Journal of Chromatography, 266 (1983) 39–47
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 032

HIGH-PERFORMANCE ION-EXCHANGE CHROMATOGRAPHY OF PLASMA PROTEINS

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SUMMARY

The use of anion-exchange high-performance liquid chromatography columns for the analysis of plasma proteins has been investigated. Mono Q and Polyanion SI were used as anion-exchangers. Several factors, including solvent composition, pH, flow-rate, sodium chloride linear concentration gradient and sample loading capacity, were examined for their effects on the resolution of protein standards and pooled human plasma (PHP). PHP was separated into ten or more protein fractions by a Mono Q column (50 × 5 mm I.D., flow-rate 2 ml/min) within 10 min. Components analysis of each fraction was performed using immunochemical methods and size-exclusion high-performance liquid chromatography. The Mono Q column was applied to the analysis of IgG myeloma and other patient plasma samples.

INTRODUCTION

Recent developments[1,2] of high-performance ion-exchange chromatography enable us to achieve the separation and purification of proteins and other biopolymers. The use of a rigid matrix, such as synthetic glycol methacrylate[3] and glycerolpropylsilyl controlled-pore glass (CPG)[4], was effective in both high-resolution and high-speed separations by allowing high mobile phase velocity and high-pressure operations. Various applications have been investigated, for example, to proteins[5–8], enzymes[9], peptides[10,11] and amino acids. Chang et *al.*[12] achieved separation of human serum within 30 min by use of an amine-coated epoxy polymer.

Recently, new commercial "fast protein liquid chromatography" (FPLC) ion exchangers have been introduced for analytical use[13], which provide high resolution and short separation time: Mono Q and Polyanion SI are anion exchangers, Mono S is a cation-exchanger and Mono P is a chromatofocusing medium.

In this paper, the use of Mono Q and Polyanion SI in anion-exchange columns has been investigated in the analysis of plasma proteins. In order to determine the best separation conditions, the effects of solvent composition, pH, flow-rates, sodium chloride concentration gradient and sample loading on the resolution and elution profile of protein standards and pooled human plasma (PHP) were studied. The elution profile of plasma proteins was analysed by immunochemical methods and

0021-9673/83/$03.00

size-exclusion high-performance liquid chromatography (HPLC) using TSK-G3000SW columns[14–16]. Furthermore the Mono Q column was applied to the analysis of immunoglobulin G (IgG) myeloma and other patient plasma samples,

EXPERIMENTAL

Apparatus

Mono Q based on monodisperse 10 μm spheres and Polyanion SI based on 17-μm high porosity silica were employed as anion exchangers. These gels were supplied packed in HR 5/5 columns (50 × 5 mm I.D.). Ion-exchange chromatographic experiments were performed with the Pharmacia FPLC system, consisting of a GP-250 gradient programer, a P-500 pump, a V-7 injection valve, a solvent mixer, a prefilter, a sample loop of *50* μl, a UV-1 UV monitor with an HR low-dead-volume flowcell, and a Rec-1 recorder, all supplied by Pharmacia (Tokyo, Japan).

Size-exclusion HPLC of plasma proteins was carried out with a high-performance liquid chromatograph (Model HLC-802, Toyo Soda) equipped with two TSK-G3000SW-type columns (60 × 0.75 cm I.D.) in tandem.

Materials

Chemicals *for* buffer solutions such as tris(hydroxymethyl)aminomethane (Tris), ethanolamine, diethanolamine, sodium acetate, acetic acid and sodium chloride were purchased from Wako (Tokyo, Japan).

Human serum albumin (HSA), human serum immunoglobulin G (IgG, Cohn Fraction II), human fibrinogen, human transferrin and human antithrombin III were fractionated by using Cohn's method and other conventional methods at the Japanese Red Cross Central Blood Center (JRCCBC)[17,18]. Pooled human plasma (PHP) was collected with acid-citrate-dextrose (A.C.D.).

Patient plasma samples were supplied by Drs. M. Inoue and H. Nakanishi, Senko Medical Instrument Mfg. Co.

All samples were filtered through a Millex-GS 0.2-μm filter unit (Millipore, Bedford, MA) prior to use.

Anti-sera and immunodiffusion plate (LC-Partigen) were purchased from Hoechst Japan (Tokyo, Japan).

Methods

High-performance ion-exchange chromatography was carried out as follows. The linear sodium chloride gradient system was prepared by combining the initial buffer (solution A) and the final buffer (solution B) in prescribed ratios. For the initial buffers, 0.05 *M* Tris–HCl (pH 7.0–8.6), 0.02 *M* acetate buffer (pH 4.0–6.0), 0.05 *M* ethanolamine (pH 8.6), and 0.05 *M* diethanolamine (pH 8.6) were employed in order to determine the best condition for the anion-exchange chromatography of plasma proteins. The final buffers (solution B) were prepared from solution A and 0.5 *M* sodium chloride.

The effects of flow-rate, gradient time and loading capacity on the anion-exchange chromatography were evaluated by using a mixture of transferrin and albumin in a ratio of 2:5. The resolution, R_s(T,A), was calculated according to eqn. 1

$$R_s(\mathrm{T,A}) = 2(V_A - V_T)/(W_A + W_T) \quad (1)$$

where V is the retention volume, w is the peak width at the baseline and suffixes T and A refer to transferrin and albumin, respectively. In order to determine the resolution, 0.7% of protein solution was injected at flow-rates of 0.5, 1.0, 2.0 and 3.0 ml/min, and linear gradient volumes (product of flow-rate and gradient time) of 5, 10, 20, 30, 40, 45 and 60 ml. The influence of load capacity on resolution was investigated by using 50 μl of protein mixture, the concentrations of which were 0.188, 0,376, 0.940, 1.88 and 3.76%.

In the size-exclusion HPLC by two TSK-G3000SW columns in tandem, 0.05 M sodium acetate (pH 5.0) containing 0.1 M sodium sulphate was used as an elution buffer. The flow-rate of the buffer solution was 1.0 ml/min and the sample volume was 500 μl[14–16].

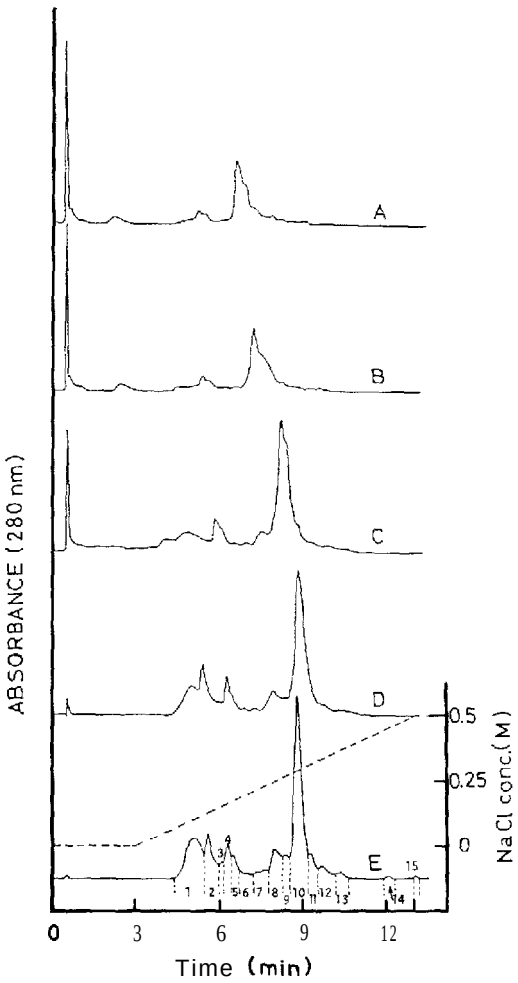

Fig. 1. The FPLC elution pattern of pooled human plasma. Anion exchanger, Mono Q, packed in HR 5/S column (50 × 5 mm I.D.). Elution buffer: 0.05 M Tris–HCl at pH 7.0 (A), 7.5 (B), 8.0 (C), 8.5 (D) and 8.6 (E). Flow-rate: 2.0 ml/min. Linear sodium chloride concentration gradient from 0 to 0.5 M, in 10 min. Sample: SO μl of 0.7% PHP. In E, numbers corresponding to each peaks indicate the fractions collected and analysed thereafter.

All chromatographic analyses were carried out at room temperature and elutes were detected by spectrophotometry at 280 nm.

The plasma protein fractions of high-performance anion-exchange chromatography were analysed by the following immunochemical methods. Counter immunoelectrophoresis was carried out using 1% agar gel in 0.05 *M* Verona1 buffer at pH 8.6. Samples and anti-sera were applied in volumes of 5 μl of each. Immunodiffusion was performed with LC-Partigen plates according to the instruction manual.

RESULTS AND DISCUSSION

Selection of elution conditions

The influence of various buffer species and hydrogen ion concentration (pH) on FPLC chromatograms was investigated using PHP. Figs. 1 and 2 show the elution patterns of PHP using 0.05 *M* Tris–HCl buffer at different pH on Mono Q and Polyanion SI, respectively. As shown in Fig. 1, when PHP was chromatographed on the Mono Q column at pH below 8.5, some of the protein fractions were eluted at void volume. However, at pH 8.6 most of protein components were sorbed on the column and then eluted according to the sodium chloride concentration. The same was true with the Polyanion SI column, as is shown in Fig. 2. As in the cases men-

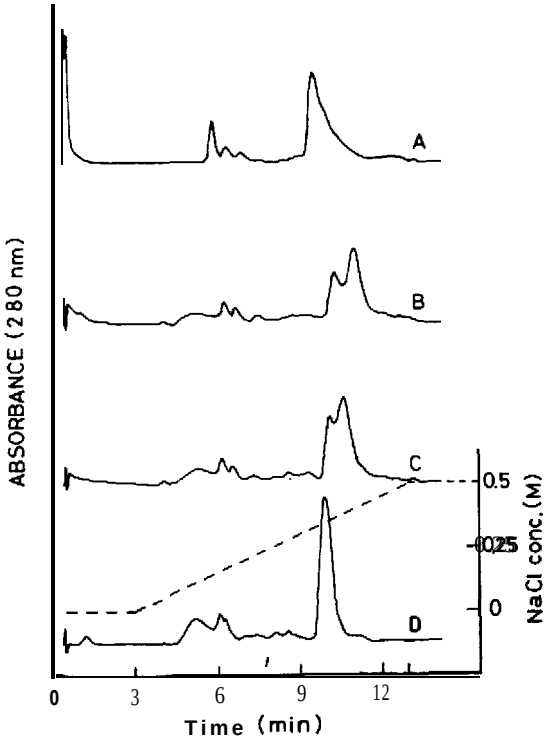

Fig. 2. The FPLC elution pattern of pooled human plasma. Anion exchanger, Polyanion SI, packed in HR 5/5 column (50 × 5 mm I.D.). Elution buffer: 0.05 *M* Tris–HCl at pH 7.0 (A), 8.0 (B), 8.5 (C) and 8.6 (D). Flow-rate: 2.0 ml/min. Linear sodium chloride concentration gradient from 0 to 0.5 *M*, in 10 min. Sample: 50 μl of 0.7% PHP.

tioned above, all other buffer systems gave poor resolution at pH below 8.5. This may be due to the fact that at pH below 8.5 most plasma proteins do not have a sufficient negative net charge to interact with Mono Q or Polyanion SI beads electrostatically. These results, therefore, indicated that FPLC of plasma proteins should be carried out at pH 8.6 or higher pH. However, chromatography at pH below 8.5 might be suitable for precise analysis of proteins of lower isoelectric points, such as albumin and transferrin. At pH 8.6, analysis on the Mono Q column gave better resolution and reproducibility compared to the Polyanion SI column. This might be due to the nature of the column packing, because the polyanion SI column, consisting of high-porosity silica, is unstable at high pH. At pH 8.6, any buffer system except Tris-HCl gave unsatisfactory peak resolution. Therefore, the Mono Q column was used with 0.05 A4 Tris–HCl buffer at pH 8.6 in further experiments.

Evaluation of the Mono Q column for albumin and transferrin

HSA and transferrin, which cannot be easily separated by size-exclusion HPLC with TSK-G3000SW columns because their molecular sizes are similar[14], were chosen

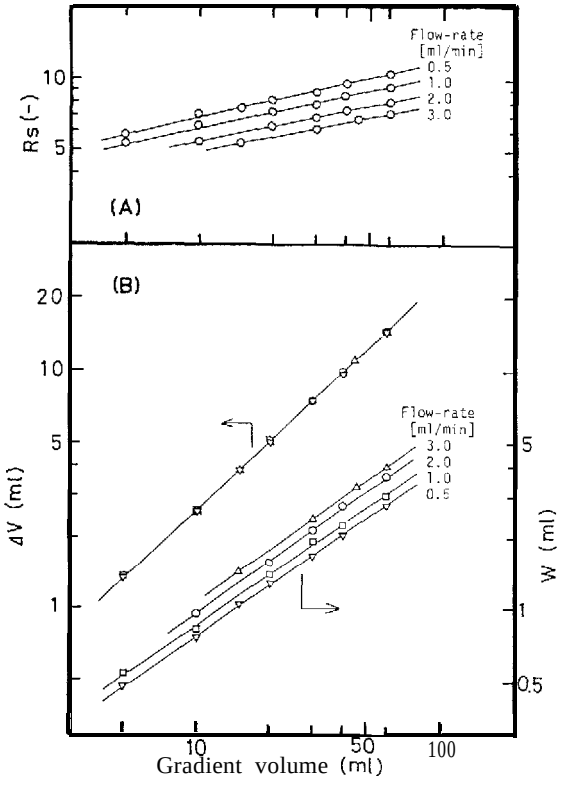

Fig. 3. (A) Effect of the sodium chloride concentration gradient on the resolution of transferrin and albumin peaks at various Bow-rates. (B) Effect of the sodium chloride gradient on the peak distance ΔV and the sum of peak widths ΔW. Column: Mono Q packed in HR 5/5 column (50 × 5 mm I.D.). Eluent: 0.05 M Tris-HCl buffer at pH 8.6. Gradient: linear from 0 to 0.5 M of sodium'chloride. Flow-rates: 0.5, 1.0, 2.0 and 3.0 ml/min. Sample: 50 μl of mixture of 0.2% transferrin and 0.5% albumin.

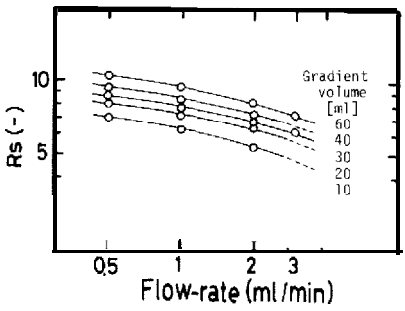

Fig. 4. The effect of flow-rate on the resolution of transferrin and albumin peaks at constant gradient time. Gradient times: 10, 20, 30 and 60 min. Other conditions were the same as in Fig. 3.

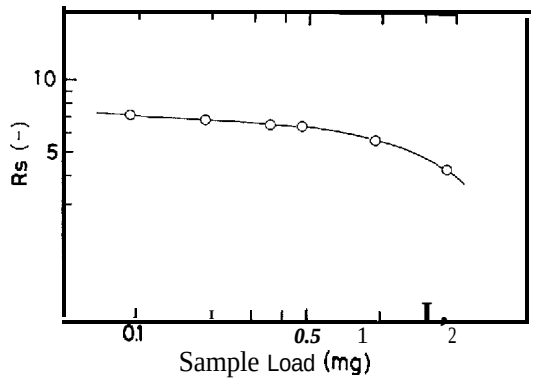

Fig. 5. Effect of sample loading on the resolution of transferrin and albumin peaks. At different concentrations of 0.188, 0.376, 0940, 1.88 and 3.76% (w/v), 50 μl of mixture was applied to the Mono Q column. Flow-rate: 2.0 ml/min. Gradient time: 10 min (= gradient volume of 20 ml).

for the evaluation of the Mono Q anion-exchange column. Chromatographic efficiency was assessed using the resolution R_s(T,A), in eqn. 1 cited above.

Fig. 3A shows the effect of the sodium chloride concentration gradient on the resolution of albumin and transferrin peaks at various flow-rates. The changes of peak distance ΔV ($= V_A - V_T$) and the sum of peak widths ΔW ($= W_T - W_A$) according to gradient volume are indicated in Fig. 3B. It was confirmed from Fig. 3 that at constant flow-rate, R_s(T,A) increased in proportion to (gradient volume)$^{0.24}$, and higher resolution was achieved with lower flow-rates at constant gradient vol-

TABLE I

RESULTS OF COMPONENT ANALYSIS OF FPLC FRACTIONS

Proteins were determined using immuno-electrophoresis. – = Negative, ± = slightly positive, + = positive, + + = strongly positive, + + + = very strongly positive

Protein	*Fraction no*															
	1	2	3	4	*5*	6	7	8	9	*10*		*11*	12	*13*	14	*15*
Albumin	–								±	+	+ + +	+	+	+	–	–
Transferrin	–		+	+ +	+	–	–	–	–	–		–				
Fibrinogen	–	–	–	–						+	–	–				
Ceruloplasmin	–											+				
Haptoglobin	–	–	–	–	–	–	±	+	+	±		–				
α_1-Acid glycoprotein							–	+	+	–		–				
α_1-Antitrypsin	–	–	–	–		–	±	+	±	–		–				
α_1-Lipoprotein	–							+	+	±		–				
α_2-HS Glycoprotein	–	–	–	–	–	–	±	+	+ t			–				
α_2-Macroglobulin	–							±	t	+		–	–	–	–	–
β-Lipoprotein	–							+	t	+		+	±	±	–	–
C3-Component		–	–	–					±	+		+				
Prealbumin	–								–	–		–	–	+	–	–
IgG*	+ + +	+	+	+								–				

* IgG was determined using immunodiffusion method

ume. This is mainly due to the change of the AW value according to the flow-rate of the elution buffer, because ΔV remains constant at different flow-rates when the gradient volume is unchanged as shown in Fig, 3B.

In order to obtain good resolution within a limited time, the effect of flow-rate on resolution was studied at fixed gradient times. The results are shown in Fig. 4. It was confirmed from Fig. 4 that the best resolution was attained with a flow-rate of *ca.* 2 ml/min at fixed gradient times. The maximum resolution thus obtained at fixed gradient times was due to the total effects on R_s(T,A), which increased with gradient volume and decreased with flow-rate at constant gradient volume.

Fig. 5 shows the effect of sample loading on resolution. It can be said from these results that sample loads ranging from 0.1 to 0.5 mg should be applied to the Mono Q column in order to obtain better resolution. Below 0.1 mg sample load, the signal-to-noise ratio increased significantly.

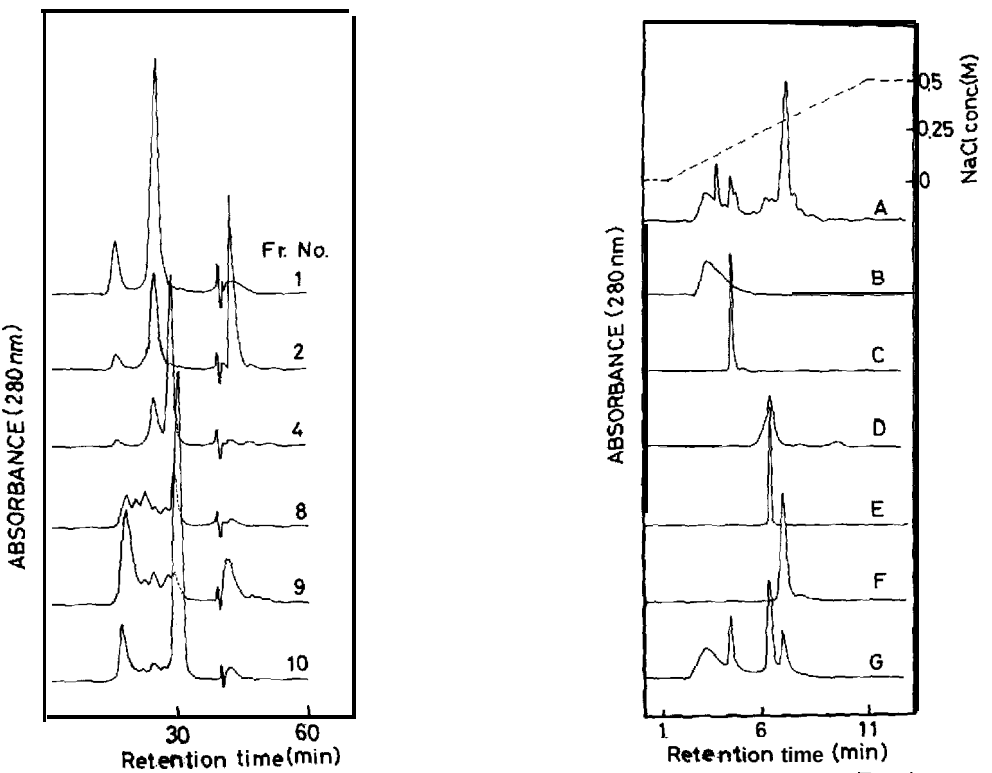

Fig. 6. Size-exclusion WPLC of plasma protein fractions, eluted from the Mono Q column. Fraction (Fr.) numbers correspond to those in Fig. 1E. Two columns (TSK G-3000SW, 600 × 7.5 mm) in tandem. Eluent: 0.05 *M* sodium acetate (pH 5.0) containing 0. I *M* sodium sulphate. Flow-rate: 1 .0 ml/min.

Fig. 7. FPLC anion-exchange chromatograms of purified standard proteins and their mixture on Mono Q column. 0.05 *M* Tris–HCl buffer (pH 8.6) at a Row-rate of 2.0 ml/min and sodium chloride linear gradient time of 10 min. A = Pooled human plasma, B = IgG, C = transferrin, D = fibrinogen, E = anti-thrombin III, G = a mixture of 0.1% IgG + 0.1% albumin + 0.04% transferrin + 0.2% anti-thrombin III.

Fractionation of plasma proteins on the Mono Q column

Fig. 1E shows a typical anion-exchange chromatogram of pooled human plasma on the Mono Q column with 0.05 A4 Tris-HCI buffer (pH 8.6) at a flow-rate of 2 ml/min and a gradient time of 10 min (= gradient volume of *20* ml). The plasma separated into fifteen protein fractions, representing much better resolution than by cellulose acetate membrane electrophoresis. In the latter case the same sample was fractionated into seven fractions such as prealbumin, albumin, α_1-globulin, α_2-globulin, B-globulin, fibrinogen and y-globulin.

Our results thus confirm that high-performance anion-exchange chromatograph on a Mono Q column under the consitions determined here can be applied to the rapid analysis of plasma proteins. The analysis time of about 10 min enables us to perform comparative analyses during therapy. Furthermore, we also expect to apply this technique to the inspection of protein fractionation processes and to the quality control of plasma protein preparations.

Each fraction in Fig. 1E was collected and analysed by immunochemical methods. The protein fractions thus identified are listed in Table I. Generally speaking, proteins having higher isoelectric points were eluted faster from the anion-exchange column. Thus, IgG was first to be eluted (fraction 1), then transferrin (fraction 4). The highest peak of fraction 10 was attributed to albumin. The a-globulins were eluted mainly in fractions 7-10, and the /?-globulins in fractions 8-13.

The second sharp peak (fraction 2) in Fig. 1E is unidentified, but not due to a protein, This was confirmed by size-exclusion HPLC of fraction 2 on TSK-G3000SW columns. As shown in Fig. 6, fraction 2 is a low-molecular-weight component of plasma.

The exact retention volumes of plasma protein components were then deter-

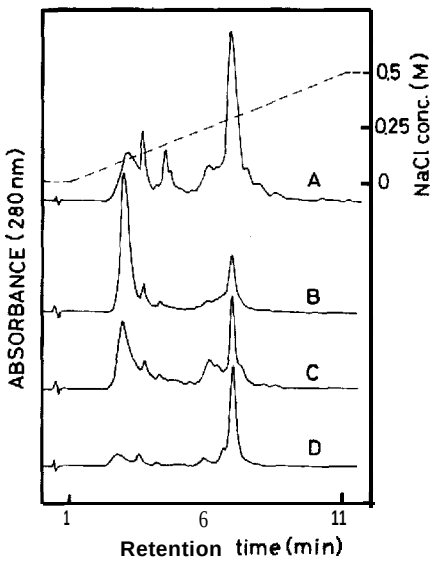

Fig. 8, Analysis of patient plasma on a Mono Q column. Conditions were the same as in Fig. 7. A = pooled human plasma (normal), B = IgG-myeloma patient plasma, C = lung-cancer patient plasma, D = lung-cancer patient plasma after membrane-plasmapheresis therapy.

mined with highly purified proteins. The results are shown in Fig. 7. The elution volume of each protein was not affected by mixing, suggesting that there were no significant interactions among the proteins, and separation mechanisms other than anion-exchange interaction were not important under the present conditions.

Analysis of patient plasma with the Mono Q column

Mono Q column chromatography was applied to the analysis of several plasma samples by using 0.05 *M* Tris–HCl buffer (pH 8.6) and a linear gradient from 0 to 0.5 *M* sodium chloride in 10 min. An abnormal pattern of IgG components was detected in the IgG myeloma plasma (Fig. 8B) as compared with that of normal plasma (Fig. 8A). Fig. 8C shows the chromatogram of plasma from a lung-cancer patient. After treatment of the patient by membrane plasmapheresis therapy, the immunoglobulin fractions decreased in concentration as shown in Fig. 8D, suggesting improvement of the protein profile as a result of the therapy.

ACKNOWLEDGEMENT

We are indebted to Pharmacia Fine Chemicals Japan for the rental of FPLC columns and instruments.

REFERENCES

1 T. D. Schlabach, A. J. Alpert and F. E. Regnier, *Clin. Chem.*, 24 (1978) 1351.
2 A. J. Alpert and F. E. Regnier, *J.* Chromatogr., 185 (1978) 375.
3 0. Mikes, P. Strop, J. Zbrozek and J. Coupec, *J. Chromatogr.*, 119 (1976) 339.
4 S. H. Chang, R. Noel and F. E. Regnier, *Anal. Chem., 48 (1976)* 1839.
5 K. M. Gooding, K.-C. Lu and F. E. Regnier, *J. Chromatogr.*, 164 (1979) 506.
6 G. Vanecke and F. E. Regnier, *Anal. Biochem.*, 121 (1982) 156.
7 J. P. Boissel, H. Wajcman, H. Fabritius, R. Cabannes and D. Labie, *Biochim.* Biophys. *Acta,* 670 (1981) 203.
8 Y. Kato, K. Komiya and T. Hashimoto, *J.* Chromatogr., 246 (1982) 13.
9 0. Mikeš, P. Štrop and J. Sedláčková, *J. Chromatogr., 148 (1978) 237.*
10 A. N. Radhakrishnan, S. Stein, A. Licht, K. A. Gruber and S. Udenfriend, *J. Chromatogr., 132 (1977) 552.*
11 M. Dizaroglu, H. C. Krutzsch and M. G. Simic, *J. Chromatogr., 237 (1982) 417.*
12 S. H. Chang, K. M. Gooding and F. E. Regnier, *J. Chromatogr., 125 (1976) 103.*
13 The Pharmacia *FPLC* System *Catalogue,* Pharmacia, Uppsala, 1982.
14 T. Tomono, S. Yoshida and E. Tokunaga, *J. Polym. Sci. Polym. Left.* Ed., 17 (1979) 335.
15 T. Tomono, T. Suzuki and E. Tokunaga, Biochim. *Biophys. Acta,* 660 (1981) 186.
16 T. Tomono, T. Suzuki and E. Tokunaga, *Anal.* Biochem.. 123 (1982) 394.
17 E. J. Cohn, L. E. Strong, W. L. Hughes, D. J. Mulford, J. N. Melin and H. L. Taylor, *J. Amer.* Chem. *Soc.,* 68 (1946) 459.
18 M. Wickerhauser, C. Williams and J. Mercer, *Vox Sang..* 36 (1979) 281.