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Impact of organic modifier and temperature on protein denaturation in hydrophobic interaction chromatography

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ABSTRACT

The goal of this study was to better understand the chromatographic conditions in which monoclonal antibodies (mAbs) of broad hydrophobicity scale and a cysteine conjugated antibody-drug conjugate (ADCs), namely brentuximab-vedotin, could denaturate. For this purpose, some experiments were carried out in HIC conditions using various organic modifier in natures and proportions, different mobile phase temperatures and also different pHs. Indeed, improper analytical conditions in hydrophobic interaction chromatography (HIC) may create reversed-phase (RP) like harsh conditions and therefore protein denaturation. In terms of organic solvents, acetonitrile (ACN) and isopropanol (IPA) were tested with proportions ranging from 0 to 40%. It appeared that IPA was a less denaturating solvent than ACN, but should be used in a reasonable range (10–15%). Temperature should also be kept reasonable (below 40 °C), to limit denaturation under HIC conditions. However, the combined increase of temperature and organic content induced denaturation of protein biopharmaceuticals in all cases. Indeed, above 30–40 °C and 10–15% organic modifier in mobile phase B, heavy chain (HC) and light chain (LC) fragments dissociated. Mobile phase pH was also particularly critical and denaturation was significant even under moderately acidic conditions (pH of 5.4).

Today, HIC is widely used for measuring drug-to-antibody ratio (DAR) of ADCs, which is a critical quality attribute of such samples. Here, we demonstrated that the estimation of average DAR can be dependent on the amount of organic modifier in the mobile phase under HIC conditions, due to the better recovery of the most hydrophobic proteins in presence of organic solvent (IPA). So, special care should be taken when measuring the average DAR of ADCs in HIC.

1. Introduction

Protein therapeutics, especially monoclonal antibodies (mAbs), Fc fusion proteins and antibody-drug conjugates (ADC) have become a particularly relevant part of the pharmaceutical industry over the past 20 years [1,2]. Those products show inherent heterogeneity which has to be evaluated by the simultaneous use of a number of different analytical techniques [3–8]. Liquid chromatographic methods, such as ion exchange (IEX) [9], size exclusion (SEC) [10], hydrophobic interaction (HIC) [11], reversed-phase (RPLC) [12] and recently, hydrophilic interaction (HILIC) liquid chromatography [13] are often used for the analytical characterization of such samples.

HIC is considered as an alternative – and historical – technique to RPLC, since the retention is mainly driven by hydrophobic interactions in both modes [14,15]. The main differences between HIC and RPLC are: (1) the non-denaturating nature of the HIC separation (native conformations and bioactivity of fractionated proteins are usually preserved), (2) the use of non-volatile mobile phases in HIC (high salt concentration), which may prevent the direct coupling of HIC with MS and (3) the high selectivity to changes of protein surface-associated hydrophobicity under HIC conditions [16]. Various applications for HIC characterization of therapeutic proteins can be found in the literature [16,17]. Probably, the greatest potential of analytical HIC is the characterization of ADCs. This new class of therapeutic proteins is supposed to combine the high specificity of monoclonal antibodies and the potency of small molecular weight cytotoxic drugs. The cytotoxic drug is conjugated to the protein via linkers. After penetrating target cells, the cytotoxic drug is released from the protein, leading to cell death. Two types of ADCs are already marketed, including the cys-

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teine conjugated brentuximab vedotin (Adcetris®) and the lysine conjugated ado-trastuzumab emtansine (Kadcyla®). Cysteine conjugated brentuximab vedotin is formed through partial reduction of the antibody's (IgG1) interchain disulfide bonds. The reduced cysteines are then alkylated with a preformed drug-linker. The process results in conjugates with a distributed drug load with 0, 2, 4, 6 or 8 drugs incorporated per antibody and an average drug to antibody ratio (DAR) of ~4. Since distribution of the loaded drugs and average DAR are critical quality attributes, their thorough characterization is necessary. The main benefit of HIC – over other denaturating modes of chromatography – is that the native Y-shape conformation of the antibody is maintained under the mild conditions, even when the disulfide bridges are reduced. Therefore, for cysteine linked ADCs, the individual DARs can be separated in HIC on the basis of the number of attached drugs. This application of HIC is unique and of great importance for the biopharmaceutical industry.

Some works on generic HIC method development for mAbs and ADCs were recently reported [18,19]. The influence of various parameters on the HIC separation, such as gradient steepness, mobile phase pH, ionic strength (salt type and concentration) and organic modifiers using various stationary phase chemistries, has been studied. It has been found, that the impact of pH and salt type (when adjusted to the same lyotropic strength) on selectivity and resolution were not significant. However, gradient steepness, mobile phase organic modifier and the nature of the stationary phase significantly affect the overall separation. The effect of organic modifier on the separation is still not well understood. Some studies justify the use of organic modifier with an increased resolution of sample components [20] or attenuation of binding to the stationary phase of the most hydrophobic species [21]. Vendors' column manuals reported that the addition of organic solvent may improve the resolution, especially for hydrophobic proteins or mAbs [22,23]. The use of isopropanol (IPA) and/or acetonitrile (ACN) in a concentration range of 5–25% seems to appear arbitrarily or being of historical origin in the literature. Most of the methods using [24–36] or not using [22,23,34,35,37–42] organic modifier in HIC separations do not discuss the advantage or drawback of the presence or absence of such additives in the mobile phase. Recently, Chen et al. reported the use of 50% ACN in mobile phase B in a hybrid form of RPLC and HIC separation, followed by direct LC–MS analysis of model proteins and *E. coli* cell lysate [43]. Moderate ammonium acetate concentration in mobile phase A seemed to play a role in the preservation of the native-like protein structure, while high organic modifier concentration in mobile phase B eluted the proteins directly to the ion source from the relatively hydrophobic (pentyl to decyl bonding) stationary phases [43]. Rodriguez-Aller et al. reported, that the use of IPA in mobile phase B can be a parameter for tuning selectivity of mAbs' separation. In the case of ADC, as expected, IPA decreased the retention of the naked host mAb and DAR2, but surprisingly increased the retention of DAR 4–8 species. Moreover, incomplete elution of higher DARs was observed above 6% IPA from some stationary phases. This was possibly due to effects of pH shift, conformational changes, or increased salting out effect in presence of high amounts of IPA (protic solvent) [18].

The aim of this study was to better understand the impact of organic modifier addition in HIC mobile phases and to clarify its possible advantages and drawbacks for therapeutic proteins (mAbs and ADCs) analysis. Proteins can yield multiple and/or irregular-shaped peaks for seemingly pure species. A variety of molecular properties as well as measurement conditions can potentially play a role in this behavior, among them, conformational changes in protein structure (e.g., denaturation) [44,45]. Indeed, high proportion of organic modifier may lead to protein denaturation, and could affect chromatographic profiles and recoveries. Temperature and pH probably influence protein denaturation, too. The improper use of organic modifiers in HIC may create RPLC-like harsh conditions and therefore protein denaturation. The effect of both aprotic and protic modifiers (ACN and IPA) was investigated in a wide concentration range using commercial mAbs of extensive hydrophobicity scale and a cysteine conjugated ADC, brentuximab vedotin. Temperature and pH effects were also investigated.

2. Experimental

2.1. Chemicals and equipment

Water was obtained from a Milli-Q Purification System from Millipore (Bedford, MA, USA). Ammonium acetate, dithiothreitol (DTT, ≥99.0%), trifluoroacetic acid (TFA, 99%) and acetic acid (≥99.7%) were purchased from Sigma–Aldrich (Buchs, Switzerland). Isopropanol (IPA, HPLC grade) and acetonitrile (ACN, HPLC grade) were purchased from Fischer Chemicals (Reinach, Switzerland).

FDA and EMA approved therapeutic IgG monoclonal antibodies including bevacizumab, infliximab, and denosumab as well as ADC brentuximab vedotin (BV) were kindly provided by the Center of Immunology Pierre Fabre (Saint-Julien en Genevois, France). MAbPac HIC-10, an alkyl amide modified silica based HIC column of 100 × 4.6 mm, 5 μm (1000 Å) was purchased from Thermo Fisher Scientific AG (Reinach, Switzerland), Acquity BEH300 C18 column of 150 × 2.1 mm, 1.7 μm (300 Å) was purchased from Waters AG (Baden-Dättwil, Switzerland).

All the experiments were performed using a Waters Acquity UPLC™ system equipped with a binary solvent delivery pump, an autosampler and fluorescence detector (FL). The system included a 5 μL sample loop and a 2 μL FL flow-cell. The loop is directly connected to the injection switching valve (no needle seat capillary). The connection tube between the injector and column inlet was 0.13 mm I.D. and 250 mm long (passive preheating included), and the capillary located between the column and detector was 0.10 mm I.D. and 150 mm long. The overall extra-column volume (V_{ext}) is about 14 μL as measured from the injection seat of the autosampler to the detector cell. The measured dwell volume is around 100 μL. Data acquisition and instrument control were performed by Empower Pro 3 Software (Waters). Calculation and data processing were achieved with Excel (Microsoft) and Statistica v12 (Statsoft) software. The mobile phase pH was measured using a SevenMulti S40 pH meter (Mettler Toledo, Greifensee, Switzerland).

2.2. Chromatographic conditions

HIC mobile phase A was 4.5 M aqueous ammonium acetate (NH_4OAc) solution (pH 7.4). Mobile phase B contained 20 mM ammonium acetate and organic modifier (ACN and IPA) at various concentrations. A general linear gradient from 100% A to 100% B was systematically performed using a flow rate of 1 mL/min on the MAbPac HIC-10 (100 × 4.6 mm, 5 μm, 1000 Å) column. Gradient time was set either to 10 min (10%B/min) or 40 min (2.5%B/min). Column temperature was set between 20 and 60 °C.

Preliminary RPLC experiments were performed using a linear gradient from 27 to 57% B in 7.5 min using an Acquity BEH300 C18 (150 × 2.1 mm, 1.7 μm, 300 Å) column. Mobile phase A was water and mobile phase B was ACN, both containing 0.1% TFA. Column temperature was set to 80 °C, and flow rate 0.4 mL/min.

A volume of sample equal to 3 μL was injected in both modes using partial loop injection. The FD detector operated at 280 nm excitation and 360 nm emission wavelengths, at 2 Hz sampling rate.