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URINARY PROTEIN PROFILING BY HIGH-PERFORMANCE GEL PERMEATION CHROMATOGRAPHY

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SUMMARY

We describe a new application of high-performance aqueous gel permeation chromatography for the analysis of human proteinuria. Separations of urinary proteins from normal subjects and patients with renal impairment were performed with TSK G 3000 SW columns. The effects of pH and ionic strength of the eluent on the separation of urinary proteins were investigated. Albumins were selectively separated from urine by affinity chromatography on Blue Sepharose CL-6B. According to the results of clinical investigations, urinary protein pattern derived from gel permeation chromatography revealed a good prediction of the site of renal involvement. Predominant excretion of proteins with lower molecular weight than albumin correlated with tubular damage. Albumin and higher molecular weight protein patterns were associated with glomerular disease. Absorbance measurements of the eluent at 280 nm were used for quantitative determination of total urinary protein. Gel permeation chromatography was compared to sodium dodecyl sulfate—polyacrylamide gel electrophoresis and the resulting protein patterns are in good agreement.

INTRODUCTION

Proteinuria (the excretion of proteins in excess of 100—200 mg/day) usually signifies either increased permeability of the glomerular capillary membrane and/or diminished tubular reabsorption. Different localizations of renal lesions are characterised by different molecular weight distributions of the urinary proteins. Therefore methods based on separation according to molecular size should be used for the characterisation of the urinary protein patterns. Sodium dodecyl sulfate—polyacrylamide gel electrophoresis (SDS—PAGE) has been proved to be a valuable tool in the diagnosis of kidney diseases [1—5]. First attempts with gel chromatography on Sephadex were too time-consuming for routine laboratory use [6]. Recently, gel permeation chromatography (GPC) columns packed with microspheres of chemically modified silica gel (TSK GEL, type SW; Varian, Darmstadt, G.F.R.), have become commercially available. These columns can be used under high pressure in aqueous systems

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and possess a large number of theoretical plates.

High-performance liquid chromatography (HPLC) with these columns has been studied for the analysis of many biological substances, such as polypeptides, proteins, lipoproteins and enzymes [7—12]. However, these GPC columns have not yet been applied to the analysis of human urinary proteins and for characterisation of proteinuria.

In the present paper we investigated the value of GPC for differentiating proteinurias and for monitoring the follow-up of a diagnosed kidney disease. In addition, the new applied analytics were compared with an established method.

EXPERIMENTAL

Apparatus

Urinary proteins were separated with column systems consisting of a precolumn TSK GWSP (10 cm × 7.5 mm I.D.) and two TSK G 3000 SW columns (particle size 10 ± 2 μm, 30 cm + 50 cm × 7.5 mm I.D.) in series with 50 mm × 0.23 mm I.D. stainless-steel tubing (Varian). The HPLC system consisted of a Waters Model 6000 A solvent delivery system and an automatic sample injection system (Model 710 WISP, Waters, Königstein, G.F.R.). The UV absorbance of all proteins was measured at 280 nm with an ISCO Absorption Monitor Model UA-5 (Colora, Lorch, G.F.R.). The detector output was also connected to a Waters Data System M 730 for the determination of retention times and peak areas.

SDS—PAGE was carried out in a vertical slab gel using a Hoefer Scientific Instruments Model SE 500 (San Francisco, CA, U.S.A.).

Chromatography

For removal of interferences of low molecular mass we used scaled-down gel chromatography of urine samples in small columns (PD 10, Pharmacia, Freiburg, G.F.R.) originally filled with Sephadex G-25. The medium was drained and refilled to a height of 5.5 cm with hydrated Sephadex G-50 fine (Pharmacia) giving a total bed volume of 9.1 ml.

Normally 100 μl of gel-filtrated urine were injected automatically in time intervals of 35 min. The mobile phase consisted of 1/15 *M* phosphate buffer (pH 6.8) containing 0.1 *M* NaCl and $6 \cdot 10^{-4}$ *M* NaN_3 and was pumped through the G 3000 SW columns at a flow-rate of 1 ml/min.

SDS—PAGE was carried out according to the description of Laemmli [13]. Fifty microliters of gel-filtrated urine were mixed with SDS containing Tris buffer and separated by electrophoresis in a 10% polyacrylamide vertical slab gel at 25 mA/gel at 10°C. The gels were fixed with 20% sulphosalicylic acid for 1 h, stained with a solution of 1.25 g of Coomassie Brilliant Blue R 250 in 454 ml of 50% methanol and 46 ml of acetic acid for 1 h, and destained by diffusion in a solution of 875 ml of water, 75 ml of acetic acid and 50 ml of methanol.

Reagents

Thyroglobulin (porcine), ferritin (horse spleen) ovalbumin, chymotrypsinogen, myoglobin (horse) and cytochrome *c* were obtained from Serva Fein-

Biochemica (Heidelberg, G.F.R.). Human γ-globulin, Cohn fraction II, was from Sigma Chemie (Taufkirchen, G.F.R.); human albumin and transferrin were from Behringwerke (Marburg, G.F.R.).

Precinorm® U was obtained from Boehringer Mannheim (Mannheim, G.F.R.). Eluent and protein solutions were filtered with 0.45-μm mean pore size filters, type HA (Millipore, Neu-Isenburg, G.F.R.).

Samples and procedures

Urine was collected with sodium azide (0.3 g/l) as preservative. Under these conditions it can be stored for several days at 4°C or for several weeks at −20°C. After centrifugation 2 ml of urine were placed on top of Sephadex G-50 columns and washed with 0.8 ml of phosphate buffer used also in the HPLC gel permeation procedure. The protein-containing fractions were eluted in 4 ml of this buffer. The columns were rinsed with 20 ml of eluent to prepare them for re-use.

We used the group-specific adsorbent Blue Sepharose CL-6B (Pharmacia) for removing albumins selectively from urine samples; 0.5 ml of urine was mixed with a suspension of Blue Sepharose CL-6B, resulting in dilution by a factor of 2.

For quantitative determination of total urinary protein we used the biuret method described by Weichselbaum [14], as well as UV spectrometry after gel chromatography. The individual peak areas eluting between 13 and 34 min were determined separately and summarized at the end of a run as a measure for total protein concentration. Precinorm® U (1:50 dilution) was used as standard for both methods.

RESULTS AND DISCUSSION

A mixture of standard proteins was examined using a G 3000 SW column (30 cm + 50 cm), and their separation patterns monitored at 280 nm are shown in Fig. 1. Though the column gave very sharp peaks, h-transferrin and h-albumin eluted as one peak.

A calibration curve of the same series of protein standards with apoferritin and myoglobin added is plotted semilogarithmically in Fig. 2. With G 3000 SW columns the exclusion limit for proteins is above a molecular weight of 600,000, and best separation efficiency is in the molecular weight range below approximately 60,000. Since retention times are printed out by the integrator, this calibration curve allows a reliable determination of the molecular weight of separated urinary proteins. For more accurate molecular-weight determinations of proteins by GPC, proteins are usually denaturated by the addition of SDS to the mobile phase. But then the exclusion limit of G 3000 SW columns decreases [15] and no separation of IgG and higher molecular weight proteins is possible.

We used gel chromatography for quantification of proteinuria by measuring absorbance at 280 nm of all eluated proteins. Interfering substances of lower molecular weight than 10,000 are separated on small Sephadex G-50 columns. Sample volume and column dimensions are adjusted to minimize the dilution during column passage to a factor of 2. The analytical recovery of diluted

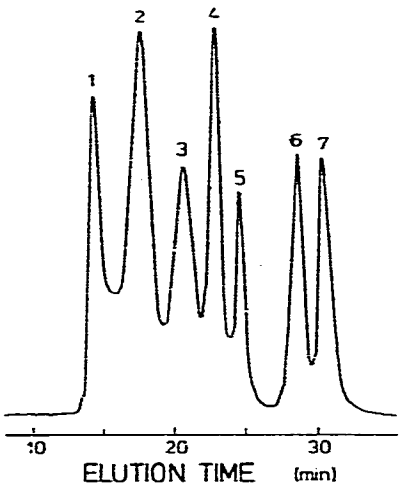

Fig. 1. Separation of a mixture of standard proteins: 1 = thyroglobulin (porcine); 2 = ferritin (horse spleen); 3 = h-γ-globulin; 4 = h-transferrin + h-albumin; 5 = ovalbumin; 6 = chymotrypsinogen; 7 = cytochrome *c*. Column: G 3000 SW, 7.5 mm I.D., 30 cm + 50 cm with precolumn. Flow-rate: 1 ml/min. Solvent: 1/15 *M* potassium phosphate buffer, pH 6.8, containing 0.1 *M* NaCl and $6 \cdot 10^{-4}$ *M* NaN_3. Sample load: 20—80 μg; charge 100 μl. Detector: UV 280 nm, 0.01 a.u.f.s.

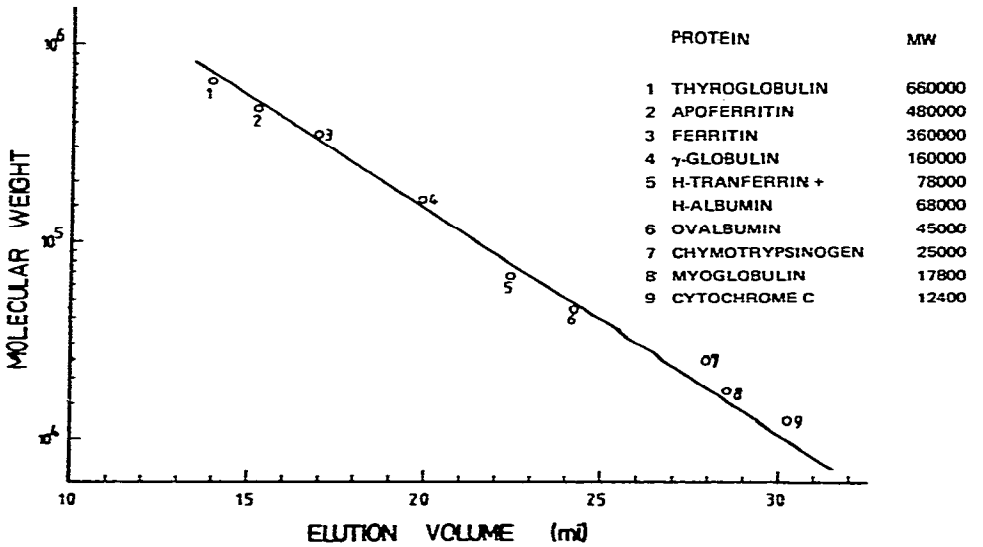

Fig. 2. Relationship between molecular weight of proteins and elution volume for G 3000 SW type columns. Conditions as in Fig. 1.

Precinorm® U (1.04 g/l) added to six different columns was 98.9%. To confirm the results obtained with the HPLC—280 nm method we also measured urinary protein concentration by the biuret method (see Fig. 3). The regression line of 62 paired measurements corresponds to the equation $y = 1.010x - 0.102$ with a correlation coefficient of 0.967, though in urine samples with a high content of lower molecular weight proteins (< 68,000) higher concentrations were

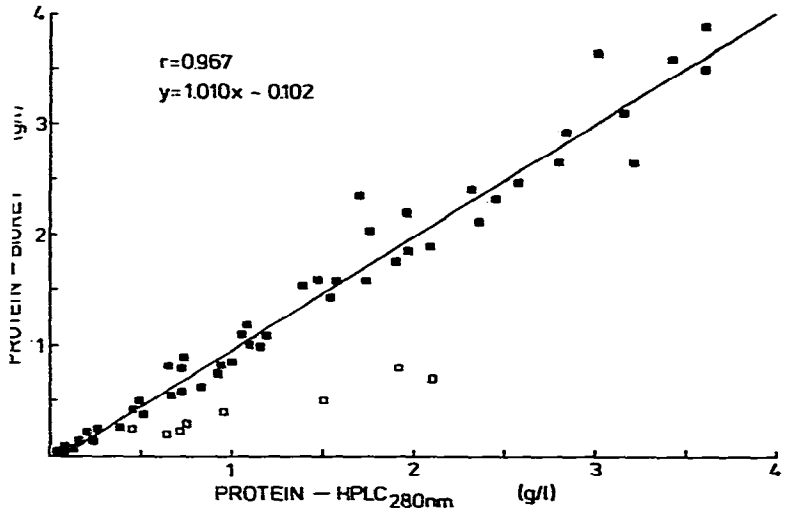

Fig. 3. Comparison of biuret and HPLC—280 nm techniques for the measurement of total urinary protein concentration. Open symbols indicate samples with more than 50% low molecular weight (< 68,000) proteins, which were included in the determination of the regression line. When these samples are excluded from the calculation, the regression line is $y = 1.024x - 0.037$ with a correlation coefficient $r = 0.988$.

found with the HPLC—280 nm method. This is probably caused by the high absorbance of a protein around 45,000 daltons at 280 nm scarcely visible in SDS—PAGE after staining with Coomassie Brilliant Blue R 250. However, there was a high correlation for quantification of Bence-Jones proteinurias using both methods.

The reproducibility of the method was tested also with diluted Precinorm® U solution (1.04 g/l). There was a within-run imprecision (C.V.) of 3.4% (n = 19) and a between-day imprecision of 3.65% (n = 13), which includes the error inherent in gel chromatography on G-50 columns. With an injection volume of 100 μl protein excretions from 40 mg/l up to 3 g/l can be measured. Since peak area does not depend on sample load in the range of 20—200 μl, optimal volumes can be injected according to the results of semiquantitative protein determinations obtained using test sticks.

For better differentiation of post-renal IgG secretion and IgG excretion caused by glomerular alteration, separation of transferrin and albumin is necessary. In both diseases albumin is always secreted, but transferrin only due to glomerular damage. In gel chromatography separation of these two proteins on the basis of their similar molecular size (78,000 vs. 68,000) can not be accomplished. However, the gel permeation media contain certain amounts of negatively charged groups which cause an additional ion-exclusion effect [16]. Since transferrin is more basic than albumin (pI 5.8 vs. 4.9), a better separation seems to be possible using a mobile phase of low ionic strength and high H^+ concentration. The elution patterns of a urine sample containing immunoglobulin, transferrin, albumin and different proteins of lower molecular weight using various eluents are shown in Fig. 4. The elution profiles with each eluent were similar, but the elution time of proteins increased with decreasing pH

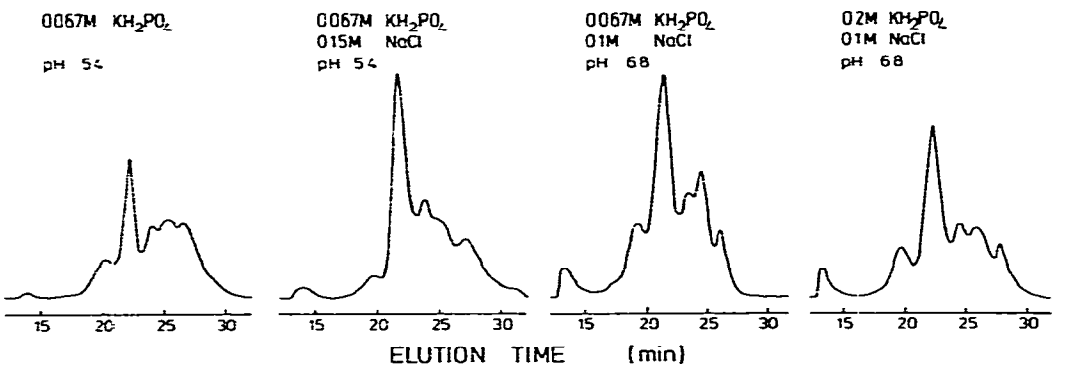

Fig. 4. Effect of pH and salt concentration of the mobile phase on separation of urinary proteins for one urine sample. Load volume: 30 µl. Detector: UV 280 nm, 0.01 a.u.f.s.

value, addition of sodium chloride and higher phosphate concentration. Absorbance of all proteins depends on the mobile phase. By decreasing the pH value further to 4.5 we observed a slight resolution between albumin and transferrin at low salt concentration but a loss in resolution in the area of microproteins. Since this condition was also not suitable for the column, we consider 1/15 *M* phosphate buffer, pH 6.8, containing 0.1 *M* NaCl as optimal for urinary protein separation.

We found affinity chromatography on immobilized Cibacron Blue E 3G-A an exceedingly efficient method for removing albumin selectively not only from plasma [17] but also from urine. Fig. 5 illustrates the removal of albumin from two urine samples with different transferrin concentrations by chromatography on Blue Sepharose CL-6B with consequent gel permeation on a G 3000 SW column.

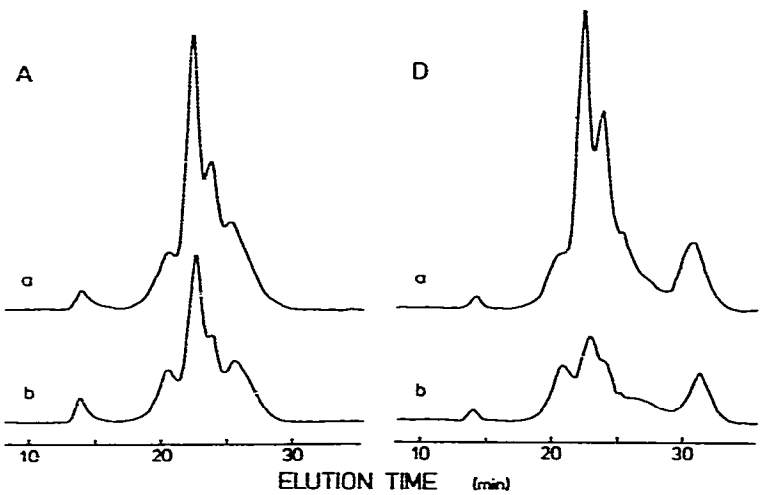

Fig. 5. Urinary pattern of proteinuria caused by glomerular and tubular involvement before (a) and after (b) treatment with Blue Sepharose CL-6B. Conditions as in Fig. 1. Sample A (50 µl injected) and sample D (100 µl injected) were also used for SDS–PAGE in Fig. 6. Dilution of samples by treatment with Blue Sepharose CL-6B was corrected for by injecting the doubled volume.

The increase in absorbance about 22 min after injection must be caused solely by transferrin (see Fig. 5, patterns b). This was judged by SDS—PAGE using samples of different protein concentrations. As can be seen from Fig. 6, albumin is removed completely by affinity chromatography on Blue Sepharose CL-6B. Urinary protein patterns using SDS—PAGE were in good agreement with the results obtained by gel chromatography on G 3000 SW columns, though resolution is higher with SDS—PAGE (see Figs. 5 and 6, samples A and D). Comparing peak heights in gel chromatography at a retention time of about 22 min before and after treatment with Blue Sepharose CL-6B as a measure of transferrin concentration is difficult to perform since the specific molar extinction coefficient is more than twice as high for transferrin as for albumin. But differences in peak areas can be used for determination of albumin concentrations.

Typical protein patterns for different types of proteinuria are shown in Fig. 7. In renal tubular diseases (Fig. 7b and c) proteins with a molecular weight lower than 70,000 are greatly increased, caused by the insufficiency of the tubules to reabsorb proteins filtered by the normal glomerulus. Among them albumin, α_1-antitrypsin (MW 54,000), free and dimer light chains (MW 22,000, 44,000), retinol binding protein (MW 21,000) and β_2-microglobulin (MW 12,400) have been identified [18].

Damage to glomerular basal membrane or degenerative diseases are associated with excretion of albumin and macroproteins in relative amounts similar

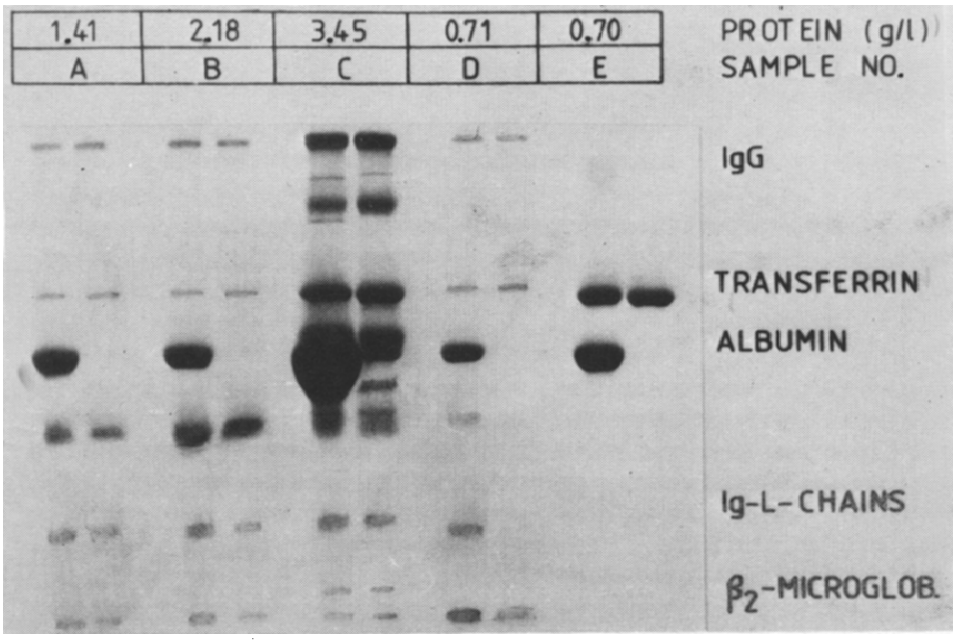

Fig. 6. SDS—PAGE patterns of different samples before and after chromatography on Blue Sepharose CL-6B. Samples A—D: mixed proteinuria with variable protein concentrations. Sample E: mixture of 0.5 g/l albumin and 0.2 g/l transferrin. Dilution by chromatography on Blue Sepharose CL-6B is corrected for by doubling the sample load.

to serum (Fig. 7d). Since the exclusion limit of the column is around MW 600,000, it is not possible to distinguish between α_2-macroglobulin and IgM. The most frequent proteinurias found were caused by glomerular and tubular alterations (Fig. 7f). Within the distinguishable tubular, glomerular, or mixed proteinurias, protein patterns can be different depending on the etiology (see Fig. 7b and c) but proved to be qualitatively constant during the course of a kidney disease. Patients with pure tubular dysfunction were much less frequent than those with glomerular disease and protein excretion was then always below 1.5 g per 24 h.

Patients with multiple myeloma often have Bence-Jones proteins passing freely across the normal glomerular wall. GPC of such urine samples (Fig. 8) seems to be particularly useful in the diagnosis of this disease and may be used for monitoring the therapy of the disease by quantification of the proteinuria. Additionally, glomerular and/or tubular alterations may be detected simultaneously (Fig. 8c).

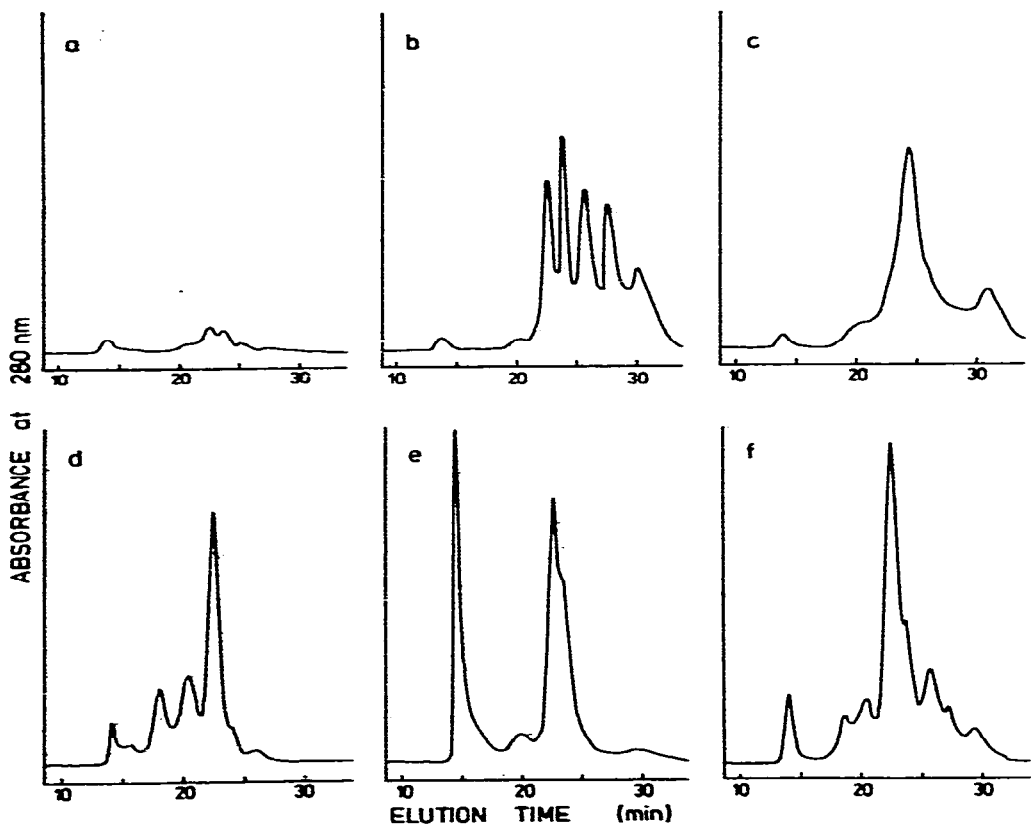

Fig. 7. Different patterns of renal proteinurias after separation on GPC: (a) physiological proteinuria; (b) tubular proteinuria in interstitial nephritis, MW 70,000—10,000; (c) tubular proteinuria in kaliopenic nephropathy, MW 50,000—10,000; (d) unselective glomerular proteinuria, MW 600,000—60,000; (e) glomerular proteinuria in plasmocytoma with excretion of albumin and polymer IgA (immunological proved); (f) mixed tubular and glomerular proteinuria in chemotherapy of a bronchial carcinoma, MW 600,000—10,000.

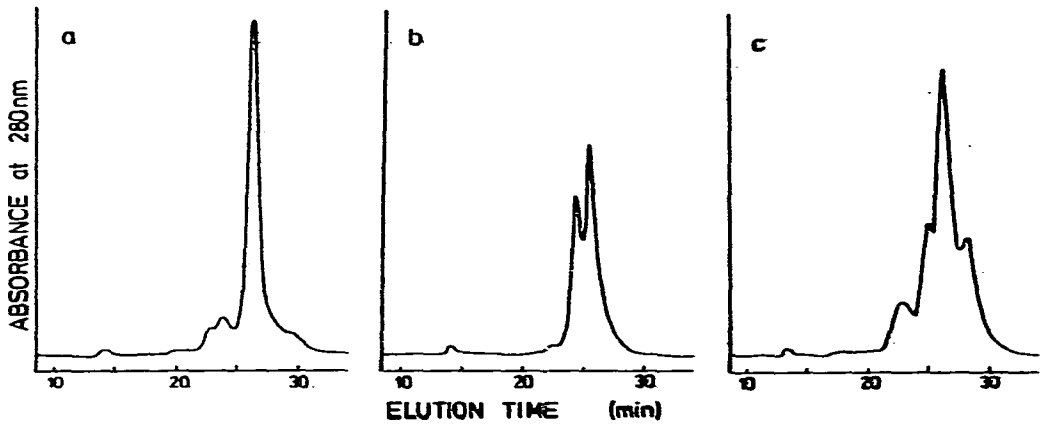

Fig. 8. Urinary pattern of patients with prerenal proteinuria: (a) plasmocytoma IgG, type kappa with excretion of monomer light chains; (b) plasmocytoma IgD, type lambda with excretion of monomer and dimer light chains; (c) mutiple myeloma IgA, type lambda with excretion of monomer and possible dimer light chains with simultaneous tubular alteration.

The chromatographic method we have described seems well suited for urinary protein profiling. Both relative and absolute changes in the protein pattern can be identified. GPC has been found to be as valuable as SDS—PAGE in differentiating physiological, tubular and glomerular types of proteinuria. Though protein discrimination might be further improved, the speed of analysis, the ability for automation, the favourable qualitative and quantitative results advocate this GPC method for routine use.

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