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.

I - Optimization of separation conditions

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Abstract

Antibody-drug-conjugates (ADCs) manufacturing leads to a mixture of species which need to be characterized during development and for further quality control. The coupling of on-line HIC x RPLC to high resolution mass spectrometry can be considered as a very efficient analytical method, providing extensive information on ADC sample, within a reduced time scale. Our intention in this first paper is to present the approach used to rationally optimize the numerous conditions that can affect the quality of the 2D-separation. HIC and RPLC conditions were therefore optimized to prevent salt precipitation due to solvent mixing and to enhance sensitivity, while limiting the total analysis time. We demonstrated that adding salt in the sample solvent before HIC injection allows a significant peak shape improvement. The gradient profile was also carefully optimized in both dimensions, leading to a two-step gradient in HIC and bracketed gradient in RPLC. This study shows that on-line HIC x RPLC hyphenated to high resolution mass spectrometry is a useful method to obtain rapid and

extensive structural information on the peaks observed in the first HIC dimension, thereby leading, in a single step requiring 75 min, to the precise determination of the average drug-to-antibody ratio (DAR) by HIC as well as the knowledge of the drug load distribution for a particular DAR. The structural characterization of ADC fragments by RPLC-QTOF will be discussed in the second part of this two-part series.

Keywords

On-line comprehensive 2D-LC, HICxRPLC-QTOF-MS, antibody-drug conjugate, ADC, brentuximab vedotin, Adcetris

1 Introduction

Monoclonal antibodies (mAbs) and their related products such as antibody-drug-conjugates (ADCs), bispecific antibodies, Fab fragments, Fc-fusion proteins and radio-immunoconjugates are the fastest growing class of human therapeutics [1]. Even if numerous mAbs and derivatives have been approved, the first generation mAbs often suffers from a limited efficacy. ADC technology combines the specificity of a monoclonal antibody (mAb) together with a potent cytotoxic drug covalently bounded via a linker to the antibody, allowing an efficacy improvement. There are two main types of ADC technology based either on cysteine or lysine linked conjugations and currently - as a third class – the so-called engineered ADCs (cysteine residues are engineered on the surface of the mAb) are often mentioned as a promising technology. Cysteine linked ADCs are made by the chemical conjugation of cytotoxins to mAbs, by first reducing inter-chain disulfide bonds, followed by the conjugation of the resulting free thiols with drugs. This process yields a controlled, but heterogeneous population of conjugated products that contains species with various numbers of drugs linked to different former inter-chain disulfide cysteine residues on the antibodies [2]. This process results in conjugates with a distribution of 0, 2, 4, 6 and 8 drugs incorporated per antibody and an average DAR of around 3-4 drugs/mAb which seems to be optimal in clinical trials [3,4]. Figure 1 shows the possible positional isomers of DAR species of a cysteine conjugated ADC. In ADC products, the DAR0 is considered as an impurity while DAR species with odd numbers (DAR1 or DAR3) are degradation products.

Among the several liquid chromatographic (LC) modes applied for the analysis of ADCs, hydrophobic interaction chromatography (HIC) is the reference technique to separate the different populations of DARs. The main advantage of HIC compared to other LC modes (reversed phase (RP) particularly) is that it is non-denaturing, so the native forms of the proteins are expected to be maintained. HIC allows the separation of the different DARs of native cysteine conjugated IgG1 type ADCs such as DAR0, DAR2, DAR4, DAR6 and DAR8

under their physiological-like conformation [5–7]. In an orthogonal way, DAR species can also be separated by RPLC under reducing conditions as well as capillary electrophoresis sodium dodecyl sulfate (CE-SDS) under both non-reducing and reducing conditions [2]. Finally, mass spectrometry (MS) is ultimately used for structural assessment [8,9].

MAbs are heterogeneous and possess several microvariants including glyco variants, charge variants and size variants [10]. In addition, the conjugation of payloads to mAb further increases the structural complexity, and requires improved methods for the characterization of drug loading, distribution, average DAR, size and charge variants or unconjugated drug linkers [11]. State-of-the-art structural characterization methods of ADCs were recently reviewed by Beck and Cianferani and case studies of cysteine-linked Brentuximab vedotin (Adcetris®) and lysine-linked Trastuzumab emtansine (Kadcyla®) were also discussed [12,13]. ADC analysis and characterization can be performed at different levels such as top-down, middle-down and bottom-up levels. Coupling of different techniques such as LC-MS or multidimensional LC separations may offer additional benefits over one-dimensional methods [14,15].

Recently, Debaene *et al.* applied HIC and off-line ion mobility native mass spectrometry (IM-MS) for the detailed characterization of Brentuximab vedotin [16]. The main advantage of using native MS for exact mass measurements of ADCs with inter-chain cysteine-linked drugs, lies in its ability to detect non-covalent associations of light and heavy chains that cannot be detected directly with classical denaturing LC-MS methods. This methodology resulted in a fast determination of DAR ratio, average DAR and naked mAb, but provided no information about the drug loading position.

An improved LC-MS peptide mapping protocol to characterize the drug loaded peptides was reported by Janin-Bussat *et al.* [17]. This protocol was developed for the commercially available Brentuximab vedotin. Because of the drug hydrophobicity, all the steps (including enzymatic digestion) were improved to maintain the hydrophobic drug-loaded peptides in solution, allowing their unambiguous identification by LC–MS. For the first time, the payloads positional isomers observed by RP-LC after IdeS-digestion and reduction of the ADC were also characterized.

Recently, an interesting work showed that multi-dimensional (2D) chromatography could be a powerful technique for ADC analysis and characterization, since it allows the hyphenation of non-compatible MS chromatographic method (i.e. HIC as the first dimension) to MS instrumentation via a suitable interface (involving the use of RPLC as the second dimension) [18]. Informative data could be obtained with intact ADC injected in non-denaturing medium in first dimension. Even DARs were separated and injected in the second dimension leading to the separation of sub-units resulting from denaturing RPLC conditions. Although this approach was efficient, this 2D-LC method, based on heart-cuts of the first dimension peaks,

requires as many injections as peaks to be analyzed, which makes this strategy sample-consuming and hence less attractive when only a limited sample amount is available. Moreover, this approach may be time consuming and requires 3 pumps, including first and second dimension pumps as well as a regenerating pump. On-line comprehensive two-dimensional liquid chromatography could therefore be an attractive alternative for reducing both sample consumption and analysis time. In addition, in on-line LCxLC, the entire first dimension separation is subjected to the second separation. As a result, the risk of missing peaks of interest which in turn may lead to lose essential information is avoided. However, it was recently shown that the optimization of on-line LCxLC conditions may turn out to be a tedious task without an efficient method development, able to find the best trade-off between peak capacity, sensitivity and analysis time [19]. The purpose of the present study is therefore to develop a straightforward on-line HICxRPLC–MS method. In the first part of this study, the conditions of the two-dimensional separation were rationally optimized to gain information that would help in enlarging the current knowledge on ADCs. Our objective is to obtain in one single injection, both average DAR determination and comprehensive identification of positional isomers, thereby allowing the characterization of both even and odd DARs. This second aspect will be extensively discussed in the second part of this study.

2 Experimental section

2.1 Material and reagents

Acetonitrile (ACN), methanol (MeOH), formic acid (FA) and trifluoroacetic acid (TFA) all LC-MS grade were purchased from Sigma-Aldrich (Steinheim, Germany). Ultra-pure water was obtained from an Elga water purification system (Veolia water STI, Le Plessis Robinson, France). For HIC mobile phase, ammonium-sulfate, ammonium-acetate (AA), ammonium-formate and sodium-chloride were purchased from Sigma Aldrich (Steinheim, Germany). Disodium-hydrogen-phosphate dodecahydrate, sodium-dihydrogen-phosphate (anhydrous), phosphoric acid and sodium hydroxide were purchased from Acros Organic (New Jersey, USA). Brentuximab Vedotin (Adcetris®) and Brentuximab B were kindly provided by the Center of Immunology Pierre Fabre (Saint-Julien en Genevois, France). Brentuximab B is a mAb differing from naked Brentuximab in three amino acids. Their concentrations were 5 mg/mL and 2 mg/mL respectively. The pH values were measured with a Standard pH meter (Radiometer analytical, Villeurbanne, France).

2.2 Columns

The HIC column was a MabPac HIC-10 (100 mm x 4.6 mm, 5 μ m, 1000 Å) from Thermo Scientific (Cheshire, UK). The RPLC column was an Acquity UPLC Protein BEH C4 (50 mm x 2.1 mm, 1.7 μ m, 300 Å) from Waters (Milford, MA, USA).

2.3 Instrumentation

The LCxLC-MS system is presented in Fig.2. It consists in a 2D-Iclass liquid chromatograph hyphenated to a quadrupole time-of-flight Xevo G2-S Q-Tof, both from Waters (Milford, MA, USA). The 2D-Iclass instrument includes two high-pressure binary solvent delivery pumps, an autosampler with a flow-through needle of 15 μ L equipped with an extension loop of 50 μ L, a column manager composed of two independent column ovens with a maximum temperature of 90°C and two 6-port high pressure two-position valves acting as interface between the two separation dimensions. A single wavelength UV detector and a diode array detector both equipped with 500 nL flow-cell withstanding pressure up to 70 bar were used for the first and second dimension respectively. The maximum pressure capability is 1285 bar for both pumps. Beyond a flow-rate of 1 mL/min, the upper pressure limit decreases depending on both flow-rate and mobile phase composition. The measured dwell volumes were 160 μ L and 100 μ L for the first and the second dimensions, respectively. It should be noted that the dwell volume of the second dimension does not take into account the volume of the sample loops used at the interface. The measured extra-volumes were 12 μ L and 17 μ L for the first and second dimensions, respectively. The volume of the sample loops used for interfacing both dimensions was 200 μ L. 1D-LC experiments were carried out with the first dimension instrument. For the LCxLC-MS experiments, an external 10-port high pressure 2-positions valve (Vici Valco Instruments, Houston, USA) was placed before the MS to avoid any salt contamination due to the injection plug. A flow splitter was also used to reduce the flow rate before entering MS.

Data acquisition and instrument controlling were performed with Masslynx software (Waters). 2D-data were exported to Microsoft Excel and Matlab V7.12.0635 was used to construct a matrix via house-made calculation tools enabling the construction of 2D-contour plots.

2.4 Experimental conditions

2.4.1 Sample preparation

Both Brentuximab Vedotin and Brentuximab B were injected as intact proteins.

2.4.1. Chromatographic conditions

Three ammonium acetate (AA) concentrations were considered to optimize the HIC separation, namely 2.5, 3 and 3.5 M. The mobile phase A was composed of the desired concentration of AA and 0.1 M phosphate buffer (Na_2HPO_4) with pH adjusted to 7 with phosphoric acid. The mobile phase B was composed of 0.1 M phosphate (Na_2HPO_4 and NaH_2PO_4) with pH adjusted to 7 with sodium hydroxide solution. For this optimization, the gradient conditions were 0 to 100 % B in 10 min at a flow-rate of 800 $\mu\text{L}/\text{min}$. Column temperature, wavelength and acquisition rate were set at 30 $^\circ\text{C}$, 280 nm and 10 Hz respectively.

The acquisition rates for UV detection were set at 10 Hz and 40 Hz for the first and second dimensions respectively. The wavelength was set at 280 nm for both dimensions. The conditions in HICxRPLC result from specific development discussed in this work.

2.4.2. Mass spectrometry conditions

Continuous data were acquired in sensitivity mode with positive polarity. A mass to charge ratio (m/z) from 500 to 3500 was used for data collection. Other mass spectrometer settings were 3.20 kV and 120 V as capillary and sample cone voltage, respectively. Source and desolvation temperature were equal to 110 and 500 $^\circ\text{C}$, respectively. Gas flow for desolvation and cone were set at 900 and 50 L/Hr, respectively. The scan time was set at 0.5 s.

3 Results and discussion

3.1 Optimization of HICxRPLC-MS separation

3.1.1 Optimization of ^1D -HIC conditions

3.1.1.1. Phase system optimization in HIC

Ammonium sulfate is the most widely used salt for HIC, as it often leads to an efficient separation of the different DARs [5–7,20]. However, it is not a good candidate for on-line HICxRPLC, as it may precipitate with organic solvent in the second RP dimension, even at very low salt concentrations, resulting in pressure elevation and risk of clogging. Thus, different combination of stationary phases and salts were evaluated.

First, the stationary phase was selected on the basis of DARs retention (elution) window and achievable peak capacity (efficiency). Among the six tested columns at disposal (i.e. Thermo MAbPac HIC-10, Thermo MAbPac HIC-20, Thermo MAbPac HIC-butyl, Thermo ProPac HIC-

10, Waters Protein-Pak Hi Res HIC and Tosoh TSKgel Ether-5PW), the Thermo Mab-Pac HIC-10 was selected since it provided appropriate retention, selectivity and peak capacity. Some of the columns (Thermo MAbPac HIC-20 and Thermo MAbPac HIC-butyl) did not allowed the elution of the most hydrophobic DAR species (DAR6 and DAR8) of brentuximab-vedotin [21].

Then, the mobile phase salt concentration and type (lyotropic strength) were optimized, since these variables are highly relevant for tuning HIC retention and selectivity. Generally, the influence of different salts on hydrophobic interactions follows the Hoffmeister (lyotropic) series for the precipitation of proteins from aqueous solutions [22]. Not only the effect of salt concentration on ADC DARs retention was evaluated, but the impact of different salts on selectivity was also studied.

First, the solubility of 1 – 4 M ammonium-sulfate, ammonium-acetate, ammonium-formate and sodium-chloride was assessed in 10, 30, 50% and 100% methanol and acetonitrile. Based on the results, ammonium-sulfate was found to be the less appropriate salt, while ammonium-acetate and ammonium-formate showed acceptable solubility in all organic mixtures. The solubility was found to be somewhat lower in neat acetonitrile than in methanol.

Ammonium-acetate and ammonium-formate were then compared. Ammonium-formate showed somewhat lower elution-strength than ammonium-acetate and therefore required higher salt concentration to sufficiently retain the DAR0 and DAR2 species. Higher salt concentration is not recommended, because of the high organic modifier content in the second RP dimension. Therefore, taking the solubility and elution strength of the four salts into account, ammonium-acetate was selected for the HIC separation.

Finally, the salt concentration was optimized. Based on the preliminary results, 2.5, 3.0 and 3.5 M ammonium-acetate were tested when applying a linear salt gradient from 100 to 0% salt concentration. . To keep sufficient retention for the least retained compounds, while minimizing the analysis time, 2.5 M was found to be the optimum concentration (see supplementary Figure S1).

3.1.1.2. Optimization of ¹D-Gradient conditions in HIC

The analysis time in the first dimension separation has to be minimized, since it represents the analysis time of the whole LCxLC separation. In the meantime, the separation power has to be maximized. Furthermore, with the objective to send in ²D, a constant number of fractions per peak of first dimension, gradient conditions must be carefully chosen, so that the widths can be kept constant for all peaks. To achieve these conflicting objectives, HIC

gradient conditions were optimized using computer assisted optimization (Osiris software). Three preliminary experiments were performed under linear gradient conditions with three different normalized gradient slopes (5%, 15% and 25%), the normalized gradient slope being the product of the gradient slope and the column dead time. It was shown that the Linear Solvent Strength model (LSS) [23] was accurate enough to describe the retention behavior of DAR species in HIC. The corresponding retention model ($\log k$) can be written as:

$$\log k = \log k_0 - S \cdot C \quad (1)$$

where $\log k_0$ is the extrapolation of the retention model to a null value of the salt concentration, C is the salt concentration and S is the slope. S and $\log k_0$ can be determined from two gradient runs. As shown in Fig. 3a, the variation of S with $\log k_0$ for the different peaks is linear which is in good accordance with the fact that only one mechanism is involved in the HIC separation process (i.e. hydrophobic interaction mechanism). The strong increase in S value with the number of drugs and hence with the hydrophobicity of DAR species (Fig. 3b) suggests that a linear gradient cannot be suitable for the purpose of keeping both the same peak width and the same peak spacing all over the separation and that a concave profile should be much more appropriate. The optimum predicted gradient separation is given in Fig. 4a, while the experimental one is shown in Fig. 4b. A first normalized gradient slope of 25% was first applied followed by a second normalized gradient slope of 5%. As expected, the predicted separation is in good agreement with the experimental one. However, it can be observed that the least retained peaks (e.g. DAR 0) are strongly distorted. This is mainly due to injection effects produced by the combination of large injection volume (20 μ L) and strong injection solvent (no salt).

3.1.1.3. Effect of salt addition in the sample solvent in HIC

To enhance sensitivity in HICxRPLC-MS, large injection volumes are mandatory. However, strong injection effects such as peak distortions and breakthrough phenomena may occur as above highlighted, particularly for the least retained compound (i.e. DAR 0 and DAR 2). Decreasing the eluent strength of the sample solvent is a good way to improve the peak shape. In HIC, the eluent strength can be decreased by increasing the ionic strength. The effect of different AA concentrations in the sample solvent on Brentuximab B (home-made naked brentuximab) peak shape can be observed in Fig. 5 for different injection volumes. As observed, the phenomenon of breakthrough appears for salt concentrations lower than 1.5 M even for very low injection volumes. On the contrary, this phenomenon no longer exists at higher salt concentrations. The obtained results clearly point out that a volume as high as 40

μL can be injected onto the column without peak distortions, provided that the concentration of ammonium acetate in the injection solvent is 2.5 M or more.

As highlighted in this study, the addition of AA into the sample solvent is very efficient to improve mAbs peak shape in HIC. Such ionic strength conditions were further applied to the ADC sample. The obtained separations for different injection volumes with 2.5 M AA added to the sample solvent are shown in Fig. 6. As expected, neither peak distortion nor peak broadening can be observed with injection volumes as high as 40 μL. Thus, these conditions (2.5 M AA in injection solvent and 40 μL as injected volume) were further selected for 2D experiments with the goal to significantly enhance sensitivity of least retained compounds.

3.1.2 Optimization of RPLC-MS conditions

3.1.2.1. Selection of organic modifier in RPLC

To avoid a potential precipitation issue when the injection plug coming from the first HIC dimension is diluted in the second RPLC mobile phase, methanol was first preferred to acetonitrile. However, with very short gradient times, the optimal flow-rate to achieve the highest possible peak capacity is much higher than the one to achieve the best plate number (van Deemter curve), thereby leading to the need for very high flow-rates in the second dimension [24] to maximize peak capacity. In this context, acetonitrile (ACN) is expected to be more attractive than methanol (MeOH) due to its lower viscosity. The two organic modifiers were therefore compared at the highest possible flow-rate compatible with the instrumentation, namely 1.2 and 1.4 mL/min for MeOH and ACN, respectively. The comparison was based on the achievable peak capacity obtained in the second RPLC dimension for a given sampling time (i.e. 1 min), considering the separation of the fractions of DAR 6. Fig. 7 illustrates the overlaid separations of the different sub-units of DAR 6 resulting from disruption of non-covalent bonds in RPLC denaturing conditions. It can be observed that the retention space was larger when acetonitrile was used as organic modifier. For a more reliable assessment of the separation power, the experimental peak capacity, 2n , was calculated in both cases according to the following relationship:

$$^2n = 1 + \Delta t / ^2w$$

Where Δt is the retention space represented by the difference in retention times between the last and the first eluted compounds and 2w is the average peak width at $4 \cdot ^2\sigma$.

The results listed in Table 1, point out a 1.5-fold gain in peak capacity when using acetonitrile rather than methanol. As a result, acetonitrile was chosen for the rest of the study.

3.1.2.2. Optimization of gradient conditions in RPLC

The ²D-RPLC method has to fulfill two major objectives: (i) minimizing the risk of precipitation which could result from the large amount of salt injected from the first dimension to the hydro-organic media of the second dimension has to be avoided and (ii) maximizing the peak capacity in order to facilitate MS spectra deconvolution.

To avoid precipitation, an easy solution may consist in injecting a very low sample volume in the second dimension. This can be done either by setting a short sampling time or by using a flow splitter to reduce the flow-rate entering the interface. However, shortening the sampling time results in decreasing the peak capacity, while increasing sample dilution. On the other hand, flow splitting allows to keep an acceptable peak capacity but also leads to high dilution and hence low sensitivity. An alternative solution consists in using a gradient elution bracketed by two water steps so that the contact of the injection plug, rich in salt, with the mobile phase of the second dimension, rich in organic solvent, can be minimized. According to this idea, the second dimension RPLC gradient conditions were modified by programming an isocratic hold (99% water) at the beginning and at the end of the gradient method, to complete the elimination of the salt in both the loop and the column before the organic solvent was in contact with the injection plug.

To maximize the peak capacity, we took into account the strong focusing power of water as injection solvent in RPLC, thereby allowing the injection of large volumes without the occurrence of peak distortion. As a result, sampling times and hence peak capacities could be maximized. To keep an acceptable sampling rate (2-3 fractions *per peak*), the sampling time was set at 1.5 min, corresponding to an injection volume of 150 µL. Accordingly, two identical sample loops of 200 µL were selected for the interface. Their volume was slightly higher than the injection volume to prevent any sample loss. It should be underlined that the sampling time must include the bracketing times in addition to the gradient time. Once again, it was of prime importance to work at the highest possible flow-rate so that the column dead time was as low as possible. The use of high column temperature in the second dimension was therefore mandatory.

Instrumental dwell volume (100 µL), loop volume (200 µL) and column dead volume (about 100 µL) were carefully considered to determine the duration of the isocratic hold, which has to be long enough to avoid any salt precipitation and as short as possible to maximize peak

capacity. Considering these two conflicting objectives, the duration of the first and the second isocratic holds were set at 0.14 min and 0.28 min respectively, corresponding to solvent volumes of 200 μ L and 400 μ L, respectively for a second dimension flow-rate of 1.4 mL/min. In these conditions, the injection plug was therefore washed out with 350 μ L of water-rich solvent (99% water). Such a volume corresponds to more than two injection volumes (350 μ L vs 150 μ L). At the end of the analysis run, the column was filled with the same solvent, thereby bracketing the next injection plug. Considering the duration of the two isocratic holds, the time available for the gradient was 1.06 min which led to a reasonable normalized gradient slope of 0.56% with 8% as composition range. The gradient conditions for the 2 D-RPLC separation are given in table 2.

3.1.2.3. Further RPLC optimization designed to enhance MS Detection

To increase sensitivity, 0.1 % FA was added to the mobile phase in addition to 0.05% TFA [25]. To prevent any salt contamination in the MS source resulting from the very high salt concentration in the injection plug, the 2 D-mobile phase coming from the UV-detector was sent to the waste, thanks to a two-positions switching valve. This was done for a period which depends on the width of the solvent peak.. The time required for the salt peak to fully return to the baseline corresponds to about 0.4 min after the injection time, thereby fixing the time required to eliminate the whole amount of salt (see supplementary Figure S2). It should be noticed that this time is more than two-fold higher than the time required for on-column injecting and column travelling (i.e. about 0.18 min) as a result of the high dilution of the injection plug in the mobile phase. PolyEthylene Glycol (PEG) was also detected from the Total Ion Current signal (MS-TIC-signal) at the beginning of the gradient run due to its presence in the ADC formulation. However, the most intense signal for PEG was found within 500 to 800 m/z as mass range which did not constitute a barrier for the MS-detection of ADC sub-units considering their higher expected mass-to-charge values. Furthermore, the flow was split post-column so that the flow-rate entering MS was 750 μ L/min only to enhance ionization, while 650 μ L/min were directed to UV detection.

3.2 *HICxRPLC-MS results*

The above optimized conditions were applied to the HIC x RPLC-MS separation of brentuximab vedotin[®]. All operating conditions were summarized in Table 3. The obtained

first dimension HIC separation is shown in Fig. 8. The successive fractions sent to the second dimension are delimited from vertical lines. As expected by our optimization approach above discussed, two to three fractions per peak could be analyzed in the second RPLC-dimension, ensuring a comprehensive on-line 2D-LC separation. The integration of the peak areas of the different even DARs allowed to determine the average DAR (avDAR) value according to the following relationship [2,26]:

$$avDAR = \frac{\sum_0^8 n A_{DARn}}{\sum_0^8 A_{DARn}} \quad (2)$$

where n correspond to the number of loaded drug and A_{DARn} corresponds to the peak area of DAR n, n being the DAR number.

The results are shown in Table 4. The obtained value of 3.9 ± 0.1 is in good agreement with those found in the literature [16,27]. Furthermore, the reliability of this measure seems to be very good, considering the obtained relative standard deviation of 1.5% calculated from 3 replicate injections (Table 4). These results demonstrate the ability of the ¹D-HIC method to provide an accurate determination of the average DAR.

The 2D-colour plots are shown in Figs. 9a and 9b for UV and MS-signal, respectively. Each horizontal dotted line shown in Fig. 9b, passes through the peak apex of the different sub-units obtained from the dissociation of a given species. Horizontal black dotted lines allow to locate the separations of the 5 fractions corresponding to the peak apex of the 5 even DARs in 1D- HIC (see also Fig. 8). As shown by the black spots in Fig. 9b, 17 peaks were found in this 2D-map. Some of them are vertically aligned suggesting that the corresponding sub-units are the same. The vertical alignment of these colored spots is noteworthy. This observation highlights the very good run-to-run repeatability of retention times and as a result, the ability of this ²D-RPLC method to discriminate between the different spots. Here, the three vertical lines in Fig. 9b indicate three series of identical sub-units. However, it appears that 6 spots are not aligned with the others thereby suggesting that the corresponding sub-units are quite different. Similarly, some horizontal dotted lines (highlighted in red in Fig. 9b) do not correspond to any peak apex of even DARs. They suggest that some additional species, unknown so far, are also separated to some extent in ¹D-HIC. It can be concluded that 2D-maps can be very helpful for further MS identification of sub-units and beyond sub-units, for identification of the different species present in the ADC product. This will be discussed in more details in the second part of this study.

Finally, it is very interesting to notice the excellent similarity between two 2D-UV-colour-plots obtained from the same brentuximab vedotin[®], but analyzed with four days between injections (Figs 9a and 9c). This result points out that, in addition to good run-to-run

repeatability of ²D separations, as above discussed, the variability of the whole developed 2D-separation method is very low. The perfect similarity between the two 2D-separations is attested by the excellent correspondence between ²D-retention times as emphasizes by the vertical dotted lines joining the spots in Fig. 9a to those in Fig. 9c. The low variability of this comprehensive 2D-separation method can further be assessed by observing the similar intensity of UV-signals between the two successive separations. The high reliability of this comprehensive 2D-separation method associated to the versatile information it can provide, suggests that such 2D-colour plots, obtained from either UV or MS signal or from both, might constitute a very convenient tool to quickly evaluate the quality of a given ADC. Knowing that two different ADCs can still deliver similar HIC profiles, 2D-plots could be indeed very useful for gathering extensive information within a single run. This feature will be more extensively discussed in the second part of this study wherein 2D-colour plots of stressed ADCs will be compared to that of the original one.

4 Conclusion

The comprehensive characterization of ADCs is a challenging task due to their high complexity. In this work, an on-line HICxRPLC-MS method was successfully developed leading to an informative 2D-separation requiring 75 min only. The objective was to get relevant information on both drug loaded profile (average DAR) and structural information on positional isomers of each DAR of a cysteine-linked commercial ADC (brentuximab vedotin, Adcetris®).

To achieve this goal, several optimization steps were tackled. ¹D-HIC conditions were carefully optimized by (i) replacing the usual salts employed in the mobile phase with ammonium acetate to improve the solubility of the salt in the hydro-organic media of the second dimension and MS hyphenation (ii) using a concave gradient thanks to a computer assisted method based on LSS model, which was found to accurately predict the retention behavior of different DARs and (iii) adding a sufficient amount of salt in the sample before

injection to reduce the injection solvent strength thereby allowing to increase the injected amount and hence sensitivity. ²D-RPLC gradient conditions were also carefully optimized. Furthermore, the gradient profile was bracketed by two water steps to prevent any salt precipitation at the injection time and both very high flow-rate and high temperature were used to maximize peak capacity. Peak capacity was also enhanced thanks to the strong column focusing effect in the second dimension, allowing a sampling time of 1.5 min and therefore very large injection volume (150% of the second dimension column dead volume). Finally, the flow-rate coming from the second dimension was first diverted to discard the salt plug coming from ¹D fractions to preserve MS detector and then decreased to ensure a sufficient sensitivity.

The obtained average DAR value was found in very good accordance with those reported in the literature. In addition, the LCxLC-UV method was found to be highly reproducible and very informative and could therefore be used for a rapid characterization of ADCs. We demonstrated that the precise determination of the average drug-to-antibody ratio (DAR) and an extensive knowledge of the drug load distribution could be obtained in a single step method. The identification of the different sub-units related to their specific DAR will be discussed in the second part of this series.

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Figure captions

Figure 1: Representation of positional isomers for the different even DARs of a cysteine conjugated ADC.

Figure 2: HICxRPLC set-up. The eluent of the first dimension is stored in the loop 1, while the content of loop 2 is injected in the second dimension. Abbreviations are defined as SV: switching valve; UV : UV detector; DAD : diode array detector; QTOF : quadrupole time-of-flight mass spectrometer.

Figure 3: Variation in HIC, of the coefficient S in Eq. 1 with (a) the calculated $\log(k_0)$ and (b) the number of drugs. Conditions: Mobile phase A: 2.5 M ammonium acetate + 0.1 M phosphate and B: 0.1 M phosphate; 800 $\mu\text{L}/\text{min}$, gradient from 0 to 100% B in 24.9 min (5%), 8.3 min (15%) and 5 min (25%). 20 μL injected; 30°C. S and $\log k_0$ were calculated from Osiris software.

Figure 4: Comparison between (a) predicted and (b) experimental ^1D HIC separations of Brentuximab Vedotin. Mobile phase A: 2.5 M ammonium acetate + 0.1 M phosphate and B:

0.1 M phosphate; 100 μ L/min, gradient from 0-90% B in 36 min and 90-100% B in 21 min. 20 μ L injected; 30°C. The straight lines represent the gradient profile (Ce is the eluent composition at the column exit). See experimental section for other conditions.

Figure 5: Effect of different concentrations of ammonium acetate in the sample solvent on the peak shape of Brentuximab B for different injection volumes. (a) 0 M ; (b) 1.5 M ; (c) 2.5 M and (d) 3.5 M . Optimized HIC conditions as in Fig. 4.

Figure 6: Effect of different injection volumes on the HIC separation of ADC. 2.5 M as ammonium concentration in the sample. Optimized HIC conditions as in Fig. 4.

Figure 7: Overlay of ²D RPLC separations of fractions of DAR 6 with (a) MeOH; 1.2 mL/min; 50 to 58% in 0.6 min and (b) ACN; 1.4 mL/min; 42 to 50% B in 0.7 min. Sampling time: 1 min; 80°C; 280 nm as wavelength. Figure 8: Optimized ¹D HIC separation. The successive fractions of 1.5 min sent in the second dimension are delimited from vertical lines. Conditions as in Fig. 4.

Figure 9: 2D-colour plots of the HIC x RPLC separation of Brentuximab Vedotin using optimized conditions displayed in Table 3. (a) first UV-signal, (b) Total Ion Current MS signal and (c) second-UV-signal obtained four days after the first one.

600 Table 1 : Comparison of the peak capacities obtained in Fig.8 for the separation of the fractions of
 601 DAR 6. MeOH and ACN as organic modifier. Conditions given in Fig.8

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	MeOH ($\Delta t=17s$)		ACN ($\Delta t=23s$)	
	$w_{4\sigma}$ (s)	2n_c	$w_{4\sigma}$ (s)	2n_c
Peak 1	1.9	8.9	1.8	12.8
Peak 2	3.2	5.3	2.7	8.5
Peak 3	3.4	5	3.4	6.8
Average value	2.8	6	2.6	9

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606 Table 2 : ²D RPLC method with bracketing water steps

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Time (min)	%B (ACN+0.05%TFA+0.1%FA)
0	1
0.14	1
0.15	32
1.21	40
1.22	1
1.5	1

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610 Table 3 : HICxRPLC conditions

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Chromatographic conditions	1 st dimension (1 ^D)	2 nd dimension (2 ^D)
Stationary phase	MabPac HIC-10	Acquity Protein BEH C4
Column geometry	100x4.6 mm, 5 µm 1000 Å	50x2.1 mm, 1.7 µm 300 Å
Mobile phase	A : 2.5 M AA (pH=7) B : 0.1 M Phosphate (pH=7)	A : Water+0.05%TFA+0.1%FA B : ACN+0.05%TFA+0.1%FA
Flow rate	100 µL/min	1.4 mL/min
Gradient	0 to 90 % (B) in 36 min, 90 to 100 % (B) in 21 min, 100 % (B) for 11 min	1 % (B) for 0.14 min, 1 to 32 % (B) in 0.01 min, 32 to 40 % (B) in 1.06 min, 40 to 1 % (B) in 0.01 min,
Sampling time		1.5 min
Injected volume (%V ₀)	40 µL (4% ¹ V ₀)	150 µL (150% ² V ₀)
UV detection	280 nm (10 Hz)	280 nm (40 Hz)
MS conditions (ESI+)		
Scan range		500-3500 m/z
Scan time		0.5 s
Capillary / Sample cone voltage		3.20 kV / 120 V
Desolvation temperature		500 °C
Desolvation / Cone gas flow		900 L/Hr / 50 L/Hr
Source temperature		110 °C

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615 Table 4 : Average DAR calculation

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Number of drug load	HIC peak area (%) (average on 3 injections)
DAR 0	6.0
DAR 2	26.1
DAR 4	42.6
DAR 6	17.2
DAR 8	8.1
Average DAR	3.9
RSD (%)	1.5

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● = Drug

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

B:MeOH+0.05% TFA

B:ACN+0.05% TFA

Figure 7

Figure 8

Figure 9