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Evaluation of recent very efficient wide-pore stationary phases for the reversed-phase separation of proteins

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ABSTRACT

In the present contribution, columns packed with fully porous widepore 1.7 μm particles (Acquity BEH300) and widepore core–shell 3.6 μm particles (Aeris WP) were evaluated for the separation of model and therapeutic proteins of varying sizes, hydrophobicity and isoelectric points. Two types of bonding were compared, namely C4 and C18 in a systematic way. The kinetic performance of these stationary phases was evaluated in a previous paper hence this new work focuses on their retention behaviour, loading capacity and selectivity. Using the Tanaka tests, model proteins, and other confirmatory experiments, it is highly probable that with proteins, strong interaction mechanisms were predominant on the Aeris WP while the hydrophobic interaction was the driving force of the retention on the Acquity BEH300 material. This explained why, despite the lower pore volume of the Aeris WP material, the apparent retention factors of proteins possessing both hydrophobic and charged amino acids residues were very close on the four investigated columns. In terms of peak widths, values for proteins were similar for all the tested stationary phases, despite the probable strong ion exchange mechanisms of Aeris WP column. This could be explained by the excellent mass transfer characteristics afforded by the thin porous layer (~0.2 μm) at the surface of the particle which probably compensates for the slow secondary ionic interaction kinetics. The loading capacity was also evaluated on all the four widepore columns, using model proteins. On average, approximately 2–4 times higher amount of proteins can be injected on the fully porous BEH300 compared to the core–shell Aeris WP columns when avoiding 10% change in peak width or in tailing. However, this result could be strongly influenced by the nature and shape of the protein, its hydrophobicity, folding, size and number of charges. Finally, all of these columns were employed for the highly efficient separation of a therapeutic protein (interferon-α-2A) and some closely related proteins and showed excellent performance and selectivity. This result confirms that RPLC gained interest in the biopharmaceutical field as it provides significantly better peak widths than size-exclusion or ion-exchange and inherent compatibility with MS.

1. Introduction

Since few years, the pharmaceutical market has evolved from chemically synthesized small molecules to products resulting from biopharmaceutical technology. There are many reasons to explain the success of biopharmaceuticals including their high specificity, high efficacy, limited side effects, wide therapeutic range, as well as exceptional chemical and biological diversity. In addition, the therapeutic market has become more lucrative, particularly through the development of biosimilar medicines [1,2].

From the analysis point of view, the characterization of therapeutic proteins is quite complex as it requires complete drug substance characterization (e.g. primary structure, and post translational modifications), along with the usual stability studies, impurity profiling, lot-to-lot and batch-to-batch comparisons... For this purpose, numerous analytical tools including chromatographic, electrophoretic, spectrophotometric, and mass spectrometric methods are commonly employed [3,4]. Liquid chromatography is a widespread technique in biopharmaceutical analysis, especially its size-exclusion (SEC), ion exchange (IEX) and reversed phase (RPLC) modes. RPLC has the additional advantage that it can be directly combined with the powerful electrospray mass spectrometry (ESI-MS), to gain supplementary information of the sample.

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During the last few years, some significant advances were brought to the field of RPLC of proteins, including new instrumentations and stationary phase technologies [5–7]. In terms of instrumentation, the old generation of LC systems was replaced by robust UHPLC systems, able to withstand pressures up to 1300 bar and more readily compatible with high-throughput separations. The dwell volume and system dead volume have been also drastically reduced to make them compatible with narrow-bore short columns packed with very fine particles [8,9]. Finally, for the analysis of therapeutic proteins, bio-inert UHPLC systems with an inert flow-path (made of titanium) have been commercialized by LC instrumentation providers.

Since the beginning of the 21st century, some significant improvements were also brought to the stationary phase technology [10]. In the beginning, monolithic supports were a revolutionary material because of its high permeability and efficiency and for their adequate specific area [11]. In addition, organic polymer-based monoliths have shown great potential for RPLC separations of intact proteins [12]. Despite these obvious advantages, the use of this type of chromatographic support is not widespread for the characterization of therapeutic proteins and most of its applications deal with complex proteomics samples [13]. On the other hand, columns packed with sub-2 μm fully porous particles or made with core–shell sub-3 μm (superficially porous) particles are certainly the most promising strategies for the analysis of therapeutic proteins and are commercially available since 2004 and 2007, respectively [14,15]. Using such columns, it is possible to attain some impressive performance with peptides and proteins, at tolerable backpressure for the current UHPLC instrumentation. However, when dealing with large proteins or even monoclonal antibodies (mAbs) and their fragments, the pore sizes of the existing stationary phases are often too small, 90–130 Å, leading to restricted diffusion of the solute (peak tailing or broadening) and inaccessibility to the total surface area of the particles [16]. As a rule of thumb, the solute molecular diameter should be approximately one-tenth the size of the pore diameter [17]. Thus, various providers have commercialized columns packed with wide-pore (160–300 Å) sub-2 μm porous or core–shell particles [18,19].

In a very recent study [20], the performance of two columns packed with porous sub-2 μm particles, namely Hypersil Gold C18 1.9 μm, 175 Å and Acquity BEH300 C18 1.7 μm, 300 Å and of two columns packed with core–shell particles, namely Ascentis Express Peptide ES C18 2.7 μm, 160 Å and Aeris WP C18 3.6 μm and other conventional fully porous 3 and 5 μm columns (Jupiter and Zorbax) were compared for various gradient times and flow rates. According to this study, the Acquity BEH300 C18 and Aeris Widepore (WP) C18 were found to be promising materials for protein analysis.

Except kinetic performance, there is a need to evaluate the retention properties, selectivity and loading capacity of these stationary phases [21] to have a comprehensive view of the possibilities offered by these materials in real-life protein separations. The present study focuses on Acquity BEH300 1.7 μm and Aeris WP 3.6 μm materials in both their C4 and C18 surface modifications. First, the different materials were compared in terms of physico-chemical properties (such as particle size distribution, hydrophobic properties, silanol activity, etc.). The retention behaviour and loading capacity were evaluated with various model proteins of different sizes and isoelectric points. Finally, the columns were employed for real-life analysis of interferon samples, containing the protein in its native form, its related impurities and its oxidized as well as reduced forms. The selectivity of these stationary phases was also compared in this example.

2. Experimental

2.1. Chemicals, columns

Acetonitrile and methanol (gradient grade) were purchased from Sigma–Aldrich (Buchs, Switzerland). Water was obtained with a Milli-Q Purification System from Millipore (Bedford, MA, USA). Small MW test analytes such as uracil, ethylbenzene, propylbenzene, butylbenzene, pentylbenzene, benzylamine, phenol and caffeine were purchased from Sigma–Aldrich. The test proteins such as insulin (from bovine pancreas, MW ~ 5.7 kDa), myoglobin (from equine skeletal muscle, MW ~ 17.7 kDa), carbonic anhydrase isozyme II (from bovine erythrocytes, MW ~ 29.1 kDa), cytochrome-c (from horse heart, MW ~ 12.4 kDa), bovine serum albumin (BSA, MW ~ 66.8 kDa), ovalbumin (albumin from hen egg white, MW ~ 44.3 kDa) and lysozyme (from chicken egg white, MW ~ 14.3 kDa) were purchased from Sigma–Aldrich (Buchs, Switzerland). Recombinant human granulocyte-colony stimulating factor (G-CSF or filgrastim, MW ~ 18.8 kDa) was obtained from Amgen (Switzerland). Recombinant interferon alfa-2A (MW ~ 19.2 kDa, Roferon) was obtained from Roche Pharma (Switzerland).

For reducing the proteins, dithiothreitol (DTT) was obtained from Sigma–Aldrich. Trifluoroacetic acid (TFA), 30% hydrogen peroxide, potassium dihydrogen phosphate (KH_2PO_4) and methionine were also purchased from Sigma–Aldrich.

Acquity BEH300 C18 and C4 columns with a particle size of 1.7 μm (150 mm × 2.1 mm) were purchased from Waters (Milford, MA, USA). The new Aeris Widepore (WP) C18 and C4 columns packed with 3.6 μm core–shell particles (150 mm × 2.1 mm) were generous gifts from Phenomenex (Torrance, CA, USA).

2.2. Equipment, software

All measurements were performed using a Waters Acquity UPLC™ system equipped with a binary solvent delivery pump, an autosampler and UV detector. The Waters Acquity system includes a 5 μL sample loop, and a 0.5 μL UV flow-cell. The connection tube between the injector and column inlet was 0.13 mm I.D. and 250 mm long (passive preheating included), while 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}) was about 13 μL as measured from the injection seat of the auto-sampler to the detector cell. The measured dwell volume was 100 μL. The average extra-column peak variance of our system was found to be around σ^2_{ec} ~ 5–6 μL^2, in the flow rate range 0.1–0.6 mL/min (1 μL injected volume). Data acquisition and instrument control were performed by Empower Pro 2 Software (Waters).Scanning electron microscopy (SEM) was performed using a JEOL JSM 5500LV instrument. Calculation and construction of the histograms were achieved by using Statistica 10 (StatSoft, Tulsa, OK, USA) software while the loading capacity plots were prepared in MS Excel.

2.3. Apparatus and methodology

2.3.1. Mobile phase composition and sample preparation

The evaluation of the silanol activity, hydrophobicity and hydrophobic selectivity was conducted according to the standard Tanaka column characterization protocol, with minor modifications, to achieve appropriate retention on each column [22]. It should be remembered that this characterization protocol has gained general acceptance for the characterization of various reversed-phase stationary phases used for the analyses of small molecular weight species. Unfortunately, no testing protocol specifically designed for the characterization of sorbents used in the analysis of proteins has been proposed to date. Therefore, the