Chromatography on DEAE ion-exchange and Protein G affinity columns in tandem for the separation and purification of proteins

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Abstract

A high-performance liquid-chromatographic method based on coupled DEAE anion-exchange and Protein G affinity columns has been developed for the simultaneous separation and purification of immunoglobulin G and albumin from mouse serum. The diluted mouse serum was injected directly into this system, and the proteins were eluted separately from the DEAE and Protein G columns, coupled in series, by the column-switching technique. The advantages of this method are that IgG and albumin can be separated and purified simultaneously, the expensive affinity column is protected from contamination by the impurities in the mouse serum, and it is fast, selective, robust, and reproducible. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Currently, blood is a main source for more than 15 therapeutic products, including albumin, immunoglobulin, various clotting factors, and protease inhibitor. Chromatographic techniques have been developed for purifying them for therapeutic use. Albumin is the major protein component of blood, making up 50–65% of the total protein content and having typical concentrations of 35–52 g/l. Ion-exchange chromatography (IEC) is commonly used for separating albumin [1]. IgG is the most abundant immunoglobulin in serum, and normal levels of IgG vary from 4.0 to 12 g/l. It can be isolated by IEC, hydrophobic-interaction chromatography (HIC), and hydroxyapatite chromatography [2].

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However, these methods do not display an exceptional high selectivity for IgG. Affinity chromatography (AC) on Protein A or/and Protein G has been considered as a more efficient method of purifying IgG. These two ligands bind to the Fc region of IgG specifically and are now commonly used on a large scale for the purification of IgG [3].

In general, albumin and IgG in serum are usually purified by a series of discrete purification steps, e.g., the effluents from the first column are collected in a number of fractions, each fraction is desalted or/and concentrated as necessary, and the concentrated fractions are applied to a second column with different specificity. Multi-step purification with manual operations is time-consuming, cumbersome, and less reproducible. In addition, the yields of the multi-step treatment are usually poor. On-line methods of columns in tandem permit automation of operation and less sample handling [4]. The system combining an IEC column and an AC column has been used for analytical and preparative separations of protein [5,6]. Analysis of a monoclonal antibody has been conducted on a Protein A and an anion-exchange column in tandem [7]. The tandem system based on two affinity columns has been used for the simultaneous determination of albumin and IgG in serum [8]. These studies have shown that the use of such multicolumn systems has several potential advantages, e.g., high sensitivity, quick separation, better reproducibility, and less sample handling.

In this paper, we have developed a tandem HPLC system, consisting of an anion-exchange column and an affinity column coupled in series, for the simultaneous separation and purification of IgG and albumin from mouse serum by the column-switching technique. Optimization of resolution, speed, and productivity was conducted experimentally as a function of several variables. In addition, methods of cleaning the system to prolong the life of the column have also been investigated.

2. Materials and methods

2.1. Materials and apparatus

Mouse serum IgG and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO, USA), the other chemicals are of analytical pure grade or biological reagents. Water was obtained from a Milli-Q system (Millipore, Bedford, MA, USA). The mouse serum was from Beijing Anapure Bioscientific (Beijing, China). Before separation and purification, it was filtered through a 0.22- μ m polysulfone filter, and diluted 1:1 (v/v) with loading buffer.

The TSP liquid chromatograph (TSP, San Jose, CA, USA) consisted of a P4000 pump, an AS3000 autosampler, a Focus PDA detector, and a data work station. Chromatographic system control, analysis, and data acquisition were performed with TSP Spectra System Software PC 1000. The configuration of the tandem system, consisting of an anion-exchange column (TSK gel DEAE-5PW, 75 $\times$ 7.5 mm I.D., TOSOH, Japan) and a Protein G affinity column (POROS G/M, 30 $\times$ 2.1 mm I.D., PerSeptive Biosystems, Cambridge, MA, USA) is shown in Fig. 1. A six-port valve (Rheodyne model 7010, Berkeley, CA, USA) between these two columns was set up for switching operation.

Fig. 1. Schematic representation of the coupled-column system.

2.2. Chromatographic procedure

At the beginning of the tandem separation, the diluted mouse serum sample was injected into the system, equilibrated with the loading buffer (A) (see Table 1), and target proteins were loaded on both columns. Once the non-retained components in the sample were eluted completely, the Protein G column was switched off-line by turning the six-port valve, as shown with a dashed line in Fig. 1. Then, the albumin was eluted from the DEAE column, using the elution buffer (B) (see Table 1), and the other impurities of proteins were eluted completely using the elution buffer (C) (see Table 1). After that, the Protein G column was switched on-line by turning the six-port valve again for eluting the bound IgG. The eluted proteins were rapidly neutralized by dropwise addition of a concentrated buffer of 1 M Tris–HCl (pH 8.0) to avoid denaturation. Finally, the system was reequilibrated with the loading buffer (A) for the

Table 1

Optimized chromatographic conditions employed for the tandem-chromatographic separation of albumin and IgG from mouse serum

Step	Action	Condition
1	The system is coupled with the IEC and AC columns on-line	Loading buffer (A): 35 mM PB (pH 7.0); 40 mM $(\text{NH}_4)_2\text{SO}_4$, 15 min
2	The AC column is taken off-line, albumin is eluted from the IEC column	Elution buffer (B): 35 mM PB (pH 7.0); 70 mM $(\text{NH}_4)_2\text{SO}_4$, 15 min
3	The AC column is taken off-line, contaminant proteins are eluted from the IEC column	Elution buffer (C): 35 mM PB (pH 2.5); 70 mM $(\text{NH}_4)_2\text{SO}_4$, 15 min
4	The AC column is put on-line and IgG is eluted	Elution buffer (C): 35 mM PB (pH 2.5); 70 mM $(\text{NH}_4)_2\text{SO}_4$, 5 min
5	The system is reequilibrated	Loading buffer (A), 10 min

next run. The proteins were monitored at 280 nm and system was operated at room temperature.

2.3. Determination of capacity of Protein G affinity sorbents for IgG

A solution of standard IgG (3 mg/ml), dissolved in loading buffer of various ionic strengths, was injected into the Protein G column at a flow-rate of 0.2 ml/min. After the equilibrium of the Protein G column with IgG solution had been reached, i.e., the elution curve reached a plateau, the elution buffer (C) was used to elute the IgG from the Protein G column at a flow-rate of 0.5 ml/min. The column capacity of Protein G for IgG can be calculated from the elution curve by frontal analysis [9,10].

2.4. Analysis of the products

2.4.1. Immunological analysis

Immunochemical analyses for IgG and albumin were carried out by radial immunodiffusion (RID). Samples of 5 μ l of IgG or albumin fractions were dripped into wells of the RID gel. The mouse serum IgG and albumin from Sigma were used as standards.

2.4.2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The purity of IgG and albumin fractions, purified by the tandem system, was tested by vertical SDS-PAGE, using 12% resolving gel and 4% stacking gel under denaturing and reducing conditions. Protein staining was performed with Coomassie Brilliant Blue R-250.

3. Results and discussion

The proteins of mouse serum include some acidic proteins, such as albumin, some neutral proteins, such as IgG, and a small portion of basic proteins. Theoretically, under neutral conditions, anion exchangers tend to bind most of the acidic proteins; IgG is completely unretained on the DEAE column but binds to the Protein G affinity column; whereas basic proteins and the other neutral proteins pass through both columns. In the tandem system, solvent compatibility of the mobile phases used in different steps is an essential condition to be considered.

3.1. Selection of buffers for different steps in tandem chromatography

3.1.1. For the loading step on the DEAE column

Theoretically, the result of optimum loading should be that the DEAE column retains albumin completely and only very little IgG. The optimization of the loading buffer was evaluated in terms of the amounts of albumin retained and IgG unretained on the column. Three types of 35 mM phosphate buffer (PB, pH 7.0) with ammonium sulfate, sodium sulfate, and sodium chloride at different concentrations were compared individually. The results, shown in Fig. 2, illustrate that 40 mM ammonium sulfate resulted in a

Fig. 2. Protein recoveries from the DEAE column with various salts at different concentrations. 20 μg standard proteins were injected individually into the column at a flow-rate of 1.0 ml/min. The chromatogram was evaluated according to its unretained peak. Complete recovery (100%) was assumed when the proteins were individually injected through a low-dead-volume connector.

better discrimination between albumin and IgG, and that the 78% of IgG and 8% of albumin was eluted, respectively.

The effects of different phosphate concentrations (20, 35 and 50 mM) in buffer solution at pH 7.0 with 40 mM ammonium sulfate on eluting albumin and IgG were also studied. The results show that in comparison of 20 mM, 50 mM with 35 mM PB as loading buffer, the former led to a poor discrimination between albumin and IgG, whereas the latter got much better improvement. Therefore, 35 mM PB (pH 7.0), containing 40 mM ammonium sulfate, was chosen in this study as the optimum loading buffer for the DEAE column. The loading buffer with higher ionic strength has three advantages: (1) increasing the recovery of IgG, (2) increasing the purity of albumin, owing to less retention of most contaminant proteins with higher pI (> 4.7) on DEAE

column, and (3) decreasing the interactions between albumin and the ion-exchange sorbents, thus increasing the recovery of albumin.

3.1.2. For elution from the DEAE column

The albumin retained on the DEAE column during the loading step was eluted by step-gradient mode. The results indicate that an increase of ammonium sulfate concentration from 60 to 100 mM in PB (pH 7.0) could increase the recovery of albumin. However, higher salt concentration might lead to elution of more contaminant proteins with lower pI (< 4.7). A salt concentration of 70 mM was chosen as a compromise between the recovery and purity of albumin.

3.1.3. For the loading step on the Protein G column

Protein G has a broader range of binding IgG than Protein A. For instance, IgG₁, a subclass of IgG, binds much more readily to Protein G than to Protein A [3]. In addition, separations of IgG by Protein A require a higher salt concentration [11], and this can limit its coupling with the ion-exchange column for binding albumin. Thus, the Protein G column was chosen in this study for purifying IgG. The IgG binding capacities of the Protein G column were determined at various binding conditions. As shown in Fig. 3, increasing the concentration of ammonium sulfate could lead to a minor decrease of the binding capacity on the Protein G column. The result is coincident with Ref. [12], in which a negative correlation was significantly found between the salt concentration of the binding buffer and the binding capacity of the column.

Fig. 3. Effect of ionic strength on the IgG binding capacity of the Protein G column. The capacity was measured by injection of IgG standard (3 mg/ml) at a flow-rate of 0.2 ml/min by frontal analysis, followed with elution buffer at a flow-rate of 0.5 ml/min.

3.1.4. For elution from the Protein G column

Two solutions were investigated for both eluting IgG from the Protein G column and regenerating the DEAE column simultaneously: (1) 150 mM sodium chloride (pH 2.5), (2) 35 mM PB (pH 2.5), containing 70 mM ammonium sulfate. The former is a recommendation by the manufacturer [13] for eluting IgG from Protein G columns. Both eluents can effectively elute IgG [14], but the latter is more efficient for regenerating the DEAE column. The solution of 35 mM PB (pH 2.5), containing 70 mM ammonium sulfate, was then chosen for eluting IgG and regenerating the DEAE column.

3.2. Operating conditions in tandem chromatography

According to the results reported above, the optimum mobile phases and the operating program are shown in Table 1. Two columns in tandem mode are illustrated in Fig. 1. The chromatographic procedure was controlled by a computer program and a total separation period needs 60 min. The flow-rate can influence the mass transfer of affinity separations due to the slower desorption kinetics [14]. Therefore, a flow-rate of 0.5 ml/min was used for eluting IgG from the Protein G column in order to obtain a higher recovery and concentration. The chromatograms of standard samples (containing 200 µg albumin and/or 200 µg IgG) separated under the optimum conditions are shown in Fig. 4a–c.

3.3. Separation and purification of albumin and IgG from mouse serum

IgG and albumin were isolated and purified from mouse serum diluted with Buffer (A) (1:1) by our system (see Fig. 4d). To test the purity of proteins separated by the tandem technique, the four major peaks in Fig. 4d were collected and analyzed by SDS-PAGE and RID. According to the results of RID, the yield was 1.1 mg of IgG and 10 mg of albumin from 0.5 ml diluted mouse serum. The recoveries of IgG and albumin were 85% and 67%, respectively. The results in Fig. 5 show the peaks of IgG and albumin with higher purity.

A series of injections of mouse serum was used to determine the reproducibility and lifetime of the system. The yields from the 2nd, 8th, and 16th injection were 1.05, 1.1, and 1.08 mg for IgG, and 11.08, 10.56, and 10.60 mg for albumin, respectively. The purity of IgG collected from various injections was not appreciably changed, as shown in Fig. 5. No signs of column deterioration (e.g., deformed peaks, increasing/decreasing peak heights or peak areas) appeared after over 20 injections. This lifetime is much longer than the typical 8–10 column cycles for the preparative chromatography of IgG on Protein G perfusion columns [15].

An advantage of our system is that the serum sample passes through the DEAE column first, and thus the expensive Protein G column is protected from contamination and has a longer lifetime. Our previous studies [15] have shown that proteins, nucleic acids, and lipids in serum can reduce the efficiency of the affinity column and cause difficulties in the clean-up, because Protein G is unstable under the harsh, basic cleaning conditions. A series of appropriate sample pretreatment procedures is normally required before serum sample injection in order to remove some contaminant components. Using

Fig. 4. Chromatograms obtained after injections of (a) albumin, (b) IgG, (c) albumin plus IgG, and (d) diluted mouse serum. The samples, chromatographic conditions, and operating procedure used are described in the text.

Fig. 5. SDS-PAGE of fractions from the coupled-column system. (1) Unretained fraction; (2) mouse serum; (3,4) fractions eluted from the DEAE column with the buffers (B) and (C), respectively; (5–7) fractions eluted from the Protein G column after the 2nd, 8th and 16th injections, respectively; (8) molecular-mass markers. The masses of standard proteins, from top to bottom, are 97.4, 68, 42.7, 30.1, 21.5, 14.4 kD.

a pre-column is the usual way to protect affinity media from excessive contamination. In our study, removal of most contaminants by the DEAE column made it possible to obtain reproducible affinity separations with a longer Protein G column lifetime.

Our chromatographic system has now been used for more than 32 runs of preparative separations of IgG and albumin. Repeated injection of mouse serum resulted in an increased pressure drop on the DEAE column, and then gradually a decreased peak

Fig. 6. The chromatogram of proteins on (a) contaminated DEAE column, and (b) cleaned DEAE column with 0.1 M NaOH in 4 M urea.

resolution, due to the accumulation of lipids, nucleic acids, and very large proteins of low solubility, as shown in Fig. 6a. For regenerating the Protein G column with little damage to its capacity, 4 M urea in 0.1 M sodium hydroxide can effectively be used [15]. We used this cleaning procedure to treat a contaminated DEAE column in this study (Fig. 6b). Comparing it with the 0.2 M sodium hydroxide, recommended by the column manufacturer, both procedures can effectively regenerate the DEAE column and recover the column efficiency. However, 4 M urea in 0.1 M sodium hydroxide is easier to use for the simultaneous cleaning of the DEAE column and the Protein G column in the tandem system.

4. Conclusions

The data presented showed that our system was effective in the isolation of IgG and albumin from mouse serum in high purity and recovery. The method has several advantages: (1) both albumin and IgG with less sample pretreatment are isolated and purified simultaneously; (2) removal of most impurities from mouse serum by the DEAE column protects the Protein G column from contamination and prolongs its lifetime; (3) the mobile phase used in this study is compatible with both IEC and AC, coupled on-line; (4) good recovery of the IgG and albumin from the corresponding column simplifies instruments and operations. The procedure developed is particularly suited to laboratory-scale applications, but it can also be applied to the purification on a large scale. In addition, this method could also be used for the separation of monoclonal antibodies from mouse ascites and hybridoma culture supernatant, since their compositions are similar to mouse serum.

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