Journal of Chromatography, 273 (1983) 107—116
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1532

SEPARATION OF URINE PROTEINS ON THE ANION-EXCHANGE RESIN MONO Q™

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SUMMARY

Proteins excreted in urine due to renal failure were separated on Mono Q™, a new strong anion exchanger designed for fast high-resolution protein separations. The separation procedure was divided into two steps. The first step involved removal of low-molecular-weight substances by rapid desalting on a Sephadex G-25 Superfine column. In the second step, the total protein fraction (3—6 ml) was loaded onto the Mono Q column with the aid of a superloop. The proteins were adsorbed onto the top of the ion-exchanger column and gradually displaced by a combined pH and salt gradient in 40 min. The choice of ion exchanger and initial operating conditions were based on data obtained from electrophoretic titration curve experiments. Identification of separated proteins was achieved by fused rocket electrophoresis and sodium dodecyl sulphate-polyacrylamide gel electrophoresis, respectively.

INTRODUCTION

The identification of serum proteins excreted into the urine in renal failure has traditionally been carried out by electrophoretic techniques such as immuno- or sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis [1]. The major drawback of all electrophoretic methods is their comparatively slow speed and results are generally not available until one day or more after sample delivery. This can be partly compensated by running several parallel samples on the same gel.

The major alternative to electrophoresis is chromatography. Protein chromatography has been used in clinical research for several decades, but has not been recognized as a substitute for electrophoresis in routine clinical chemistry, mainly due to the comparatively low resolution and slow speed

0378-4347/83/0000—0000/$03.00

of operation of conventional chromatography [2]. This situation has been dramatically changed by recent developments in gel materials suitable for high-performance liquid chromatography (HPLC) of proteins [3]. New media with an optimal combination of rigidity, porosity, hydrophilicity and inertness enable complex protein mixtures in urine to be separated in an hour or less. The patterns of the various absorbance peaks as they elute can provide important information on the likely pathophysiological state of the urinary tract. HPLC is not only faster than electrophoresis, but also allows fractions to be collected for subsequent multiparametric analysis of the separated proteins.

Recently Pharmacia Fine Chemicals has introduced a new hydrophilic, polyether resin with a narrower particle size distribution (9.8 ± 2%): Mono Q (containing $CH_2N^+(CH_3)_3$ groups) which appears suitable for solving the above problem.

MATERIALS AND METHODS

Materials

Materials were obtained from the following sources: Sephadex G-25 Superfine, Mono Q HR 5/5 prepacked columns, PD-10 columns, Pharmalyte 3—10, Agarose A, gradient gels PAA 4/30 and antibodies against κ-light chains, λ-light chains, IgG, transferrin, albumin, α_1-antitrypsin and whole blood serum from Pharmacia Fine Chemicals (Uppsala, Sweden); Bis Tris propane chloride (1,3-bis[tris(hydroxymethyl)-methylamino]-propane) from Sigma (St. Louis, MO, U.S.A.); antibodies against β_2-microglobulin, retinol-binding protein and α_1-acid glycoprotein from Dako (Copenhagen, Denmark).

Buffer solutions

Standard starting buffer for the ion-exchange chromatography step was Bis Tris propane chloride (6.25 m*M*) adjusted to pH 7.5 with hydrochloric acid. Standard limit buffer was Bis Tris propane chloride (6.25 m*M*) adjusted to pH 9.5 with hydrochloric acid with the addition of sodium chloride (0.35 *M*). In initial experiments the buffer salt, pH and ionic strength of the starting and limiting buffers were varied systematically to determine the optimum separation conditions.

Equipment

All chromatographic runs were performed using an FPLC System from Pharmacia Fine Chemicals. This system avoids contact of the sample and eluent with stainless steel. The use of standard HPLC equipment had to be abandoned due to rapid corrosion of the pumps at high pH in the presence of salt and subsequent degradation of column performance.

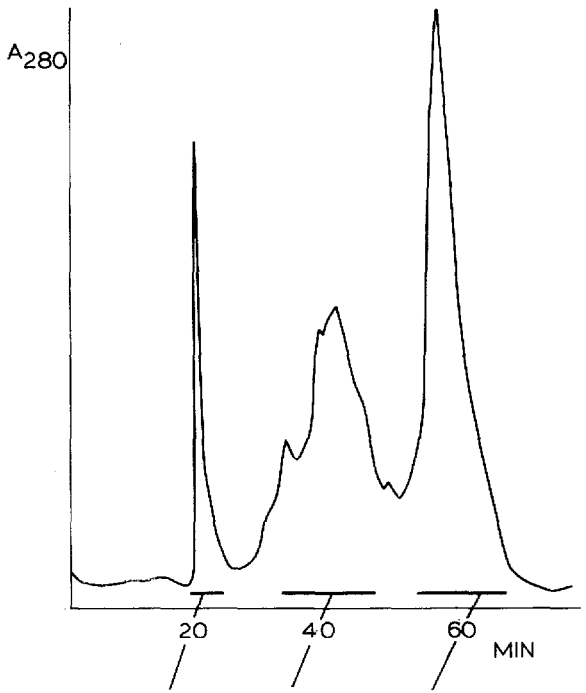
Electrophoretic techniques

SDS-polyacrylamide gradient electrophoresis and fused rocket immunoelectrophoresis were carried out by standard methods [4]. Electrophoretic titration curves were performed by the method of Rosengren et al. [5] using Pharmalyte 3—10 in place of Ampholine 3.5—10.

RESULTS AND DISCUSSION

Removal of low-molecular-weight components

To free the proteins from the low-molecular-weight salts and degradation products in urine, we used a rapid desalting step on Sephadex G-25 Superfine. The gel was swollen for 1 h in the starting buffer for the ion-exchange step



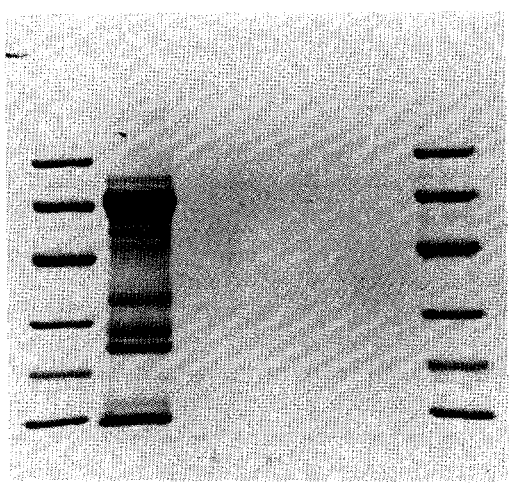


Fig. 1. Gel chromatography of urine (0.5 ml) on Sephadex G-25 Superfine. Column C 16/40, bed height 34 cm. Freeze-dried, pooled fractions were examined by SDS-electrophoresis. The total protein content elutes in the first peak. The outside lines are low molecular weight marker proteins.

and slurry-packed into a C 16/40 column at a flow-rate of 5 ml/min. Centrifuged urine (0.5 ml) was applied to the column and eluted at a flow-rate of 1.35 ml/min. The purified protein fraction eluted after 20 min in a volume of 3—6 ml depending on the amount of protein present (see Fig. 1). The recovery of proteins from this step was > 90%.

Prediction of the optimal separation conditions for ion-exchange chromatography

In ion-exchange chromatography of molecules with a very high net charge, e.g. proteins, there is a correlation between the ionic strength needed for elution and the net charge of the protein [6]. Since the net charge on a protein, and consequently the ionic strength at which it will be eluted, varies with pH as the ionogenic groups on the protein are titrated, titration curves are of great assistance in predicting the elution pattern over a range of pH. The titration curves of proteins in a complex mixture can be determined by an electrophoretic technique of Rosengren et al. [5].

Fig. 2 shows a schematic of electrophoretic titration curves for a mixture of urine proteins. To identify the curves for the individual proteins, immunoprints were obtained by placing acetate paper wetted with the corresponding antibody on the separation gel. The strips were then stained with Coomassie Blue-R. Fig. 3 shows examples of the immunoprints for β_2-microglobulin and albumin. As can be seen from the schematic titration curves in Fig. 2, the biggest differences in charge between the four main proteins in this pathological urine (β_2-microglobulin, retinol-binding protein, α_1-acid glycoprotein and albumin) are found in the basic region, pH 7—10. The optimum pH for separation of these major components should, therefore, be within this pH range.

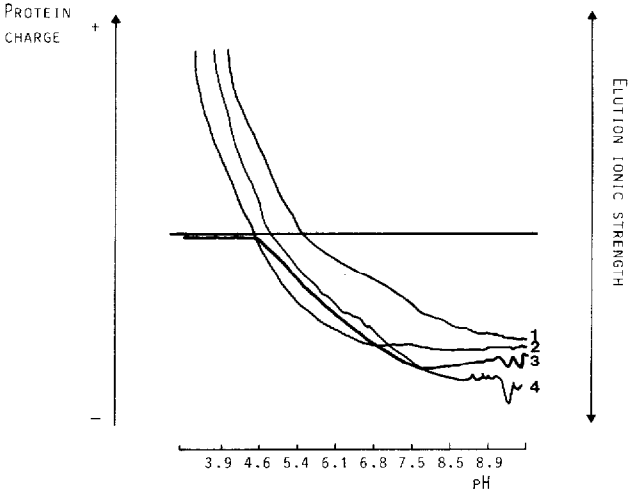
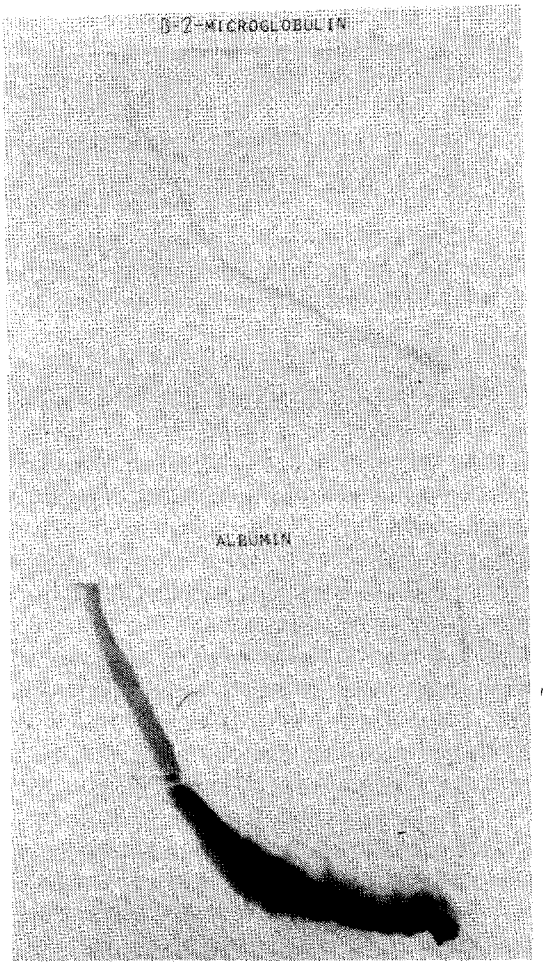


Fig. 2. Schematic electrophoretic titration curves. The numbered curves are (1) β_2-microglobulin, (2) retinol-binding protein, (3) α_1-acid glycoprotein, (4) albumin.



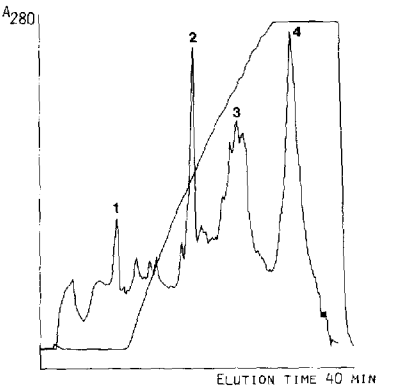


Fig. 3. Immunoprints from the electrophoretic titration curves.

Fig. 4. The elution of β_2-microglobulin (peak 1) at an ionic strength of 12.5 m*M* Bis Tris propane (pH gradient from 7.5 to 9.5, sodium chloride gradient to 0.35 *M*). Compare to Fig. 8.

Ionic strength and pH of the starting buffer

The low ionic strength of the starting buffer for the ion-exchange separation is necessitated by the elution characteristics of β_2-microglobulin. The elution of this protein is very sensitive to both the pH and the ionic strength if a sharp peak is required. At a lower pH than 7.5, it is not retarded on the column and elutes in the front. At higher pH values, β_2-microglobulin tends to elute as a broad peak with lower separation efficiency as a result. A higher starting ionic strength than 6.25 m*M* also results in a broader peak. Fig. 4 shows β_2-microglobulin eluting in 12.5 m*M* Bis Tris propane pH 7.5 as starting buffer.

Optimization of pH

Separations at three different pH values in the range 7—10 with the same gradient of sodium chloride concentration are shown in Figs. 5—7. At pH 7.5, there is optimal resolution between β_2-microglobulin and retinol-binding protein and no separation between α_1-acid glycoprotein and albumin. The optimal resolution for these two proteins is at pH 9.5. To obtain good resolu-

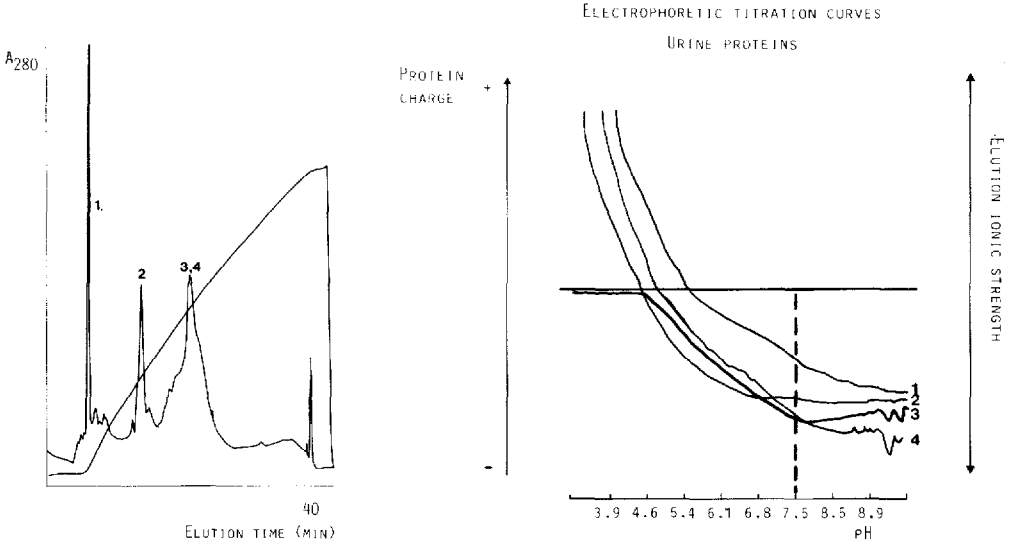


Fig. 5. Separation of urine proteins at pH 7.5 (6.5 m*M* Bis Tris propane, sodium chloride gradient to 0.35 *M*). Injection volume 3 ml. The dashed line on the titration curve indicates the charge differences between the four proteins at the pH of chromatography. Compare Figs. 6—8. Peak identification as in Fig. 2.

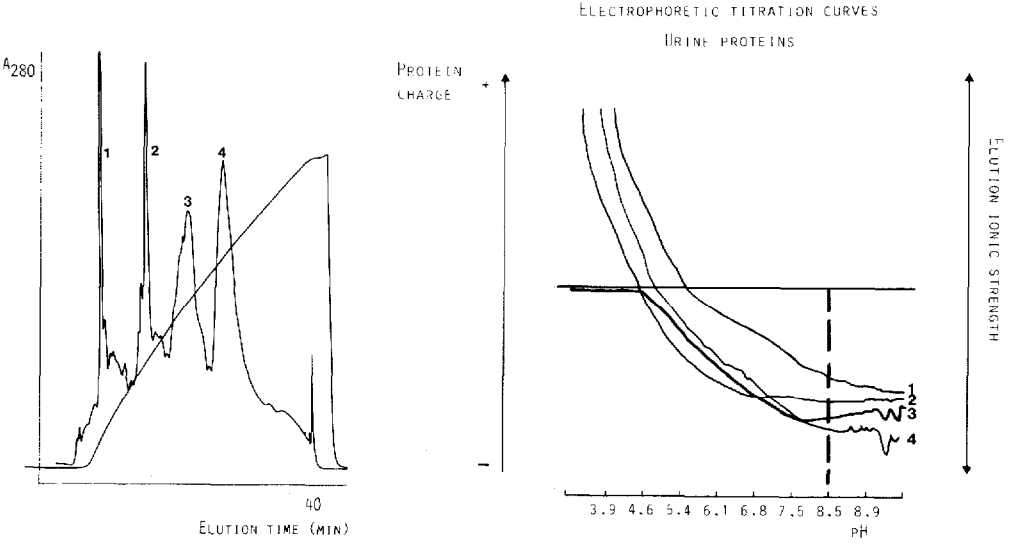


Fig. 6. Separation of urine proteins at pH 8.5 (gradient as in Fig. 5). Injection volume 3.5 ml. Peak identification as in Fig. 2.

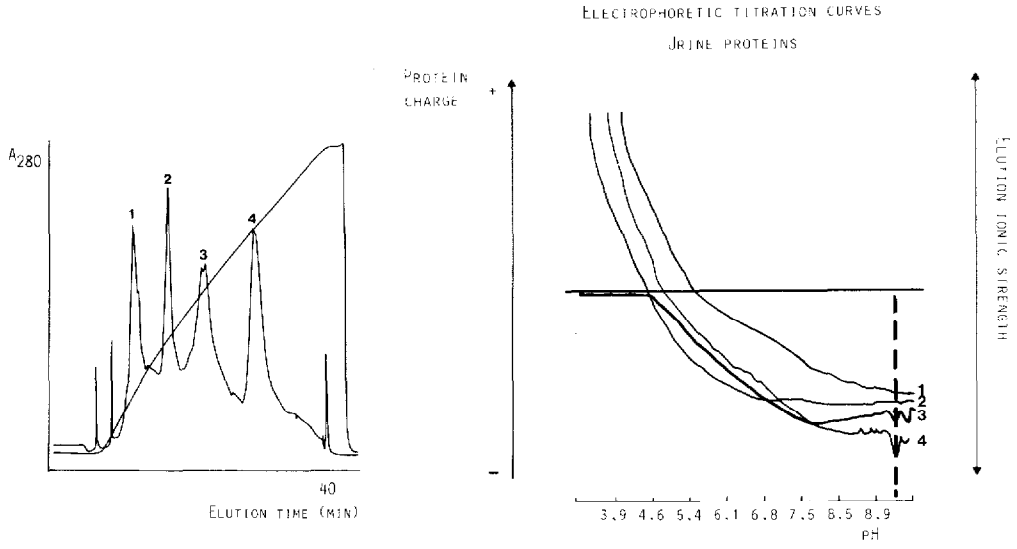


Fig. 7. Separation of urine proteins at pH 9.5 (gradient as in Fig. 5). Injection volume 3 ml. Peak identification as in Fig. 2.

tion between all four proteins, a combined pH (7.5—9.5) and salt gradient (0—0.35 *M* sodium chloride) was used in Fig. 8. Bis Tris propane with pK_a 6.8 and 9.0 gives a linear pH gradient between 7.5 and 9.5 [7]. It is important that the buffer has the same ionic strength at both the start pH and at the final pH in the absence of added salt otherwise a concave or convex pH gradient will form.

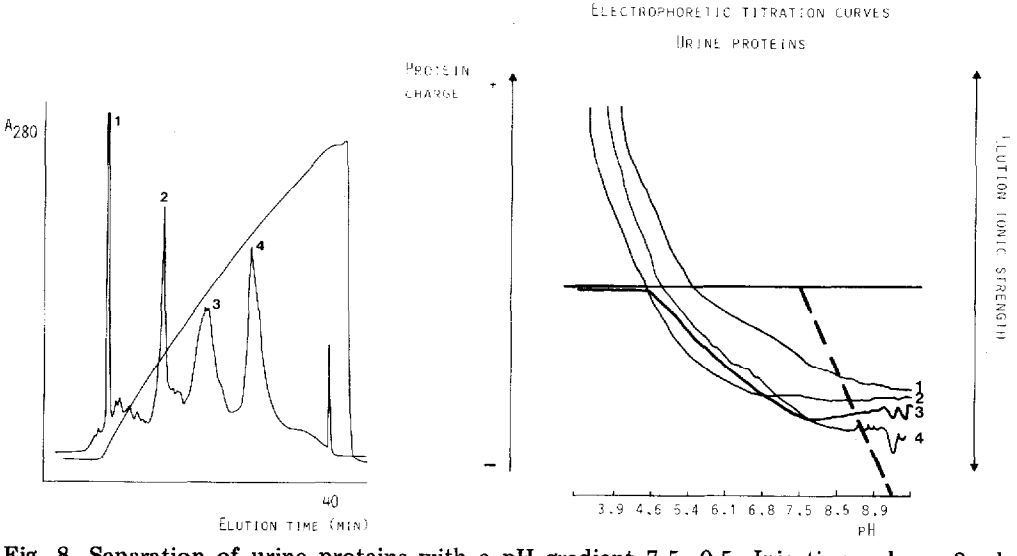
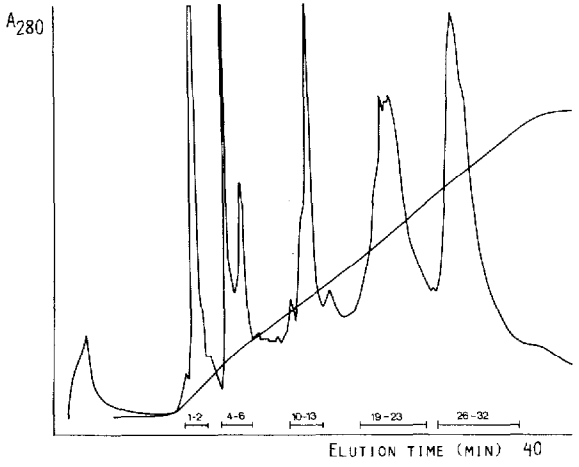


Fig. 8. Separation of urine proteins with a pH gradient 7.5—9.5. Injection volume 3 ml. Peak identification as in Fig. 2.

Identification of the individual proteins

Fig. 9 shows a typical chromatogram for urine excreted in renal disease. Fractions (1 ml) were collected, desalted on a PD-10 column, concentrated



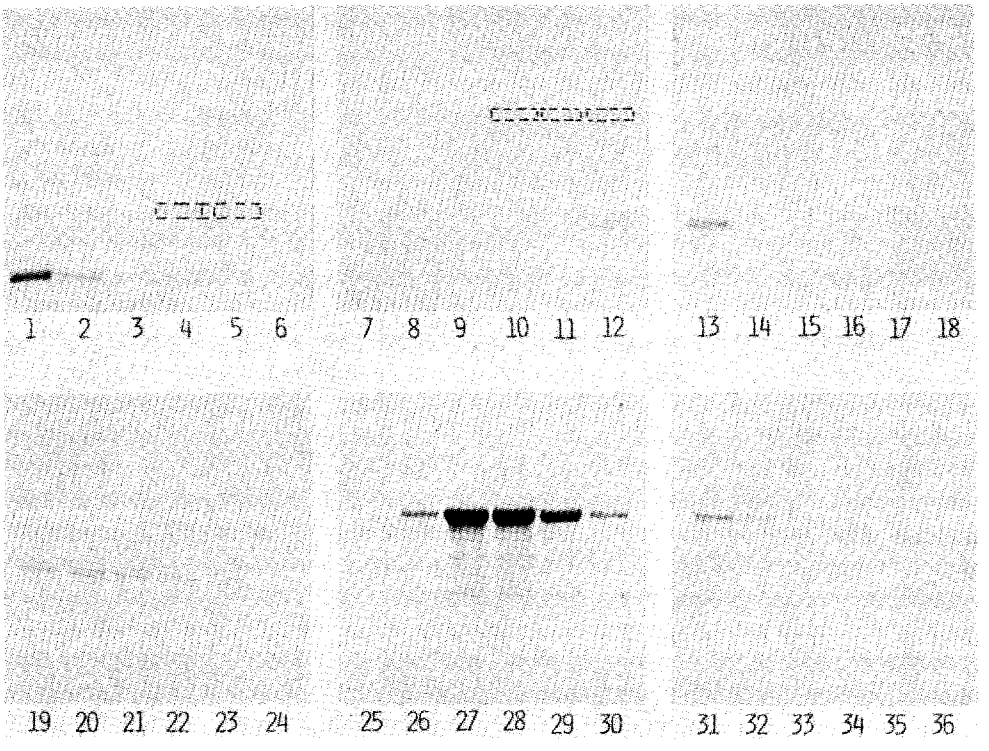
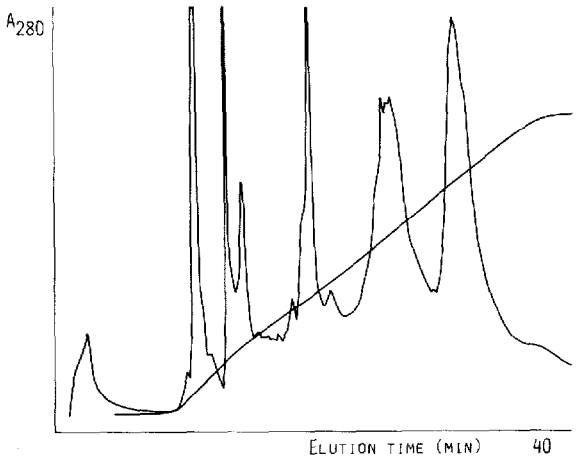


Fig. 9. Urine from chronic pyelonephritis on Mono Q column, 1-ml fractions analysed by SDS-gel electrophoresis. Lanes 1, 2, 4, 5: β_2-microglobulin. Lanes 4, 5: κ- and λ-chains. Lanes 10—12: transferrin. Lanes 12, 13: retinol-binding protein. Lanes 18—21: α_1-microglobulin. Lanes 19—23: α_1-acid glycoprotein. Lanes 20—24: α_1-antitrypsin. Lanes 26—32: albumin.



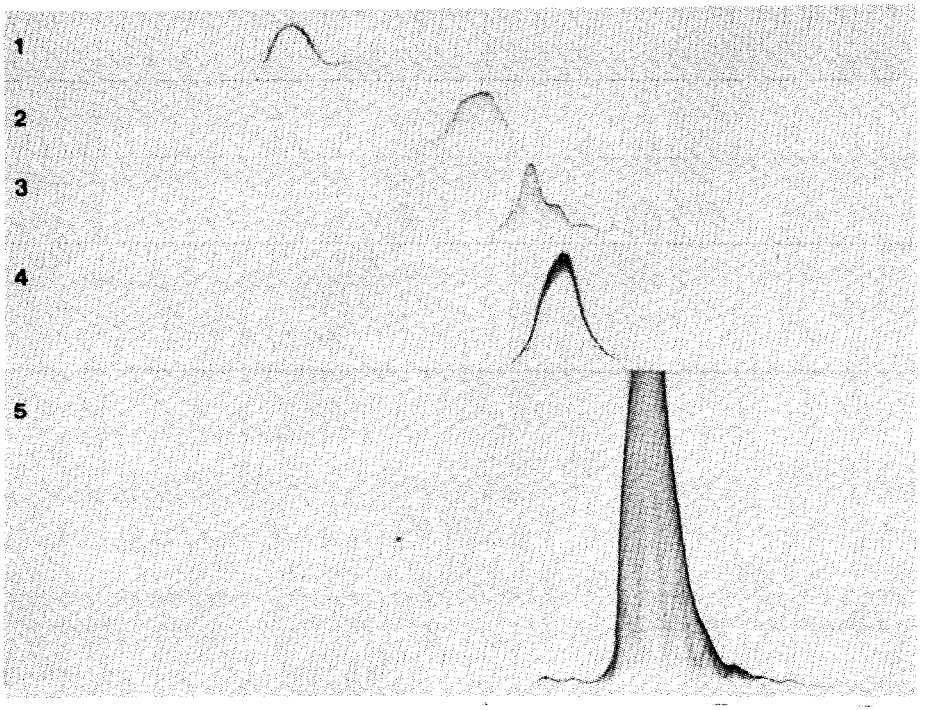


Fig. 10. Urine from chronic pyelonephritis on Mono Q column, 1-ml fractions analysed by fused rocket immunoelectrophoresis. 1 = β_2-microglobulin, 2 = transferrin, 3 = α_1-antitrypsin, 4 = α_1-acid glycoprotein, 5 = albumin.

by freeze-drying and dissolved in aliquots for analysis by SDS-polyacrylamide gel electrophoresis and fused rocket immunoelectrophoresis (Figs. 9 and 10).

Injection volume and lowest detectable limit

Since the efficiency of ion-exchange chromatography is limited by the total protein load and not by the injection volume, large volumes of dilute

protein solution can be concentrated on the column. The way to increase the sensitivity of detection for this application is thus to pool the protein fractions from a number of gel filtration runs and inject the pooled fractions directly onto the Mono Q column via a superloop.

CONCLUSIONS

Several major protein peaks can be resolved by two-step chromatography of urine. Immunochemical analysis and SDS-polyacrylamide gel electrophoresis have shown that each peak contains several proteins usually with one predominant or characteristic single protein.

Clinical investigations by one of us (E.H.C.) indicate that the peak profiles can be correlated to certain pathophysiological changes in renal function. The optimization technique is being applied to obtain maximum separation between protein fractions that are of major interest in different forms of proteinuria. The system can provide a basis for the separation of individual proteins as well as reveal information about the relative concentration of a wide variety of plasma proteins in the urine.

ACKNOWLEDGEMENTS

The authors wish to thank General Manager Jan Rosengren and Dr. Klaus Hiesche, Pharmacia Diagnostic AB, for initial support and valuable discussions. We also gratefully acknowledge the valuable comments on the manuscript provided by Dr. John Brewer and Dr. Jan-Christer Janson.

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