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Separation of antibody drug conjugate species by RPLC: A generic method development approach

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ABSTRACT

This study reports the use of modelling software for the successful method development of IgG1 cysteine conjugated antibody drug conjugate (ADC) in RPLC. The goal of such a method is to be able to calculate the average drug to antibody ratio (DAR) of and ADC product. A generic method development strategy was proposed including the optimization of mobile phase temperature, gradient profile and mobile phase ternary composition. For the first time, a 3D retention modelling was presented for large therapeutic protein. Based on a limited number of preliminary experiments, a fast and efficient separation of the DAR species of a commercial ADC sample, namely brentuximab vedotin, was achieved. The prediction offered by the retention model was found to be highly reliable, with an average error of retention time prediction always lower than 0.5% using a 2D or 3D retention models. For routine purpose, four to six initial experiments were required to build the 2D retention models, while 12 experiments were recommended to create the 3D model. At the end, RPLC can therefore be considered as a good method for estimating the average DAR of an ADC, based on the observed peak area ratios of RPLC chromatogram of the reduced ADC sample.

1. Introduction

Antibody drug conjugates (ADCs) are chemotherapeutics constituted of a cytotoxic chemical drug linked covalently – via peptide linker – to a monoclonal antibody (mAb) [1]. They are used for the treatment of cancer by combining the proven antigen-specific selectivity and activity of mAbs with the potency of highly cytotoxic small molecules [2]. Drug conjugation can be achieved via reactions at different amino acid residues such as at lysine side chains amines (lysine conjugation), at cysteine thiol groups after reduction of the interchain disulfide bonds (cysteine conjugation), or at engineered cysteine residues at specific sites on a mAb [3,4]. The addition of the drugs results in a heterogeneous population of ADCs that differ in the number of drugs per antibody, and this mixture can be described by an average drug to antibody ratio (DAR) [4]. Structurally, immunoglobulin G1 (IgG1) mAbs contain four interchain disulphide bridges. Among those, two are located in the hinge region and connect the two heavy chains (H) while the other two connect the H and light chain (L). Therefore, a cysteine linked IgG1 ADC is composed of a heterogeneous mixture of 0, 2, 4, 6 or 8 drug conjugations [5]. An odd number of conjugated drugs on an ADC is typical indicative of degradation [6,7]. In this study, we focus only on a commercially available cysteine linked IgG1 ADC, namely brentuximab vedotin.

One of the most important critical quality attributes (CQA) of an ADC is the average DAR, since it determines the amount of "payload" that can be delivered to a tumor cell and can affect efficacy and safety [3]. A variety of analytical methods have been used to measure the average DAR, including UV–vis spectroscopic, liquid chromatographic, and native mass spectrometric methods [4,8–19]. Among liquid chromatographic methods, hydrophobic interaction chromatography (HIC) and reversed phase chromatography (RPLC) are routinely used. The former approach separates the intact DAR species under non-denaturating conditions, while RPLC is mostly used to separate the DAR species of reduced ADC sample related to the H or L chains as L0, L1 and H0, H1, H2 and H3 species.

In addition to information about the average DAR, multiple methods have been applied to analyze the distribution of drug linked forms and their positional isomers. This is again an important characteristic because different forms may have different pharmacokinetic and toxicological properties [3,16]. A number of methods for this task are based on the use of mass spectrometry (MS) [20–23]. The separation of intact DARs in HIC also allows the isolation of pure species and permits further characterization (e.g.

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by capillary electrophoresis-sodium dodecyl sulphate and capillary iso-electric focusing (CE-SDS, cIEF) or cell based bioassay) [3,5]. The complete characterization of ADC DAR positional isomers can also be performed on-line, by peptide mapping LC–MS or two dimensional (HIC x RPLC)-MS analysis of the native ADC [6,7,24,25]. In addition to average DAR, DAR distribution and positional isomer analysis, a number of complementary methods can be applied for further characterization of ADCs to determine residual free drugs, size variants or charge variants [25].

As mentioned earlier, RPLC is mostly used to separate the DAR species of reduced ADC related to H and L, but can also be applied on the native ADC to separate L, H, HH, HL and HHL fragments. The latter one can be useful, as a second dimension separation prior to MS. In the literature, only "historical" methods are reported, using a mobile phase containing 0.1% trifluoroacetic-acid (TFA), an acetonitrile gradient and elevated temperature of 70–80 °C. However, nothing is reported about systematic method development, optimization procedure or retention behavior. In this study, we offer a possible generic method development approach for IgG1 cysteine conjugations. Both 2D and 3D retention models are discussed. The impact of temperature and mobile phase ternary composition was also studied. Finally, four, six and twelve experiments based designs were suggested for routine purposes. This new approach allows a fast and accurate systematic method development for the efficient separation of ADC DAR species and fragments.

2. Experimental

2.1. Chemicals and columns

Water was obtained from a Milli-Q Purification System from Millipore (Bedford, MA, USA).

Acetonitrile (ACN) and methanol (MeOH) were ULC–MS grade and purchased from Biosolve (Valkenswaald, Netherlands). Dithiothreitol (DTT, ≥99.0%) and trifluoroacetic acid (TFA, 99%) were purchased from Sigma–Aldrich (Buchs, Switzerland).

Brentuximab vedotin as therapeutic monoclonal antibody drug conjugate (IgG1 cysteine linked ADC) was kindly provided by the Center of Immunology Pierre Fabre (Saint-Julien en Genevois, France).

The AdvanceBio RP-mAb C4 (3.5 μm, 150 mm × 2.1 mm, 450 Å) column was purchased from Agilent (USA).

2.2. Equipment and software

Measurements were performed using a Waters Acquity UPLC™ I-Class system equipped with a binary solvent delivery pump (maximum flow rate of 2 mL/min and upper pressure limit of 1200 bar), an auto-sampler and UV detector. The system includes a flow through needle (FTN) injection system with 15 μL needle and a 0.5 μL UV flow-cell. The overall extra-column volume (V_{ext}) was about 7.5 μL as measured from the injection seat of the auto-sampler to the detector cell. The dwell volume was measured as V_d = 0.09 mL.

Data acquisition and instrument control were performed by Empower Pro 3 software (Waters). Calculations and data transferring were achieved with Excel (Microsoft). Retention and resolution modelling was performed with DryLab® 4 software (Molnár-Institute, Berlin, Germany).

2.3. Mobile phase composition and sample preparation

For the gradient separation of ADC DARs and its fragments, the mobile phase "A" consisted of 0.1% TFA in water, whereas the mobile phase "B" was 0.1% TFA in organic solvent having different possible compositions of acetonitrile and methanol.

Native brentuximab vedotin was injected as received (5 mg/mL) using low volume insert vials.

Reduced brentuximab vedotin sample was prepared by the addition of DTT solution (10 mM) and incubation at 30 °C for 60 min. The ADC was completely converted into the light and heavy chain components. Then, sample was injected from low volume insert vial.

2.4. Investigation of retention properties of ADC species (preliminary studies)

Native brentuximab vedotin was eluted in RPLC mode using linear gradients. The effect of the gradient profile, temperature and organic modifier on the retention and selectivity of ADC species was studied. Detection was carried out at 280 nm, and 1 μL sample was injected.

First, the effect of gradient steepness (gradient time, t_G) on retention and selectivity was evaluated. A generic linear gradient, starting from 25% to 50% B was applied at a flow rate of 0.3 mL/min. For mobile phase B, acetonitrile was used as organic modifier and the experiments were performed at T = 90 °C. In our previous study, the validity of linear solvent strength model (LSS) was proved for mAbs and subunits in the practically relevant design space (DS) [26]. The gradient time was varied as t_G = 6, 12 and 18 min. The observed apparent (or gradient) retention factors (k_{app}) were plotted against t_G and the change in selectivity (elution order) was followed.

Based on previous experiments, high temperature seemed to be mandatory for correct peak shape and recovery [27]. Therefore, all the experiments were performed at mobile phase temperature of T ≥ 70 °C. Short gradient runs (t_G = 6 min) were carried out at 70, 75, 80, 85 and 90 °C (up to the upper temperature limit of the column). Our purpose was to illustrate the effect of temperature on the retention and recovery. Plots of log k_{app} *vs.* 1/T and recovery *vs.* T were made. For the estimation of recovery, peak area values were normalized to the ones observed at the highest temperature (90 °C).

At last, the impact of the organic modifier was studied by running gradients with mixtures of acetonitrile and methanol. Aprotic and protic solvents may have an impact on the solvation of ADC species and can interact in different ways with the proteins and also with the residual acidic silanols of the silica based stationary phase. Therefore, differences in retention and selectivity were expected. Ternary mobile phases were prepared using water, acetonitrile and methanol. The ternary composition (*tC*) of mobile phase B was set as 0, 20, 40 and 50% MeOH in ACN. Since MeOH has a lower eluent strength than ACN, the gradients were run in an extended%B range (30–80%B), to elute all the species with all mobile phase compositions. The experiments were performed at T = 90 °C, and t_G = 6 min. Higher than 50% MeOH content resulted in unacceptable recovery and too high retention.

2.5. Systematic method optimization

Snyder et al. recommended initial basic runs for multifactorial experimental designs already in the 90's [28]. A general approach is to simultaneously model the effect of temperature and gradient steepness (t_G) on selectivity with a previously selected RPLC column [29,30]. Then, with the help of resolution maps generated by modelling software – which show the critical resolution of the peaks to be separated [31] – the gradient program and column temperature can be rapidly and efficiently optimized. Today – thanks to new developments of modelling software – it is also possible to simultaneously optimize three method variables. Beside temperature and gradient steepness, ternary mobile phase composition or mobile phase pH are often selected as third variable [32–34]. Since