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DETERMINATION OF THE MOLECULAR SIZE DISTRIBUTION OF IMMUNOGLOBULIN G (IgG) IN INTRAVENOUS IgG–ALBUMIN FORMULATIONS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Recent reports have expressed concern about the safety of intravenous human immunoglobulin G (IgG) preparations. Evidence seems to indicate that aggregates in these formulations are responsible for several adverse reactions in some patients, including anaphylaxis and dyspnea. Therefore, monitoring the molecular size distribution of IgG in these products is essential for ensuring their safety.

This paper describes a sensitive and precise two-stage high-performance liquid chromatographic method for determining the molecular size distribution profile of IgG in an intravenous formulation stabilized with albumin. In the first step, all molecular forms of IgG are separated from all molecular forms of albumin by anion-exchange chromatography. In the second stage, a portion of the collected IgG fraction is re-chromatographed by size-exclusion chromatography and separated into its aggregate, dimer, and monomer components. Although some minor losses of aggregate do occur in the procedure, the overall molecular size distribution is not significantly affected. The method described is both time-efficient and accurate, and represents an improvement over existing methods.

INTRODUCTION

Monitoring the molecular size distribution of human immunoglobulin G (IgG) in intravenous formulations is very important in maintaining the safety of these products. Intravenous administration of human IgG has been known to induce adverse side reactions in some individuals. Although no specific cause has been identified, it is generally thought that IgG aggregates account for these reactions, which may include anaphylaxis and dyspnea[1]. Barandun *et al.*[2] have indicated that IgG aggregates may cause the release of anaphylatoxins into the bloodstream via complement

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activation, while other workers have also mentioned the possibility of harmful side effects due to aggregates[3]. Although no specific limit or safe level of IgG aggregates is known, above which any of these possible side effects would be induced, it would appear that the manufacturers of these products are obligated to monitor aggregate levels as well as the overall IgG molecular size distribution.

In past studies, several investigators have utilized either anion-exchange chromatography (AEC) or size-exclusion chromatography (SEC) for the separation of proteins. Kadoya *et al.*[4] were able to separate ten proteins, including human IgG, on a DEAE polymer-based column by either isocratic elution or by elution with a linear salt gradient. They reported excellent resolution with high recoveries. Other advantages were the high column capacity, speed, and recovery of biological activity. Other workers[3,5–8] have demonstrated the utility of SEC in separating the various molecular forms of IgG. Two groups of workers[3,7] used SEC to fractionate IgG in several commercial intravenous preparations. Law and Painter[7] showed the presence of monomer, dimer, and aggregates in three preparations and were able to quantitate the levels of each. However, none of these formulations contained albumin as an IgG stabilizer. Suomela *et al.*[3] showed separation profiles for six IgG preparations containing albumin. Their chromatograms showed that albumin interfered with the IgG monomer peak, and this would make quantitation of that component difficult. Similar attempts in our laboratories, in which only a one-step analytical-scale SEC of an IgG formulation containing albumin was used, failed due to overlapping IgG and albumin peaks, particularly IgG and albumin aggregates.

Recently, Andrade and Mankarious[9] developed a combined procedure for determining the complete IgG molecular size profile in a human IgG solution containing albumin, but their method is difficult and time-consuming. After a lengthy preparative-scale SEC to separate the molecular forms of IgG from albumin while collecting only the relatively pure portion of each peak, they concentrated each fraction. They were able to show by repeated analytical-scale SEC and AEC that several dimeric forms of IgG, as well as the major portion of monomeric IgG, were separated from each other and from albumin by the initial preparative SEC system. However, the IgG aggregate peak contained multimeric albumin, and the albumin monomer peak contained some IgG monomer. These were easily separated by AEC, thus providing an essentially complete IgG molecular profile. In addition, they demonstrated that the molecular forms of IgG could be separated as a single peak from the molecular forms of albumin (also as a single peak) in the AEC system.

The method described here is a more time-efficient approach in which the initial chromatographic step is AEC, followed by SEC of the collected IgG fractions. A complete molecular size profile can be determined in two steps and in much less time than by the method of Andrade and Mankarious[9]. Despite some minor losses of IgG aggregate in the procedure, the overall molecular size distribution of IgG was not significantly affected. These factors will be discussed in this paper.

EXPERIMENTAL

Materials

Samples of human albumin (Albuminar®-25, U.S.P., 25%) and human immune globulin G (Gammar®, U.S.P., containing 165 ± 15 mg IgG/ml) were obtained

from Armour Pharmaceutical Company (Kankakee, IL, U.S.A.). Molecular weight calibration standards, carbonic anhydrase, bovine albumin, alcohol dehydrogenase, β-amylase, thyroglobulin, and Blue Dextran, ranging in molecular weight from 29 000 to 2 000 000 were purchased from Sigma (St. Louis, MO, U.S.A.). Samples of intravenous IgG (IVGG) consisted of 5% IgG, stabilized with 2.5% albumin (lyophilized sample, Armour). Sodium chloride (certified ACS grade) and sodium azide (purified grade) were purchased from Fisher (Fair Lawn, NJ, U.S.A.), while dibasic potassium phosphate and tris(hydroxymethyl)aminomethane (Tris) (electrophoresis purity reagent) were purchased from Mallinckrodt (St. Louis, MO, U.S.A.) and Bio-Rad Labs. (Richmond, CA, U.S.A.), respectively.

Anion-exchange chromatography

The AEC system consisted of a Waters Protein Pak DEAE 5 PW column (7.5 cm × 7.5 mm), a DuPont Instruments gradient pump (set at 1.0 ml/min) and Series 8800 gradient controller, a Kratos Spectroflow 783 programmable absorbance detector (set at 280 nm and 1.0 a.u.f.s.), a Waters WISP Model 710B autoinjector (set at 200-μl injections), and a Houston Instruments Omniscribe recorder (set at 0.1 in/min). A Hewlett-Packard 3357 LAS computer was used for data acquisition.

Separation of IgG and albumin was accomplished at ambient temperature with a run time of 50 min. The mobile phases consisted of 20 m*M* Tris (pH 8.5) (solvent A) and 20 m*M* Tris (pH 7.0) containing 0.3 *M* sodium chloride (solvent B). Gradient conditions were 100% A to 100% B (0–30 min, linear), 100% B to 100% A (30–35 min, linear), and 100% A (35–50 min) for column re-equilibration. Typical collection times for the IgG and albumin (including sodium acetyltryptophanate, albumin stabilizer) fractions were 9.5–20 min and 20–31.5 min, respectively. The entire elution process was monitored on the chart recorder as individual fractions were collected over ice. Each IgG fraction was thoroughly mixed and 200 μl was immediately injected into the SEC system. The albumin fraction was similarly analyzed after the IgG fraction.

Size-exclusion chromatography

The SEC system consisted of two Beckman Spherogel™ TSK 3000 SW columns connected in series (60 cm × 7.5 mm combined length), a Waters Model 6000 A pump (set at 00.5 ml/min), a Waters Model 440 absorbance detector (set at 280 nm and 0.5 a.u.f.s.), a Waters WISP Model 710B autoinjector (set at 200-μl injections), and a Houston Instruments Omniscribe recorder (set at 0.1 in/min). A Hewlett-Packard 3357 LAS computer was used for data acquisition. Separation of the IgG monomer, dimer, and aggregate components was accomplished at ambient temperature by isocratic elution with a mobile phase consisting of 0.2 *M* dibasic potassium phosphate (pH 7.0) containing 0.02% sodium azide. A typical run time was 40 min. The separation of the corresponding albumin components was accomplished with the same system but with a run time of 60 min to allow for complete elution of the sodium acetyltryptophanate.

The IgG aggregate, dimer, and monomer peaks were typically eluted at retention times of 18.2, 21.6, and 25.8 min, respectively. Corresponding peaks for albumin were eluted at 18.4, 25.5, and 30.1 min, respectively.

Preliminary evaluation of IgG and albumin molecular size profiles

An initial molecular size profile of both Albuminar (untreated) and Gammar (IgG without albumin, stressed at 37°C for 16 h to produce aggregates[6,8,10]) was obtained with the SEC system. Following this evaluation, a 2:1 (w/w) mixture of the two proteins was prepared (stressed Gammar/Albuminar) and separated by AEC. After collection, 200 μl of each fraction was re-injected into the SEC system. The molecular size profile obtained for each was compared to the original profiles.

IVGG sample preparation

The lyophilized IgG–albumin sample (IVGG, Armour) was placed in an ice bath and reconstituted with 25 ml of deionized water by constant, gentle stirring with a magnetic stirrer. The final concentration of IgG was *ca.* 100 mg/ml. A 200-μl sample, containing about 20 mg IgG and 10 mg albumin, was then injected into the AEC system, followed by fraction collection over ice, as previously described. Studies indicated that *ca.* 15–20 mg of IgG was a convenient amount for detection of aggregates. The AEC column capacity was 100 mg totaal protein, as specified by the manufacturer.

Since approximately 10–11 ml of eluate was collected from the AEC system for each fraction, the final IgG and albumin concentrations injected into the SEC system were 2.0 mg/ml and 1.0 mg/ml, respectively.

Calibration of the size-exclusion system: determination of molecular weights

The molecular weights of the IgG and albumin components (molecular forms) were determined from a calibration plot obtained with known molecular weight standards (Sigma) according to the following procedure: Approximately 50 mg of each molecular weight standard was dissolved in 500 μl of the dibasic potassium phosphate buffer and injected into the SEC system. The ratio of the retention time of each standard, R_p, to that of the Blue Dextran standard (MW 2 000 000), R_{bd}, was calculated. A calibration curve was obtained by plotting this ratio *vs.* molecular weight ($\times 10^3$). The result was a linear plot, from which the molecular weights of the different forms of IgG and albumin were determined[11].

RESULTS AND DISCUSSION

Size-exclusion chromatography

Fractionation of the molecular forms of IgG and albumin can be obtained quite readily by using the described SEC system. Figs. 1 and 2 show the SEC chromatograms of stressed Gammar and Albuminar, respectively. Monomer, dimer, and aggregates were eluted as single peaks for each protein, and the molecular size distribution was easily determined.

However, a single size-exclusion chromatogram of a sample of IgG containing albumin would be subject to significant interference by components of similar molecular weight, as shown in the overlay SEC plots of Fig. 3. The positions of the arrows in Fig. 3 show where IgG aggregates would be eluted with albumin aggregates and where IgG monomer would be eluted with albumin dimer. This elution of different molecular forms at the same time is due to the similarities in their molecular weights, summarized in Table I. The molecular weights were determined from the

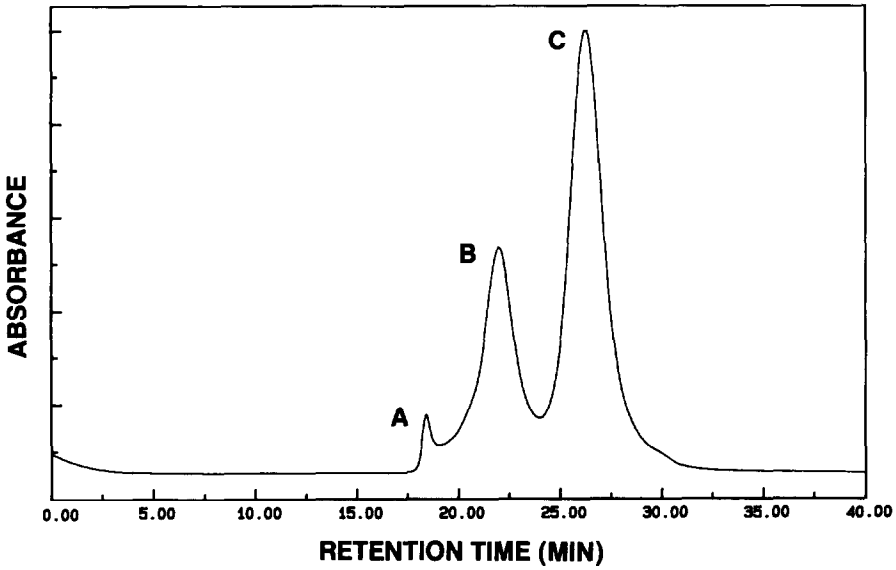

Fig. 1. SEC chromatogram of stressed IgG (37°C for 16 h) on two TSK 3000 SW columns in series. (A) Aggregates; (B) dimer; (C) monomer. Chromatographic conditions as explained in text.

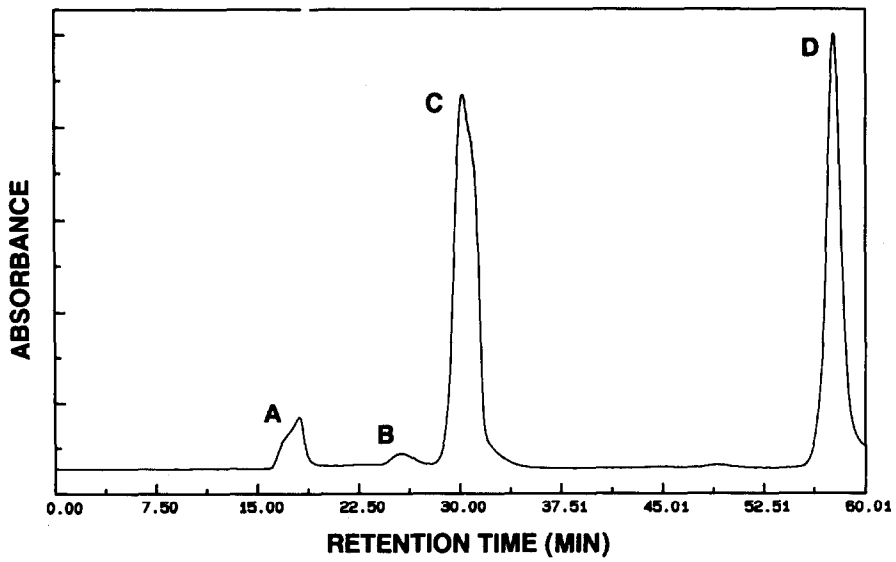

Fig. 2. SEC chromatogram of Albuminar on two TSK 3000 SW columns in series. (A) Aggregates; (B) dimer; (C) monomer; (D) sodium acetyltryptophanate (albumin stabilizer). Chromatographic conditions as explained in text.

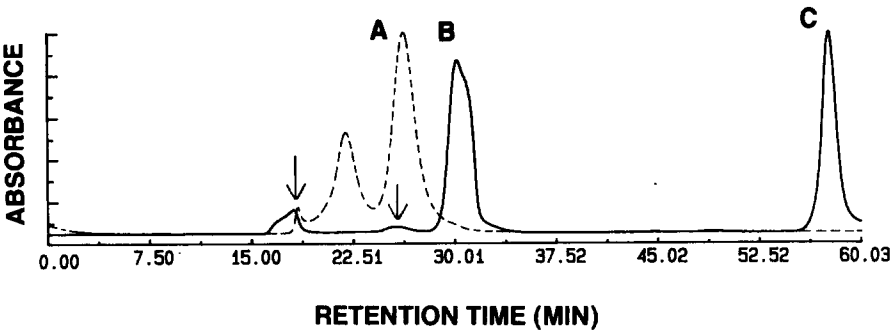

Fig. 3. Overlay plots of Figs. 1 and 2. (A) IgG; (B) albumin; (C) sodium acetyltryptophanate (albumin stabilizer). Time scale for A extended for illustrative purposes. Arrows indicate where the IgG and albumin molecular forms would co-elute.

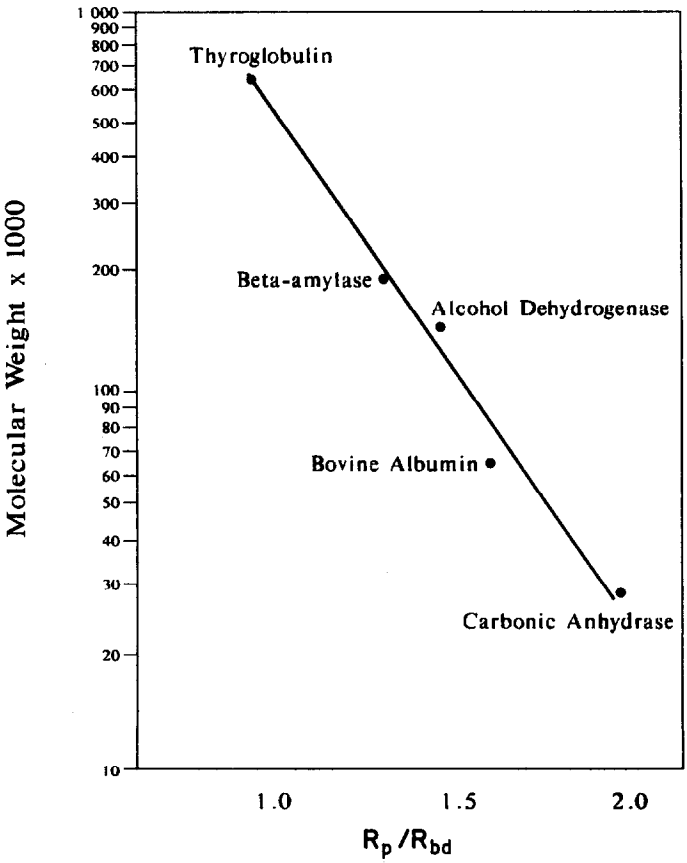

Fig. 4. Semi-log calibration curve for size-exclusion system. R_p/R_{bd}, ratio of retention time of protein standard to that of Blue Dextran.

TABLE I

MOLECULAR WEIGHTS DETERMINED BY SIZE-EXCLUSION CHROMATOGRAPHY

SEC of each molecular weight standard was performed, as explained in the text. Fig. 4 shows the calibration plot obtained.

Component	*Molecular weight found**	*Literature value***
IgG aggregates	> 500 000	> 400 000
IgG dimer	370 000	> 300 000
IgG monomer	175 000	160 000
Albumin aggregates	> 500 000	> 400 000
Albumin dimer	195 000	220 000
Albumin monomer	87 000	72 000

* Determined from Fig. 4.
** From Andrade and Mankarious[9].

calibration plot of protein standards shown in Fig. 4. Separation of similar molecular weight components by SEC is difficult, making it extremely difficult or impossible to obtain molecular size profiles. Therefore, in order to determine the molecular size distribution of IgG accurately, IgG first had to be separated from albumin.

Anion-exchange chromatography

It has been shown that the molecular forms of IgG and albumin are eluted as broad but separated peaks when AEC is used[9]. Fig. 5 shows that baseline separation of IgG and albumin was obtained with the AEC system described in the text. During separation, both the IgG and albumin fractions were collected over ice for SEC analysis. Typical collection times for the IgG component ranged from 9.5 to 20 min,

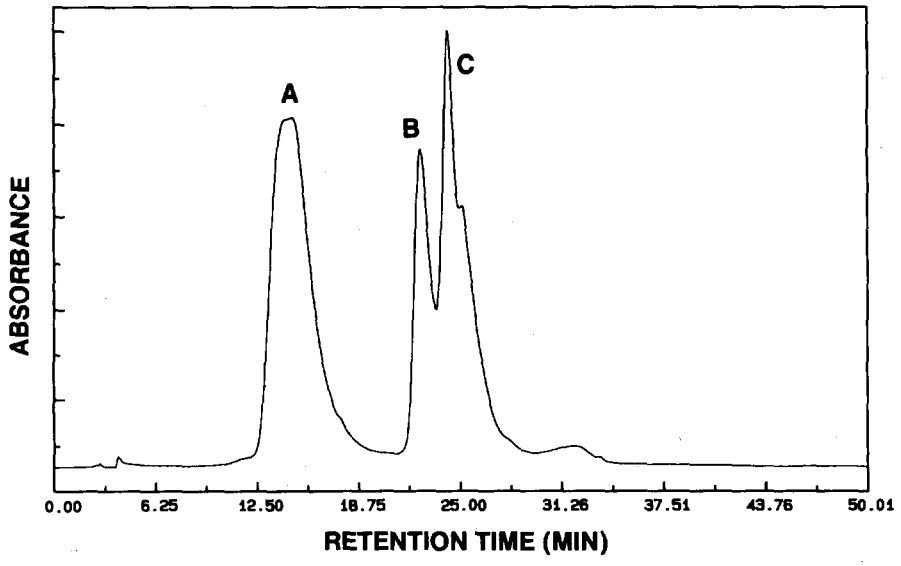

Fig. 5. AEC of a mixed sample of Gammar and Albuminar. (A) IgG; (B) sodium acetyltryptophanate (albumin stabilizer); (C) albumin. Chromatographic conditions as explained in text.

while collection times for albumin ranged from 20 to 31.5 min. Sodium acetyltryptophanate was collected with the albumin fraction.

Molecular size distribution of IgG before and after anion-exchange chromatography

To ascertain that all the molecular forms of IgG were collected with the AEC system, and to ensure that no changes in the molecular size profile occurred during AEC, several SEC determinations were performed on a stressed Gammar sample to establish an initial molecular size distribution profile. This sample was then mixed with Albuminar to simulate actual commercial preparations and chromatographed in the AEC system.

Subsequent SEC analyses indicated that a slight loss of IgG aggregates had occurred. However, this did not significantly affect the overall molecular size distribution, as shown in Fig. 6. Table II summarizes the results of several determinations. Good correlation of the molecular size distribution profile was obtained for IgG before and after AEC separation.

At this time, it is not known whether (a) a selective loss of aggregates occurred on the anion-exchange column, or (b) the dilution of IgG in the fraction collected and its separation from the albumin stabilizer led to a conversion of aggregates to monomer and/or dimer. Some reports give evidence that such a conversion does occur. Whitaker[12], in his work on gel filtration on Sephadex, cited Gutfreund[13], who indicated that hemoglobin can dissociate into subunits in very dilute solutions. In a more recent article, Zini *et al.*[14], who used high-performance liquid chromatography in frontal analysis, showed that a reversible equilibrium between monomer and polymer species in human serum albumin is dependent on dilution. An increase in mon-

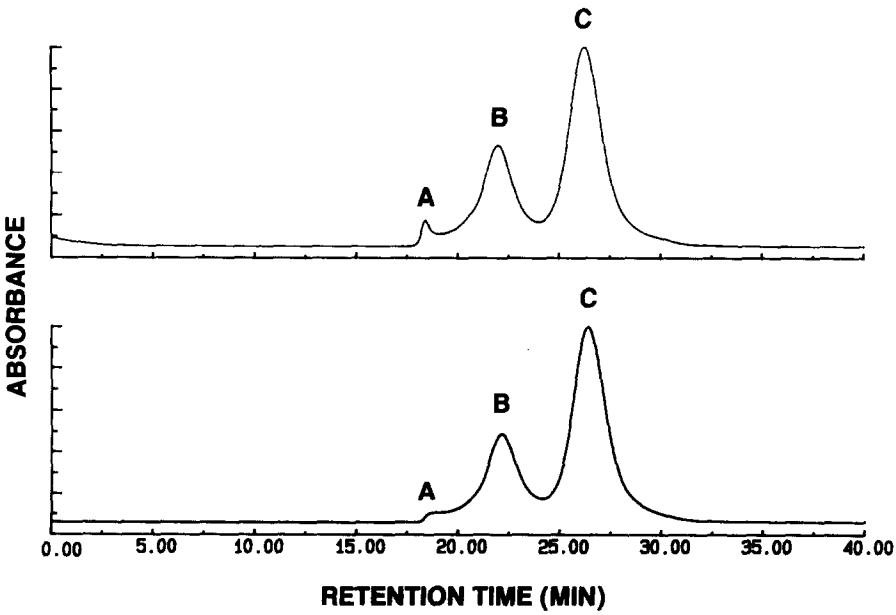

Fig. 6. Dual plot of size-exclusion chromatograms of stressed Gammar before (top) and after (bottom) AEC. (A) Aggregates; (B) dimer; (C) monomer.

TABLE II

MOLECULAR SIZE DISTRIBUTION OF IgG BEFORE AND AFTER ANION-EXCHANGE CHROMATOGRAPHY

Four samples from a prepared stressed Gammar–Albuminar solution in a weight ratio of 2:1 were each subjected to AEC. The molecular size distribution of the collected IgG fraction was determined by SEC and compared to the initial molecular size distribution before AEC.

Sample No.	*Percent**			*Ratio (monomer/dimer)*
	Monomer	*Dimer*	*Aggregates*	
1	76.4	22.7	0.92	3.37
2	76.7	22.3	1.05	3.44
3	75.4	23.6	1.00	3.20
4	75.6	23.4	1.04	3.23
Mean	76.0 ± 0.62	23.0 ± 0.60	1.00 ± 0.06	3.30 ± 0.11
Before AEC**	75.5 ± 0.83	22.6 ± 0.80	1.95 ± 0.04	3.36 ± 0.15

* Derived from peak areas.

** Average of 3 determinations on stressed Gammar alone.

omer content would occur at increasing dilution and *vice versa*. In their study, the technique of frontal analysis was based on a saturation method in which the concentration of a given protein remained constant during elution. Based on their findings and the fact that IgG undergoes dilution in both chromatographic systems, it is not surprising that some small decrease in IgG aggregate levels was observed.

TABLE III

MOLECULAR SIZE DISTRIBUTION OF ALBUMIN BEFORE AND AFTER ANION-EXCHANGE CHROMATOGRAPHY

Six samples from a prepared stressed Gammar–Albuminar solution in a weight ratio of 2:1 were each subjected to AEC. The molecular size distribution of the collected albumin fraction was determined by SEC and compared to the initial molecular size distribution before AEC.

Sample No.	*Percent**			*Ratio** (monomer/dimer)*
	Monomer	*Dimer*	*Aggregates*	
1	70.0	21.3	8.7	3.3
2	72.3	17.7	10.0	4.1
3	71.5	20.2	8.3	3.5
4	71.7	20.3	8.0	3.5
5	70.5	18.5	11.0	3.8
6	67.6	21.5	10.9	3.1
Mean	70.6 ± 1.7	19.9 ± 1.5	9.5 ± 1.3	3.5 ± 0.36
Before AEC***	90.6	2.3	7.1	39.4

* Derived from peak areas.

** See Fig. 7. The area of the component thought to be due to fragments was not included in these calculations.

*** Based on one determination.

Molecular size distribution of albumin before and after anion-exchange chromatography

In contrast to the IgG results, the overall molecular size distribution of albumin was significantly altered during analysis. These results are summarized in Table III. Fig. 7 shows the size-exclusion chromatograms of a sample of albumin before and after AEC. On a comparative basis, following AEC, there was a slight increase in the aggregate level, a significant decrease in the monomer level, and a significant increase in the dimer level. A fourth peak, appearing at 45 min, is thought to be due to albumin fragments. Dilution effects during analysis, as shown by Zini *et al.*[14], may be contributing to the significant change in the distribution profile. However, the profile changes somewhat differ from their observations. Another possible cause, in conjunction with the dilution effects, may be the separation of albumin from its stabilizer, sodium acetyltryptophanate, during SEC analysis. It is interesting to note that the separation of IgG from its stabilizer, albumin, during AEC did not produce the significant changes seen for albumin. It is also interesting that albumin seems to produce a significant level of fragments following AEC. This would indicate that the conditions for the AEC analysis, although adequate for IgG, may be too severe for albumin, causing protein fragmentation.

Limit of detection for aggregates

The limit of detection for IgG aggregates in the SEC system was considered to be that concentration corresponding to a peak area approximately twice the baseline noise at the aggregate retention time. For the system described herein, a detection

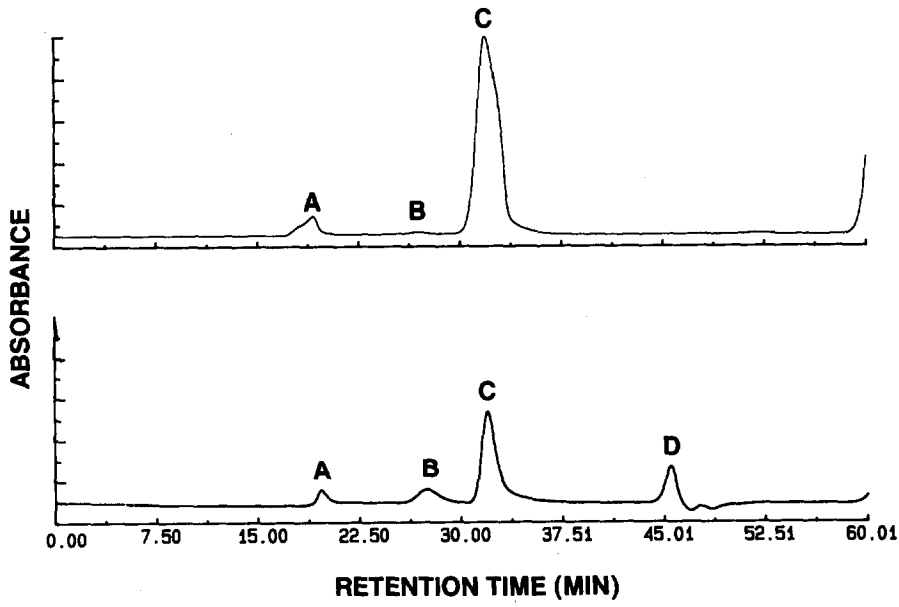

Fig. 7. Dual plot of size-exclusion chromatograms of Albuminar before (top) and after (bottom) AEC. (A) Aggregates; (B) dimer; (C) monomer; (D) possibly fragments generated during the procedure. The component beginning to elute on the right of each chromatogram is sodium acetyltryptophanate.

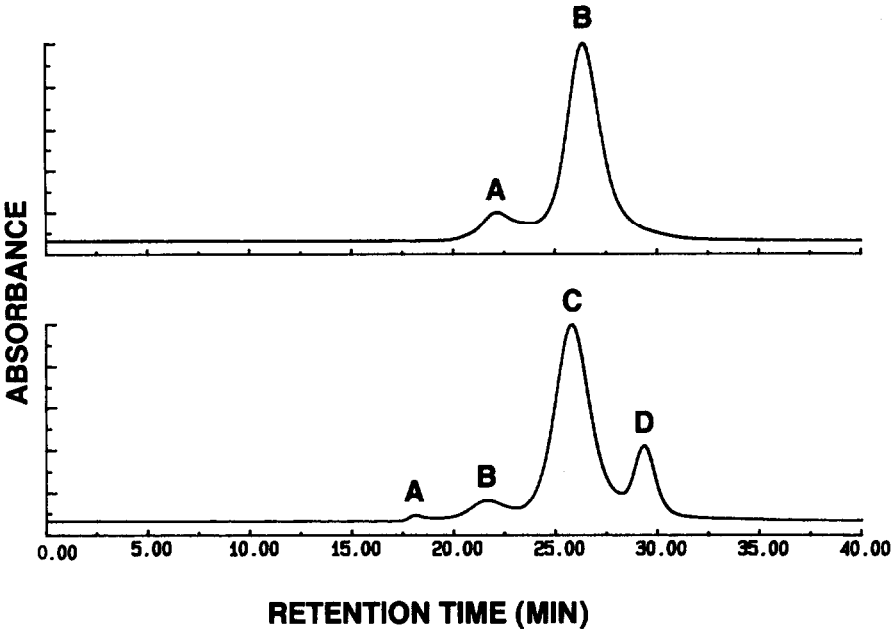

Fig. 8. Dual plot of a commercially available sample of intravenous IgG (albumin stabilized) analyzed by the AEC–SEC procedure (top) and by SEC only (bottom). Top: (A) IgG dimer; (B) IgG monomer. Bottom: (A) aggregates; (B) IgG dimer; (C) IgG monomer and albumin dimer; (D) albumin monomer.

limit of approximately 0.4 μg of aggregate was found. This represents the aggregate content in a 200-μl sample of a diluted IgG fraction, collected with the AEC system. The total IgG concentration in the diluted fraction was 0.15 mg/ml.

Analysis of a commercial IVGG sample: combined AEC–SEC method vs. SEC

Fig. 8 is a dual plot of a size-exclusion chromatogram (top) of a commercially available sample of intravenous IgG (albumin stabilized) analyzed by the AEC–SEC procedure, compared with a similar sample analyzed by SEC only (bottom). The size-exclusion chromatogram shows significant overlap of IgG and albumin components, making the determination of the molecular size distribution of IgG impossible. However, utilizing the AEC–SEC procedure, the molecular size profile can easily be obtained.

Of particular interest is the absence of aggregates in the IVGG sample (top chromatogram) as compared to the stressed Gammar sample shown in Fig. 1. However, no aggregates were found in unstressed Gammar during initial analysis.

CONCLUSION

A sensitive, precise, and accurate two-stage high-performance liquid chromatographic method has been developed for the determination of the molecular size distribution of IgG in intravenous formulations stabilized with albumin. The method consists of initially separating all IgG forms from all albumin forms in an anion-

exchange system by a binary pH and salt gradient. A portion of the collected IgG fraction is then re-chromatographed in a size-exclusion system and separated into its aggregate, dimer, and monomer components. Some minor losses of IgG aggregates occurred during analysis. However, this did not have a significant effect on the overall molecular size distribution. This method can be used to determine the molecular size distribution of IgG in both fresh and stored intravenous formulations containing albumin.

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