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URINARY PROTEIN ISOLATION BY HIGH-PERFORMANCE ION-EXCHANGE CHROMATOGRAPHY

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SUMMARY

Proteins excreted in urine, following renal failure, were analysed by high-performance anion-exchange chromatography and chromatofocusing. The analysis involved three steps: (1) removal of the low-molecular-weight fraction by rapid desalting, (2) anion exchange of the high-molecular-weight fraction by using combined salt and pH gradients and (3) further separation of the main peaks by chromatofocusing.

The selection of the column and conditions were based on data obtained from electrophoretic titration curves. The purity of selected peaks was evaluated by sodium dodecyl sulphate polyacrylamide gel electrophoresis.

INTRODUCTION

The level of various proteins in urine and its relation to renal function is becoming an area of significant clinical interest. Various techniques have been used to characterize urinary proteins, and the location of renal lesions has been characterized by molecular weight distributions of urinary proteins[1]. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) has been used in the diagnosis of kidney disease[2,3]. Most recently, high-performance ion-exchange chromatography (HPIEC) has been found to be a valuable tool for urinary protein analyses[4].

In this work, we have investigated the ability of HPIEC to separate the proteins in urine into single components. HPIEC columns packed with 10-μm monobeads of a hydrophilic polymer (MonoBeads Q and P; Pharmacia, Uppsala, Sweden) were used. Mono Q is a strong anion exchanger (quaternary amino function); Mono P is a weak anion exchanger derivatized with groups having different buffering capacities. The Mono P column separates the proteins on the basis of their isoelectric point (p*I*)[6].

Our investigation deals only with the separation parameters and not with the clinical significance of this work. As a result, the experimental procedures and conditions are the most valuable information supplied by this paper.

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EXPERIMENTAL

Apparatus

A Pharmacia Fast Protein Liquid Chromatography (FPLC) system was used, consisting of two P-500 pumps, a V-7 valve injector with a 10-ml Superloop, a GP-250 gradient programmer, a UV-1 monitor with an HR 10 cell, a UV-2 monitor, a FRAC-100 fraction collector and an REC-482 recorder. Sephadex G-25 SF packed in a 340 × 16 cm I.D. column was used to remove the low-molecular-weight (LMW) (< 5000) compounds from the urine. Anion-exchange chromatography was performed on a Mono Q column (50 × 5 mm I.D.). A Mono P column (200 × 5 mm I.D.) was used for the chromatofocusing separations.

SDS-PAGE was carried out on a vertical gel electrophoresis apparatus. The electrophoretic titration curves used for predicting separation conditions were carried out according to Rosengren[5] on a Pharmacia IEF flat-bed system.

Reagents

The buffers were obtained from Sigma (St. Louis, MO, U.S.A.) and all salts from Fluka (Buchs, Switzerland). Polybuffer 74, gradient gels PAA 4/30, Pharmalytes 3–10, Agarose A and LMW protein marker kits were obtained from Pharmacia.

Samples

Urine samples were obtained from a patient with chronic pyelonephritis. The samples were preserved with sodium azide (0.3 g/l).

Chromatography

Rapid desalting was performed in order to separate the high-molecular-weight (HWM) proteins from the LMW components in urine (Fig. 1). Sephadex G-25 SF was swollen for 1 h in 10 m*M* Bis–Tris propane (pH 7.5) adjusted with hydrochloric acid and then slurry-packed into a Pharmacia C 16/40 column at a flow-rate of 5 ml/min. Centrifuged urine was applied to the column and the HMW protein fraction eluted after 20 min in a volume of 6 ml.

Anion-exchange chromatography was used to separate the proteins in peak 1 from the gel filtration step on Sephadex G-25 SF. The SDS-PAGE results for each of the fractions are given in ref. 4.

Fractions containing the main peaks from the Mono Q run were desalted on Pharmacia PD-10 columns and re-equilibrated with starting buffer of the appropriate pH. Samples applied to the Mono P column varied in size from 3.5 to 10 ml, depending on the volume of the fraction from the Mono Q run. Samples were applied to the column in starting buffer and then eluted isocratically with the eluent (Figs. 5–9).

SDS-PAGE results accompany each chromatofocusing separation. The technique was performed according to ref. 7. Lane 1 shows proteins from the LMW protein marker kit which consists of (1) phosphorylase *b* (94,000), (2) albumin (67,000), (3) ovalbumin (43,000), (4) carbonic anhydrase (30,000), (5) trypsin inhibitor (20,000) and (6) α-lactalbumin (14,000). The other lanes are fractions from the main peaks under which they are located.

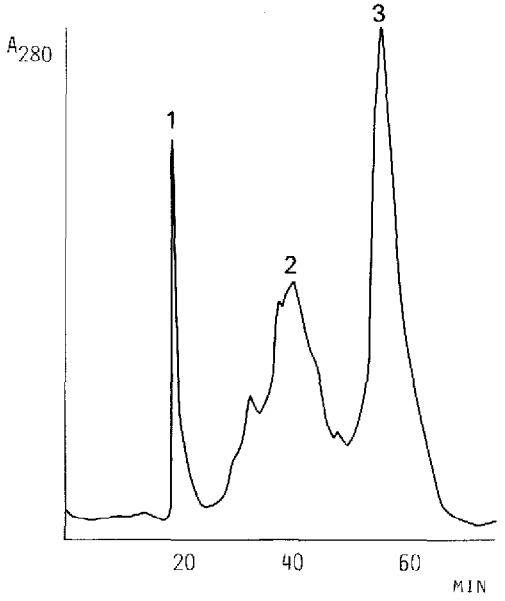


Fig. 1. Chromatogram of desalting step, performed on Sephadex G-25 SF. Chromatographic conditions: column, Sephadex G-25 SF packed in a C 16/40 column, 1.6 × 34 cm; eluent, 10 m*M* Bis–Tris propane (pH 7.5) with HCl; flow-rate, 1.35 ml/min; sample, 0.5 ml of centrifuged urine; detection, 280 nm; 0.2 a.u.f.s.

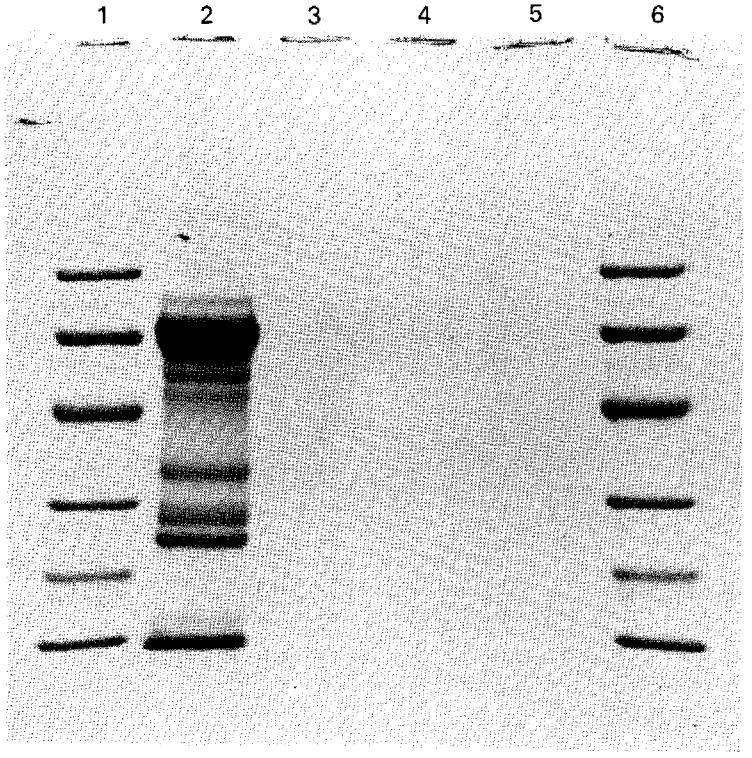


Fig. 2. SDS-PAGE of fractions from the desalting step in Fig. 1. Lanes 1 and 6 are LMW standards; 2 is peak 1; 3, 4 and 5 are fractions from peaks 2 and 3.

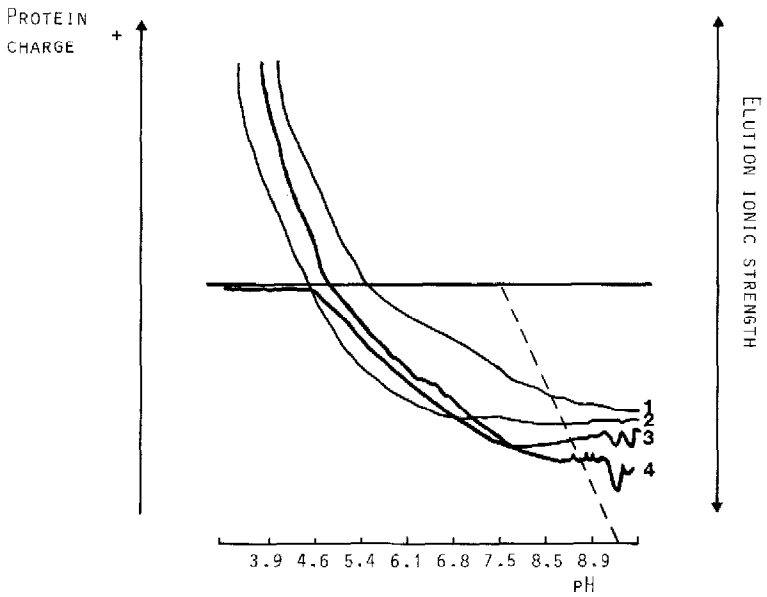


Fig. 3. Schematic representation of an immunoprint from the electrophoretic titration curve of urine from a patient with chronic pyelonephritis. 1 = β-2-Microglobulin; 2 = retinol-binding protein; 3 = α_1-acid glycoprotein; 4 = albumin.

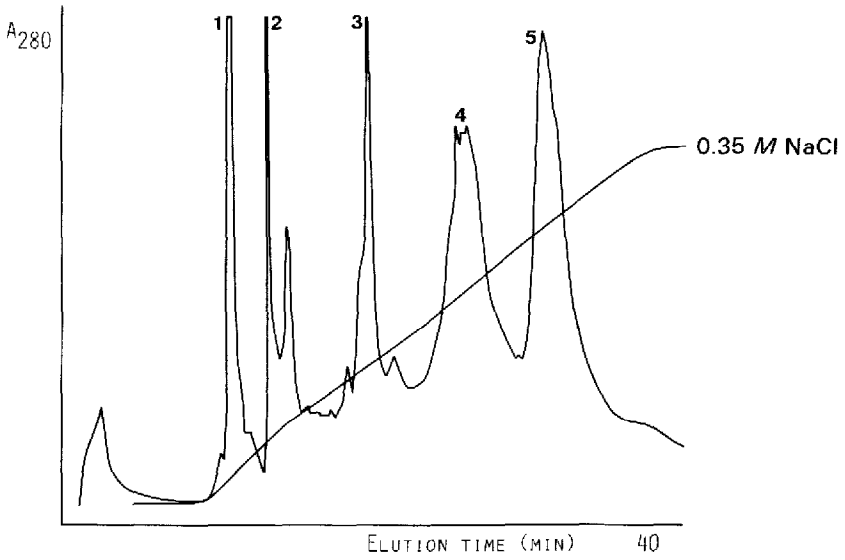


Fig. 4. Anion-exchange separation, performed on Mono Q HR 5/5. Peak numbers correspond to the following proteins: 1 = β-2-microglobulin; 2 = K-λ-light chain; 3 = transferrin and retinol binding protein; 4 = α_1-microglobulin, α_1-antitrypsin, α_1-acid glycoprotein; 5 = albumin. Electrophoretic analyses are given in ref. 4. Chromatographic conditions: column, Mono Q prepacked HR 5/5; buffer A, 10 m*M* Bis–Tris propane (pH 7.5) with HCl; buffer B, 10 m*M* Bis–Tris propane (pH 9.5) with HCl plus 0.35 *M* NaCl; gradient, 0 to 100% B in 40 ml; flow-rate, 1.0 ml/min; sample, 6 ml of HMW fraction from the gel chromatography step; detection, 280 nm; 0.05 a.u.f.s.

RESULTS AND DISCUSSION

SDS-PAGE showed that all proteins, detectable by staining with Coomassie Blue were collected in the first fraction from the gel filtration column (Fig. 2). Fig. 3 explains the selection of the optimal conditions for the anion-exchange experiment.

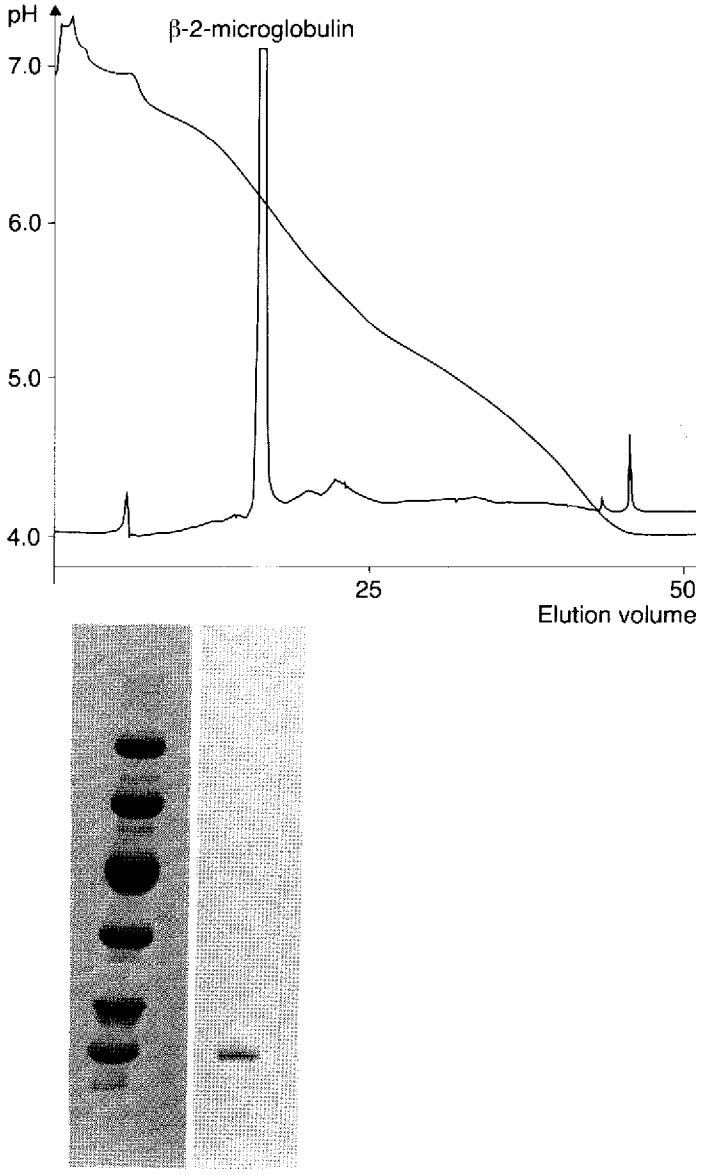


Fig. 5. Chromatofocusing profile from Mono P HR 5/20 chromatography and SDS-PAGE of β-2-microglobulin. Chromatographic conditions: column, Mono P HR 5/20; starting buffer, 25 m*M* Bis–Tris (pH 7.1) with iminodiacetic acid (saturated); eluent, Polybuffer 74 (1:10) (pH 4.0) with iminodiacetic acid; flow-rate, 1.0 ml/min; sample, variable; detection, 280 nm; 0.02 or 0.05 a.u.f.s.

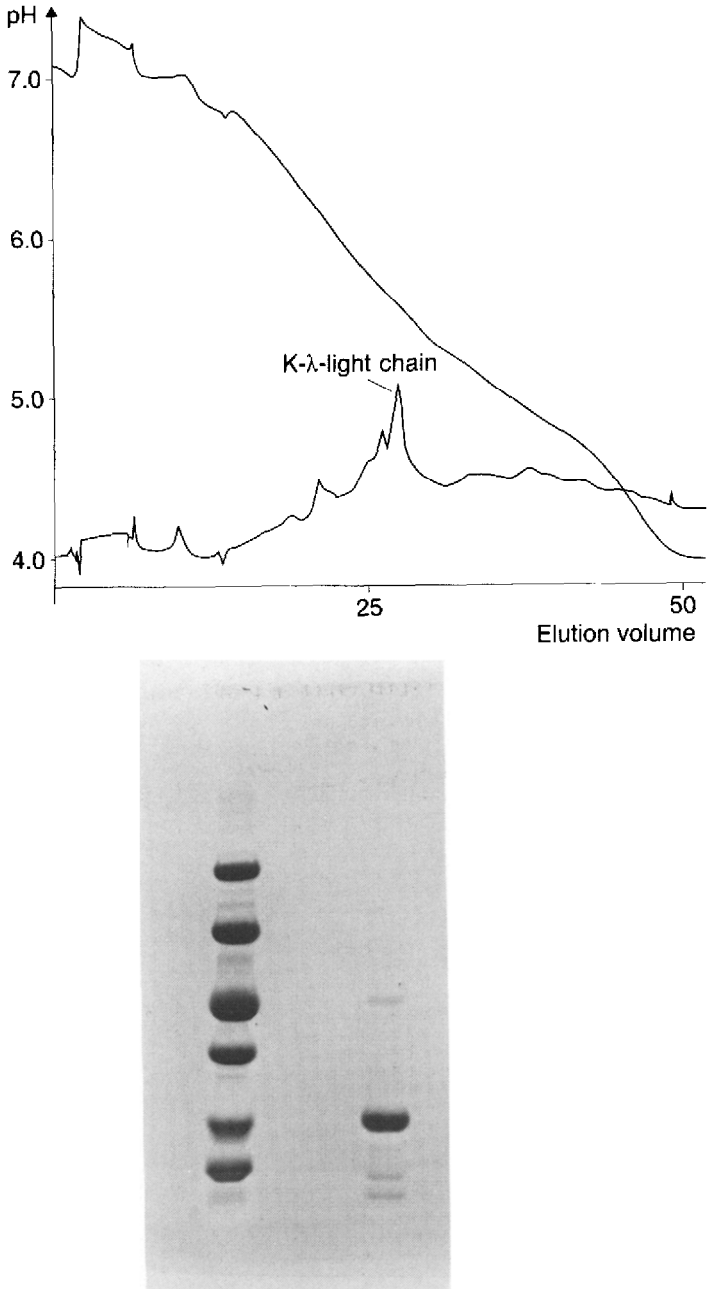
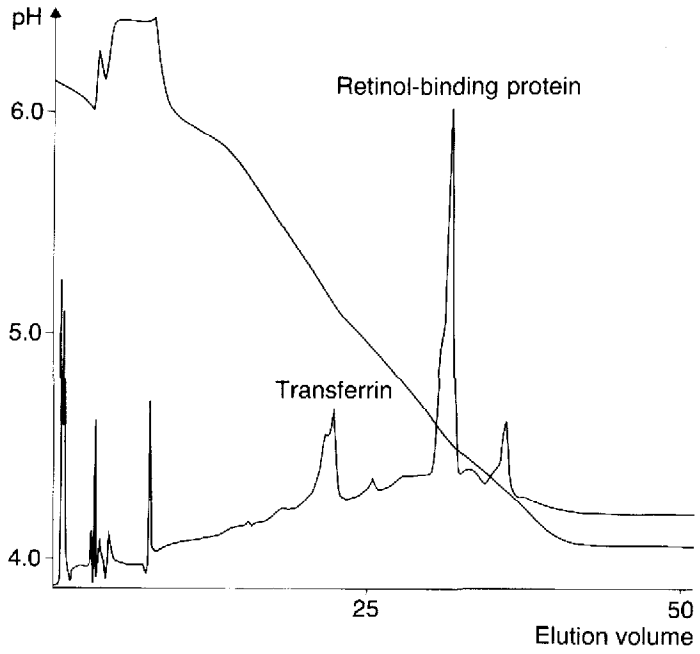


Fig. 6. Chromatofocusing profile from Mono P HR 5/20 chromatography and SDS-PAGE of the K-λ-light chain peak. For chromatographic conditions, see Fig. 5.



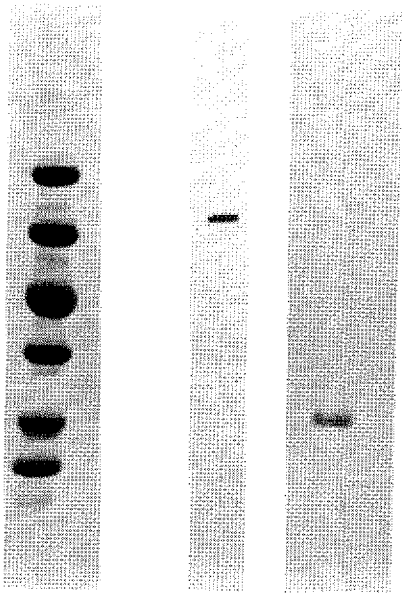


Fig. 7. Chromatofocusing profile from Mono P HR 5/20 chromatography and SDS-PAGE of retinol binding protein. Chromatographic conditions: column, Mono P HR 5/20; starting buffer, 25 m*M* Bis–Tris propane (pH 6.3) with HCl; eluent, Polybuffer 74 (1:10) (pH 4.0) with HCl; flow-rate, 1.0 ml/min; sample, variable; detection, 280 nm; 0.02 or 0.05 a.u.f.s.

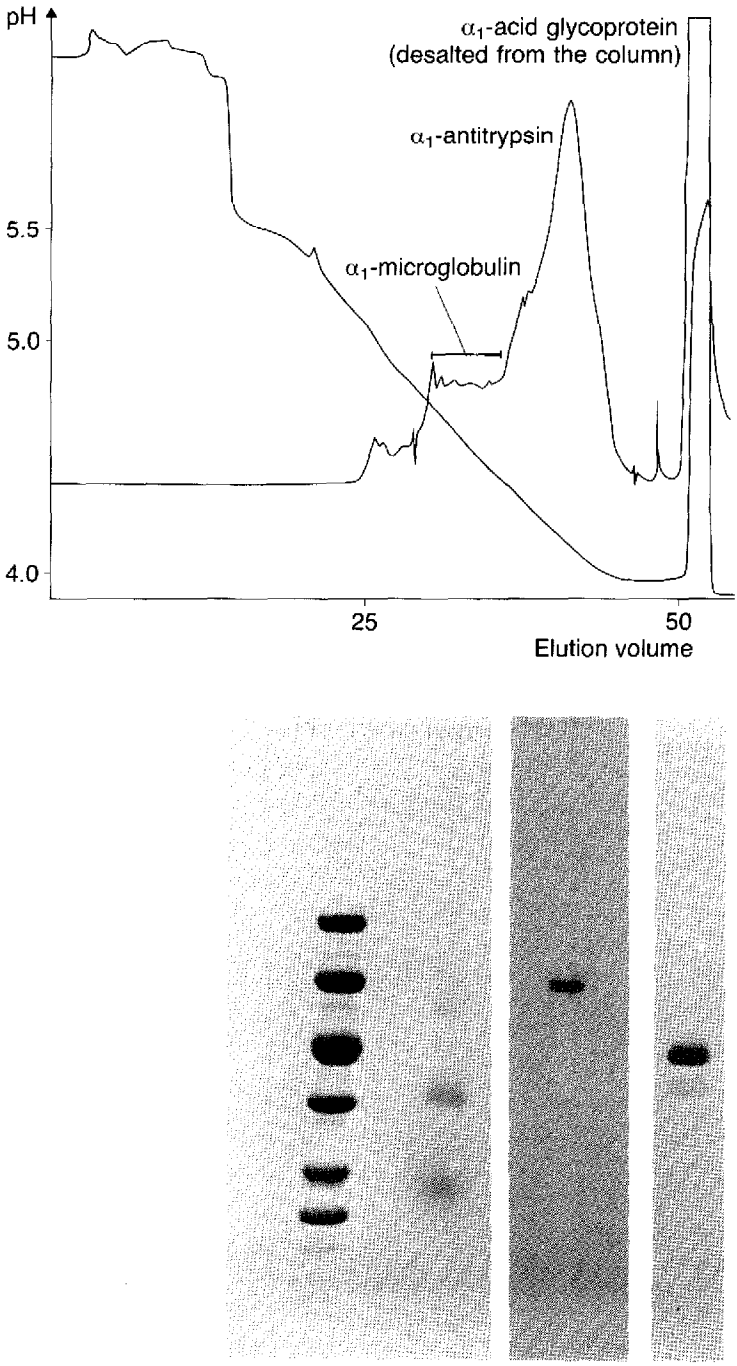


Fig. 8. (A) Elution pattern from the chromatofocusing separation performed on Mono P HR 5/20. For chromatographic conditions, see Fig. 7. (B) SDS-PAGE of (from left to right) α_1-microglobulin, α_1-antitrypsin and α_1-acid glycoprotein peaks.

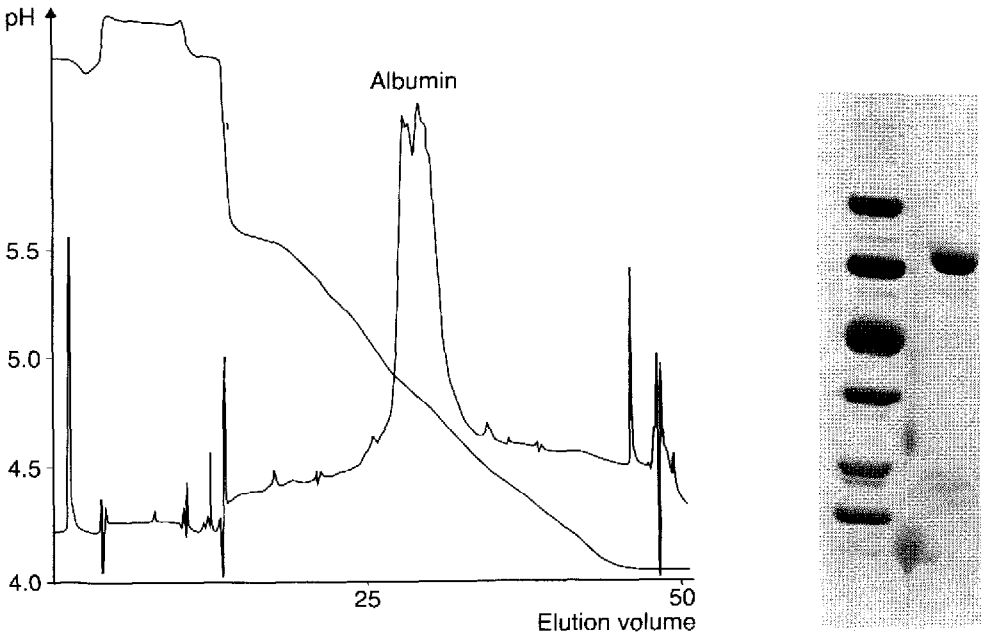


Fig. 9. Elution pattern from the chromatofocusing separation performed on Mono P HR 5/20 and SDS of the albumin peaks. For chromatographic conditions, see Fig. 7.

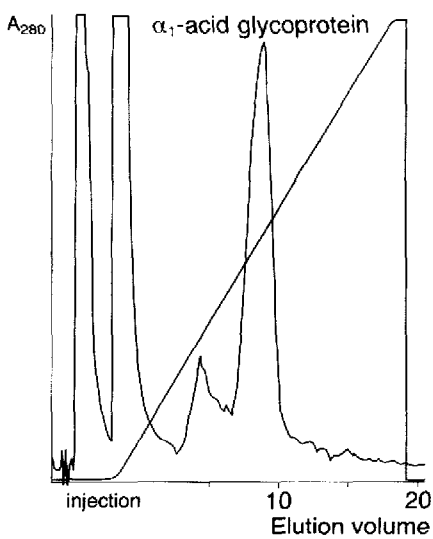


Fig. 10. Chromatogram of the anion-exchange separation of α_1-acid glycoprotein performed on Mono Q HR 5/5. Chromatographic conditions: column, Mono Q; buffer A, 20 m*M* 4-(2-hydroxyethyl)-1-piperazinepropanesulphonic acid (EPPS) (pH 3.8) with HCl; buffer B, 20 m*M* EPPS (pH 3.8) with HCl and 0.35 *M* NaCl; gradient, 0 to 100% in 20 ml; flow-rate, 1 ml/min; sample, 2.5 ml; detection, 280 nm; 0.1 a.u.f.s.

Because Mono Q is a strong anion exchanger (no buffering capacity), it is possible to use a pH gradient together with a salt gradient to obtain an optimal separation. Note that the elution sequence on Mono Q (Fig. 4) was predictable from the electrophoretic mobility of the proteins, as seen on the electrophoretic titration curve (Fig. 3). Analysis by SDS-PAGE of the fractions collected from the anion-exchange separation is shown in ref. 4. As those results showed that every peak consisted of more than one protein, further purification on Mono P was attempted. In this step, the separation occurred close to the isoelectric points of the various peak components. The selection of the pH interval was based on the p*I* of the main component of each fraction. The p*I* of the protein of interest should lie somewhere in the middle third of the interval used. The experiments represented in Figs. 5–9 were performed on Mono P at various pH intervals. The SDS-PAGE results showed that further separation was achieved by chromatofocusing.

The α_1-acid glycoprotein was eluted from the Mono P column with a salt gradient after the pH gradient was completed. This particular separation is more suitably performed on Mono Q with low pH buffer as in Fig. 10.

CONCLUSION

HPIEC could prove to be as valuable as gel chromatography and SDS-PAGE in the clinical analyses of urine. As the resolving power of ion-exchange chromatography is greater than that of gel chromatography[1], GPC and HPIEC should perhaps be used as complementary techniques. Using an electrophoretic titration curve to predict the pH gradient for optimal separation proved beneficial. Further correlation studies need to be completed before clinical significance can be attributed to these results. The chromatographic conditions and the overall separation scheme are useful as model systems.

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