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Extending the limits of size exclusion chromatography: Simultaneous separation of free payloads and related species from antibody drug conjugates and their aggregates

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ABSTRACT

Size exclusion chromatography (SEC) is commonly performed in isocratic conditions to separate partially excluded molecules from the pores of the stationary phase, based on their difference in hydrodynamic volume. In this work, a baseline resolution was obtained between the monomeric antibody drug conjugate (ADC) and high molecular weight species (HMWS). Besides HMWS, small free payloads, linkers and linker-payloads of ADCs, which would not be discriminated solely based on their size (MW < 1.5 kDa), were also separated on the same SEC column by applying sequentially an acetonitrile gradient after the elution of the largest species. Such an approach allowed a simultaneous i) measurement of the HMWS amount under native conditions, and ii) quantitation of the free payloads, within one generic SEC run. For this purpose, a state-of-the-art 150 × 4.6 mm SEC column packed with 2.0 μm particles and 250 Å pore size, was selected to achieve fast separations of the species within 10 min. A second dimension (RPLC) was also developed to further extend the possibility offered by this experimental setup. The SECxRPLC multiple heart cutting mode was operated by using a modern 2D-LC instrument containing twelve 120 μL sampling loops. Repeatabilities (0.01% < RSD < 3.68%) and recoveries (between 82% and 107%) were found to be suitable with both approaches (SEC and SECxRPLC), whereas the LOQs remain similar. Finally, the SEC method was applied for the screening of ADC crude reaction mixtures, whereas the SEC x RPLC method facilitated separating some additional impurities. The streamlined methodology will further support the development and characterization of ADC products.

1. Introduction

Based on the strong development of monoclonal antibodies, with more than 70 candidates approved by the Food and Drug Administration (FDA) and European Medicines Agency (EMA) to date [1], antibody-drug conjugates have also experienced a tremendous activity over the last few years, to increase or extend the benefits of targeted therapy. Next to the approval of brentuximab vedotin in 2011 and trastuzumab emtansine in 2013, two additional ADCs were approved in 2017, namely inotuzumab ozogamicin and gemtuzumab ozogamicin. In 2016, trastuzumab emtansine (brand name Kadcyla) and brentuximab vedotin (brand name Adcetris) generated $831 million and $520 million in global sales, respectively [2]. The success of these two ADCs has paved the way to further developments, with currently more than 60 ADCs in clinical development [3]. The first generation of ADCs involved a linker-payload conjugated to basic lysine residues. In the second and third ADC generations, the conjugation takes place on the cysteine of the disulfide bridges or on engineered cysteine sites (site-specific conjugation), respectively. These improvements in ADC design have led to an increased therapeutic window, thanks to reduced off-target toxicity [3].

In addition to the usual Critical Quality Attributes (CQAs) of antibody products, such as the measurement of HMWS, several additional CQAs, such as the average drug to antibody ratio (DAR), the drug loading distribution, the levels of the free drug and free antibody also have to be determined for ADC products. The quantitation of free payloads has attracted major attention over the last few years and can be evaluated with two reference methods, namely Enzyme Linked ImmunoSorbent Assays (ELISA) [4,5] and RPLC after the removal of the protein with various approaches. The isolation of the free payload is often achieved after protein precip-

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itation [6,7], but other approaches have also been developed, such as solid phase extraction [8] or online cleaning with a guard column [9]. To overcome these time consuming pretreatments that may also result in unacceptable method performance [10], 2D-LC methods have been developed and evaluated [10,11]. The proposed heart cutting SECxRPLC methods involved an isocratic SEC elution with the addition of 20% of acetonitrile in the mobile phase to elute the free payloads and related species together and collect the corresponding fraction, which can then be sent to a second dimension RPLC separation [10,11].

Alternatively, an in-house method involving SEC and RPLC columns in series allowed separating the free payload related species from the proteins by SEC whereas a diverting valve located between the two columns enabled the proteins removal and transfer only the free payloads to the RPLC column (unpublished results). However, the overall performance and robustness of this approach was limited in the case where non-specific interactions occurred between the ADC and the SEC material. Indeed, if the SEC separation mechanism is not solely based on size, protein adsorption and chromatographic peak tailing can occur, leading to an incomplete protein removal before the RPLC analysis. Therefore, the goal of this study was to develop an effective and straightforward strategy that could be applied to a broad range of ADC products.

In this work, four ADCs in pre-clinical or clinical phases were initially characterized by hydrophobic interaction chromatography (HIC) and cation exchange chromatography (CEX). The goal of these initial runs was to characterize and classifiy the four ADC products against two FDA and EMA approved ADCs, namely trastuzumab emtansine (T-DM1) and brentuximab vedotin (BV). Based on the hydrophobic and ionic properties of these ADC products, the performance of two "state-of-the-art" SEC columns (known to have limited non-specific interactions) [12] was compared. A gradient elution was then developed in SEC, and care was taken to limit the amount of organic modifier during ADC elution in order to avoid protein denaturation. A multiple heart-cutting SECxRPLC method was finally implemented to check the purity of the multiple peaks originating from small species (MW < 1.5 kDa) in the first dimension. The performance of the SEC and SECxRPLC methods was then compared, and ADC samples at various stages of the purification process were analyzed from the crude reaction mixture, after tangential flow filtration, as well as the final ADC products.

2. Experimental

2.1. Chemicals and reagents

Water was obtained from a Milli-Q Purification System from Millipore (Bedford, MA, USA). LC–MS grade acetonitrile, methanol and LC grade 2-propanol were purchased from Merck (Darmstadt, Germany). BioUltra grade potassium chloride, potassium phosphate monobasic, potassium phosphate dibasic, and LC–MS grade ammonium acetate and formic acid were all purchased from Sigma-Aldrich (Buchs, Switzerland). Aqueous mobile phases were filtered through 0.22 μm polyethersulfone (PES) membrane filter (Sartorius, Göttingen, Germany) and used within one week. Novartis Pharma AG (Basel, Switzerland) supplied the mAbs and ADC products currently in preclinical and clinical phases (these products are named ADC1 to ADC4 for the purpose of this study). Some of the important properties (i.e. isotype, conjugation chemistry, size, lipophilicity and pK_a) of ADC components (i.e. unconjugated mAb, linker, payload, linker-payload) are reported in Table 1.

2.2. Sample preparation

ADC and mAb products were diluted to a concentration of 5 mg/mL for CEX and HIC, while a concentration of 10 mg/mL was used in SEC. Water was used as the sample diluent for SEC and CEX experiments, while HIC mobile phase A containing 1 M ammonium sulfate and 0.1 M potassium phosphate buffer in water (pH = 6.8) was employed as the sample diluent in HIC. Stock solutions of the free payloads, linkers and linker-payloads were prepared at 1.0 mg/mL in acetonitrile for the lipophilic species (logP > 0) and N,N-dimethylformamide for the hydrophilic (logP < 0) ones. Spiking solutions and standards solutions for the calibration curves were then prepared in water/acetonitrile (80:20 v/v).

2.3. Equipment

Experiments were performed on an Agilent 1290 Infinity II 2D-LC system equipped in the first dimension with a binary solvent delivery pumps, an autosampler, a column oven, and a diode array detector (DAD) operated simultaneously at eight wavelengths (sampling rate 2.5 Hz). A second binary solvent delivery pump and DAD also operating at eight wavelengths (sampling rate 10 Hz) were involved in the second dimension. The autosampler was thermostated at 8 °C, and the seat-back and needle wash were flushed using acetonitrile/water (80:20 v/v) for 5 s, 2-propanol/water (80:20 v/v) for 10 s and acetonitrile/water (80:20 v/v) for 15 s.

The 1290 Infinity II 2D-LC system includes a flow-through needle (FTN) injector, 10-mm DAD flow-cell (1.0 μL), and a 2D-LC valve connected to two Multiple Heart-Cutting (MHC) valves. Each MHC valve is composed of a cluster of six 120 μL sampling loops providing two parking decks (A and B) with 12 loops position. The 2D-LC valve was connected to the ^{1}D DAD detector by means of a Pressure Release Kit (PRK) containing 0.12 mm I.D. tubing (void volume of 13.4 μL). The MHC valve was connected to the 2D-LC valve with a 0.12 mm I.D. × 170 mm tubing (1.9 μL). A common setup (with sampling loops of 40 μL) of the 2D-LC can be found in the literature [13].

2.4. Chromatographic conditions

2.4.1. Columns

To characterize the hydrophobic and ionic properties of antibodies, HIC and CEX analyses were performed with the MAbPac HIC-10 (100 × 4.6 mm, 5.0 μm, 1000 Å) and the MAbPac SCX-10 (150 × 4.0 mm, 5.0 μm) columns, purchased from Thermo Fisher Scientific AG (Sunnyvale, CA, USA), respectively.

For the SEC experiments, the TSKgel UP-SW3000 (150 mm × 4.6 mm, 2.0 μm, 250 Å) and AdvanceBioSEC column (150 mm × 4.6 mm, 2.7 μm, 300 Å) were purchased from Tosoh (Tokyo, Japan) and Agilent Technologies (Wilmington, DE, USA), respectively. The calibration range for proteins is linear from around 10 kDa to 500 kDa based on the SEC columns specifications and our experience. The RPLC column used in the second dimension was an Acquity BEH C18 (100 mm × 3.0 mm, 1.7 μm, 130 Å) purchased from Waters (Milford, MA, USA).

2.4.2. Separation conditions

2.4.2.1. HIC and CEX. To understand the effects of protein hydrophobicity in SEC and elucidate our observations, relative hydrophobicities of mAb products were determined by HIC at pH 6.8. HIC mobile phase A contains 0.1 M potassium phosphate + 1 M ammonium sulfate in water, whereas mobile phase B was composed of 0.1 M potassium phosphate in water. The mobile phase B composition was increased from 0% to 100% in 20 min during the HIC run. Injection volume was 5.0 μL.

Similarly, the effects of protein ionic properties in SEC were investigated by CEX at pH 6.0. CEX mobile phase A contains 10 mM of 2-(*N*-morpholino)ethanesulfonic acid (MES) in water, whereas mobile phase B was composed of 10 mM MES + 200 mM sodium