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Note

Separation of intravenous IgG containing albumin by high-performance size-exclusion chromatography on TSKgel G3000SWXL

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Intravenous IgG has been widely applied for clinical purposes such as hypo-and agammaglobulinaemia, antibiotic therapy and thrombocytopenia[1–4]. Some of these intravenous IgG contain albumin as a stabilizer and it is therefore important to determine both IgG and albumin for quality control. There have been many reports of the separation of monoclonal IgG and albumin by high-performance liquid chromatography (HPLC), ion-exchange chromatography (IEC)[5–14], hydroxyapatite chromatography (HAC)[15,16], hydrophobic interaction chromatography (HIC)[9] and affinity chromatography (AFC)[17–20]. None of these methods, however, is satisfactory for quality control owing to the long analysis time and complex elution as in the gradient method. On the other hand, size-exclusion chromatography (SEC) has been widely used for quality control purposes owing to its simple operation, in spite of the lower resolution, and it has been employed for the separation of monoclonal antibodies[5,6,21] and immunoglobulin in serum[22]. Although chromatographic variables for protein separation have been extensively studied[23–29], there are no reports of the study of the separation of IgG and albumin by SEC. Lee *et al.*[30] reported the separation of intravenous IgG containing albumin by SEC. However, they removed albumin from the sample by IEC prior to the SEC analysis.

Recently, a new SEC column, TSKgel G3000SWXL, has become commercially available[31]. According to the manufacturer, this column has twice the number of theoretical plates as the conventional G3000SW, and IgG could be separated quantitatively within 15 min. We examined the elution conditions for the separation of IgG and albumin in intravenous IgG by SEC on TSKgel G3000SWXL and the results are reported in this paper.

EXPERIMENTAL

Human IgG was purchased from Miles Labs. (Kankakee, IL, U.S.A.) and human albumin from Sigma (St. Louis, MO, U.S.A.). The intravenous IgG sample Gammagard was obtained from Baxter (Tokyo, Japan), Venilon from Fujisawa Pharm. (Osaka, Japan) and Venoglobulin-I from Green Cross (Osaka, Japan). According to the manufacturers, the ratios of IgG to albumin in the samples were 50:1, 20:1 and 5:1, respectively.

All chromatographic procedures were performed with an HPLC system con-

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sisting of a CCPM pump (TOSOH, Tokyo, Japan), a Model 7125 sample injector (Rheodyne, Cotati, CA, U.S.A.) with a 100-μl sample loop and a UV-8000 detector (TOSOH). UV detection was applied at 280 nm (0.64 a.u.f.s.). The samples were separated on a TSKgel G3000SWXL column (300 × 7.8 mm I.D.) at a flow-rate of 1.0 ml/min; 50 mM sodium phosphate of various pH values containing 0.1 M Na_2SO_4, and the same buffer containing 0.3 M NaCl or 0.3 M $NaClO_4$, were used for the evaluation of the elution conditions. Amounts of 100 μg of IgG and albumin in 100 μl of buffer were separated on the column for evaluation of the elution conditions, and 250 μg of an intravenous IgG sample in 5 μl of solution were separated. The total recoveries were calculated from the peak areas on the chromatograms.

The resolution (R_s) between IgG and albumin was determined using the equation

$$R_s = 2(V_2 - V_1)/[(W_1 + W_2)(\log M_1 - \log M_2)]$$

where V_1 is the elution volume of IgG monomer (ml), V_2 the elution volume of albumin monomer (ml), W_1 the peak width of IgG monomer (ml), W_2 the peak width of albumin monomer (ml), M_1 the molecular mass of IgG monomer (155 000) and M_2 the molecular mass of albumin monomer (67 000).

RESULTS AND DISCUSSION

Fig. 1 shows the dependence of the difference in the elution volumes of IgG and albumin on the pH of the buffer containing three kinds of salts. The ionic strengths of the salts in buffer were adjusted to be the same (*ca.* 0.3), as ionic interactions between the sample and the packing materials were presumed to occur. The difference in the elution volumes of the two proteins was small and was slightly dependent on the pH of the buffer containing 0.3 M NaCl or $NaClO_4$. On the other hand, when 0.1 M Na_2SO_4 was used, the difference in elution volumes was fairly large and was also slightly dependent on the pH of the buffer. The difference in elution volumes increased with decreasing of pH of the buffer from neutral to 5.0, as elution of albumin was delayed. At pH 4.0, however, IgG was eluted as a broad peak and the resolution

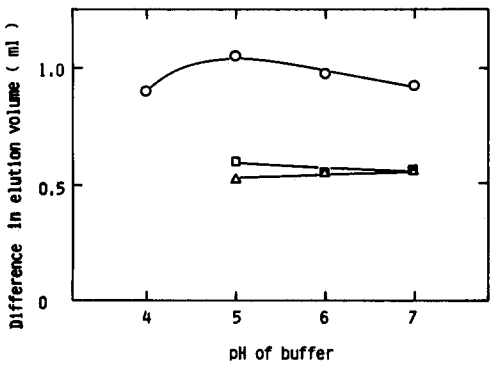

Fig. 1. Dependence of difference in elution volumes of IgG and albumin on pH of buffer on TSKgel G3000SWXL. IgG and albumin were separated with 50 mM sodium phosphate at various pH containing (○) 0.1 M Na_2SO_4, (□) 0.3 M NaCl or (△) 0.3 M $NaClO_4$.

of the two proteins became worse. The change in elution volume suggests that a change in the steric conformation of the proteins or some interaction with the packing materials occurs, depending on the pH of the buffer. It was found that 50 m*M* sodium phosphate (pH 5.0) containing Na_2SO_4 was the optimum elution buffer for the separation of the two proteins. We also examined acetate and citrate buffers containing Na_2SO_4 but a better resolution could not be obtained.

Fig. 2 shows the dependence of the resolution between IgG and albumin on the concentration of Na_2SO_4 in the buffer. Although a buffer of pH 6.8 was also examined, the R_s value could not be determined as it was too low. Fig. 2 indicates that the two proteins could be separated with higher resolution in pH 5.0 than pH 6.0 buffer. The dependence of resolution on the salt concentration in buffer was greater at pH 6.0 than pH 5.0. Accordingly, the optimum concentration of Na_2SO_4 was found to be 0.1 *M* in both buffers, where the ionic interaction of the sample with the packing materials would be negligible. At a 0.2 *M* concentration of Na_2SO_4 the R_s value was lower, which suggests a hydrophobic interaction of the sample with the packing material. Above 0.5 *M* Na_2SO_4, IgG showed a broad peak and the R_s value could not be determined. The resolution also decreased with salt concentrations below 0.1 *M*, which suggests an ionic interaction between the proteins and the packing material. Accordingly, the optimum separation buffer for the two proteins was found to be 50 m*M* sodium phosphate (pH 5.0) containing 0.1 *M* Na_2SO_4.

Figs. 3–5 show comparisons of the separation of intravenous IgG with conventional and optimum elution conditions. The sample in Fig. 3 contains a trace amount of albumin (IgG: albumin = 50:1). Albumin in the sample was recognized as a small peak under the optimum conditions, in spite of a shoulder obtained with conventional eluents. The separation was completed within 15 min. The recovery was 96% under the optimum conditions.

Fig. 4 shows the separation of intravenous IgG containing albumin (IgG: albumin = 20:1). The albumin peak appeared clearly under the optimum conditions and the peak was also recognized with the conventional eluents. The recovery was 92% under the optimum conditions.

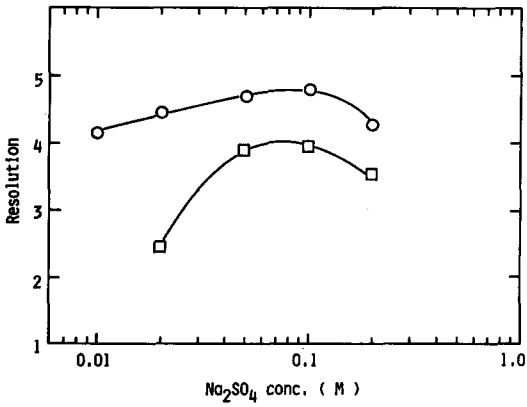

Fig. 2. Dependence of resolution between IgG and albumin on concentration of Na_2SO_4 in buffer on TSKgel G3000SWXL. IgG and albumin were separated with 50 m*M* sodium phosphate at (○) pH 5.0 or (□) pH 6.0 containing various concentrations of Na_2SO_4.

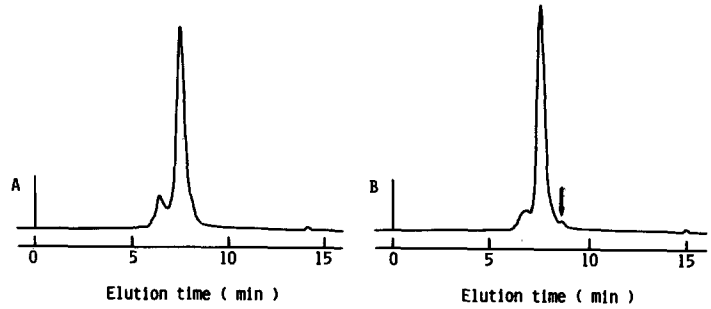

Fig. 3. Separation of intravenous IgG containing albumin by high-performance SEC on TSKgel G3000SWXL: 5 μl (250 μg) of Gammagard were separated with (A) 50 m*M* sodium phosphate (pH 6.7) containing 0.3 *M* NaCl or (B) 50 m*M* sodium phosphate (pH 5.0) containing 0.1 *M* Na_2SO_4 at a flow-rate of 1.0 ml/min at 25°C. UV detection at 280 nm. Total recoveries were (A) 102% and (B) 96%.

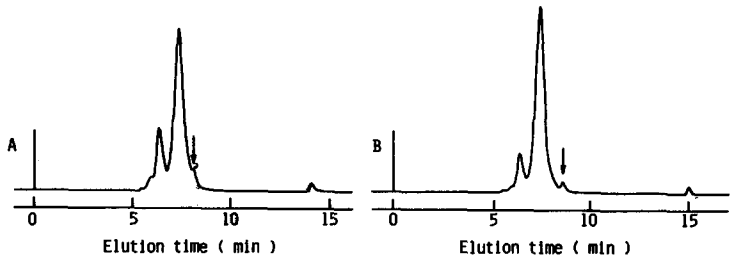

Fig. 4. Separation of intravenous IgG containing albumin by high-performance SEC on TSKgel G3000SWXL: 5 μl (250 μg) of Venilon were separated. Conditions as in Fig. 3. Total recoveries were (A) 94% and (B) 92%.

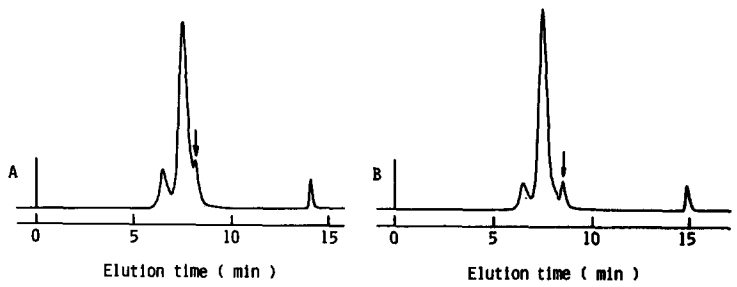

Fig. 5. Separation of intravenous IgG containing albumin by high-performance SEC on TSKgel G3000SWXL: 5 μl (250 μg) of Venoglobulin-I were separated. Conditions as in Fig. 3. Total recoveries were (A) 100% and (B) 101%.

In Fig. 5, intravenous IgG (IgG: albumin = 5:1) occurs as a sharp albumin peak under the optimum conditions. The recovery was quantitative. In addition, IgG monomer and dimer were also separated well in all the chromatograms. The compositions of the dimer and monomer, however, differed slightly with the two elution conditions. An intermolecular change in IgG might occur, depending on the pH of the eluent.

In conclusion, the separation of albumin from IgG in intravenous IgG was achieved by SEC on TSKgel G3000SWXL with 50 m*M* sodium phosphate (pH 5.0) containing 0.1 *M* Na_2SO_4. The separation was completed within 15 min with high resolution and the recovery was more than 92%. Accordingly, TSKgel G3000SWXL would be very useful not only for determining the composition of IgG components and albumin in intravenous IgG, but also for separating IgG such as monoclonal antibodies from albumin in ascites fluid or supernatants.

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