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Analysis of antibody-drug conjugates by comprehensive on-line two-dimensional hydrophobic interaction chromatography x reversed phase liquid chromatography hyphenated to high resolution mass spectrometry. II- Identification of sub-units for the characterization of even and odd load drug species

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ABSTRACT

This paper is the second part of a two-part series dedicated to the development of an on-line comprehensive HICxRPLC-UV/MS method for the characterization of a commercial inter-chain cysteine-linked ADC (brentuximab vedotin, Adcetris®). The first part focused on the optimization of the chromatographic conditions. In the second part of this series of papers, the structural characterization of the Brentuximab Vedotin was extensively discussed. With the combination of HIC and RPLC–MS data, the average DAR was easily measured in HIC and, at the same time, the predominant positional isomers were identified in RPLC–MS in one single injection. It was also demonstrated that the retention data obtained in the first and second dimensions was particularly useful to assist ADC characterization through the identification of sub-units. Using this methodology, the presence of odd DARs (1, 3 and 5) and their relative abundance was assessed by a systematic evaluation of HIC x RPLC-UV/MS data for both commercial and stressed ADC samples. Finally, once the exhaustive characterization of ADC was completed, MS could be conveniently replaced by UV detection to quickly assess the conformity of different ADCs batches.

1. Introduction

Hydrophobic interaction chromatography (HIC) is one of the most popular modes of chromatography that is widely applied for the characterization of antibody-drug conjugates (ADCs). This reference technique enables the separation of the different populations of ADC molecules that differ in their number of drugs *per* antibody which are often known as DAR (drug-to-antibody ratio) species [1,2]. For cysteine linked ADCs, thiol conjugates are produced by the partial reduction of disulphide bridges and followed by the conjugation with a drug linker, resulting in a heterogeneous population that differs with respect to the site of conjugation and the number of drugs *per* antibody (DAR) [1].

The drug-loading distribution and conjugation sites of ADCs have been reported to influence pharmacokinetic, toxicity, clearance and therapeutic index [3–5], therefore it is important to determine the average DAR and distribution of the different populations. One of the most important quality attributes of an ADC is the average number of drugs that are conjugated, because this determines the amount of "payload" that can be delivered to the tumor cell. A fully conjugated IgG1 ADC has a maximum DAR of 8 and is composed of a heterogeneous mixture of 0, 2, 4, 6, and 8 DARs [6]. An odd number (e.g. DAR 1 or DAR 3) of conjugated drug is typically indicative of incomplete conjugation or degradation, and odd DARs are therefore mostly observed in very small amount [1].

Obviously, other techniques than HIC can also be used to determine the average DAR and DAR distribution [7]. UV–vis spectrophotometry, reversed-phase liquid chromatography (RPLC) or sodium-dodecyl-sulfate capillary gel electrophoresis (CGE-SDS) are often used for cysteine linked ADCs, while ion-exchange

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chromatography (IEX) or capillary isoelectric focusing (cIEF) are routinely used for lysine conjugations [6,7]. With RPLC, not only the average DAR, but also the amount of each positional isomer can be determined. Native mass spectrometry (MS) is also an important tool for the determination of average DAR and DAR distribution [8].

Multi-dimensional (2D) chromatography could be a powerful technique for ADC characterization, since it allows the hyphenation of MS incompatible chromatographic modes to MS instrumentation. Three approaches are applied for multi-dimensional separations of ADC samples: (i) off-line sample collection (LC–MS), (ii) heart-cutting approach (LC–LC–MS) and (iii) fully comprehensive on-line approach (LCxLC–MS). Debaene et al. applied HIC for off-line native MS characterization of ADC (brentuximab vedotin) [9]. HIC fractions were collected and then analyzed by ion mobility (IMS) combined with native MS. On-line multi-dimensional LC of HIC and MS using an RPLC desalting step prior to MS was applied by Birdsall et al. [10]. Their heart-cutting setup provided the identification of positional isomers and the drug conjugation site confirmation. This approach however requires as many injections as peaks to be analyzed. On-line comprehensive two-dimensional liquid chromatography could therefore be an attractive alternative for reducing both sample consumption and analysis time.

It has been shown in the first part of this study that combining HIC in the first dimension of an on-line comprehensive 2D-separations and RPLC in the second dimension was a promising solution for MS-coupling, given the fact that the salt can be completely eliminated in the second dimension [11]. In addition, the whole sample is subjected to two different separation modes in comprehensive 2D-LC which dramatically increases the separation power provided of course that the degree of orthogonality of the combination is sufficient. Indeed, we demonstrated that the second RPLC-dimension permitted to obtain a good separation of the different sub-units of the DAR positional isomers. It was also shown that 2D retention times were highly repeatable and hence able to provide useful additional information for completing MS identification. As a result, an extensive information on the peaks observed in the first HIC-dimension can be expected from 2D-data. The objective of this second study was therefore to cross information from retention and MS data to clearly identify the peaks obtained in the second dimension, thus tracking the ADC structure. Non-stressed and stressed brentuximab vedotin samples were analyzed to assist the ADC characterization. The final objective was to demonstrate that, once the characterization was completed, MS could be conveniently replaced by UV detection to quickly assess the conformity of different ADC batches.

2. Experimental section

For detailed explanations, the reader is kindly referred to Part I [11] of the present study, in particular to Table 3 for the description of the HIC x RPLC-UV/MS method and to Fig. 2 for instrumental set-up.

2.1. MS-data processing

Mass spectra were deconvoluted using the MaxEnt 3 algorithm of Waters MassLynx.

2.2. Protocol to obtain stressed ADC samples

The Brentuximab vedotin ADC sample was analyzed as soon as received (non-stressed sample) and after having been subjected at 40 °C during one month (one-month stressed sample) and two months (two-month stressed sample).

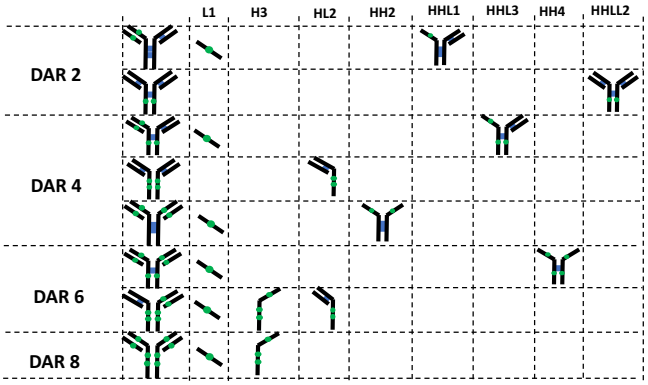

Fig. 1. Expected sub-units for the positional isomers of even DARs resulting from denaturing conditions in RPLC. Abbreviations are defined as "L" for light chain; "H" for heavy chain with numbers 1, 2, 3 and 4 referring to the number of drugs.

3. Results and discussion

3.1. Structural elucidation of even DAR positional isomers by on-line HICxRPLC–MS

Brentuximab vedotin is a cysteine-conjugated ADC. The conjugation of drugs to the antibody results in a heterogeneous population of DARs. Drug loads are usually expected in even intervals (2, 4, 6 and 8). The positional isomers of even DARs are shown in Fig. 1 as well as the expected sub-units resulting from disruption of non-covalent interaction in a denaturing medium such as RPLC hydro-organic mobile phase. For sake of convenience, abbreviations for the different sub-units have been used all along this paper in the following way: "L" and "H" refer to light and heavy chains respectively; a combination of "L" and "H" is used when several chains are present; the number at the end of the abbreviation indicates the number of payloads without specification of their location in the sub-unit. For example, HHL1 refers to two heavy chains, one light chain and one drug. HHL1 corresponds to a theoretical mass of 125675 Da, as calculated from the work of Janin-Bussat et al. [12]. Theoretical masses for both sub-units and even DARs are given in Table 1.

The different steps leading to identification of the different peaks obtained in the second RPLC dimension have been reported in Fig. 2 for the particular case of DAR 6, whose peak apex was located at a retention time of 58.5 min in HIC (Fig. 2a). The fraction between 58.5 and 60 min was sent to the second RPLC-dimension and led to the separation of four major sub-units, as highlighted by MS-Total Ion Current (MS-TIC) chromatogram (Fig. 2b). Sub-units could be identified after deconvolution of mass spectra (Fig. 2c), leading to measured masses of 25040.4 Da (peak 1) matching with L1, 76676.4 Da (peak 2) matching with HL2, 105915 Da (peak 3) matching with HH4 and 54271.3 Da (peak 4) matching with H3. In the cases of HL2 and H3, two masses differing by 162 Da were observed as a result of microheterogeneity in the sub-units (difference in glycoforms G1F/G0F). MS-sensitivity was limited for HH4 and prevented from unambiguously identifying glycosylation (G1F). Measured masses can be compared to theoretical ones in Table 1 for all the identified sub-units. The observed errors were 24, 26, 63 and 123 ppm for L1, HL2, H3 and HH4, respectively. The increase in error with the mass of the sub-unit is likely to be due to a lower sensitivity for larger sub-units. Increase in error on measured masses may also be the result of less efficient de-adducting of the higher loaded species [9]. Fig. 3 shows the HICxRPLC-UV analysis of brentuximab vedotin. Even DARs are given on the left side of the 1D-HIC separation. The corresponding sub-units that were further identified by mass spectra deconvolution are indicated at the top of