements for blank sample runs between samples (SI, Figure S1).

For peak identification, additional analysis was required. It is well-known that some species of the resulting ADCs do not possess covalent bonds between the heavy and light chains; therefore, we predicted
that several peaks would be observed in the chromatogram due to dissociation from ADCs into the corresponding subunits (Figure 2b). To assign these peaks, we evaluated naked trastuzumab (Figure 2a) by the same HPLC method (condition C, see experimental section). Wirth group reported that the peak characterization by both intact RP-HPLC and RP-HPLC coupled with quadrupole time-of-flight mass spectrometry (Q-TOF MS), would show four main peaks (L1, H3, HH2, HL2 and HL1) (Figure 3b). Theoretically, the other compounds illustrated in figure 3c should be observed, but they were not detected by Wirth's analysis. To understand some peaks obtained from our present study, RP-HPLC for fully reduced ADC was also investigated utilizing the same HPLC condition (condition B in experimental section). These comparison studies helped assign three peaks as shown in figure 2b. The earliest main peak (retention time \(= 6.08 \text{ min} \)) corresponded to light chain conjugated with MMAE (L1) based on comparison with reduced ADC sample. The second earliest peak (retention time \(= 6.66 \text{ min} \)) was identified with naked trastuzumab (D0). The latest peak (retention time \(= 12.3 \text{ min} \)) was assigned as heavy chain conjugated with MMAE (H3). The three peaks (retention times \(= 7.25, 8.37 \text{ and } 9.10 \text{ min} \)) were observed between the D0 and H3, however, they did not match the retention time for naked trastuzumab or fully reduced trastuzumab-MMAE. Based on Wirth's report, these peaks may be assigned as HH2, HL2 and HL1, but to clarify the identity of these remaining peaks, Q-TOFMS investigation is required. Considering the application to Q-TOF MS, the mobile phases required modification to enhance ionization efficacy. We tested formic acid (FA) containing mobile phases which indicated showing that the additional investigation was needed due to insufficient peak separation. Typically, FA has negative impact for peak resolution. The mobile phase optimizations to overcome this issue are currently underway. Additionally, some additional analytical and purification studies are ongoing for this system. Analytical studies to elucidate the site occupancy of these ADCs using recently established analytical methods such as peptide mapping and subunit analysis are underway. Purification studies to separate DAR species are also started to obtain simpler ADC composition which enables further analysis simplification.
Next, the reproducibility of this analysis was confirmed by three different ADCs that were synthesized utilizing three different TCEP equivalents to provide different DARs (Figure 4).

The peak area of H3 compound was reflected in the TCEP amount, therefore, we expected the peak area of H3 generated from this intact analysis to predict the DAR generated from traditional RP-HPLC analysis of purified ADCs.

**DAR prediction from area % of H3 peak**

The relationship area % of H3 and DAR was evaluated by three trastuzumab-MMAE ADCs with different DARs utilizing three different TCEP equivalents (Figure 4). These DARs were confirmed by the RP-HPLC for reduced ADCs (condition A in experimental section). In addition to trastuzumab derived ADCs, we also included rituximab-based ADCs and infliximab-based ADCs in this comparison study (Figure S2, S3 and in Supplementary material).

In general, reactivity of an antibody is dependent on its PI. Therefore, these three antibodies showed different conjugation efficacy, as reflected by the DAR (Figure 4). A discussion of these differences in reactivity was recently published in another peer-reviewed journal. In the case of all three antibodies, the H3 peak obtained by intact ADCs showed a trend similar to that of DAR, suggesting that RP-HPLC analysis without pretreatment has the potential to be used for a variety of ADCs (Table 1).

We also applied this methodology to Kadcyla (T-DM1), a commercially available lysine-based ADC. These types of ADCs are highly heterogeneous and contain more than eighty isomers, making analysis exceptionally challenging. Naked trastuzumab provided a single sharp peak but the analytical result of T-DM1 was extremely complicated, indicating that this analytical approach is not ideal for lysine-based ADCs.
(Figure S4). This limitation was also found in traditional HIC-HPLC and reduced RP-HPLC, which further demonstrates the difficulty in analyzing these types of ADCs even with various HPLC methods.\textsuperscript{30,31}

**in-situ RP-HPLC analysis**

Finally, to evaluate the potential to find utility in conjugation reaction monitoring by intact RP-HPLC, an *in-situ* analysis without any purification was investigated. RP-HPLC can separate ADC compounds and drug-linker related impurities in the RP-HPLC column, while omitting the pretreatment sample preparation required for DAR determination. The ADC sample was directly injected from the reaction mixture into the RP-HPLC system (condition C, see material and method section) without any purification. An *in-situ* RP-HPLC analysis of trastuzumab-MMAE provided a peak distribution that was identical to the analysis using a purified ADC (Figure 5a). The MC-VC-MMAE drug-linker was also evaluated and demonstrated that drug-linker separated from ADCs in the chromatogram (Figure 5b and c), indicating that RP-HPLC is useful for the *in-situ* monitoring of conjugation efficacy. In addition to MMAE based ADCs, trastuzumab-DM1 and trastuzumab-MMAF, both of which have differing hydrophobicity compared to MMAE, were also evaluated by this intact HPLC method and showed the compatibility of this analysis with a variety of ADCs (Figure 5d-5i).
Investigation of RP-HPLC analysis for intact ADCs was conducted. Peak distribution results provided the same trends as DAR results produced by well-established HPLC techniques, and compatibility was confirmed by three different ADCs. A feasibility study of an in-situ RP-HPLC analysis showed the potential of this method for conjugation reaction monitoring. This manuscript reporting first application of in-situ RP-HPLC to DAR characterization and reaction monitoring of cysteine-based ADCs has the potential to serve as a gateway to initiate further investigations of HPLC strategies for ADC analysis.
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Supporting Information

This material is available free of charge on the Web at https://www.jsac.or.jp/analsci/.
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Table 1. Comparison of H3 species and DAR

| TCEP equivalent | Area % in intact ADC analysis | DAR in reduced ADC analysis |
|-----------------|------------------------------|----------------------------|
| **Trastuzumab-MMAE** |                              |                            |
| 2.2 eq          | 9.7                          | 3.6                        |
| 2.4 eq          | 11.9                         | 3.8                        |
| 2.6 eq          | 16.1                         | 4.0                        |
| **Rituximab-MMAE** |                              |                            |
| 2.2 eq          | 6.0                          | 3.0                        |
| 2.4 eq          | 8.4                          | 3.3                        |
| 2.6 eq          | 9.3                          | 3.4                        |
| **Infliximab-MMAE** |                              |                            |
| 2.2 eq          | 7.6                          | 3.4                        |
| 2.4 eq          | 9.8                          | 3.6                        |
| 2.6 eq          | 12.2                         | 3.8                        |
Fig. 1 Overview of the conjugation reaction.

Fig. 2 Comparison of RP-HPLC results; a) analysis of trastuzumab under intact conditions, b) analysis of trastuzumab-MMAE under intact conditions, c) analysis of trastuzumab-MMAE under reduced conditions (condition B in experimental section), d) abbreviation and illustration for peak assignments
Fig. 3 Abbreviation and illustration of cysteine-based ADC; a) proposed ADC species after cysteine conjugation b) possible structure in RP-HPLC for intact ADCs (observed in Wirth’s analysis); c) possible structure in RP-HPLC for intact ADCs (not observed in Wirth’s analysis).

Fig. 4 Relationship between area % of H3 in intact analysis and DAR generated by reduced analysis, a) trastuzumab-MMAE, b) rituximab-MMAE, c) infliximab-MMAE.
Fig. 5 An in-situ RP-HPLC analysis: a) trastuzumab-MMAE (post purification); b) trastuzumab-MMAE (in-situ analysis); c) MC-VC-MMAE; d) trastuzumab-DM1 (post purification); e) trastuzumab-DM1 (in-situ analysis); f) SMCC-DM1; g) trastuzumab-MMAF (post purification); e) trastuzumab-MMAF (in-situ analysis); f) MC-MMAF