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A size exclusion-reversed phase two dimensional-liquid chromatography methodology for stability and small molecule related species in antibody drug conjugates

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ABSTRACT

Antibody drug conjugates (ADCs) are complex therapeutic agents combining the specific targeting properties of antibodies and highly potent cytotoxic small molecule drugs to selectively eliminate tumor cells while limiting the toxicity to normal healthy tissues. One unique critical quality attribute of ADCs is the content of unconjugated small molecule drug present from either incomplete conjugation or degradation of the ADC. In this work, size exclusion chromatography (SEC) was coupled with reversed-phase (RP) HPLC in an online 2-dimensional chromatography format for identification and quantitation of unconjugated small molecule drugs and related small molecule impurities in ADC samples directly without sample preparation. The SEC method in the 1st dimension not only separated the small molecule impurities from the intact ADC, but also provided information about the size variants (monomer, dimer, aggregates, etc.) of the ADC. The small molecule peak from the SEC was trapped and sent to a RP-HPLC in the 2nd dimension to further separate and quantify the different small molecule impurities present in the ADC sample. This SEC–RP 2D-LC method demonstrated excellent precision (%RSD < 2.0), linearity ($r^2 = 0.9999$), sensitivity (LOQ of 0.05 μg/mL of free drug in ADC sample) and accuracy (95–105% recovery of spiked samples). The 2D-LC method was further utilized to study the stability of an ADC drug product at different temperatures and pHs. Both small molecule degradation products and aggregation of the conjugate were observed in the stability samples and the degradation pathways of the ADC were investigated. This 2D-LC method offers a powerful tool for ADC characterization and provides valuable information for conjugation and formulation development.

1. Introduction

Antibody drug conjugates (ADCs) are complex biotherapeutics combining the specific targeting properties of monoclonal antibodies with the extreme potency of small molecule drugs [1–4]. With the approval of Kadcyla [5] and Adcetris [6], the therapeutic benefits of antibody drug conjugates are increasingly clear. By utilizing the selectivity of monoclonal antibodies, an efficacious dose of the cytotoxic drug can be targeted to the tumor site to kill the tumor cells while limiting the general toxicity of the highly potent cytotoxic agents to normal, healthy tissues [7–10]. ADCs are comprised of three components: the monoclonal antibody, a cytotoxic drug, and a linker that holds the antibody and small molecule drug together. As ADCs have more complex and heterogeneous structures than the corresponding antibodies and free drugs, they require special characterization [11,12]. One unique critical quality attribute of ADCs is the content of unconjugated small molecule drug whose cytotoxicity is no longer targeted and can increase the side effects and adverse events in patients.

Several approaches have been reported for the analysis of the free drug content in ADCs. For instance, competition ELISA assays have been developed and implemented utilizing free drug-specific antibodies [13,14]. However, detection and quantitation of all of the small molecule impurities in the ADCs cannot be assured with this technique, since the antibodies used in these assays are specific to the free drug and may not bind degradation products of the free drug. RP-HPLC is the standard method for the assay and impurity analysis of small molecule drugs [15,16] and therefore could enable complete characterization of the unconjugated small molecule impurity profile of an ADC. However, direct injection of protein-containing ADC samples onto RP HPLC columns can cause column deterioration due to the irreversible binding of protein to

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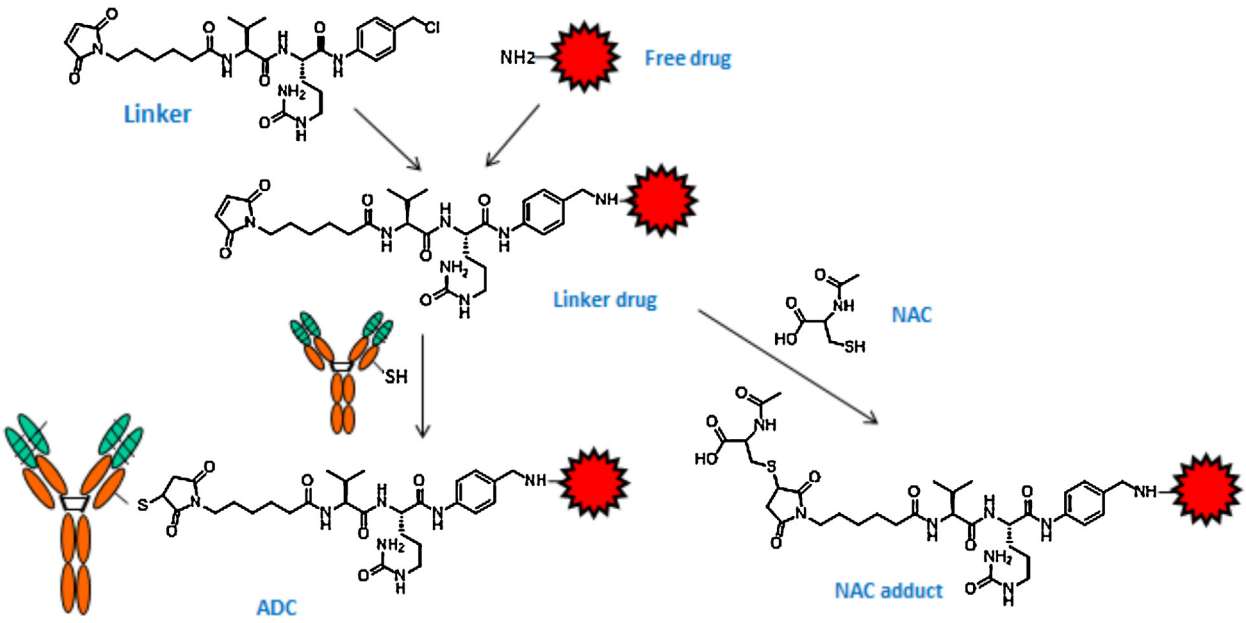

Fig. 1. ADC conjugation procedure and related small molecular impurities that can be derived in the process.

the stationary phase. Guard columns installed before the analytical column can help prevent contamination and possible damage to the analytical column [17]. In addition to using guard columns, various sample clean up procedures have been applied in order to remove protein from the ADC prior to HPLC analysis. For example, solid phase extraction (SPE) can be used to retain the analytes on the SPE stationary phase while protein passes through the column unbound. The free drug species is then recovered and analyzed by RP HPLC [18]. Another method includes isolating the small molecules by protein precipitation, a widely-used sample pretreatment in the biopharmaceutical industry [19,20]. In this approach, organic solvent, such as methanol, acetonitrile or isopropyl alcohol, is added to the ADC to precipitate the protein, while the free drug and small molecule impurities remain soluble in the organic extract. The precipitated sample is then centrifuged to remove the protein, and the supernatant is analyzed by RP HPLC for quantitation of the small molecular impurities. However, all of these sample pretreatment approaches are labor intensive and time consuming; therefore, developing a method that can automatically and directly quantitate the free drug and related impurities in ADCs is highly desirable.

2D-LC represents a powerful tool to overcome the challenges of analyzing complex samples and enhance resolving power by using orthogonal separation mechanisms in each dimension. In recent years, numerous 2D-LC applications have been reported in proteomic and small molecule pharmaceuticals [21–24]. Comprehensive and heart-cutting modes are two commonly used configurations in 2D-LC. In the comprehensive mode, eluent from the first dimension (1st D) is constantly cut and transferred into the second dimension (2nd D) to obtain a comprehensive sample profile in a short amount of time [25–27]. In heart-cutting mode, only the eluent that contains the peak or peaks of interest from the 1st D separation is redirected to the 2nd D. This makes heartcutting mode more suitable for the separation and quantitation of the target analytes in complex sample matrices [28–30].

In this work, a novel approach using SEC coupled with RP-HPLC in a 2-dimensional chromatography format was developed for the direct analysis of free drug and related small molecules in ADC samples. The SEC method in the 1st dimension provided information about the size variants (monomer, dimer, aggregates, etc.) of the ADC and also separated the small molecular impurities from the intact ADC. On-line heart-cutting of the small molecule impurities was used to avoid sample loss from the first dimension and to allow gradient analysis on the second dimension to obtain the full small molecule impurity profile.

The ADC conjugation procedure and the small molecule impurities analyzed in this study are depicted in Fig. 1 (the chemical structure of the free drug is not shown due to proprietary concerns). Generally, a reactive linker is first attached to the free drug to form the linker drug, at which point the linker drug is introduced to the protein to form the final ADC. Often when working with maleimide containing linker drugs, *N*-acetylcysteine (NAC) is added to the solution at the end of the conjugation to deplete the excess of reactive linker drug and aid in free drug clearance. Therefore, the small molecule impurities that need to be monitored include the free drug (no linker present), linker drug, and the NAC adduct of the linker drug as all of these impurities may remain in the final ADC drug product as a result of incomplete removal by purification steps downstream of the conjugation reaction. During the storage of the ADCs, new small molecule degradation products may also be released from the conjugate. Therefore, a mass spectrometry (MS) detector was incorporated into the 2nd D and used to identify the structures of the small molecular species. The stability of an ADC drug product was studied using this 2D-LC method, and the major degradation pathways of the ADC were first reported.

2. Experimental

2.1. Reagents and materials

Acetonitrile (ACN, HPLC grade) was purchased from J.T. Baker (Phillipsburg, NJ, USA). Formic acid, trifluoroacetic acid (TFA), sodium phosphate, and histidine acetate were purchased from Sigma–Aldrich (St. Louis, MO, USA). Deionized water was from an in-house Milli-Q system (Millpore, Billerica, CA, USA). The ADC conjugate samples and the small molecule free drug, linker drug and NAC adduct standards were provided by Genentech (South San Francisco, CA, USA).

The stock standard solutions of the small molecule free drug, linker drug, and NAC adduct were prepared at concentration of 0.1 mg/mL in a diluent of ACN:water = 1:1. The ADC samples were