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Practical method development for the separation of monoclonal antibodies and antibody-drug-conjugate species in hydrophobic interaction chromatography, part 1: optimization of the mobile phase

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ABSTRACT

The goal of this work is to provide some recommendations for method development in HIC using monoclonal antibodies (mAbs) and antibody-drug conjugates (ADCs) as model drug candidates. The effects of gradient steepness, mobile phase pH, salt concentration and type, as well as organic modifier were evaluated for tuning selectivity and retention in HIC. Except the nature of the stationary phase, which was not discussed in this study, the most important parameter for modifying selectivity was the gradient steepness. The addition of organic solvent (up to 15% isopropanol) in the mobile phase was also found to be useful for mAbs analysis, since it could provide some changes in elution order, in some cases. On the contrary, isopropanol was not beneficial with ADCs, since the most hydrophobic DAR species (DAR6 and DAR8) cannot be eluted from the stationary phase under these conditions.

This study also illustrates the possibility to perform HIC method development using optimization software, such as Drylab. The optimum conditions suggested by the software were tested using therapeutic mAbs and commercial cysteine linked ADC (brentuximab-vedotin) and the average retention time errors between predicted and experimental retention times were ~1%.

1. Introduction

Due to the increasing number of monoclonal antibodies (mAb) and antibody-drug-conjugates (ADCs) in clinical phases and the number of biosimilars potentially entering the market, the need for analytical techniques adapted for their detailed characterization has increased [1].

Among the several liquid chromatographic (LC) modes applied for the analysis of mAbs and ADCs, hydrophobic interaction chromatography (HIC) is the reference technique to determine the relative hydrophobicity of mAbs and to separate the different populations of ADC molecules that differ in the number of drugs per antibody which are often known as DAR (drug-to-antibody ratio) species [2,3]. The main advantage of HIC compared to other LC modes (reversed phase (RP)) is that it is non-denaturating, so the native forms of the proteins are expected to be maintained, and moreover the separated proteins can be collected for further activity measurements. HIC is able to separate the different DARs of native ADCs such as DAR0, DAR2, DAR4, DAR6 and DAR8 of an IgG1 type ADC under their physiological-like conformation [4].

The separation concept of HIC is based on the protein salting-out principle and was first described by Tiselius [5]. Later on, Hjerten called this mode of separation hydrophobic interaction chromatography [6]. In HIC, proteins lose their hydrate shell in the highly concentrated salt solution (mobile phase A) and are repelled to the hydrophobic surface of the stationary phase. Mobile phase B is an aqueous solvent containing no or low salt concentration and elute proteins by allowing a reassembly of the water shell and enable the elution of the proteins by floating down the column. The retention in HIC is solely dictated by the hydrophobic interaction between amino acid residues of the proteins and the alkyl chain – or other non-polar functional groups – located at the surface of the stationary phase. In contrast to RP, there are no additional secondary interactions in HIC. Therefore, the elution order enables to rank the proteins on the basis of their relative hydrophobicity. Hydrophobicity generally means the repulsion between a non-polar moiety of the protein and of the polar aqueous environment of water [7]. The structure of water is highly ordered and stabilized by dipol–dipol interactions over a 3-dimensional structure, characterized by the high surface tension of water (~72 mN/m at 25 °C). Each oxygen atom has four hydrogens as neighbors in a tetraeder and each

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hydrogen forms a bridge between two oxygen atoms. When dissolving hydrophobic moieties of a protein in an aqueous system, the neighboring water molecules have to be separated to form a cavity for the protein. This requires the investment of energy. The surface of the cavity multiplied with the surface tension is representing this energy. If two or more partners are associating, their hydrophobic contact surface areas are reduced, and energy is released. The energy is proportional to the size of the hydrophobic contact surface area of the protein. The larger this area, the higher is the chromatographic retention in HIC. In other words, the interaction between two or more hydrophobic molecules in aqueous solutions proceeds spontaneously and is mainly determined by the change in entropy [8–10]. The retention properties of proteins in HIC was explained by the thermodynamic model of Melander et al. [11,12]. The solvophobic theory relates the retention of proteins to the molar surface tension increment of the salt [13–15]. However, the model requires that there is no specific binding between the salt and protein and no change in the protein structure under the operating conditions.

Today, HIC appears as a key technique for mAb and ADC analysis. Several methods were developed for the determination of DAR distribution of ADCs [16–18]. Debaene et al. applied HIC for off-line native mass spectrometry (MS) characterization of brentuximab vedotin [19]. HIC is also often used for protein purification based on the apparent hydrophobicity of impurities and is a valuable tool in downstream purification procedure [6,7,20].

Retention mechanism in HIC is well studied and understood but practical method development aspects are rarely reported in the literature [21]. Our purpose was to study and find the most important variables impacting selectivity and to propose a generic method development approach for mAbs and ADCs' DARs separations. Therefore, the effects of gradient steepness, mobile phase pH, salt concentration and type, and organic modifier, on the retention were systematically evaluated and a computer assisted method development was applied. Method optimization for real life samples such as therapeutic mAbs (denosumab, palivizumab, pertuzumab, rituximab and bevacizumab) and a cysteine linked ADC (brentuximab-vedotin), approved both by the Food and Drug Administration (FDA) and the European Medicine Agency (EMA) is presented, and the reliability of retention time prediction is also discussed in this study.

2. Experimental

2.1. Chemicals and columns

Water was obtained with a Milli-Q Purification System from Millipore (Bedford, MA, USA). Sodium dihydrogen phosphate, Disodium hydrogen phosphate, ammonium sulfate, ammonium acetate, ammonium formate, sodium chloride and 1 M sodium hydroxide (NaOH) solution were purchased from Sigma–Aldrich (Buchs, Switzerland). Isopropanol (HPLC grade) was purchased from Fischer Chemicals (Reinach, Switzerland).

FDA and EMA approved therapeutic IgG monoclonal antibodies including bevacizumab, rituximab, palivizumab, denosumab and pertuzumab and ADC brentuximab vedotin were kindly provided by the Center of Immunology Pierre Fabre (Saint-Julien en Genevois, France).

MAbPac HIC-10 100 × 4.6 mm, 5 μm (1000 Å) column was purchased from Thermo Fisher Scientific AG (Reinach, Switzerland).

2.2. Equipment and software

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 Waters Acquity 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 auto-sampler to the detector cell. The measured dwell volume is around 100 μL. Data acquisition and instrument control was performed by Empower Pro 2 Software (Waters). Calculation and data transferring was achieved by using Excel templates.

The mobile phase pH was measured using a SevenMulti S40 pH meter (Mettler Toledo, Greifensee, Switzerland).

Method optimization was performed using DryLab® 4 chromatographic modelling software (Molnar-Institute, Berlin, Germany) and Excel macros (Microsoft).

2.3. Apparatus and methodology

2.3.1. Mobile phase composition and sample preparation

Generally, for the separation of mAbs and ADC DARs, the mobile phase "A" consisted of 2–5 M aqueous salt solution (e.g., ammonium sulfate, ammonium acetate, ammonium formate or sodium chloride) containing 0.1 M phosphate buffer, while mobile phase "B" was 0.1 M phosphate buffer. The mobile phase pH was set by adjusting the ratio of sodium dihydrogen phosphate and disodium hydrogen phosphate and then was measured and further adjusted if required. The pH was set between 6.3 and 7.0.

Both the mAb and ADC samples were diluted in water to 2 mg/mL and stored at 4 °C. Then, the samples were directly injected using low volume insert vials.

2.3.2. Selection of stationary phase and flow rate

Preliminary study was performed to select the most appropriate stationary phase for ADC DAR separations. Several butyl and ether phases of 10, 5 and 2.5 μm particle sizes were tested. Finally, the MAbPac HIC-10 phase provided suitable elution window for all DARs and performed the highest peak capacity and was thus selected for this study. The details of phase system optimizations will be explained in a following study as the second part of this paper.

The impact of flow rate on peak capacity and analysis time was also studied and 1 mL/min (on 4.6 mm I.D. column) was found to be a good compromise between efficiency and analysis time.

2.3.3. Investigation of retention properties of mAbs and ADC DARs (brentuximab vedotin)

Intact antibodies and ADC DARs were eluted in linear inverse salt gradient mode. For studying the retention properties of intact mAbs, five of the available antibodies were selected based on their type (IgG class and isotype), calculated isoelectric point (p*I*) and hydrophobicity. The purpose was to cover the common p*I* range of mAbs and to include chimeric (ch), humanized (hz) and human (hu) reference IgG1 and IgG2 isotypes, to draw overall and reliable conclusions. Relative hydrophobicity of 19 different mAbs was previously measured by a generic HIC method and then, the less and most hydrophobic ones (denosumab and bevacizumab, respectively) as well as three other ones were selected to cover the whole hydrophobicity range. The selected mAbs were the followings in the order of their hydrophobicity (HIC elution order): denosumab (huIgG2, p*I* = 8.8), palivizumab (hzIgG1, p*I* = 9.0), pertuzumab (hzIgG1, p*I* = 8.7), rituximab (chIgG1, p*I* = 9.1) and bevacizumab (hzIgG1, p*I* = 8.5).

For studying the retention properties of ADC DARs, brentuximab vedotin was selected as it is the first approved cysteine linked ADC available on the market. Brentuximab vedotin is a chIgG1 class anti-

body including peptide linker and auristatin E as cytotoxic drug [22,23].

First, the effect of salt gradient steepness on the retention was evaluated. Different gradient times were tested at a given mobile phase pH (pH 7) and ionic strength (2 M ammonium sulfate in mobile phase "A"). A generic linear gradient, starting from 0% to 100% B (equivalent to 2–0 M ammonium sulfate gradient) was applied at a flow rate of 1 mL/min for all samples. The gradient time (t_g) was varied as 10, 15, 20, 30 and 40 min (at $T = 25\,^{\circ}C$). The observed apparent retention factors ($k_{app} = (t_r - t_0)/t_0$ with t_r as retention time and t_0 as column dead time) were plotted against the gradient time (steepness).

For the investigation of mobile phase organic modifier, the above mentioned experiments were repeated by adding 5%, 10% and 15% isopropanol to mobile phase "B". Apparent retention factors (k_{app}) were plotted against the gradient time at different isopropanol concentrations and against the isopropanol concentration at given gradient times.

The impact of mobile phase pH was evaluated by performing the generic gradients at various gradient times (10, 15, 20, 30 and 40 min), but adjusting the mobile phase pH to 7, 6.7 and 6.3. The commonly applied mobile phase pH in HIC for mAbs and ADCs is between 6.4 and 7, therefore our selected range represents the conditions of real-life separations.

Finally, the impact of salt concentration and type was studied by varying the salt concentration of mobile phase "A" as 2–4 M. The following commonly used salts were applied: ammonium acetate, ammonium formate and sodium chloride. The experiments were carried out at various gradient times (10, 15, 20, 30 and 40 min) and constant pH (pH 7). The observed k_{app} values were plotted versus the gradient time and versus the mobile phase molality at a given gradient time.

2.3.4. Systematic method optimization

Initial basic runs for multifactorial experimental designs were already suggested in the 90's for reversed phase liquid chromatographic method optimization [24]. A general approach consists in modelling simultaneously the effect of the most important factors i.e., gradient steepness and temperature on selectivity on a previously selected column [25,26]. Then, with the help of resolution maps generated by modelling software – which show the critical resolution of the peaks to be separated [27] – the selected variables can be rapidly and efficiently optimized. This approach was currently applied for the reversed phase and ion-exchange separation of antibody variants [28–30].

Based on the observed effects of the factors on retention and resolution of mAbs peaks, an experimental design with 4 runs was suggested for HIC method optimization. It was indeed found that the impact of pH and salt type (and molarity) on selectivity and resolution were not significant. However, gradient steepness and mobile phase organic modifier (isopropanol) significantly affected the overall separation. Performing gradient runs with two gradient times (as $t_{g1} = 10$ min, $t_{g2} = 30$ min) and with two isopropanol concentrations (0 and 10% in mobile phase "B") on a 100 × 4.6 mm column allowed a reliable optimization of the separation. The latter was performed by computer simulation using DryLab and a custom made model. A mixture of reference mAbs was injected to build up the DryLab model and study the prediction accuracy error.

On the other hand, for ADC DARs separations, only the gradient steepness was found to be an important variable for method optimization. Adding organic modifier to mobile phase "B" was found to be not useful since it drastically increased the retention of DAR6 and DAR8 species and caused irreversible adsorption. DAR8 peaks were not eluted when isopropanol content was higher than 8% in mobile phase "B". So finally, performing two initial gradients with gradient times of $t_{g1} = 10$ min and $t_{g2} = 30$ min allowed the optimization of DARs separation. Again, optimization was supported by computer simulation (DryLab and M.S. Office Excel macro). Retention time prediction accuracy was finally evaluated.

3. Results and discussion

Stationary phases applied for HIC separations are similar to those used in RPLC, except that the bonded phase is less hydrophobic (e.g., ether or butyl) due to shorter alkyl ligands and a less dense bonding [26]. The mobile phase is typically an aqueous solution of a non-denaturing (antichaotropic) salt such as ammonium sulfate and a buffer to control pH (usually phosphate, $6.4 \leq pH \leq 7$). The combination of a less hydrophobic packing with purely aqueous mobile phases minimizes protein denaturation and usually allows the recovery of native (non denatured) proteins from separated fractions, especially for separations carried out at ambient temperature.

Retention and selectivity in HIC can be tuned by means of several operating parameters. To achieve an optimal HIC separation of mAbs and ADC DARs, the influence of various parameters on separation, such as gradient steepness, pH, ionic strength and organic modifiers has to be taken into account. In contrast to other separation modes (RP or IEX), the temperature has to be kept at ambient in order to maintain non-denaturating conditions and the native conformation of the proteins. Therefore, mobile phase temperature is not a parameter that can be considered for method optimization (or only in a limited range).

3.1. The effect of salt gradient time (gradient steepness) on mAbs and ADC DARs retention

For HIC analysis of mAbs and ADC DARs, the gradient elution mode is preferred in practice. The retention of compounds in the inverse salt gradient mode is strongly dependent on the salt concentration (gradient steepness) and a small change could lead to significant shift in the retention. For this reason, isocratic conditions are impractical in real-life mAb and ADC separations.

The dependence of the retention on the concentration (ionic strength) of the salt (C_S) determines the applicability of the linear solvent strength (LSS) model of gradient elution for HIC [26]. In isocratic elution mode, a log (k) versus C_S function typically shows linear behavior and can be expressed as:

$$\log k = \log k_0 + A_{HIC} \times c_s \quad (1)$$

were k_0 refers to the value of k for $C_S = 0$, and A_{HIC} is the slope of the plot log (k) versus C_S ($A_{HIC} = d(\log k)/d(C_S)$). In HIC, k increases with increased C_S, so that C_S must decrease during the gradient elution. Eq. (1) can be transformed into gradient mode based on LSS model:

$$\log k* = \log k_{ini} - S_{HIC} \times \phi_{HIC} \quad (2)$$

where k^* is the median value of k during gradient elution when the band has reached the column mid-point, k_{ini} is the value of k observed for the initial (highest) salt concentration, S is a constant for a given compound (slope of log (k) versus Φ_{HIC} plot) and Φ_{HIC} is the volume fraction of the B solvent (generally contains no salt). It is practical to show the dependence of k^* on the gradient time (t_g). For this purpose, the following equation can be derived:

$$k* = \frac{t_g \times F}{1.15 \times V_m \times \Delta\phi \cdot S_{HIC}} \quad (3)$$

where F is the mobile phase flow rate and V_m is the column dead volume.

It was experimentally shown by Szepesy and Karger that the retention of some common proteins in HIC gradient mode is in quantitative agreement with the LSS model, suggesting that

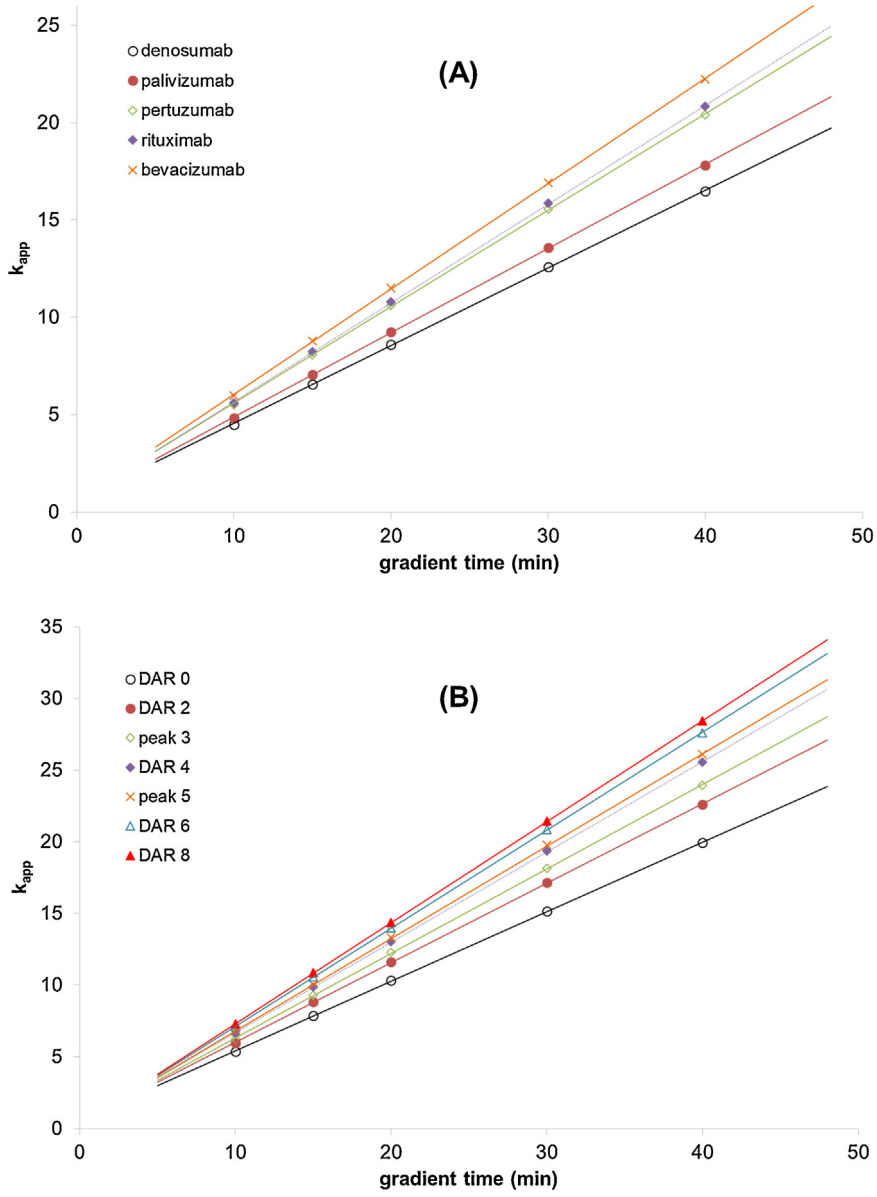

Fig. 1. The impact of gradient time (gradient steepness) on ADC DAR species retention (A) and on intact mAbs retention (B). Column: Thermo MAbPac HIC-10, 100 × 4.6 mm, mobile phase "A": 2 M ammonium-sulfate + 0.1 M phosphate (pH 7), "B": 0.1 M phosphate (pH 7). Flow rate: 1 mL/min, gradient: 0–100% B. Mobile phase temperature: 25 °C.

method development approaches applied in RP can be directly applied in HIC [21,31].

The effect of gradient steepness (gradient time) on the apparent retention of intact mAbs and ADC DARs variants was practically investigated. The gradient time was varied as 10, 15, 20, 30 and 40 min (at $T = 25\,^{\circ}C$ and at pH 7 using 2 M ammonium sulfate in mobile phase "A"). The retention of the five selected mAbs and the ADC DARs of brentuximab vedotin showed the same behavior. Fig. 1 illustrates the effect of gradient time on the apparent retention (k_{app}). The relationship between k_{app} and t_g can be perfectly described by fitting a linear function ($R^2 > 0.999$ for all solutes). Moreover, the observed results suggest that using longer gradients increase the selectivity. On the other side, as shown in [28], the retention behavior of mAb fragments showed some deviation from linear relationship in RPLC. Experimental points in k_{app} versus t_g representation followed a slightly concave curvature. It can be concluded that a LSS type model perfectly describes the retention behavior of mAbs and ADC DARs.

The results suggest that only two gradient runs are required (e.g., with $t_{g1} = 10$ min and $t_{g2} = 30$ min) for the optimization of an inverse salt gradient, then the retention times can be predicted for any gradient program.

Another interesting finding is that in HIC mode compared to RP, S_{HIC} values of mAbs are significantly smaller – by a factor of around 5 – than the corresponding S values in RP. This suggests that for a required range of retention and selectivity, a steeper gradient can be applied in HIC versus RP.

3.2. The effect of salt concentration and type on the retention and selectivity

Ammonium sulfate is the most commonly applied salt in HIC [32]. Alternative salt systems were studied and it was found that sodium acetate increased the retention of hydrophobic proteins, while it decreased retention of the hydrophilic ones [33]. In other early studies, sodium citrate was found also to be a good candidate, but was left out because of solubility issues [34,35]. It has also

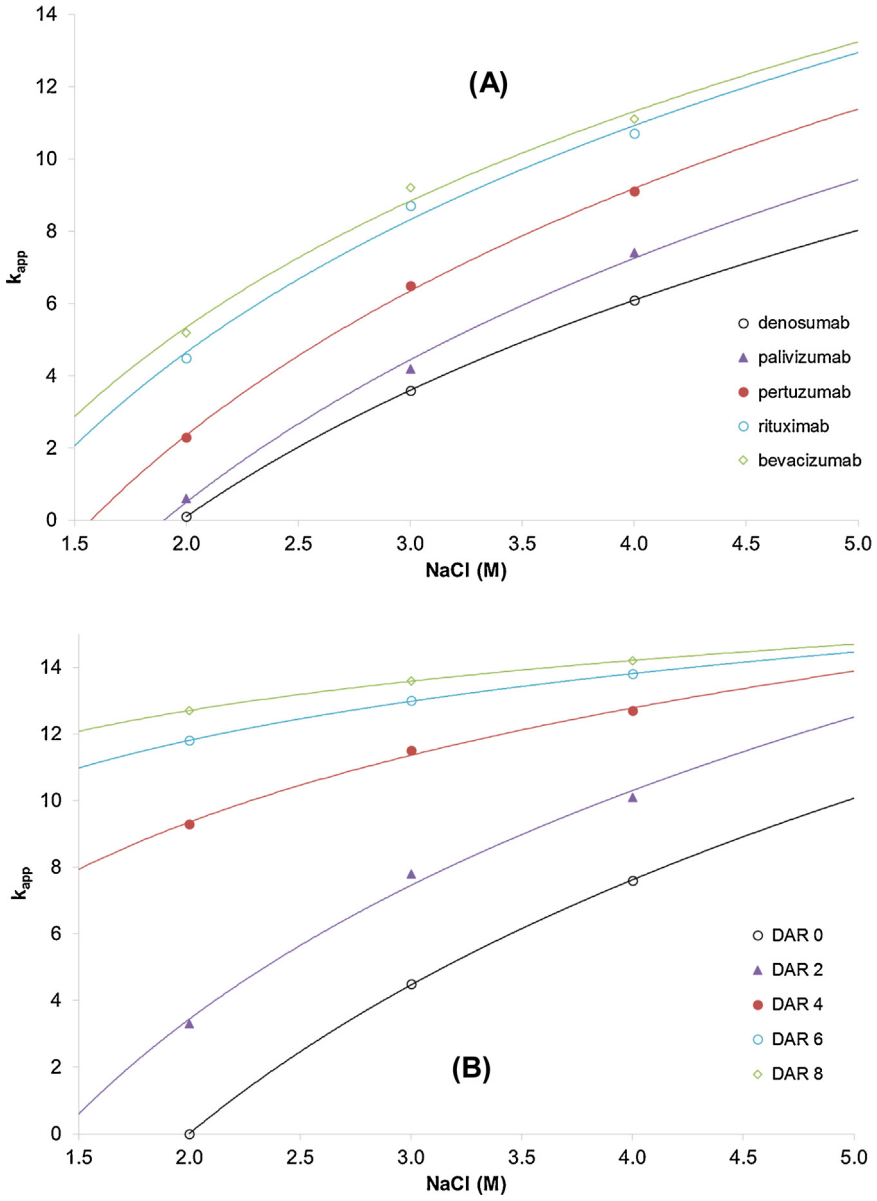

Fig. 2. The impact of mobile phase salt concentration on ADC DAR species retention (A) and on intact mAbs retention (B). Column: Thermo MAbPac HIC-10, 100 × 4.6 mm, mobile phase "A": 2–4 M sodium-chloride + 0.1 M phosphate (pH 7), "B": 0.1 M phosphate (pH 7). Flow rate: 1 mL/min, gradient: 0–100% B in 20 min. Mobile phase temperature: 25 °C.

been demonstrated that salt nature affects differently the retention on different stationary phases. It can both increase and decrease the retention of hydrophobic and hydrophilic proteins, respectively [34,35]. The above results also imply that the effect of a salt cannot be predicted in advance but should always be determined experimentally as an early step of method development [21].

One of the primary variables for varying HIC retention is the mobile phase salt concentration. Our purpose was not only to qualify the effect of salt concentration on mAbs and ADCs retention but to see the impact of different salts on selectivity. The influence of different salts on hydrophobic interactions follows the Hoffmeister (lyotropic) series for the precipitation of proteins from aqueous solutions [36]. Based on this series, salt types may be interchangeable if their lyotropic strength is considered. However, it was not reported whether salt type impacts the selectivity between closely related proteins or just play a role in the absolute retention. Therefore, salt concentration and type of mobile phase "A" was varied such as 2–4 M and based on their solubility, the following salts were selected beside the reference ammonium sulfate: ammonium acetate, ammonium formate and sodium chloride. The experiments were carried out at various gradient times and constant pH.

As expected on the basis of Eq. (1), the apparent retention factors showed logarithmic dependence over salt concentration at any gradient steepness. Moreover, very similar tendency was observed with all salts. Fig. 2 shows the results observed with sodium chloride at t_g = 20 min for intact mAbs and for ADC DARs. At first sight, the results suggested no significant difference in selectivity when salt concentration is compensated for lyotropic strength. Fig. 3. illustrates the impact of increasing salt concentration on ADC DARs' retention and shows a head-to-head comparison of the elution profile obtained with 2 M ammonium sulfate and 5 M sodium chloride. It can be clearly seen that by changing the salt concentration in mobile phase "A" the elution window and selectivity can be adjusted in a very efficient way. On the other hand, peak widths also vary with salt concentration since it impacts the concentra-

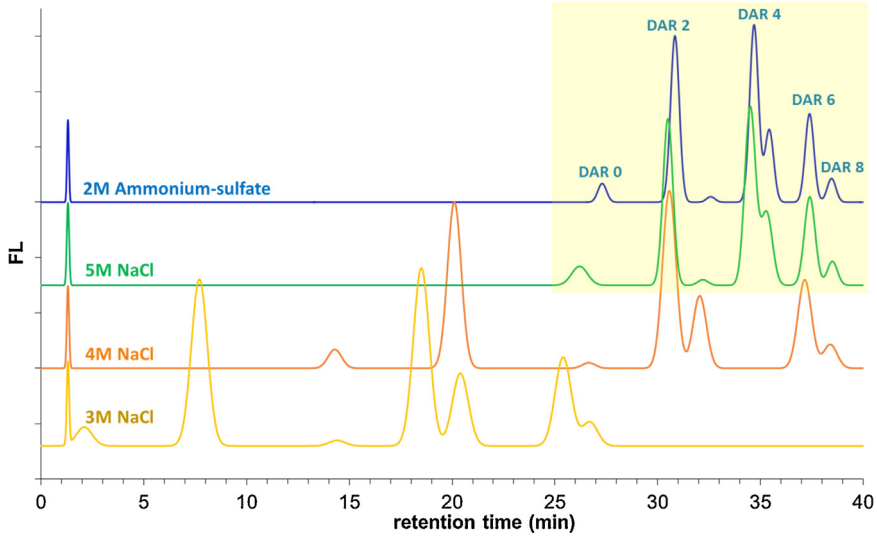

Fig. 3. The impact of mobile phase salt concentration and type on ADC DAR species retention. Column: Thermo MAbPac HIC-10, 100 × 4.6 mm, mobile phase "A": 3–5 M sodium-chloride or 2 M ammonium-sulfate + 0.1 M phosphate (pH 7), "B": 0.1 M phosphate (pH 7). Flow rate: 1 mL/min, gradient: 0–100% B in 40 min. Mobile phase temperature: 25 °C.

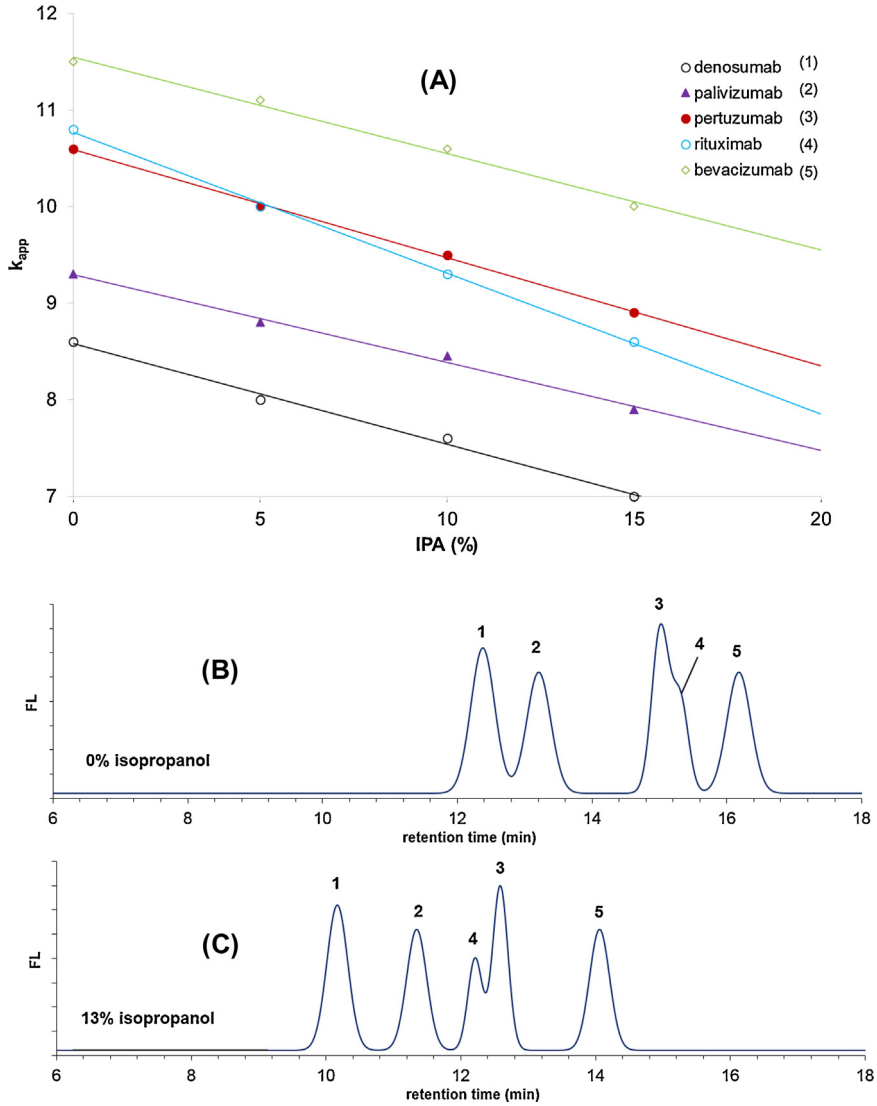

Fig. 4. The impact of organic modifier (isopropanol) on mAbs retention (A) and model chromatograms obtained for 0% isopropanol (B) and 13% isopropanol (C). Column: Thermo MAbPac HIC-10, 100 × 4.6 mm, mobile phase "A": 2 M ammonium-sulfate + 0.1 M phosphate (pH 7), "B": 0.1 M phosphate (pH 7) + isopropanol. Flow rate: 1 mL/min, gradient: 0–100% B in 20 min. Mobile phase temperature: 25 °C. Peaks: denosumab (1), palivizumab (2), pertuzumab (3), rituximab (4), bevacizumab (5).

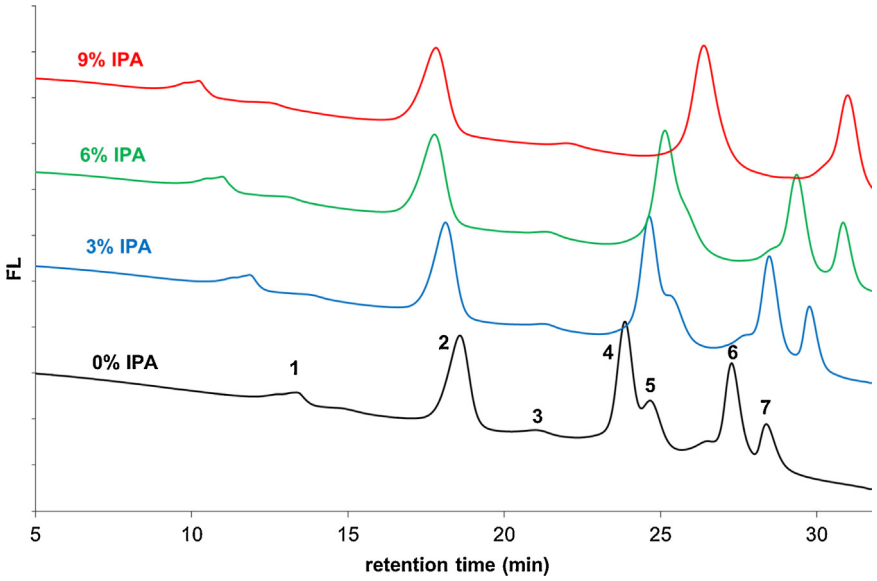

Fig. 5. The impact of organic modifier (isopropanol) on ADC DAR species retention. Column: Thermo MAbPac HIC-10, 100 × 4.6 mm, mobile phase "A": 1.2 M ammonium-sulfate + 0.1 M phosphate (pH 7), "B": 0.1 M phosphate (pH 7) + isopropanol. Flow rate: 1 mL/min, gradient: 0–100% B in 30 min. Detection: FL (λ_{ex}: 280 nm, λ_{em}: 360 nm), mobile phase temperature: 25 °C. Sample: Brentuximab-vedotin. Peaks: DAR0 (1), DAR2 (2), DAR4 (4), DAR6 (6), DAR8 (7), unknown (3,5).

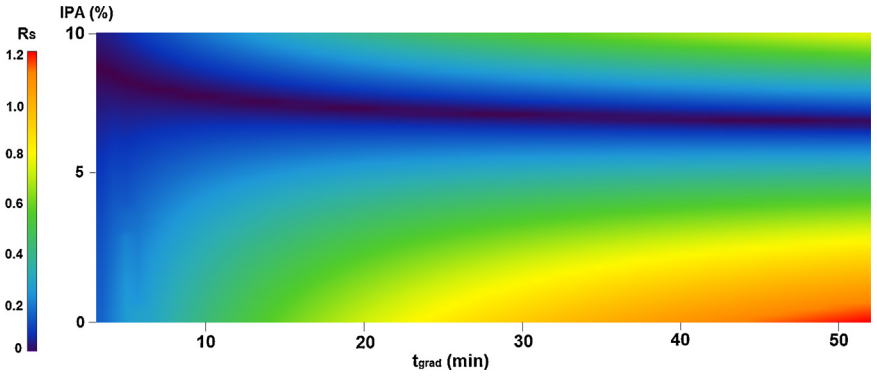

Fig. 6. Two dimensional resolution map for the optimization of mAb separation. Variables: gradient time (t_{grad}) and isopropanol% in mobile phase B (IPA%).

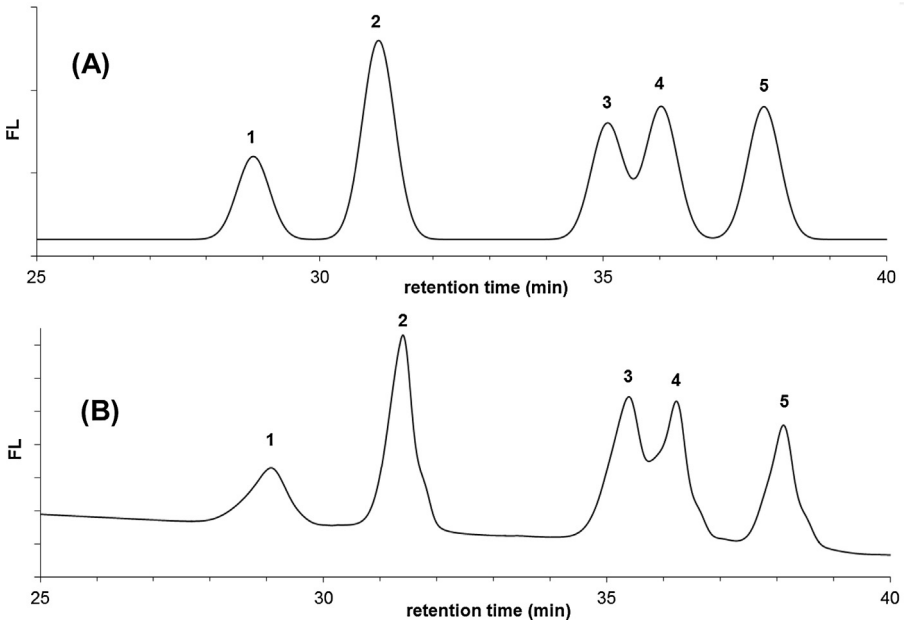

Fig. 7. Comparison of predicted (A) and experimental (B) chromatograms for the separation of intact mAbs. Column: Thermo MAbPac HIC-10, 100 × 4.6 mm, mobile phase "A": 2 M ammonium-sulfate + 0.1 M phosphate (pH 7), "B": 0.1 M phosphate (pH 7). Flow rate: 1 mL/min, gradient: 0–100% B in 50 min. Detection: FL (λ_{ex}: 280 nm, λ_{em}: 360 nm), mobile phase temperature: 25 °C. Peaks: denosumab (1), palivizumab (2), pertuzumab (3), rituximab (4), bevacizumab (5).

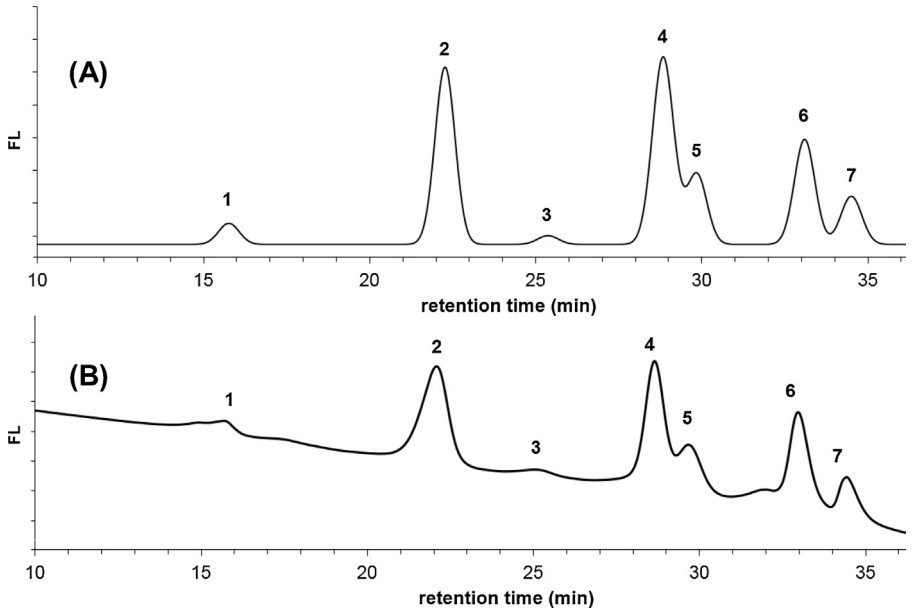

Fig. 8. Comparison of predicted (A) and experimental (B) chromatograms for the separation of ADC DAR species. Column: Thermo MAbPac HIC-10, 100 × 4.6 mm, mobile phase "A": 1.2 M ammonium-sulfate + 0.1 M phosphate (pH 7), "B": 0.1 M phosphate (pH 7). Flow rate: 1 mL/min, gradient: 0–100% B in 37 min. Detection: FL (λ_{ex}: 280 nm, λ_{em}: 360 nm), mobile phase temperature: 25 °C. Sample: Brentuximab-vedotin. Peaks: DAR0 (1), DAR2 (2), DAR4 (4), DAR6 (6), DAR8 (7), unknown (3,5).

tion gradient (steepness) and therefore the gradient band focusing effect.

Our results also suggest that similar selectivity can be set with any type of salts. As shown in Fig. 3, the selectivity obtained with 2 M ammonium sulfate and 5 M sodium chloride is practically identical, however some slight differences were observed in the achievable peak capacity (peak width) and therefore in the resolution (see for example the DAR4 peaks). A 5.5 M ammonium acetate and 5.5 M ammonium formate buffers also provided identical selectivity. Similar tendencies were observed with the intact mAbs (data not shown). Our observations are in good agreement with previous findings, namely that similar values of A_{HIC} and S_{HIC} were found for salts other than ammonium sulfate [13].

To conclude on the impact of salt type and concentration, salt concentration in mobile phase "A" (that corresponds to the gradient steepness) is an important variable for tuning the retention and selectivity of closely related proteins. Regarding salt type, similar selectivity can be obtained with different salts, when their lyotropic strength is corrected. In our study we observed that 2 M ammonium sulfate was roughly equivalent with ~4.5–5.0 M sodium chloride, or 5.0–5.5 M ammonium acetate and 5.0–5.5 M ammonium formate (that is in good agreement with the Hoffmeister series) for mAbs and ADC on our MAbPac HIC-10 stationary phase. From a practical point of view, during method development, the first step should be the selection of salt type and then the tuning of elution window by adjusting salt concentration.

3.3. The impact of isopropanol as mobile phase additive

Adding low proportion of water-miscible alcohols results in weakening the protein-ligand interactions in HIC, leading to the elution of the bound solutes [37]. The non-polar parts of alcohols (particularly isopropanol) compete effectively with the bound proteins for the adsorption sites on the HIC stationary phase resulting in the displacement of the proteins. Alcohols also decrease the surface tension of mobile phase thus weakening the hydrophobic interactions to give a subsequent dissociation of the ligand–solute complex [37]. Although additives can be used in the elution buffer to affect selectivity during desorption, there is a risk that proteins could be denatured or inactivated by exposure to high concentrations of such chemicals.

Addition of organic modifiers, such as isopropanol, is often cited as an advantageous parameter in HIC method development for decreasing the retention of hydrophobic compounds and to tune selectivity [38]. Therefore, the impact of isopropanol content in mobile phase "B" on the retention of both mAbs and ADC DARs was systematically studied. Generic HIC gradients were applied and the isopropanol content of mobile phase "B" was set to 0, 5, 10 and 15%.

Fig. 4A shows the change in retention of intact mAbs as a function of isopropanol content. As expected, the retention decreased when increasing isopropanol concentration but in a different extent, depending on the mAb. The slopes of the fitted curves are indeed slightly different and the curves of pertuzumab and rituximab cross each other at ~5% isopropanol content, suggesting the coelution of the two species. This behavior demonstrates the possibility to change the elution order (selectivity) of these two mAbs by adjusting the isopropanol content of the mobile phase. On the other hand, a simple linear model describes well the dependence of retention on isopropanol content, which makes it simple to predict the retention of mAbs for any isopropanol concentration on the basis of only two initial runs (e.g., performed with 0% and 10% isopropanol). Fig. 4B presents the effect of isopropanol content on retention and selectivity between 0% and 13% isopropanol content (based on simulation). The chromatograms clearly show the change of selectivity between all peaks and the elution order change of peaks 3 and 4. Based on these observations, the isopropanol content of the mobile phase is indeed a valuable parameter to tune the selectivity of mAbs separation in HIC.

For ADCs, a different behavior was observed. As expected, a retention decrease was observed for DAR0 and DAR2 species but surprisingly the retention of DAR 4, DAR6 and DAR8 increased with the isopropanol concentration (Fig. 5). Moreover at 9% of isopropanol, the DAR8 cannot be eluted from the column and the selectivity between peaks 4 and 5 clearly decreased. We still have not a clear explanation for this specific elution behavior of DARs but some possible explanations are (i) a shift in mobile phase pH and proteins p*I* when adding isopropanol, taking into account that the pH shift could be different depending on the amount of salts

that is continuously changing during the gradient; (ii) conformational changes of ADCs in presence of alcohols. To conclude on the addition of isopropanol for ADC analysis as a parameter of method development, it does not seem to be useful since it drastically increases the retention of the most hydrophobic ADC DAR species and does not permit the elution of DAR6 and DAR8 from the column.

3.4. The effect of mobile phase pH on mAbs and ADC retention

The effect of pH in HIC is not straightforward [37,39,40]. In this study the mobile phase pH was set between 6.3 and 7, while the p*I* of mAbs and ADC is ranged between 8 and 9. Therefore we were quite far from their p*I*. When increasing the mobile phase pH, a retention increase is expected due to increased hydrophobic interactions thanks to a shift to a more neutral state of the protein (close to its p*I*). Hjerten et al. found that the retention of proteins changed more drastically at pH values above 8.5 and/or below 5 than in the range pH 5–8.5 [41].

In our study, the impact of mobile phase pH was evaluated by performing generic gradients at various gradient times and adjusting the mobile phase pH to 7, 6.7 and 6.3. In the studied pH range, no difference or shift in retention was observed for intact mAbs and ADC species, whatever the gradient steepness. The chromatograms observed at different pH perfectly overlapped each other (data not shown). Such a behavior was expected since mAbs and ADCs possess relatively high p*I* values (typically between 8 and 9). These findings suggest that pH is not an important separation parameter in the optimization of HIC separation of mAbs and ADC species. On the other hand, in the investigated pH range, the methods were robust.

3.5. Method optimization for mAbs HIC separation by using two dimensional retention models

Optimization software packages generally employ linear models for the simultaneous optimization of two or three variables. The polynomial equation for two variables can generally be written as:

$$y = b_0 + b_1 x_1 + b_2 x_2 \quad (4)$$

where y is the response (retention time or its transformation such as log k), x_1 and x_2 are the model variables (here gradient time (t_g) and isopropanol content (IPA%)) while b_0, b_1, b_2 are the model coefficients.

In this study, DryLab was used for further method optimization and determination of the unknown coefficients of the model. The software implements an interpretive approach, where the retention behavior is modelled using experimental information from initial runs, and the retention times at other conditions are predicted in a selected experimental domain. This allows calculating the critical resolution, and accordingly, the optimal separation can be found [42,43].

As shown in Sections 3.2 and 3.4, the type of salt and mobile phase pH are minor variables, which do not influence significantly the retention and selectivity. On the contrary, the salt gradient steepness and isopropanol content of mobile phase appear as the most important factors for adjusting selectivity and resolution. Finally, mobile phase temperature is not a separation parameter in HIC, since it is important to maintain the physiological-like conformation of the proteins.

A new two dimensional mode was created in Drylab software. Retention times were transformed into retention factors, and linear models were chosen for the modeling of retention as functions of t_g and IPA%. The modeling takes place in a rectangular region in the t_g—IPA% plane, determined by 2 gradient times (steepness) and two levels of IPA%. Hence, this approach necessitates only 4 initial experimental runs for creating the model. Gradient times were set to t_{g1} = 10 min and t_{g2} = 30 min, while IPA% was varied as 0 and 10%. (Please note, that here the low level of IPA% was set to zero, that is only acceptable and feasible when having a perfectly linear behavior.) Following the execution of the input experimental runs, the figures of merit (i.e., retention times, peak widths and peak tailing values) were imported into DryLab and peak tracking was performed. Peak tracking was carried out on the basis of peak areas and individual injections of the mAbs. Next, the optimization was carried out on the basis of the created resolution map. In the resolution map, the smallest resolution (*Rs*) value of any two critical peaks in the chromatogram was plotted as a function of the two simultaneously varied experimental parameters (Fig. 6).

As shown, a 50 min gradient was found to provide the highest resolution when the mobile phase contains no isopropanol. The predicted optimum condition was set and experimental chromatograms recorded. Fig. 7 shows the comparison of predicted and experimental chromatograms.

To evaluate the accuracy of this approach, the predicted and experimental retention times were compared. The predicted retention times were in good agreement with the experimental ones and the average retention time relative errors were ~1.0%.

Despite the fact, that in this example 0% isopropanol provided the highest resolution, isopropanol content may have significant impact on selectivity (see in Section 3.3). It is highly probable that for other selected mAbs, higher isopropanol content will perform better separation.

Finally, this method optimization approach can be considered as reliable and the suggested initial experiments (10 and 30 min gradient on a 100 mm long standard bore column with 0% and 10% IPA) can be applied in daily routine work, resulting in time saving. The time spent for method development in this example was approximately 6 h (2 gradient times × 2 IPA% × 5 samples + equilibration), and then the predicted method was experimentally verified.

However, please note that for more complex samples, the optimum conditions for high resolution separations can be shifted to longer gradient time ranges. Therefore, for high resolution separations, an extended model might be useful.

3.6. Method optimization for ADC DARs HIC separation by using one dimensional retention model

Based on our findings for ADC DAR species separation, the only relevant parameter for tuning selectivity is the gradient steepness (gradient program). Addition of organic modifier to the mobile phase was found to be ineffective. Then, the method optimization was quite simple, and necessitates only two experimental runs. Gradient times were set to t_{g1} = 10 min and t_{g2} = 30 min, and mobile phase pH was kept constant (pH 7), while no organic modifier was added. The required ionic strength was determined by preliminary scouting runs. It was found that on our preferred stationary phase, an inverse salt gradient from 1.2 to 0 M ammonium sulfate provided appropriate retention for all compounds and therefore was used for further optimization. An excel macro was prepared for the modelization, and a 37 min long linear gradient performed the optimal separation of the DAR species of intact brentuximab vedotin. Fig 8. shows a comparison of the experimental and predicted chromatograms under the optimal conditions. The predicted retention times were again in good agreement with the experimental ones; the average retention time relative errors were below 0.5%.

This optimization procedure took only 1 h (2 gradients × 1 sample + equilibration). However, it has to be mentioned that the scouting runs for finding the appropriate salt concentrations may be more time consuming (depending on the number of columns used for the screening procedure).

4. Conclusion

The purpose of this study was to provide guidelines for method development in HIC, when analyzing mAbs or ADCs. Numerous HIC variables impacting selectivity and retention, were tested in a systematic way. The tested parameters include gradient steepness, mobile phase pH, salt concentration and type, and organic modifier. As demonstrated, a mobile phase pH variation in the range 6.3–7.0 was found to be ineffective for tuning selectivity in HIC mode. The type of salt employed for performing the reversed salt gradient has a significant impact on retention, but if lyotropic strength is considered (through the Hoffmeister series), it is easy to achieve similar retention, by adjusting the ionic strength. In this work, it has been demonstrated that 2 M ammonium sulfate was roughly equivalent with ~4.5–5.0 M sodium chloride, or 5.0–5.5 M ammonium acetate and 5.0–5.5 M ammonium formate (that is in good agreement with the Hoffmeister series). Under these conditions of equivalent lyotropic strength, the selectivity was virtually not affected by a modification of salt nature. However, the gradient steepness plays a major role for tuning retention and selectivity and should be optimized both for mAbs and ADCs. Finally, the possible addition of organic modifier (namely isopropanol) to the mobile phase in HIC, was found to be particularly useful for mAbs separations, as some elution order changes between peaks were noticed in the range 0 to 13% isopropanol. On the contrary, when dealing with the analysis of ADC DAR species in HIC, the addition of isopropanol was never beneficial since the most hydrophobic species (DAR6 and DAR8) cannot be eluted in presence of ~10% isopropanol.

At the end, a HIC automated method optimization procedure was developed using Drylab software. For therapeutic mAbs analysis, both the gradient steepness and isopropanol content were simultaneously optimized after 6 h of initial experiments (2 gradient times × 2 IPA% × 5 samples + equilibration), resulting in substantial time saving. The optimum conditions were tested and predicted retention times were in good agreement with the experimental ones, with average retention time relative errors of ~1.0%. A cysteine linked ADC (brentuximab-vedotin), was also used to evaluate the capability to modelize retention in HIC. Again, the reliability of retention time prediction after optimization of gradient steepness was found to be excellent and this optimization procedure took only 1 h (2 gradients × 1 sample + equilibration).

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