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Journal of Chromatography A, 676 (1994) 331–335

JOURNAL OF CHROMATOGRAPHY A

Rapid, automated, two-dimensional high-performance liquid chromatographic analysis of immunoglobulin G and its multimers

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First received 25 October 1993; revised manuscript received 11 March 1994

Abstract

It is important to determine the amount of IgG multimers in immunoglobulin-containing pharmaceuticals because these aggregates can cause adverse reactions in patients. Previous methods for determining aggregates either suffered from interference of other proteins or required fraction collection and sample purification. A new, automated two-dimensional approach has been developed in which size-exclusion chromatography is performed in the first dimension followed by protein A affinity chromatography in the second dimension. This method is robust in that the aggregates are not disturbed by a preliminary purification step. Further, the presence of contaminating proteins has no effect on the analysis since affinity chromatography is used to determine the presence of IgG in the second dimension. The entire automated two-dimensional analysis can be performed in *ca.* 1 h.

1. Introduction

Intravenous immunoglobulin G (IgG) has been used for clinical purposes such as hypo and agammaglobulinaemia, antibiotic therapy and thrombocytopenia [1–4]. However, dimers and aggregates of IgG have harmful side effects which include anaphylaxis and dyspnea [5]. The cause of these side effects is thought to be due to the activation of the complement reaction as postulated by Barandum *et al.* [6]. Others have also mentioned the possibility of harmful side effects due to aggregates [7]. IgG dimers and aggregates are often formed during pasteurization of the pharmaceutical. Pasteurization is often a necessary step, and therefore, it is important to monitor levels of IgG aggregates in pharmaceuticals which contain immunoglobulin.

A number of methods for the quantitation of IgG dimers have already been published [8–10]. However, these techniques suffer from either interference of albumin aggregates [7] and/or the necessity of collecting fractions for rechromatography. The more recent paper [10] uses a two-stage HPLC separation in which IgG is first separated from the albumin by ion-exchange chromatography and then analyzed for dimers and aggregates by size-exclusion chromatography (SEC). The disadvantage of this approach is that there can be some dissociation of the IgG aggregates during the ion-exchange step. Thus, the results may not reflect the solution concentration of the aggregates. Light scattering may also be

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0021-9673/94/$07.00
SSDI 0021-9673(94)00304-R

used for monitoring IgG aggregation but requires pure IgG samples [11–13].

A new two-dimensional chromatographic analysis is presented which is totally automated and robust. The sample is analyzed by SEC in the first dimension in order to determine molecular size. Fractions from the SEC column are then automatically transferred to the protein A column for determination of IgG. Since protein A affinity chromatography is used in the second dimension, albumin and other contaminating proteins can not interfere with the IgG determination. A sample containing induced IgG dimers was used as a model system to test the method's capability to resolve the aggregate IgG from the monomeric IgG. The system was also challenged with high-molecular-mass serum proteins to show that these non-IgG proteins would not interfere with the aggregate determination.

2. Experimental

2.1. SEC

SEC separations were achieved on a 300 × 8 mm column packed with TSK G3000 SW packing material (TosoHaas, Montgomeryville, PA, USA) on a Shandon column packer (Shandon Southern Instruments, Sewickley, PA, USA) at 2000 p.s.i. (1 p.s.i. = 6894.76 Pa). The mobile phase for SEC was 100 m*M* potassium phosphate with 100 m*M* sodium sulfate both from Mallinckrodt (Paris, KY, USA), pH 7.0.

2.2. Protein A affinity chromatography

The immobilized protein A column, POROS A/M (30 × 4.6 mm) (PerSeptive Biosystems, Cambridge, MA, USA), was packed on a Shandon column packer at 2000 p.s.i. The loading buffer was the same as the SEC buffer and the desorption buffer was 0.3 *M* magnesium chloride (Mallinckrodt) with 2% acetic acid (J.T. Baker, Phillipsburg, NJ, USA).

2.3. Apparatus

A BioCAD perfusion chromatography workstation (PerSeptive Biosystems) was configured for tandem columns with the SEC column as the first column and the protein A column as the second (see Fig. 1).

2.4. IgG dimer induction

To induce dimer formation, 100 μl of 1 mg/ml rabbit anti-(bovine IgG) was incubated with 500 μl bovine IgG 1 mg/ml, both proteins from Sigma (St. Louis, MO, USA). Incubation was at room temperature in 20 m*M* potassium phosphate pH 7.0 for 1 h before use. Excess bovine IgG was used to produce what is expected to be mostly bovine–rabbit heterodimers of IgG with excess bovine IgG monomer remaining (see Fig. 2).

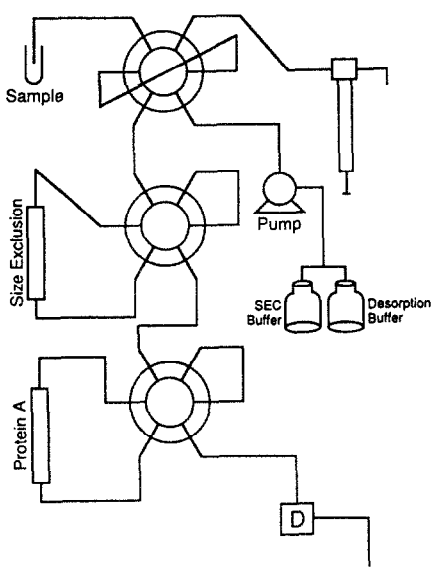

Fig. 1. Column configuration for the two-dimensional separation of IgG monomers and multimers. The SEC column is followed by the protein A column. Switching valves allow either column to be in-line with the mobile phase flow by itself, both columns to be in-line, or both columns to be off-line. Switching both columns off-line allows high-speed pumping of a new mobile phase in order to automate system flushing.

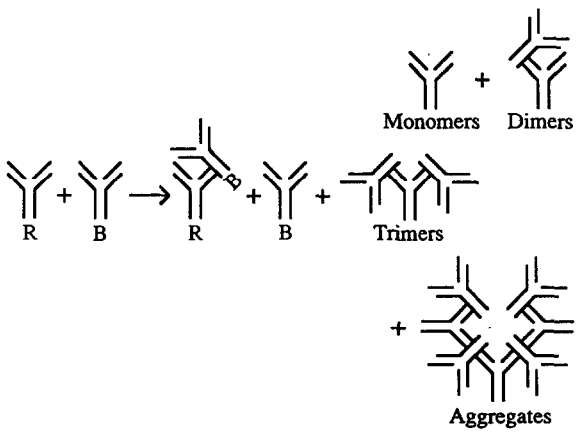

Fig. 2. Antibody reaction in which an excess of bovine IgG, B, is incubated with rabbit anti-(bovine IgG), R, to produce IgG multimers and left over bovine IgG monomers.

2.5. Reconstructed chromatograms

Reconstructed chromatograms were produced by plotting protein A peak areas against fraction volume from the SEC column. In the case of the serum sample, a non-retained peak area accounted for non-IgG protein. It was summed with the area of the retained IgG peak to produce a plot of total protein, and the areas of the IgG peaks were also plotted to produce the IgG reconstructed chromatogram. For the IgG dimers, there was no non-retained peak.

3. Results

3.1. SEC of IgG and induced dimers

Bovine IgG and the rabbit anti-(bovine IgG) were analyzed by SEC. Both eluted with the same retention volume (Table 1) and were judged to be monomeric as determined by the SEC calibration curve (data not shown). However, the induced dimers produced two peaks on the SEC chromatogram indicating that aggregation had indeed occurred and also that some monomers remained (Table 1).

Table 1
Retention times for bovine IgG (B), rabbit anti-(bovine IgG) (R) and multimers on the size-exclusion and protein A columns

Species	SEC retention volume (ml)	Protein A retention time (min)
Bovine IgG	4.5	1.35
Rabbit IgG	4.5	1.46
Aggregate IgG	3.6 and 4.5	1.35 and 1.46

3.2. Protein A chromatography of IgG and induced dimers

Monomeric bovine IgG and rabbit anti-(bovine IgG) were analyzed on the protein A column. Rabbit IgG was found to have a higher affinity for protein A than bovine IgG, eluting later in the desorption gradient (see Table 1).

3.3. Two-dimensional analysis of induced dimers

The BioCAD was configured in the tandem column configuration with the SEC column followed by the protein A column. After injection of the sample, 3 ml of buffer were passed through the SEC column (slightly less than the void volume). Then, a 200-μl fraction was transferred from the SEC column to the protein A column by switching valves. The SEC column was switched off-line and the protein A column eluted with the desorption buffer. Bovine IgG eluted first, followed by rabbit IgG. The protein A column was re-equilibrated with SEC buffer and the SEC column was placed back in line so that another 200-μl fraction could be passed onto the protein A column. Peak areas for the total IgG, rabbit IgG and bovine IgG were calculated and plotted (Fig. 3). The profile of the reconstructed chromatogram for total IgG matched closely with the SEC chromatogram for the dimer mixture as expected. Most of the rabbit IgG eluted as a multimer, because it was the limiting reagent. The bovine IgG was present

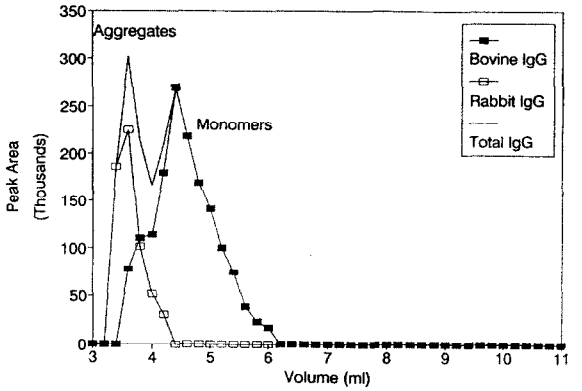

Fig. 3. Reconstructed SEC chromatogram for total IgG, rabbit IgG and bovine IgG.

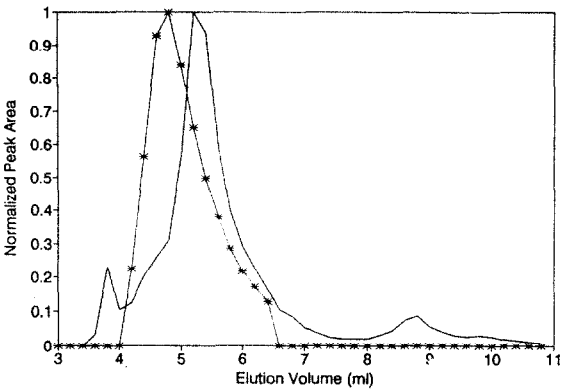

Fig. 4. Reconstructed SEC chromatogram of bovine serum in which total protein is plotted as a solid line and IgG is plotted as a dotted line. From previous work, it is known that the major peak is bovine serum albumin.

partly as an aggregate but was mostly monomeric. Although the dimers were not separated from the other aggregate forms, this separation could be made if the appropriate SEC column were used. The SEC column used in these experiments was chosen for its stability. In order to resolve larger proteins, the pore size of the SEC packing must be larger. Large pores reduce the mechanical strength of the beads which will in turn reduce the life time of the SEC column. Since all forms of IgG aggregates are undesirable, there was no reason to separate them further, especially since doing so would reduce the SEC column life.

3.4. Two-dimensional analysis of serum

To show that the presence of other proteins would not interfere with the dimer determination, the two-dimensional analysis was also applied to a serum sample that contained a number of contaminating proteins, the predominant of which is serum albumin. A reconstructed chromatogram (Fig. 4) of total peak area produces a profile comparable to the SEC chromatogram of serum (data not shown). Plotting area of the IgG peak from the protein A separation yields a peak at $M_r \approx 150\,000$, as expected. No IgG dimers were detected in this serum sample demonstrating that other non-IgG high-molecular-mass proteins would not yield a false positive result for IgG dimers.

4. Discussion

The ratio of rabbit IgG to bovine IgG in the dimer reconstructed SEC chromatogram (Fig. 3) appears to be much greater than one to one in the multimer peak. There are several reasons why one can not determine IgG ratios in this way. First, there is steric hinderance at the bovine IgG Fc region where both the rabbit IgG and protein A can bind. Since rabbit IgG is already bound to the Fc region and the binding is essentially irreversible, the protein A will only bind the Fc region of the rabbit IgG and not the bovine IgG. Second, when the IgG is desorbed from the protein A, the UV absorbance for the rabbit and bovine IgG is summed, because the multimer has not been disrupted. This will result in an overestimation of the rabbit IgG, however it will be an accurate reflection of the total multimer concentration in terms of the aggregates-to-monomer ratio. The primary purpose of plotting both rabbit and bovine IgG is to indicate that all of the rabbit IgG is in the form of multimers while some bovine IgG remains in the monomer form as predicted from the incubation of excess bovine IgG.

It is also important to note that if dimerization occurs through the Fc region, protein A or G may not be appropriate. A more general affinity technique should be applied. Polyclonal antibodies to the specific IgG can be immobilized

and used instead of a protein A or G. The polyclonal antibody to IgG will recognize multiple portions of the molecule whereas protein A and G have a somewhat "monoclonal" interaction with IgG in that they only recognize the Fc region of the IgG molecule.

Detection limits for protein A affinity chromatography have already been determined by previous authors [14,15]. They have found limits on the order of 50 ng. This means that this assay should be able to detect less than 1% aggregate in a 100-μg IgG sample.

5. Conclusions

A robust two-dimensional HPLC determination of IgG and its aggregates has been presented. It is possible to avoid IgG dimer disruption by performing size exclusion in the first dimension followed by protein A affinity chromatography in the second dimension. The analysis may be automated on a single HPLC system and performed in *ca.* 1 h. Further, purity and concentration of IgG may also be determined in the same analysis. It is probably better to use a smaller pore size SEC packing as long as the separation of dimers from aggregates is not important.

Acknowledgements

The authors would like to thank Gretchen Leuck for technical support and valuable discussions. This research was supported by NIH Grant 25431.

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