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Practical method development for the separation of monoclonal antibodies and antibody-drug-conjugate species in hydrophobic interaction chromatoraphy, part 2: Optimization of the phase system

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ABSTRACT

The goal of this second part was (i) to evaluate the performance of commercially available HIC columns and (ii) to develop a fast and automated "phase system" (i.e. stationary phase and salt type) optimization procedure for the analytical characterization of protein biopharmaceuticals. For this purpose, various therapeutic mAbs (denosumab, palivizumab, pertuzumab, rituximab and bevacizumab) and a cysteine linked ADC (brentuximab-vedotin) were selected as model substances. Several HIC column chemistries (butyl, ether and alkylamide) from different providers were evaluated in four different buffer systems (sodium acetate, sodium chloride, ammonium acetate and ammonium sulfate). As stationary phases, the historical TSK gel Butyl NPR phase and the brand new Thermo MAbPac HIC-10 were found to be the most versatile ones in terms of hydrophobicity, peak capacity and achievable selectivity. As salt types, ammonium sulfate and sodium acetate were found to be particularly well adapted for the analytical characterization of mAbs and ADCs, but it is important to keep in mind that a concentration 2 to 3-times higher of sodium acetate versus ammonium sulfate is required to achieve a similar retention in HIC. After selection of the most appropriate phase systems, the optimization of the separation can be carried out by computer assisted retention modeling in a high throughput manner.

1. Introduction

Hydrophobic interaction chromatography (HIC) is a reference technique to separate proteins on the basis of their hydrophobicity under native conditions [1,2]. The separation concept of HIC is based on the protein salting-out principle explained in details in the first part of this study [3].

Practical method development in HIC is rarely discussed in literature. In the first part of this work, a systematic study was presented on the optimization of gradient program, mobile phase pH, salt concentration and organic modifiers for the separation of monoclonal antibodies (mAbs) and antibody-drug conjugate (ADC) species [3]. Here, we focus on the selection of stationary phase and salt type. The combination of these two parameters is often referred to as "phase system" of HIC separations [4–6].

In the past 10–20 years, the number of commercially available analytical scale HIC stationary phases was quite limited, but this number has recently grown, thanks to the recent developments in column technology and strong interest for protein biopharmaceuticals. Standard (4.6 mm i.d.) and narrow bore (2.0–2.1 mm i.d.) columns packed with 2.5, 3, 5, 7 or 10 μm particles are available in various lengths. In addition, several chemistries including butyl, ether, phenyl, amide, amide/ethyl groups can be used for tuning retention (hydrophobicity) and selectivity [7]. Analytical scale HIC columns are based either on silica or polymeric particles, and both porous and non-porous particles are available. Highly cross-linked non-porous poly(styrene–divinylbenzene) (PS/DVB) and polymethacrylate-based particles are often used in protein separations due to their advantageous mass transfer properties, since the main contribution to the band broadening of large biomolecules, namely trans-particle mass transfer resistance is negligible. These materials can withstand pressure drop up to 100–400 bar. One of the most popular HIC column for mAbs and ADCs analysis in the pharmaceutical industry is the 35 × 4.6 mm, 2.5 μm TSKgel Butyl-NPR from Tosoh [8–12]. This is quite surprising since this material

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is available from the 90s and there are a significant number of alternative columns that have been commercialized since then. It is however worth mentioning here that there is a lack of 150 × 2.1 mm HIC column format, which is often applied for the analysis of proteins in modern chromatographic practice.

The functional groups on HIC phases are more sparsely distributed than in RP, producing moderately hydrophobic surfaces and resulting in mild interactions [4]. Retention in HIC mostly depends on the ligand type, ligand chain length and ligand density. Szepesy and Rippel showed that salt type can have different effects on retention depending on the hydrophobicity of the protein to be separated, and emphasized the importance of a well selected phase system [4]. This work also illustrated that working in HIC with gradient elution is more appropriate than isocratic elution conditions [4,13,14].

Today, the selection of phase system and operating conditions are mostly based on subjective and historical references, while the optimization is mostly carried out by trial-and-error approach. As an example, in most of the HIC applications, the above mentioned TSKgel Butyl-NPR column is almost exclusively operated in ammonium sulfate buffer. However, all the salts having salting out properties (and appropriate solubility) can be considered as potential buffer components for HIC separations. A systematic study showed the possibility to combine different salts (binary and ternary salt systems) to modify selectivity and retention in HIC [15]. In this phase system optimization concept, the experimental design was based on gradient experiments performed on three different columns and with three different buffers (salts).

In the present work, the purpose was to suggest a fast and automated phase system optimization procedure for the HIC separation of mAbs and ADCs species. In addition, the most recent stationary phases were compared to each other including the historical reference column (TSKgel Butyl-NPR). Four different salt systems were evaluated and after selection of the most appropriate phase system, the optimizations were carried out by computer assisted retention modeling. Real life samples such as denosumab, palivizumab, pertuzumab, rituximab and bevacizumab and a cysteine linked ADC (brentuximab-vedotin), all approved by the Food and Drug Administration (FDA) and the European Medicine Agency (EMA) are presented here as case studies.

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 and sodium chloride were purchased from Sigma–Aldrich (St. Louis, MO, USA). Sodium acetate was purchased from Fluka Chemie (Buchs, Switzerland). 1 M sodium hydroxide (NaOH) solution was purchased from VWR International S.A.S. (Fontenay-sous-Bois, France).

FDA and EMA approved therapeutic IgG monoclonal antibodies including bevacizumab (Roche, Germany), rituximab (Roche, Germany), palivizumab (Abbot, USA), denosumab (Amgen, USA) and pertuzumab (Roche, Germany) and ADC brentuximab vedotin (Seattle Genetics, USA).

MAbPac HIC-10 100 × 4.6 mm, 5 μm (1000 Å), MAbPac HIC-20 100 × 4.6 mm, 5 μm (1000 Å) and MAbPac HIC-Butyl 100 × 4.6 mm, 5 μm columns were purchased from Thermo Fisher Scientific Inc (Sunnyvale, CA, United States). Protein-Pak Hi Res HIC 100 × 4.6 mm, 2.5 μm column was a generous gift from Waters (Milford, MA, USA). TSKgel Butyl-NPR 35 × 4.6 mm, 2.5 μm column and TSKgel Ether-5PW 75 × 2.0 mm, 10 μm column were purchased from Tosoh Bioscience GMBH (Stuttgart, Germany).

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 3 Software (Waters). Calculation and data transferring was achieved by using DryLab 2010 (Molnar Institute, Berlin) and Excel templates (MS Office).

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

2.3. Apparatus and methodology

2.3.1. Mobile phase composition and sample preparation

Generally, for the separation of mAbs and ADCs, the mobile phase "A" consisted of 1.5–4.1 M aqueous salt solution (namely 1.5 M ammonium sulfate, 3 M sodium acetate, 3.7 M sodium chloride and 4.1 M ammonium acetate) also 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 further adjusted by adding 1 M NaOH solution. The pH was set at 7.0. The salt concentration was selected on the basis of Hofmeister lyotropic series of salts.

The mAb samples were diluted in water to 2 mg/mL and ADC sample was diluted in water to 2.5 mg/mL and both stored at 4 °C in refrigerator. Then, the samples were directly injected using low volume insert vials.

2.3.2. Selection of stationary phase, flow rate and gradient time

Preliminary measurements were carried out on seven different columns. Among these columns, one was clearly not acceptable for mAb and ADC separations (because of the distorted peak shape and too high hydrophobicity). Therefore, only six columns were finally characterized. Table 1 shows some important features of the selected columns.

Generic linear gradients, starting from 0% to 100% B were applied at a constant linear velocity, corresponding to 1 mL/min and 0.21 mL/min flow rate on 4.6 mm and 2 mm ID columns, respectively. The gradient time (t_G) was varied depending on the columns length: 10 and 30 min for 4.6 × 100 mm columns, 3.5 and 10.5 min for 4.6 × 35 mm columns and 6.5 and 22.5 min for 2.0 × 75 mm columns, in agreement with the rules of geometrical method transfer [16].

2.3.3. Calculations

In gradient elution mode, the observed apparent retention factor (k_{app}) is often used to compare the retention of various compounds on different columns independently on column dimensions:

$$k_{app} = \frac{(t_r - t_0)}{t_0} \quad (1)$$

where t_r is the retention time (in gradient elution mode) and t_0 is the column dead time.