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PURIFICATION OF HUMAN SERUM GAMMA GLOBULINS BY HYDROPHOBIC INTERACTION HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

STEVEN C. GOHEEN* and ROBERT S. MATSON
Bio-Rad Laboratories, 2200 Wright Avenue, Richmond, CA 94804 (U.S.A.)

SUMMARY

Fresh, whole human serum was fractionated on a Bio-Rad Protein Chromatography System, equipped with a Bio-Gel TSK Phenyl-5PW column, by utilizing a descending linear gradient of ammonium sulfate in 0.1 *M* sodium phosphate buffer, pH 7.0, at 0°C. Two major peaks were isolated corresponding to albumin and gamma globulin. The identity of these protein peaks was substantiated by chromatography of an albumin–gamma globulin standard mixture. The purity of the individual fractions was verified by high-performance size exclusion chromatography (HPSEC) on either a Bio-Sil TSK-250 or a Bio-Gel TSK-40 column. The applicability of these HPSEC columns to the molecular weight characterization of the Bio-Gel TSK Phenyl-5PW column fractions was compared. Typically, the Bio-Gel TSK Phenyl-5PW column (75 × 7.5 mm I.D.) was used to purify gamma globulin from 100 μl of plasma. This corresponded to approx. 1.5–2.0 mg of the globin fraction. Unidentified contaminants in this fraction had molecular weights of approx. 1000–3000 daltons and 260 000–300 000 daltons.

INTRODUCTION

Hydrophobic interaction chromatography is being rapidly adopted for high-performance liquid chromatography (HPLC) of proteins. Most of the results reported thus far have involved some overall characteristics in the chromatography of protein standards[1,2]. Goheen and Engelhorn[2] have also demonstrated that certain proteins are more strongly retained at higher temperatures. Hydrophobic interaction chromatography differs from reversed-phase chromatography[3–5] in the selection of solvent conditions. In reversed-phase chromatography, an increasing gradient of organic solvent is used to help unfold the proteins and enhance their hydrophobic interaction with the solid phase. In hydrophobic interaction chromatography, a decreasing salt gradient[6] is employed to rehydrate the proteins. This process affects protein structures less than the intentional denaturation produced by reversed-phase chromatography. As a result, hydrophobic interaction chromatography has sometimes been referred to as "reversed-phase without denaturation"[7].

munoglobulins[8]. Both of these are often thought of as hydrophobic proteins. Albumin has at least one large, exposed hydrophobic binding site for the transport of hydrophobic molecules, such as free fatty acids in serum[9,10]. Immunoglobulins precipitate from serum in 2 *M* ammonium sulfate[11].

Immunoglobulins can be isolated from whole serum by HPLC on the Bio-Gel HPHT Hydroxylapatite column (Bio-Rad, Richmond, CA, U.S.A.)[12] or an open column with either DEAE Affi-Gel Blue[13] or Protein A MAPS[14]. However, immunoglobulin purifications by hydrophobic interaction HPLC have not yet been reported.

For the identification of proteins by molecular weight, a common chromatographic procedure is silica-based high-performance size exclusion chromatography (HPSEC). Silica-based gel columns are generally preferred over polymer-based HPSEC columns, because they produce better resolution. However, one disadvantage of silica-based columns is their structural instability under alkaline conditions. Under those circumstances, the polymer-based HPSEC columns must be used, regardless of their characteristically lower resolution. In the present study, the content and purity of the various protein fractions from the Bio-Gel TSK Phenyl-5PW column were determined by using both a silica-based and a polymer-based column for comparison.

MATERIALS AND METHODS

All reagents were of the highest purity available. HPLC-grade ammonium sulfate was from Bio-Rad Labs. Proteins were purchased from Sigma (St. Louis, MO, U.S.A.). All chromatograms were obtained with the Bio-Rad HPLC protein Analysis System, including a Model 1305A variable-wavelength UV monitor and a Model 1322 dual pen strip chart recorder. Gradients were monitored with a Bio-Rad conductivity monitor and a standard flow cell. The Bio-Gel TSK Phenyl-5PW, Bio-Sil TSK 250, and Bio-Gel TSK-40 columns were obtained from Bio-Rad. All buffers were passed through 0.45-μm filters. The temperature of the Bio-Gel TSK Phenyl-5PW HPLC column was regulated by immersing it in an ice bath. All other chromatographic procedures were carried out at ambient temperature.

Human whole blood was obtained from healthy volunteers. The blood was allowed to clot at room temperature for 30 min and centrifuged at room temperature at 600 *g* for 15 min. The serum, separated from the pellet containing cellular debris, was refrigerated and used within 24 h.

RESULTS

Under the conditions for hydrophobic interaction HPLC, the elution profile for serum albumin is improved by either lowering the temperature or adding methanol to the eluents[2]. Nevertheless, using either lowered temperatures or methanol did not dramatically improve the elution profile of whole human serum (Fig. 1). Since the profile at 0°C was slightly better resolved than at 25°C, subsequent studies were carried out under these elution conditions.

As shown, whole human serum separated into two major peaks at 0°C when detected at 280 nm (Fig. 2). These protein-rich peaks (A and B) each contained three main components. In the early eluted peak (A), these had molecular weights of

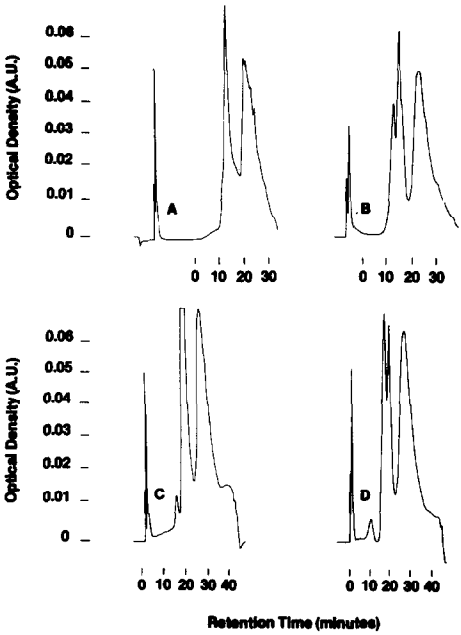

Fig. 1. Hydrophobic interaction chromatography of human serum: the effects of temperature and organic solvent. Fresh, whole human serum (20 μl) was injected into a Bio-Rad Protein Chromatography System, equipped with a Bio-Gel TSK Phenyl-5PW column, and eluted with a 30-min linear decreasing salt gradient at 1.0 ml/min from 1.7 *M* to 0 *M* ammonium sulfate with 0.1 *M* sodium phosphate (pH 7.0). In A and B, the serum sample was chromatographed isocratically in 1.7 *M* ammonium sulfate (pH 7.0) for a few minutes before the linear gradient was started to elute all unbound samples. Otherwise, the samples were chromatographed similarly, except: A, 25°C; B, 0°C; C, 5%; and D, 10% methanol in both eluents.

300 000, 65 000, and 1000, respectively (Fig. 2). The column used to determine these molecular weights was the Bio-Sil TSK 250 (Fig. 3), which has a fractionation range of 1000 to 300 000 daltons. Therefore, the high-molecular-weight peak could represent aggregates or proteins of much higher molecular weight. Likewise, the 1000-dalton peak may have contained much smaller components. We suspected that the 65 000-dalton peak was albumin, since it was the predominant component of both serum and this fraction. Later analysis confirmed this identification when pure albumin was injected into the column under identical conditions. The major constituent of the second peak (B) was similarly identified as immunoglobulin (Figs. 3–5).

Comparisons between the silica-based and hydroxylated polyether-based HPSEC columns demonstrated that the silica-based columns gave better resolution than the polymer-based columns (Figs. 3 and 4). This difference has been attributed to the narrower distribution of pore size in the silica column than in the polymer column. However, in both cases results were similar.

DISCUSSION

The fractionation of major serum proteins, such as albumin and immunoglobulins, continues to be a significant concern to researchers in the life sciences.

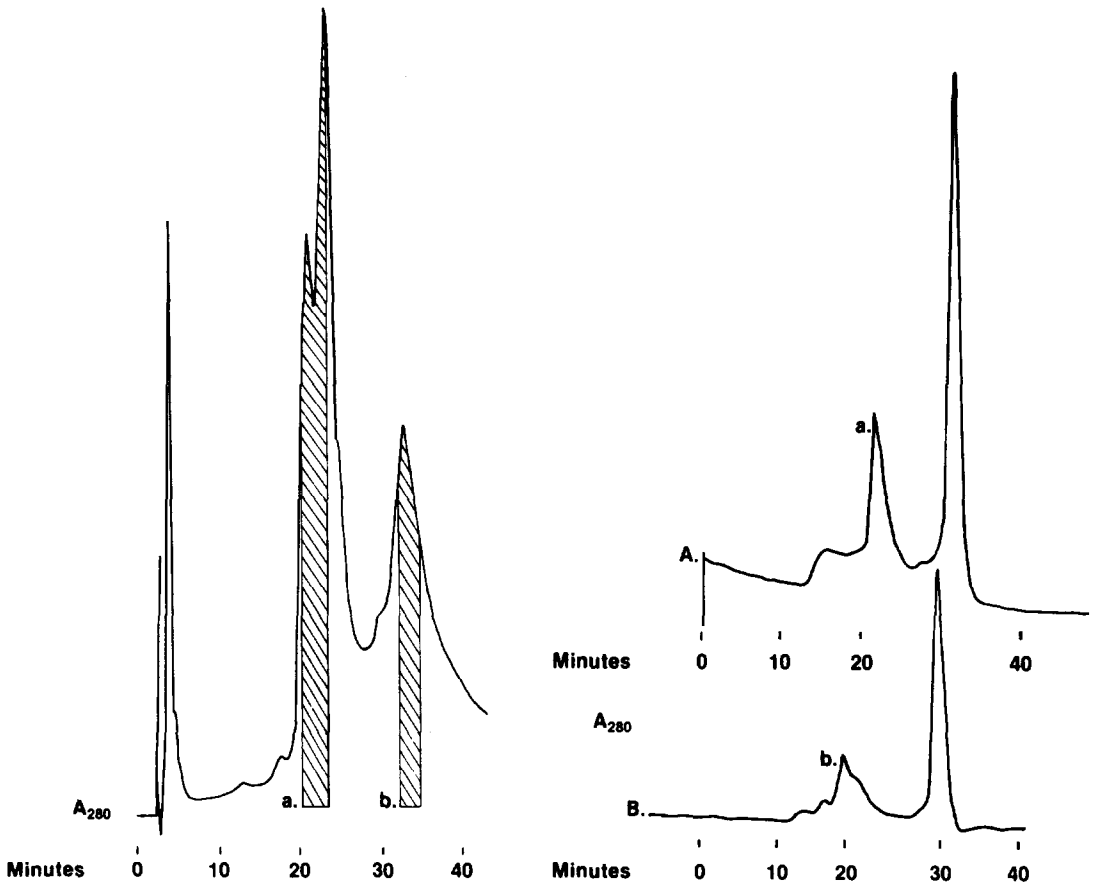

Fig. 2. TSK phenyl-5PW fractionation of human serum for HPSEC. Fresh, whole human serum (20 μl) was injected and a 30-min linear gradient of descending salt content was applied from 1.7 *M* to 0 *M* ammonium sulfate in 0.1 *M* sodium phosphate at pH 7.0. The column temperature was kept at 0°C with an ice bath. Fractions a and b were collected and rechromatographed in two different HPSEC columns. Detection was at 280 nm with 0.16 a.u.f.s. The flow-rate was 1.0 ml/min.

Fig. 3. Bio-Sil TSK 250 HPSEC of human serum fractions. Aliquots (20 μl) of Fractions A and B, described in Fig. 2, were rechromatographed. Molecular weights of the major fractions shown in A: 300 000, 65 000 and 1000. Major fractions shown in B: 280 000, 125 000 and 1100. Peaks a and b correspond to albumin and immunoglobulin, respectively. For both chromatograms, the flow-rate was 0.5 ml/min and detection was at 280 nm with 0.04 a.u.f.s. Molecular weights were calibrated with the Bio-Rad Gel Filtration Protein Standard. The elution buffer contained 0.1 *M* sodium phosphate (pH 6.5) and 0.02% sodium azide.

Several ion-exchanges and affinity chromatographic techniques are presently available that adequately accomplished this goal. For example, DEAE Affi-Gel Blue[13] in conventional open-column chromatography applications is a widely used, well established method. However, with the advent of HPLC, other column materials, such as Bio-Gel HPHT (hydroxylapatite) and the TSK DEAE-5PW resin offer the advantage of rapid separations with good recovery of serum components.

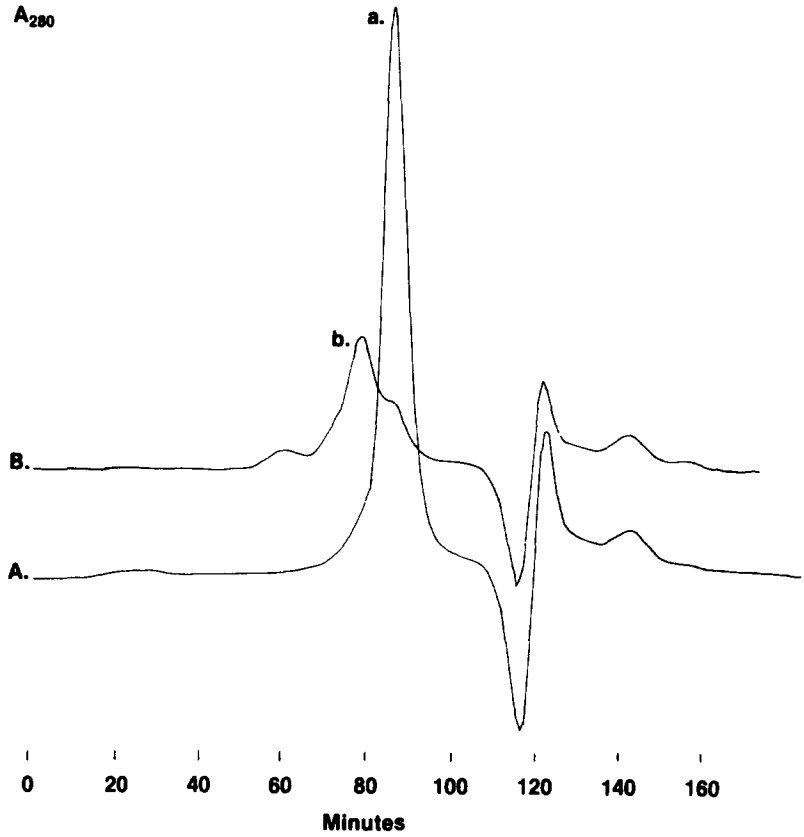

Fig. 4. Bio-Gel TSK-40 HPSEC of human serum fractions. The molecular weight of the predominant peak (a) in Chromatogram A corresponds to that of albumin, while the molecular weight of the major peak (b) in Chromatogram B corresponds to that of gamma globulin. The shoulder in peak b may be albumin. For both chromatograms, the flow-rate was 0.1 ml/min and detection at 280 nm with 0.04 a.u.f.s. Molecular weights were calibrated with the Bio-Rad Gel Filtration Standard with and without added albumin standard. The elution buffer contained 0.1 *M* sodium phosphate (pH 6.5) an 0.02% sodium azide.

Recently, it has been shown that the relative hydrophobicity of proteins can be assessed by utilizing Bio-Gel TSK Phenyl-5PW HPLC[2]. This paper presents a useful alternative HPLC separation that exploits the hydrophobic differences between serum albumin and gamma globulin. On an arbitrary scale, where 1.0 represents the most hydrophobic proteins, albumin = 0.6[2] and gamma globulin = *ca.* 0.9.

Due to the morse hydrophobic character of immunoglobulins, the separation of these components from serum albumin is easily achieved. The resolution obtained offers a convenient method for use in quality control of serum immunoglobulin preparations to quantitative albumin contamination. Moreover, the best resolution is achieved at 0°C. This should be optimal for preserving serum component stability during chromatography in ammonium sulfate or other salt gradients. A further advantage of this method is that ammonium sulfate is essentially absent from the fraction in which immunoglobulins are eluted. Therefore, it could be used as a desalting step after ammonium sulfate precipitation.

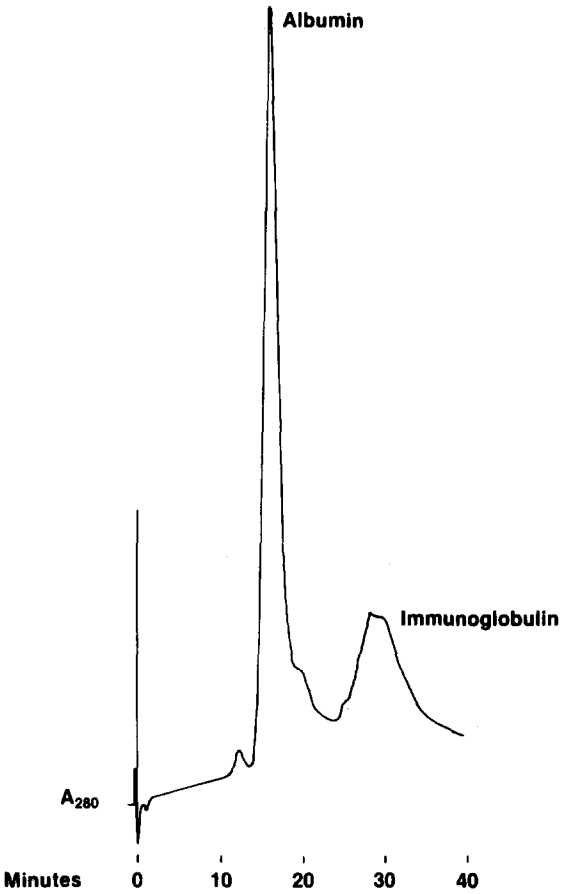

Fig. 5. Fractionation of an albumin–immunoglobulin standard mixture. A volume of 20 μl of a mixture of 40 mg serum albumin/ml and 13 mg immunoglobulin/ml in 0.1 *M* sodium phosphate (pH 6.5) was injected into a Bio-Gel Phenyl-5PW column, which was kept at 0°C. Elution conditions were as in Fig. 1. These proteins were also injected individually to determine the identity of the peaks.

Analysis of the individual immunoglobulin and albumin fractions by HPSEC on two different columns revealed that these fractions were not devoid of other serum components. No attempt was made to identify these contaminants or to separate them from the immunoglobulin- and albumin-enriched fractions. However, from these analyses, it is clear that the polymer-based HPSEC column gave an adequate separation of albumin from immunoglobulins. Thus, the combination of hydrophobic-interaction HPLC and HPSEC, as described, provides a convenient approach to the purification of IgG. In this regard, although the use of Bio-Sil TSK-250 offers a distinct advantage over the Bio-Gel TSK-40 column in terms of time and resolution, the Bio-Gel TSK-40 column is preferable when chromatography is performed at alkaline pH.

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