Separation of serum proteins by high performance liquid chromatography[1]

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This article describes the separation of serum proteins by isocratic HPLC using a polyvinyl alcohol gel as the stationary phase. The system separates immunoglobulins and other proteins by size exclusion and weak affinity bonding and gives a chromatogram that can be evaluated qualitatively, in a manner similar to serum protein electrophoresis scans. Peak heights measured for IgG, IgA, and IgM correlate closely with concentrations measured by radial immunodiffusion (RID). The separation, carried out without sample pretreatment, may prove to be a valuable technique for quantifying several serum proteins. The method may be used to screen hybridoma cultures for antibody production and to purify proteins without risk of denaturation.

Index terms: Blood proteins • Chromatography, high pressure liquid • Radioimmunoassay

Cleve Clin Q 53:181–187, Summer 1986

Chromatographic techniques for separating proteins are well established: Column chromatography has been used to purify proteins that differ in molecular size and to separate hemoglobins and isoenzymes that differ in charge. Liquid chromatography has been used to separate and purify peptides and low-molecular-weight proteins, but most absorbates cannot separate proteins in the range of molecular sizes found in serum. With gel permeation or filtration, however, chromatographic separation over a wide range of molecular weights is possible but difficult for abnormal samples because paraproteins tend to occlude columns. Gel filtration methods may prove to be a useful diagnostic adjunct to established methods for evaluating patients with dysproteinemias.

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0009-8787/86/02/0181/07/$2.75/0

We have shown that gel filtration HPLC can produce a clinically useful separation of serum proteins. The technique does not produce baseline resolution, characteristic of HPLC procedures for drugs, hormones, and small metabolites, but it does produce a chromatogram that can be used in a manner similar to densitometric scans of serum protein electrophoresis. The procedure can be optimized to quantitatively separate immunoglobulins and separate albumin from other proteins with baseline resolution. Separation, which is based primarily on differences in molecular size, has the potential to detect immunoglobulin monomers, immune complexes, and cryoglobulins and to isolate these for further investigation. For this reason we chose to analyze samples from patients with a wide range of dysproteinemias.

Materials and methods

Serum samples from normal persons and those with various paraprotein and inflammatory disorders were frozen at −20° C. Samples were analyzed by high-resolution agarose gel electrophoresis (Helena Laboratories, Beaumont, TX) and classified according to the electrophoretic pattern and clinical diagnosis. Immunoelectrophoresis (Corning Medical, Medfield, MA) was performed on all samples suspected of containing monoclonal protein and on all samples that were difficult to classify by protein electrophoresis. Radial immunodiffusion (RID) for quantifying immunoglobulins was performed on agarose gel plates (Kallestad, Chaska, MN) according to Fahey and McKelvey's method.[1]

We separated serum proteins with high performance liquid chromatography (HPLC) using a new size-exclusion, weak-affinity gel, PGP-2000 (Asahi Medical Co., Tokyo, Japan). The HPLC apparatus consisted of a Knauer Model 64 solvent delivery system; Rheodyne 7125 rotary valve loop injector; JASCO, UVDEC III ultraviolet detector; and Knauer dual-channel 732 strip chart recorder. The stationary phase separates macromolecules by size exclusion with a limit of 8×10^5 daltons as determined by calibrating columns with PEG-Pullulan, and has a resolution of 10,000 theoretical plates per 25 cm as determined by analysis of polyethylene glycol under specified conditions.[2] By separating jack bean urease tetramer from other molecular-weight markers (BioRad Laboratories, Richmond, CA), we confirmed the exclusion limit to be greater than 4.8×10^5 daltons. Our chromatograms showed an IgM peak just after the void volume of the column, which would be predicted by such an exclusion limit. The mobile phase was 0.03*M* sodium phosphate, 0.15 *M* sodium sulfate, pH 7.00. The buffer was filtered through HT-200 particle filters (Gelman, Ann Arbor, MI), degassed, and pumped at a rate of 1.0 mL/min. The column dimensions were 0.75 cm (inner diameter) × 200 cm, and the void volume was measured to be 35.2 mL.

Table 1. Recovery of immunoglobulin from concentrated normal serum using a PGP-2000 column

Ig peak	Retention time (min)
IgM	37
IgA	44
IgG	48

Retention times for immunoglobulins were determined using 20 μL of normal human serum that had been concentrated fivefold using CF50A filtration membranes with an exclusion size of 50×10^3 daltons (Amicon, Lexington, MA). Concentrated samples were injected with the nonstop-flow loop injector, and elution volumes were collected in four-minute aliquots. These were concentrated and assayed for IgG, IgA, and IgM by RID.

Chromatography of all other samples was carried out using 10 μL of unconcentrated, untreated sample. Proteins were detected by ultraviolet light absorption at 254 nm. This wavelength was chosen instead of 280 nm (the conventional absorbance maximum for protein) because it detected a creatinine peak that signaled the complete elution of sample from the column. Elution volume was measured to be 70 mL. The detector was set to a sensitivity of 0.02 ABU, and peak height was measured manually to the nearest 0.5 mm.

Results

Determining Ig Retention Times

Elution volumes containing IgG, IgA, and IgM were isolated from normal human serum that was concentrated fivefold using CF50A filtration membranes. A concentrated sample was injected into the column, and elution volumes were collected in 4.0-mL aliquots. These were concen-

trated onto CF50A membranes and the protein reconstituted in normal saline. Each sample was tested for IgG, IgA, and IgM using the kinetic RID method. *Table 1* summarizes the retention times of IgG, IgA, and IgM from the concentrated sample. *Fig. 1* shows the positions of peaks that correspond to elution volumes containing IgG, IgA, and IgM.

IgM and IgA were detected in one elution volume only. IgG was distributed in two aliquots collected over 42 to 50 minutes. Approximately 62% of the IgG was found in the 46- to 50-minute aliquot and the remainder in the 42- to 46-minute fraction. The base of the peak at 44 minutes varied from 3.5 to 4.0 minutes, and, therefore, we expected some carryover. We concluded that the peaks containing IgM and IgG were free of other immunoglobulins and that the peak corresponding to IgA contained all of the IgA and some of the IgG.

Comparison to RID

A set of 20 samples was assayed by RID for IgG, IgA, and IgM. These samples were used to evaluate the ability of the column to provide

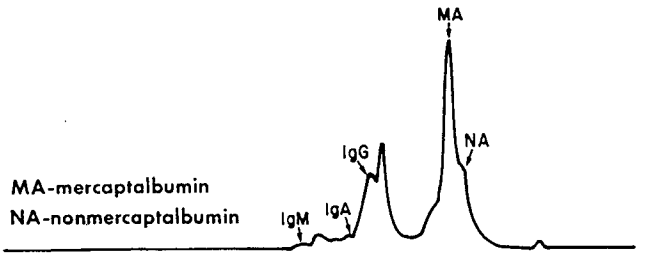

Fig. 1. Chromatogram of normal serum.

useful quantitative data for immunoglobulin class. Results comparing immunoglobulin concentrations determined by RID to peak heights obtained with HPLC are shown in *Table 2*.

The correlation coefficients for IgG, IgA, and IgM are r = 0.913, r = 0.980, and r = 0.882, respectively. Immunoglobulin concentrations below 25 mg/dL could not be measured by HPLC without using an electronic integrator. This produced poor low-end sensitivity for IgM; however, the range of concentrations that could be measured was approximately 2.5-fold greater than that afforded by RID.[3] Scatter plots and regres-

Table 2. Comparison of Ig levels (RID) with peak height for IgG, IgA, and IgM

Specimen number	IgG* RID (mg/dL)	IgG* Peak ht. (mm)	IgA† RID (mg/dL)	IgA† Peak ht. (mm)	IgM‡ RID (mg/dL)	IgM‡ Peak ht. (mm)	Clinical classification
1	835	29.5	90	5.0	156	2.0	normal
2	1995	74.5	532	20.5	157	2.0	polyclonal
3	1800	60.0	298	12.5	150	2.0	polyclonal
4	460	19.5	272	11.0	705	6.0	hypogammaglobulinemia
5	2141	80.0	380	16.5	354	4.0	monoclonal Gκ
6	9800	—§	6	00.0	15	0	monoclonal Gκ
7	1750	58.0	29	—‖	25	6.0	monoclonal Gλ
8	4500	297.0	51	13.0	20	2.0	monoclonal Gκ
9	210	29.5	1665	118.0	15	0	monoclonal Aκ
10	2150	100.0	490	29.0	700	13.5	polyclonal
11	640	43.5	170	16.0	1200	35.5	polyclonal
12	1775	100.0	330	19.0	500	6.0	polyclonal
13	210	16.0	55	5.0	600	10.5	cryoglobulinemia
14	165	4.5	28	1.0	0	0	hypogammaglobulinemia
15	1050	34.0	125	7.0	85	2.0	normal
16	6800	241.0	45	7.0	16	0	monoclonal Gκ
17	270	41.5	1605	98.5	16	0	monoclonal Aκ
18	600	36.5	375	25.5	460	5.5	polyclonal
19	580	32.5	90	7.5	35	0	monoclonal Gκ
20	2500	83.0	435	18.0	280	2.5	normal

* n = 19; r = 0.913.
† n = 19; r = 0.980.
‡ n = 20; r = 0.882.
§ Off scale.
‖ Peak not resolved due to carryover of monoclonal Ab or fragment. Peak height measured to the nearest 0.5 mm.

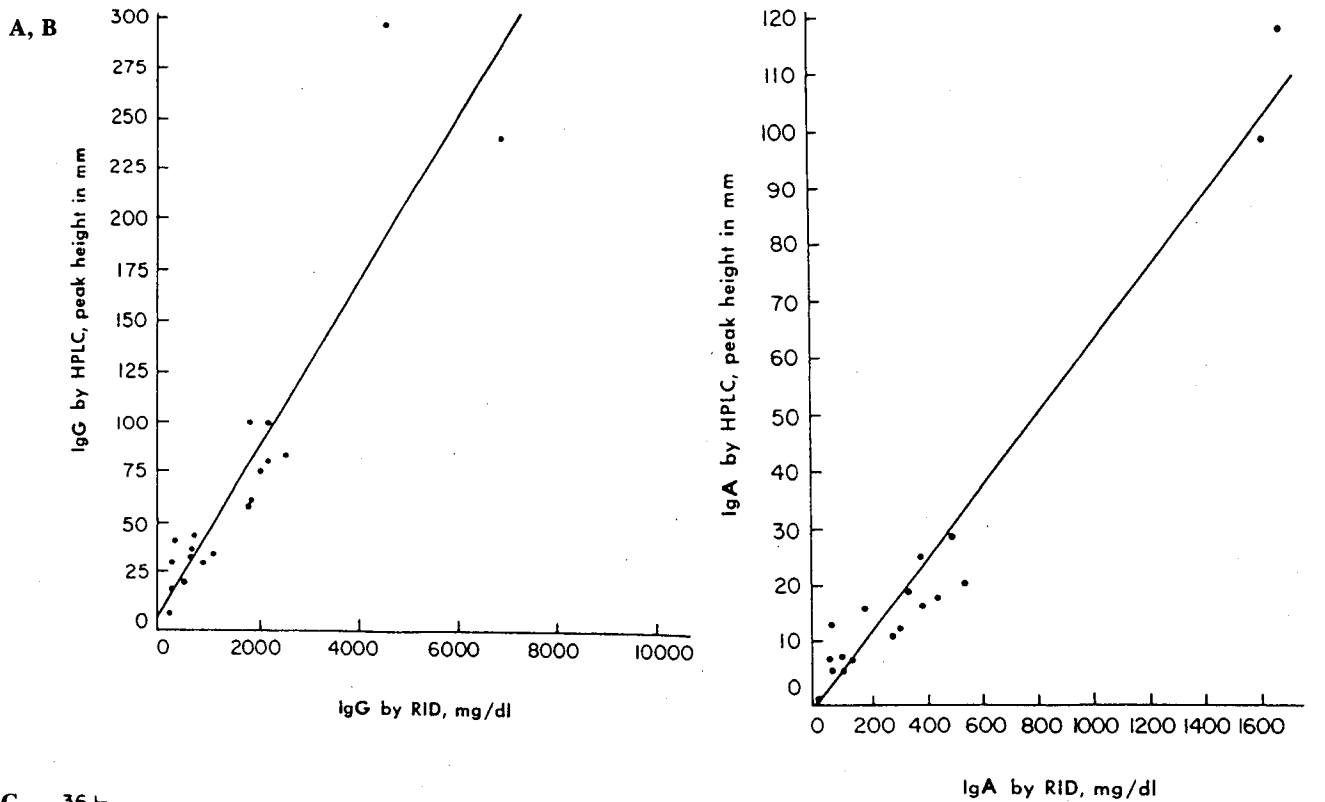

Fig. 2. **A.** Plot of IgG measured by HPLC versus IgG measured by RID.

B. Plot of IgA measured by HPLC versus IgA measured by RID.

C. Plot of IgM measured by HPLC versus IgM measured by RID.

sion lines are shown in *Fig. 2.* They indicate good agreement between methods for measuring IgG and IgA, but significant variation between those for IgM.

Linear regression analysis was performed on each set of data, and equations are presented in *Table 3.* A t test of Ho: slope = 0 is significant in all cases ($P < 0.0001$). Since the units used for HPLC measurements were millimeters (for peak height) and mg/dL for RID measurements, the data were normalized to a Z score to produce a least-squares regression independent of these units. These regression equations show all three slopes to be within ± 0.05 of 1.00 and no bias at the y intercept. The squared, multiple correlation coefficient (R square) shows that more than 95% of the plotted data can be explained by the regression line for IgG and IgA, but only 85% by the regression line for IgM. Evaluation of residual plots for IgM showed a parabolic tendency, and the data for IgM were plotted using a quadratic regression model. The R-squared expression for the slope of this line explains 96% of the plotted data. The normalized equation for this line is $\hat{y} = -0.31 + 0.68x + 0.30x^2$. The IgM

Table 3. Statistical analysis, HPLC vs. RID

	IgG	IgA	IgM
Linear regression	$\hat{y} = 8.64 + 0.034x$	$\hat{y} = -2.86 + 0.066x$	$\hat{y} = -0.27 + 0.014x$
Normalized regression	$\hat{y} = 2.9E-17 + 0.98x$	$\hat{y} = -2.6E-17 + 0.98x$	$\hat{y} = 8.9E-17 + 0.95x$
R-square	0.955	0.969	0.846
T for Ho:slope = 0	17.12	21.69	9.07
CV for RID	99.8	120.1	101.8
CV for HPLC	88.2	136.5	119.8
Sample size (n)	16	17	17

peaks were difficult to measure manually since peaks for IgM were small and asymmetrical. We concluded that IgM could be more accurately measured using peak area integration, and this is supported by the regression analysis.

In each case, the coefficient of variation (CV) for HPLC was within 15% of RID. The distribution of HPLC results for IgA and IgM show more variance, and the distribution of HPLC results for IgG less variance than RID. However, the coefficient of variation for both methods was very high because of the wide range of immunoglobulin levels present in abnormal samples and, therefore, should not be viewed as a measure of relative precision. A within-run and out-of-run replicate study using normal and abnormal reference-assayed controls would be required to determine the precision of HPLC.

Evaluating Chromatograms

Normal samples and those from patients with dysproteinemias were used in this study to evaluate the ability of chromatograms to provide clinically useful qualitative information. Chromatograms can be classified into normal and abnormal groups in a manner similar to densitometric scans of serum protein electrophoresis gels. Visual evaluation of chromatograms revealed hypo- and hypergammaglobulinemia and hypoalbuminemia. Monoclonal gammopathies produce gross elevations in peak height corresponding to the class specificity of the monoclonal protein; however, the retention time for these is typical, and, therefore, the monoclonal nature of the protein must be confirmed by immunoelectrophoresis. None of the samples we tested demonstrated 7S IgM monomer, aggregated IgG or IgA, or free light or heavy chains by either immunoelectrophoresis or chromatography.

Fig. 3A is a chromaotgram characteristic of hypogammaglobulinemia (low serum IgG and

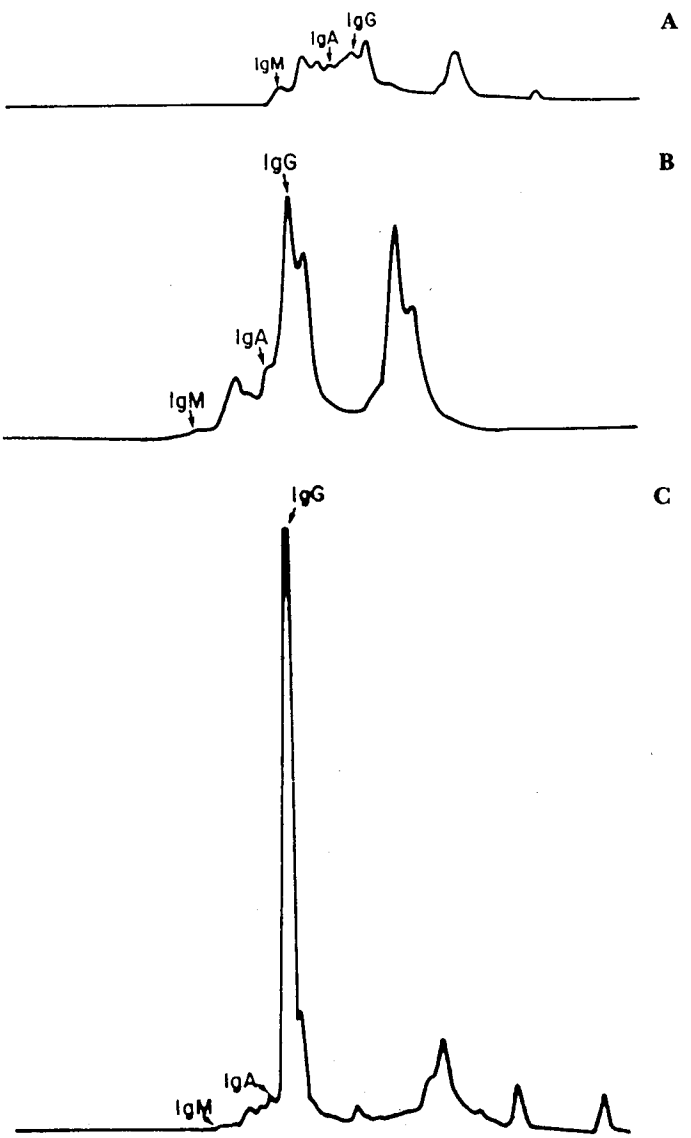

Fig. 3. A. Chromatogram of serum from patient with hypogammaglobulinemia.

B. Chromatogram of serum from patient with polyclonal gammaopathy.

C. Chromatogram of serum from patient with IgGκ myeloma (IgA peak height discordant with RID results).

low total protein), *Fig. 3B* is a chromatogram characteristic of polyclonal gammopathy, and *Fig. 3C* is a chromatogram of serum that contained monoclonal IgGκ. This sample gave a peak height for IgA that was higher than expected, presumably because of carryover of IgG, which measured 4,500 mg/dL by RID. We did not attempt to evaluate other serum proteins, but several others can be detected by increasing sensitivity.

Discussion

PGP-2000 size-exclusion columns can measure IgG, IgA, and IgM in human serum without sample pretreatment or extraction. The coefficient of correlation between RID measurements and peak heights is high, and regression statistics show good agreement. However, there are three concerns: IgA elution volume is influenced by IgG elution volume because of its elution vicinity. Some negative bias, variance, and proportional error is apparent in *Fig. 2C*. This problem may be overcome by using an electronic area integrator. In this regard, a study by Asahi Medical Co. showed excellent correlation ($r = 0.990$) comparing IgM values (for normal-range samples) determined immunochemically to peak area using PGP-2000 columns.[4] Our sample sizes were small and included a sparse number of data points in the high-abnormal range. Since our samples did not contain abnormal monomers or aggregates of immunoglobulin, we could not determine whether these would be found in an elution volume different from that characteristic of IgG, IgA, and IgM. We presume that any deviation in molecular size would affect retention, and this would lead to falsely low values in such cases. On the other hand, the ability of the column to identify and separate such proteins would be an invaluable diagnostic aid.

Daniels et al.[5] compared RID and other conventional methods of measuring immunoglobulin levels in patients with monoclonal gammopathies. They noted a significant difference in test results, which in some cases approached fivefold; they concluded that immunochemical methods may be inappropriate for these samples because subclass-specific antibodies fail to bind the monoclonal protein. HPLC may provide a more accurate measurement of monoclonal protein, which is valuable in anticipating malignancy and in following the course of treatment in these patients.

The procedure cannot be standardized with purified proteins since these do not reproduce retention times characteristic of a serum matrix. A calibration curve for immunoglobulins could be produced using WHO reference sera with IgG, IgA, and IgM levels determined by established immunochemical assays.[6] An internal standard would improve precision by compensating for slight variation in injection volume; creatinine elutes from the column at 77 minutes and may be an acceptable internal standard for samples that are not uremic.

Jun and Ruckenstein reported a method for separating immunoglobulins using potential barrier HPLC.[7] This technique required ammonium sulfate precipitation, washing, and dialysis. The dialysate was separated using a cation exchange adsorbent, Dupont Zorbax 300 SCX, at pH 7.50. They were able to recover a mixture of IgG and IgA in one elution volume and IgG without IgA in a second. They concluded that HPLC is much less likely to result in denaturation than immunoadsorbent methods because interactions between the column packing and proteins are physical rather than chemical. However, the potential barrier method did not recover IgM, and both immunoglobulin peaks were contaminated with traces of other serum proteins. Size-exclusion chromatography offers the advantage of being simpler to perform and capable of resolving IgM. We were able to recover IgG, IgA, and IgM from the PGP-2000 column, and it is likely that sample size could be increased to provide milligram quantities of several purified proteins.

Adding sulfate to the solvent separates albumin into two components that do not resolve in phosphate buffer (see *Fig. 1*). Sogami et al.[8] showed these albumins differ by the presence or absence of a mixed disulfide linkage, possibly at CYS-34. The quantity of nonmercaptalbumin varied greatly from sample to sample, sometimes being the dominant form. The clinical significance of this has yet to be determined, but nonmercaptalbumin levels may be inversely related to the quantity of heavy metals, drugs, or metabolites that form mixed disulfide linkages with albumin.[9] Such a relationship would be diagnostically useful in evaluating a wide range of toxic conditions.

Measuring immunoglobulin by HPLC is not immunochemical and thus eliminates the use of costly, class-specific antisera. The procedure can be performed in 90 minutes and may be prefer-

able to RID or nephelometry for measuring monoclonal proteins. Size-exclusion HPLC is simpler to perform than other methods of immunoglobulin purification. Recovery of purified immunoglobulins from concentrated samples is possible and may prove to provide a source of immunoglobulin suitably pure for immunization or reference material. The method uses small samples and may be applied to screening hybridoma cultures for immunoglobulin production. The method has been used to separate mercaptalbumin and nonmercaptalbumin from human serum and may be useful in characterizing the structure of other serum proteins. Size-exclusion HPLC produces a chromatogram from serum that can be used in the same manner as electrophoresis scans in evaluating and diagnosing inflammatory diseases, gammopathies, and other clinical conditions. It has the potential to become a powerful clinical tool.

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