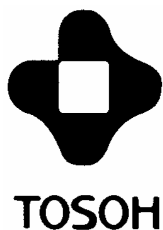

No. 062

SEPARATION REPORT

Separation of IgG and Albumin by High Performance Gel Filtration Chromatography Using TSKgel G3000SW$_{XL}$ (Application to IgG Preparation for Intravenous Injection)

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TOSOH CORPORATION

1. Introduction

Human gamma immunoglobulin G (IgG, polyclonal) is effective as a blood preparation for intravenous injection in combined use with antibiotics in hypogammaglobulinemia/agammaglobulinemia or severe infectious diseases as well as sudden thrombocytopenic purpura, and 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 applied.

Numerous reports have been made as to IgG and albumin separation by HPLC, and ion exchange chromatography (IEC)[(5) − (11)], hydroxyapatite chromatography (HAC)[(12)(13)], hydrophobic interaction chromatography (HIC)[(9)], and affinity chromatography (AFC)[(14) − (17)] were used as separation mode. However, separation time is long in any of these separation modes, and they use the gradient elution method which is not suited to quality control in which simple and rapid quantification is required. On the other hand, gel filtration chromatography (GFC) involves easy operation procedure although resolution may not be well, and separation of IgG has been reported [(5)(6)(18) − (20)]. It is also adopted widely in quality control. Though there have been reports which dealt with protein elution conditions using GFC[(21) − (27)], examination of IgG and albumin separation conditions has not been made in details. Only very recently Lee, et al.[(18)] 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 separation and it requires two hours.

In this study, we examined the elution conditions in separation of IgG and albumin using TSKgel G3000SW_{XL}[(29)], a high performance GFC column, with a goal of using only gel filtration method to obtain high resolution in short time. This document reports on the favorable results obtained in IgG preparation separation.

2. Experiment Conditions

Computer controlled excellent pump CCPE, UV/VIS detector UV-8010 were used for the HPLC system, and detection was made at 280nm. TSKgel G3000SW_{XL} (300 × 7.8mmI.D.) was employed. For eluent, phosphate buffer, acetate buffer, and citrate buffer were used and Na_2SO_4, NaCl, and $NaClO_4$ were used as the salts to examine the elution conditions with buffers of pH5.0 to pH7.0. All measurements were performed at the flow rate of 1.0mL/min and temperature of 25°C.

Examination of 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

The prepared human IgG and prepared human albumin used were commercial products manufactured by Miles Laboratories and Sigma, respectively.

Venoglobulin-I from Midori Juji, Venilon from Fujisawa Pharmaceutical, and Gummaguard from Baxter, which are commercial IgG preparations, 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 in the phosphate buffer eluent on the difference of elution volumes of IgG and albumin from the column.
The salt concentration of eluent is adjusted so that the ionic strength is 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. On the other hand, when 0.1mol/L Na_2SO_4 is used as the salt, the difference in elution volume was large, and it became larger as the pH became lower. This is ascribed to the fact that albumin elution position is delayed compared to the IgG elution position which moves only little. However, when an eluent of pH4.0 is used, albumin peak was expanded and separation of IgG and albumin became insufficient. Thus it is surmised that eluent of pH5.0 which contains Na_2SO_4 as the salt is suited for separation of IgG and albumin. When separation was examined as to buffer type using phosphate buffer, acetate buffer, and citrate buffer, it resulted in slightly better performance by phosphate buffer.

Figure-2 shows the Na_2SO_4 concentration dependency of IgG and albumin resolution when phosphate buffer solutions (pH5.0 and 6.0) are used.
Though examination was also conducted with pH6.8 eluent, resolution was small and Rs could not be calculated. It is clear that separation is higher when eluent of pH5.0 is used. Salt concentration dependency of resolution is larger for pH6.0, and resolution was highest when Na_2SO_4 concentration was 0.1mol/L for either pH. Resolution decreased with salt concentration of 0.1mol/L or higher, calculation of Rs was no longer possible with concentration of 0.2mol/L and higher. With concentration of 0.5mol/L or higher, hydrophobic interactions between the packing material and the sample became prominent, the peak was broadened and elution was delayed. Moreover, resolution was deteriorated with salt concentration of 0.1mol/L or lower due to the effect of ionic interactions.
Based on the above results, it was discovered that 50mmol/L phosphate buffer solution eluent (pH5.0) including 0.1mol/L Na_2SO_4 yields the best resolution when IgG and albumin are separated.

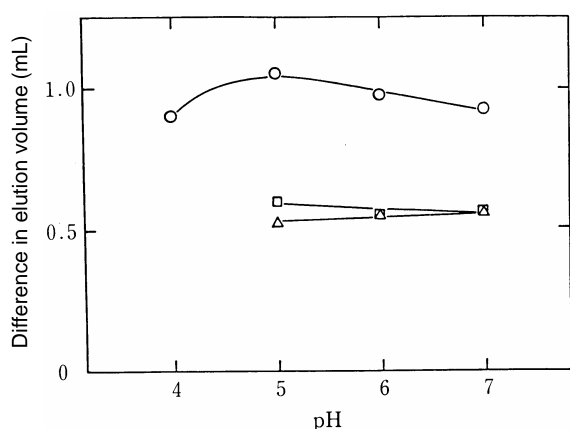

Figure-1 Effect of eluent salt and pH on separation of IgG and albumin

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

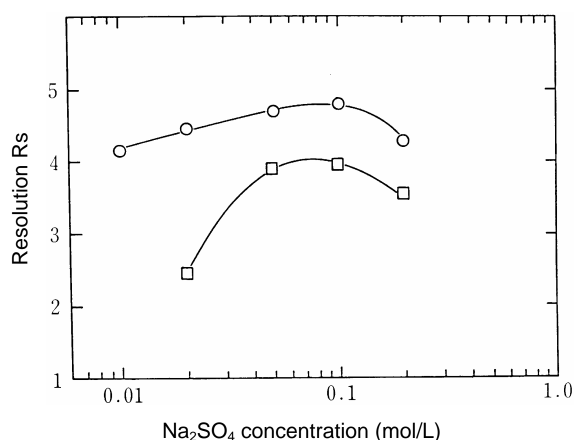

Figure-2 Effect of Na_2SO_4 concentration in eluent on separation of IgG and albumin

IgG and albumin were eluted in 50mmol/L phosphate buffers containing various concentrations of Na_2SO_4 at pH5.0 (○) or pH6.0 (□).

Figures-3, -4 and -5 show separation of IgG preparation under optimal elution conditions obtained in this study in comparison with the general elution conditions of GFC, in which separation was conducted by 50mmol/L phosphate buffer eluent (pH6.8) containing 0.3mol/L NaCl. Figure-3 shows separation of a sample with IgG-albumin ratio of 50:1. While albumin peak is seen as only a shoulder hidden by the IgG peak under the conventional general elution conditions, albumin is seen as a peak under the conditions obtained in this study. In Figures-4 and -5, albumin peaks are recognized under the conventional general elution conditions.

However, the optimal elution conditions obtained in this study shows the peaks more clearly. It is considered that the reason why the chromatogram's peak area ratio is larger than the IgG-albumin volume ratio is that the 280nm absorption constant of IgG is about 3 times higher than that of albumin. It is also evident that IgG dimmer and monomer are favorably separated. Analysis time was within 15 minutes, and recovery was 92% or higher for all components.
As to column durability, there was no effect on separation even after 200 times of sample injection.

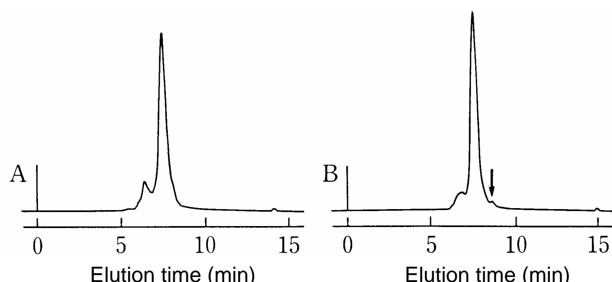

Figure-3 Separation of IgG preparation for intravenous injection containing albumin

Column: TSKgel G3000SW$_{XL}$ (7.8mmID × 30cm)
Sample: Gammaguard (50g/L, 5μL)
Eluent: A: 50mmol/L phosphate buffer (pH6.8) + 0.3mol/L NaCl
B: 50mmol/L phosphate buffer (pH5.0) + 0.1mol/L Na2SO4
Flow rate: 1.0mL/min
Temperature: 25°C
Detection: UV (280nm)
Recovery: A; 102%, B; 96%

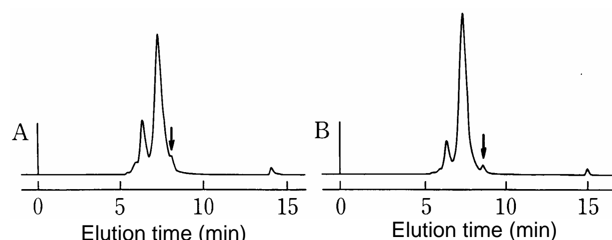

Figure-4 Separation of IgG preparation for intravenous injection containing albumin

Column: TSKgel G3000SW$_{XL}$ (7.8mmID × 30cm)
Sample: Venilon (50g/L, 5μL)
Elution conditions: Same as Figure-3.
Recovery: A; 94%, B; 92%

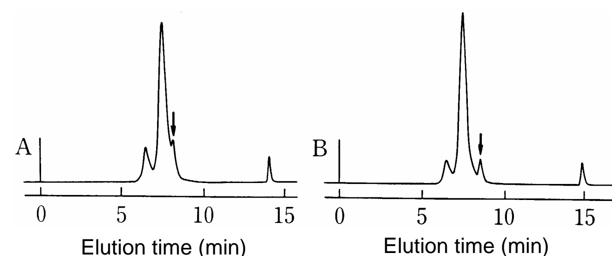

Figure-5 Separation of IgG preparation for intravenous injection containing albumin

Column: TSKgel G3000SW$_{XL}$ (7.8mmID × 30cm)
Sample: Venoglobulin-I (50g/L, 5μL)
Elution conditions: Same as Figure-3.
Recovery: A; 100% B; 101%

4. Conclusion

We were able to separate IgG and albumin in immunoglobulin preparation rapidly and quantitatively with high resolution using 50mmol/L sodium phosphate (pH5.0) containing 0.1mol/L Na_2SO_4 in TSKgel G3000SW_{XL}. TSKgel G3000SW_{XL} is considered feasible for application in not only separation of blood products in terms of quality control but also separation of monoclonal antibody and albumin in mouse ascites using the elution conditions obtained in this study.

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