Separation Report No. 062

Separation of IgG and Albumin by High Performance Gel Filtration Chromatography Using TSKgel G3000SW$_{XL}$

(Application of IgG Preparation for Intravenous Injection)

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1. Introduction

Human gamma immunoglobulin G (IgG, polyclonal) is effective as a blood preparation for intravenous injection for treatment of hypogammaglobulinemia/agammaglobulinemia or severe infectious diseases, as well as sudden thrombocytopenic purpura, in combined use with antibiotics. It is applied widely in clinical fields. Such IgG preparations include those in which albumin is added as a stabilizer[1-4]. Rapid quantification of IgG and albumin is important for quality control of these preparations and high performance liquid chromatography (HPLC) is often the method of choice.

Numerous reports describe the separation between IgG and albumin by HPLC using ion exchange chromatography (IEC)[5-11], hydroxyapatite chromatography (HAC)[12-13], hydrophobic interaction chromatography (HIC)[9], and affinity chromatography (AFC)[14–17] as the separation mode. However, separation time is long in any of these separation modes and a gradient elution method is used, which is not suited to quality control in which simple and rapid quantification are required. On the other hand, gel filtration chromatography (GFC) is a simple and fast procedure, although it may not always provide optimum resolution. The separation of IgG by GFC has been reported[5-6, 18-20] and the use of GFC has also been widely adopted in quality control methods. Though there have been reports which dealt with protein elution conditions using GFC[21-27], the conditions for the separation of IgG and albumin by GFC have not been examined in detail. Only very recently Lee et al[21] examined the separation of IgG preparation for intravenous injection containing albumin. However, a two-step chromatography method, consisting of IEC and GFC, was used for this separation and the method required two hours.

In this study the elution conditions for the separation of IgG and albumin using TSKgel G3000SW$_{XL}$[29], a high performance GFC column, was examined. The goal of this study was to obtain high resolution in a short time period using only gel filtration chromatography. This document reports on the favorable results obtained with a TSKgel G3000SW$_{XL}$ column in separating the components of an IgG preparation.

2. Experimental Conditions

A computer controlled pump (CCPE) and a UV/VIS detector (UV-8010) were used with an HPLC system with detection at 280nm. TSKgel G3000SW$_{XL}$ (7.8mm ID × 30cm) was employed as the separation column. For eluent, phosphate buffer, acetate buffer and citrate buffer were used, along with the salts Na_2SO_4, NaCl and $NaClO_4$, to examine the elution conditions with buffers of pH 5.0 to pH 7.0. All measurements were performed at the flow rate of 1.0mL/min and temperature of 25°C. Examination of the elution conditions was conducted with 100μg each of IgG and albumin. Recovery of IgG preparation from the column was calculated from the chromatogram's peak area.

Resolution (Rs) of IgG and albumin was calculated using the following formula:

$$Rs = \frac{2\,(V_2 - V_1)}{W_1 + W_2} \quad \frac{1}{\log[MW_1] - \log[MW_2]}$$

V_1: Elution volume of IgG monomer (mL)
V_2: Elution volume of albumin monomer (mL)

W_1: Peak width of IgG monomer (mL)
W_2: Peak width of albumin monomer (mL)
MW_1: Molecular weight of IgG monomer
MW_2: Molecular weight of albumin monomer

Sample solutions of human IgG and human albumin were prepared from commercial products manufactured by Miles Laboratories and Sigma, respectively.

Commercial IgG preparations of Venoglobulin-I from Midori Juji, Venilon from Fujisawa Pharmaceutical and Gummaguard from Baxter were used. The ratio of IgG and albumin in each IgG preparation was labeled as 5:1, 20:1, and 50:1 respectively.

3. Results and Discussion

Figure 1 shows the effects of pH and salt for a phosphate buffer eluent on the elution volumes of IgG and albumin. The salt concentration of the eluent was adjusted so that the ionic strength was nearly equal. When 0.3mol/L NaCl and 0.3mol/L $NaClO_4$ are used as salts, the difference in elution volume between IgG and albumin were nearly equal, and little effect of eluent pH was seen. Conversely, when 0.1mol/L Na_2SO_4 was used as salt, the difference in elution volume was significant, and it became larger as pH was reduced from 7.0 to 5.0, as albumin elutes later compared to the IgG elution position, which moves only slightly. However, when an eluent of pH 4.0 is used, the albumin peak became broader and the separation of IgG and albumin became insufficient. The conclusion is that eluent of pH 5.0, containing Na_2SO_4 as the salt, is ideal for separation of IgG and albumin. When separation was examined as to buffer type using phosphate buffer, acetate buffer and citrate buffer, the phosphate buffer resulted in overall better resolution between albumin and IgG.

Figure 2 shows the effect of Na_2SO_4 concentration on the resolution of IgG and albumin when phosphate buffer solutions of pH 5.0 and pH 6.0 are used. When the eluent pH was adjusted to pH 6.8, resolution was minimal and Rs could not be calculated.

It is clear that the separation is better when an eluent of pH 5.0 is used. Resolution is optimal at pH 5.0 at 1.0M Na_2SO_4 concentration. Resolution decreased at salt concentrations above 0.1mol/L; calculating Rs was no longer possible at a concentration of 0.2mol/L and higher. At concentrations of 0.5mol/L or higher, hydrophobic interactions between the packing material and the sample became prominent, which resulted in peak broadening and longer elution times. Moreover, resolution deteriorated at salt concentrations below 0.1mol/L due to increasing importance of ionic interactions.

Based on the above results, it is observed that an eluent

containing 50mmol/L phosphate buffer and 0.1mol/L Na_2SO_4, adjusted to pH 5.0, yields optimal resolution for the separation of IgG and albumin.

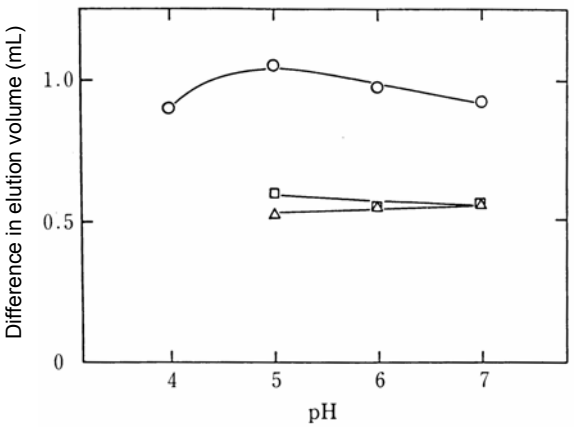

Figure 1 Effect of eluent salt and pH on separation of IgG and albumin

IgG and albumin were eluted in 50mmol/L phosphate buffer with various pH values containing 0.1mol/L Na_2SO_4 (O), 0.3mol/L NaCl (□) or 0.3mol/L $NaClO_4$ (△) as the salt.

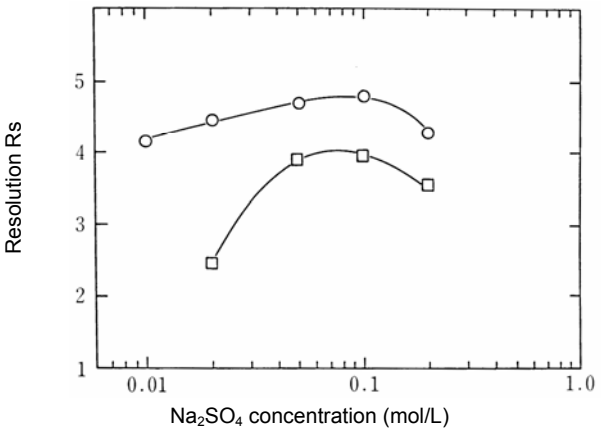

Figure 2 Effect of Na_2SO_4 concentration in the eluent on separation of IgG and albumin

IgG and albumin were eluted in 50mmol/L phosphate buffer containing various concentrations of Na_2SO_4 at pH 5.0 (O) or pH 6.0 (□).

Figures 3, 4 and 5 show the separation of an IgG preparation under the elution conditions determined in this study to be optimal in comparison with the general elution conditions for SW_{XL}-type columns, which consist of 50mmol/L phosphate buffer eluent (pH 6.8) containing 0.3mol/L NaCl.

Figure 3 shows the separation of a sample with an IgG to albumin ratio of 50:1. While the albumin peak is seen as only a shoulder on the IgG peak when using conventional general elution conditions, albumin is seen as a distinct peak when using the optimized conditions determined in this study. In Figures 4 and 5, albumin peaks were visible when the samples were analyzed using the optimized as well as the general elution conditions.

It was found that when the separation is run under the optimal elution conditions as determined in this study, the albumin peaks are more clearly distinguishable. It is believed that the reason why the chromatogram's peak area ratio is larger than the IgG to albumin volume ratio is that the 280nm absorption constant of IgG is about three times higher than that of albumin. It is also evident that the IgG dimer and monomer are favorably separated. Worthy of note is the fact that total analysis time was less than 15 minutes and recovery was 92% or higher for all components.

As to the column lifetime, the separations shown in this report remained intact even after 200 sample injections.

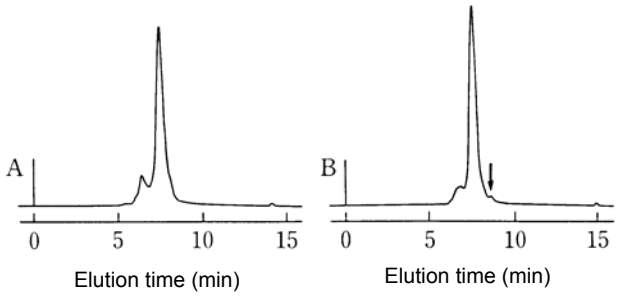

Figure 3 Separation of IgG preparation for intravenous injection containing albumin

Column:	TSKgel G3000SW_{XL} (7.8mm ID × 30cm)
Sample:	Gammaguard (50mg/mL, 5μL)
Eluent:	A: 50mmol/L phosphate buffer (pH 6.8) + 0.3mol/L NaCl B: 50mmol/L phosphate buffer (pH 5.0) + 0.1mol/L Na_2SO_4
Flow rate:	1.0mL/min
Temperature:	25°C
Detection:	UV@ 280nm
Recovery:	A: 102%, B: 96%

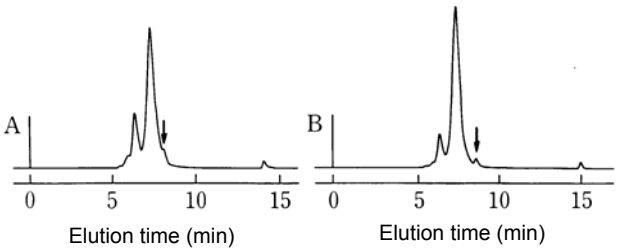

Figure 4 Separation of IgG preparation for intravenous injection containing albumin

Column:	TSKgel G3000SW_{XL} (7.8mm ID × 30cm)
Sample:	Venilon (50g/L, 5μL)
Conditions:	As in Figure 3.
Recovery:	A: 94%, B: 92%

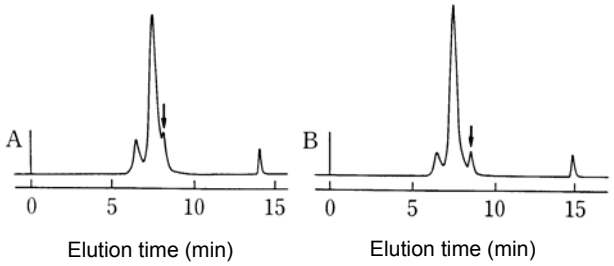

Figure 5 Separation of IgG preparation for intravenous injection containing albumin

Column: TSKgel G3000SW$_{XL}$ (7.8mm ID × 30cm)
Sample: Venoglobulin-I (50g/L, 5μL)
Conditions: As in Figure 3.
Recovery: A: 100% B: 101%

4. Conclusion

Using only a high performance gel filtration column, TSKgel G3000SW$_{XL}$, IgG and albumin in commercial immunoglobulin preparations were rapidly and quantitatively separated with acceptable resolution. Optimal elution conditions for this type of separation were determined to be a 50mmol/L sodium phosphate buffer (pH 5.0) containing 0.1mol/L Na_2SO_4. Monoclonal antibodies and albumin in mouse ascites fluid can also be separated using this same column and these elution conditions.

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