Journal of Chromatography A, 1217 (2010) 5092–5102

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Quantification of immunoglobulin G and characterization of process related impurities using coupled Protein A and size exclusion high performance liquid chromatography

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ARTICLE INFO

Article history:
Received 12 January 2010
Received in revised form 6 May 2010
Accepted 1 June 2010
Available online 9 June 2010

Keywords:
Protein A
Size exclusion
Bradford
Cell culture supernatant
Antibody purification
Protein quantification
DNA
Dynamic binding capacity
Static binding capacity

ABSTRACT

The present work describes two HPLC-UV methods for multi-protein quantification using (i) only a Protein A sensor cartridge (Protein A HPLC) and (ii) the same Protein A cartridge in combination with a size exclusion HPLC column (PSEC-HPLC). The possibility to simultaneously quantify immunoglobulin G (IgG) besides a non-binding protein such as bovine serum albumin (BSA) increases the applicability of Protein A HPLC. Its most pronounced feature is its independence of the buffer system, pH-value and salt content of the investigated sample solvent, which includes cell media. A comparison with the state-of-the-art, the photometrical Bradford method, shows that Protein A HPLC is as sensitive as Bradford, but that it comes with an extended linear range of 4 orders of magnitude, ranging from 0.15 [μg abs] to 1 [mg abs] absolute injected protein amount. The applicability of the PSEC-HPLC method is demonstrated for the analysis of real cell culture feed samples. While Protein A binds IgG, the SEC-column distributes the feed impurities by their molecular weight. The peak area ratios of IgG and the feed impurities of interest are then plotted against the collected sample fraction. These Protein A-Size-Exclusion-Chromatographic diagrams (PSEC-plot) combine the performance information of feed impurities and IgG in a single plot. Further it is shown that both methods are suitable for the performance evaluation of antibody purification media using static as well as dynamic binding experiments performed on DEAE-Fractogel and Capto Adhere. The investigated test samples were "mock" protein solutions with increasing complexity ranging from simple PBS buffer to serum free cell media and "real" cell culture feed solutions.

1. Introduction

In recent years, the demand for clinically applicable proteins, in particular human monoclonal antibodies, especially human or humanized immunoglobulin G (h-IgG) for medical purposes is increasing rapidly. Since decades, affinity materials based on Protein A or Protein G may it be in analytical scale [1–4], in semi-preparative lab-scale or process production scale [5,6] can be reckoned as state-of-the art for the purification of IgG [7]. The fact that Protein A was already immobilized onto a variety of different supports, e.g. sepharose [8], silica gel [9,10], monoliths [11–13], membranes [14] and magnetic beads [15] proof its dominance in the field of antibody purification.

The high cost of Protein A media, especially when employed in production scale processes and the increasing demand for pharmaceutically pure human monoclonal antibodies (HuMab), makes the development of newer less expensive antibody purification adsorbents such as bio-mimetica [16,17] and peptide-mimetica [18], or multi-step purification strategies without the use of Protein A adsorbents [19], highly interesting for researchers and industry alike.

Therefore, besides the development of new adsorbents for protein purification, also the implementation of simple, fast and thorough analytical screening methods for their characterization is of high importance. Hence it is of interest to not only measure the binding capacity of the adsorbent for IgG but also to be able to determine their cross-selectivity towards the presence of other non-target proteins under near-real condition.

Current methods to quantify proteins can be classified into three main groups, the photometrical, the electrophoretical and the chromatographic methods. The classic protein quantification techniques are surely the photometrical ones. While the direct quantification at 280 nm [20] is very simple, it requires fairly high protein amount to provide accurate results. Methods employing the formation of strongly absorbing protein-dye complexation products such as Bradford [21,22], Lowry [23], BCA [24], Cu(II)-neocuproine [25] or fluorescence based methods [26,27] show significantly increased sensitivity, enabling the detection of even

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doi:10.1016/j.chroma.2010.06.007

trace amounts of proteins. However, these methods are also highly susceptible to interferences. Not only other proteins but also non-proteinageous sample components e.g. phenolic compounds [28,23] and polysaccharides [29,30] or even the buffer, pH-value and salt content of the sample [31] may lead to strong deviations, which necessitate own calibration curves for each and every sample matrix composition.

Also frequently used is the semi-quantitative detection of proteins, separated by their electrical charge differences on a SDS-Page slab gel, followed by visualization of the obtained protein bands by coomassie staining [32,33] or silver-staining [34–36,33]. Although this method does not provide distinct quantitative results, it gives the user a fast insight in the semi-quantitative molecular weight distribution of proteins for a fairly large number of samples. The use of sensitive staining methods e.g. silver-staining enables the detection of trace amounts of proteins down to the lower nanogram levels of 0.5–10 ng. More thorough quantitative information can be achieved in the combination with mass spectrometry [33].

Alternatively, chromatographic methods e.g. affinity chromatography [37,1,38], hydrophobic charge induction chromatography (HCIC) [39,40,38] and size-exclusion chromatography (SEC) [41–45] enable the separation as well as quantification of proteins from a more complex protein-mixture. Concerning complexity and diversity, real cell culture supernatants are among the most difficult samples to analyze and characterize. These multi-component mixtures comprise host-cell-proteins (HCP) [46–48], DNA [49,50,47,51], endotoxins [52,53], viruses [54,55] and others constituents [56].

Therefore the choice, which analytical method to employ depends strongly on the amount of information that one desires among other factors such as time, labor and grade of accuracy.

While size-exclusion-chromatography fractionates proteins by their molecular weight, affinity chromatography and ion-exchange chromatography capture only certain proteins depending on their physiological properties and charge distribution. The latter two are therefore more target specific compared to SEC, but may come with a slight loss on information. Protein A based affinity chromatography is for instance a simple, but highly effective method to isolate and quantify IgG from highly complex sample solutions.

Nonetheless, one has to consider that Protein A binds a number of different immunoglobulins, IgG1, IgG2 and IgG4, while IgG3 is not being bound [57–59]. Additionally, Protein A captures traces of IgA and IgM from different species. The diversity increases, when one considers the different isoforms of monoclonal antibodies, which differ in their disulfide connectivity, glycosylation, deamination, etc. [60–62]. This lead to the fact that even for Protein A-based affinity materials, the obtained quantitative results for IgG resembles a sum of data for a variety of different IgG-proteins, which only differ in their degree of variability and affinity towards the ligand protein.

The main advantage of analytical Protein A perfusion columns is the short analysis time of as low as 3 min per cycle for the determination of trace amounts of IgG from biological samples. Although a nice tool, their field of application is nonetheless limited by the fact that Protein A does only bind one species of protein, namely IgG.

Hence the aim of this study was to enhance the applicability of a simple analytical Protein A cartridge to simultaneously quantify up to two proteins, the Protein A binding protein IgG and a second flow-trough protein under near-real sample background conditions. Presently, performance evaluations of adsorbents suitable for antibody purification are conducted with one-protein solutions in standardized buffer systems using photometrical methods for protein quantification. The possibility to employ standard protein mixtures in serum-free cell media enables a more realistic screening of new adsorbents for antibody purification. A direct comparison of the Bradford method and Protein A HPLC will demonstrate the clear superiority of the latter.

A new instrumental set-up, implementing a size exclusion HPLC column in series after the Protein A cartridge (PSEC-method) enables not only the analysis of multi-protein mixture with more than two proteins. It also allows the characterization and comparison of different batches of real cell culture feed solutions using a PSEC-Plot. The value of the so-obtained information becomes clearer, when the same sample fractions used to set up a PSEC-Plot are visualized by simple size exclusion chromatograms and SDS-Page slab gels.

2. Experimental

2.1. Chemicals and standard solutions

Bovine serum albumin (BSA, Fraction V, 99%), ortho-phosphoric acid, phenol red, 10% Pluronic® F-68 solution, potassium dihydrogen phosphate, di-sodium hydrogen phosphate, sodium azide, sodium chloride, triethylamine, tris(hydroxymethyl)aminomethane and hydrochloric acid were purchased from Sigma–Aldrich (Vienna, Austria). HPLC-grade methanol was from VWR (Vienna, Austria) and HPLC-grade 2-propanol from Roth (Karlsruhe, Germany). Bovine gamma globulin (BGG; 2 mg/mL) from Pierce was purchased through THP Medical Products GmbH (Vienna, Austria), polyclonal human-immunoglobulin G, Gammanorm (h-IgG; 165 mg/mL) was from Octapharma (Germany). Bradford protein assay dye reagent was obtained from BIO-RAD (Vienna, Austria). EX-CELL^{TM}VPRO serum free cell medium (SFCM) without glutamine, containing 0.1% Pluronic® F68, 6 g/L glucose and 2.7 g/L sodium bicarbonate besides an undefined amount of hypoxanthine and thymidine was obtained from SAFC Biosciences Limited (Andover, UK). Cell supernatant containing human monoclonal antibody, h-IgG1 from CHO-cell expression system, with pH 7.5 and a conductivity of 17 mS/cm at 33 °C were from ExcellGene (Monthey, Valais, Switzerland). Note that a conductivity of 17 mS/cm corresponds to approximately 150 mM of sodium chloride. The exact properties of cell culture batches A to I are listed in Table S1 in the electronic supplementary material [63].

Bi-distilled water and all buffer solutions used in this study were distilled in-house and filtered through a 0.22 μm cellulose acetate membrane filter from Sartorius purchased through Wagner&Munz (Vienna, Austria) and ultrasonicated prior to use.

Stock solutions of polyclonal IgG (Gammanorm), BGG and BSA solution were prepared at a concentration of 10 mg/mL in the corresponding buffer and subsequently diluted to 1, 0.1 and 0.01 mg/mL in order to establish calibration curves with 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 20, 50, 70, 100, 150, 200, 500, 700 and 1000 μg/mL. Sodium azide solutions in PBS and serum-free cell medium with 0.5, 1, 2 and 3 mg/mL were diluted from 10 mg/mL stock solutions. If not otherwise stated, a PBS buffer containing 10 mM phosphate and 150 mM sodium chloride with pH 7.20 and a TRIS buffer with 20 mM TRIS-HCl and pH 7.00 were used.

2.2. Instrumentation

2.2.1. HPLC-chromatography

All chromatographic measurements were performed on an Agilent 1100 series HPLC system equipped with a binary pump, column oven, temperature controlled sample tray, a multi-wavelength detector and a 2-position-6-port switching valve from Agilent (Vienna, Austria).

For the quantification of IgG a Protein-A ImmunoDetection® sensor cartridge from Applied Biosystems (Vienna, Austria) was