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Determination of proteins in urine by high-performance liquid chromatography with spectrophotometric detection using a pyrogallol red–molybdate complex

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ABSTRACT

A high-performance liquid chromatographic method with spectrophotometric detection was developed for the determination of proteins in urine. The proteins were separated on an anion-exchange column and eluted with a Tris–HCl buffer with a gradient of sodium chloride concentration and pH. The separated proteins were mixed with a pyrogallol red–molybdate complex reagent and determined spectrophotometrically. Urinary proteins were well seperated without desalting the urine. The reproducibility was satisfactory.

INTRODUCTION

Several methods, including high-performance liquid chromatography (HPLC), agarose gel electrophoresis, polyacrylamide gel electrophoresis (PAGE) and isoelectric focusing, have been used for the determination of proteins in urine [1]. Commonly used chromatographic techniques are gel permeation [2], ion-exchange [3,4] and reversed-phase high-performance liquid chromatography (HPLC) [5]. However, these methods usually require desalting of urine samples before HPLC analysis.

Here we describe an HPLC method for the determination of urinary proteins that does not require previous desalting. Proteins are separated on a ProPac PA1 anion-exchange column and eluted with a Tris–HCl buffer with a gradient of sodium chloride concentration accompanying the pH change. When a urine sample that had not been desalted was applied to an HPLC column and the eluate was monitored with a UV detector, many interfering substances were observed in the protein fractions. However, it has been found that a pyrogallol red (PR)–molybdate complex reacts exclusively with proteins and the intensity of the colour developed with this PR reaction is very high [6], so separated proteins could be measured spectrophotometrically using this reaction without desalting of urine samples before HPLC.

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The principle of a PR reaction is as follows:

$$\text{protein} + \underset{(\lambda_{max}\ 467\ \text{nm})}{\text{PR–molybdate complex}} \rightarrow \underset{(\lambda_{max}\ 604\ \text{nm})}{\text{protein–PR–molybdate complex}}$$

Using this method, characteristic chromatographic patterns of urinary proteins were found in patients with various renal diseases, and several peaks in the β_2-microglobulin (β_2M) fraction and also in the retinol-binding protein (RBP) fraction in urine could be recognized.

EXPERIMENTAL

Specimens

Random urine samples were collected from patients with different diseases, centrifuged and then filtered through a 0.22-μm filter. Filtered samples were kept frozen at −80°C until analysis. The thawed urines were centrifuged and filtered through a 0.22-μm filter and a 100-μl aliquot of the filtrate was analysed by HPLC.

Reagents

Lysozyme, transferrin (Tf), β_2M, α_1-acid glycoprotein (α_1AG), albumin (Alb), prealbumin (TBPA) and haptoglobin (Hp) in human plasma were purchased from Sigma (St. Louis, MO, U.S.A.). α_2-Macroglobulin (α_2M) and α_1-antitrypsin (α_1AT) in bovine plasma were purchased from Boehringer (Mannheim, Germany) and α_1-microglobulin (α_1M) in human plasma was kindly supplied by Eiken (Tokyo, Japan). Chromatographically purified human immunoglobulin G (IgG), IgA and IgM were purchased from Organon Teknica (West Chester, PA, U.S.A.). Antisera to human κ-light chain, λ-light chain, lysozyme, β_2M, RBP and α_1M were purchased from Dakopatts (Glostrup, Denmark). Sodium dodecylsulphate polyacrylamide gel plates and silver stain kits were supplied by Daiichi (Tokyo, Japan) and PR was purchased from Dojindo Labs. (Kumamoto, Japan). Other reagents were of analytical-reagent grade.

To prepare the PR reagent, 0.1 g of PR, 0.14 g of ammonium molybdate tetrahydrate, 0.5 g of sodium oxalate and 0.6 g of tartaric acid were dissolved in 1 l of 0.2 mol/l glycine–HCl buffer (pH 1.9) containing 0.1% Triton X-100. The final concentrations of the reagents were the same as those used in the modification [7] of the method reported by Watanabe *et al.* [6].

Apparatus

The pump, spectrophotometric detector, ProPac PA1 (50 mm × 4 mm I.D.) as a guard column and ProPac PA1 (250 mm × 4 mm I.D.) as the analytical column were obtained from Dionex (Sunnyvale, CA, U.S.A.). The pump for the PR reagent and a Model C-R6A integrator were obtained from Shimadzu (Kyoto, Japan).

Methods

For HPLC, 0.02 mol/l Tris–HCl buffer (pH 8.6) (buffer A) and 0.02 mol/l Tris–HCl buffer (pH 7.5) containing 1.0 mol/l NaCl (buffer B) were used as mobile phase component with linear gradient elution as follows: 0–10 min, 100–82% buffer A, 0–18% buffer B; 10–20 min, 82–77% buffer A, 18–23% buffer B; 20–30 min, 77–50% buffer A, 23–50% buffer B. These elution conditions were chosen because the absorbance of the reagent blank at 600 nm increased as the pH or NaCl concentration increased, the separation of proteins by this method was almost the same at pH 7.5, 8.0 or 8.6, as shown in Fig. 1, and many low-molecular-mass proteins (LMP) were eluted between 10 and 20 min. The flow-rate of the mobile phase was 1.0 ml/min at room temperature. The PR reagent flowed into the eluate at a flow-rate of 0.5 ml/min. The mixture was heated at 40°C for 1.5 min while flowing into the reaction coil and then the absorbance of the mixture was measured spectrophotometrically at 600 nm.

The concentration of total protein (TP) in urine was determined by the method of Watanabe *et al.* [6]. For sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE), two kinds of SDS gradient slab polyacrylamide gels (10–20%T and 17–27%T)[a] were used. SDS-PAGE was run according to the method of Laemmli [8].

For enzymatic immunoblotting, proteins were transferred electrophoretically from the gel to a poly(vinylidene difluoride) (PVDF) microporous membrane. These proteins were detected by an enzymatic immunoassay using primary antibody, secondary antibody combined peroxidase and 3,3′-diaminobenzidine [9].

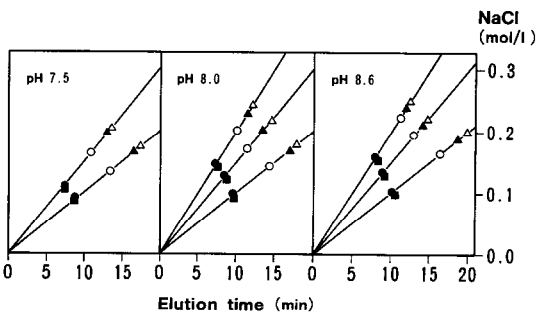

Fig. 1. Effect of pH and NaCl concentration on elution time. (○) Alb; (●) IgG; (△) TBPA; (▲) α_2M; (■) Tf.

[a] T = g acrylamide + g N,N′-methylenebisacrylamide per 100 ml of solution.

RESULTS

Elution times of various proteins

Using pure materials, the elution times of twelve proteins were determined. The elution times of Tf, β_2M, RBP, α_1M, α_1AG, Alb and TBPA in patients' body fluids, which were identified by SDS-PAGE and enzymatic immunoblotting, were the same as those obtained using pure materials. The relationship between elution time, isoelectric point (p*I*) and NaCl concentration is shown in Fig. 2.

Sensitivity of the method

The limits of detection (signal-to-noise ratio = 3) with this system were 0.6 μg for AlB, 0.5 μg for β_2M and 0.3 μg for Tf.

Reactivity of proteins towards PR

The concentrations of Alb, IgG, Tf, β_2M, α_1AG and TBPA in the respective solutions were determined by measuring the corresponding peak areas on the chromatogram and the results were compared with those obtained by immunonephelometry [10]. When the ratio of the concentrations calculated from peak areas to those given by immunonephelometry for Alb was assigned the value of 100, those for IgG, Tf, β_2M, α_1AG and TBPA were 56, 102, 84, 79 and 92, respectively.

Lack of desalting procedure

A portion of urine sample from a patient with glomerular damage was desalted through a Sephadex G-25 column (PD-10; Pharmacia, Uppsala, Sweden) [4]. However, the protein in the urine was not completely recovered by the desalting

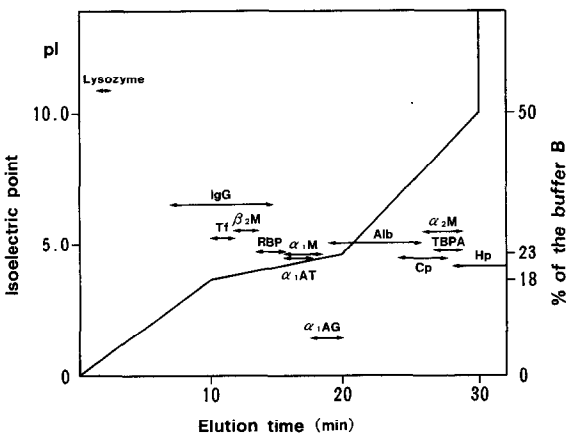

Fig. 2. Relationship between the elution time and p*I* of various proteins using the proposed method. The diagonal line shows the percentage of buffer B.

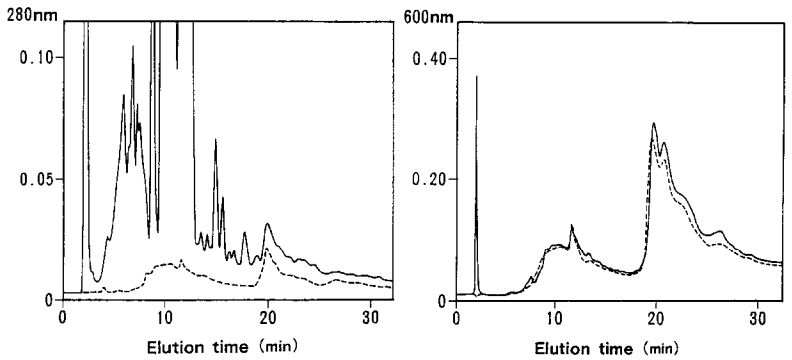

Fig. 3. Effect of desalting of urine on chromatograms obtained with UV detection (left) and the PR method (right). The concentration of TP in the urine sample was 499 mg/dl. The solid line is the trace for the filtered urine and the dashed line that for the desalted urine.

procedure. The concentration of TP in the desalted urine was measured and the volume of desalted urine that had the same amount of TP in 100 μl of original urine was applied to the ProPac PA1 HPLC column. As shown in Fig. 3, the UV profiles of the desalted urine and that of the filtered urine were entirely different (left). However, using the PR method they were almost identical with a higher absorbance (right). Therefore, the desalting procedure was not necessary for the analysis of urine by this method.

Precision

In the chromatogram of urine from a patient with renal tumor, six peaks were separated by the vertical cut method with the Shimadzu C-R6A integrator as shown in Fig. 4. Within-assay relative standard deviations (R.S.D.s) (n = 5) for

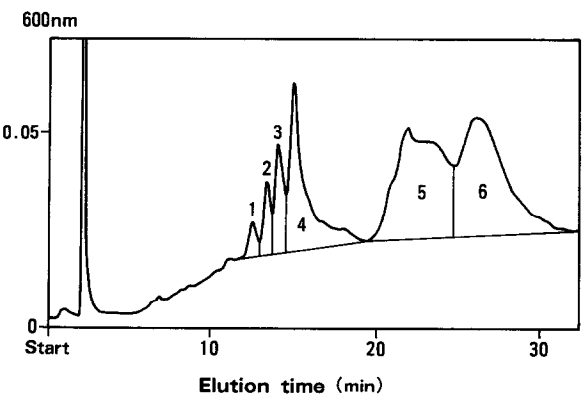

Fig. 4. Reproducibility. The concentration of TP in the urine sample was 117 mg/dl. Within-assay R.S.D.s (n = 5) for the areas of peaks 1, 2, 3, 4, 5, and 6 were 7.1, 4.1, 5.6, 3.3, 5.1 and 4.2%, respectively.

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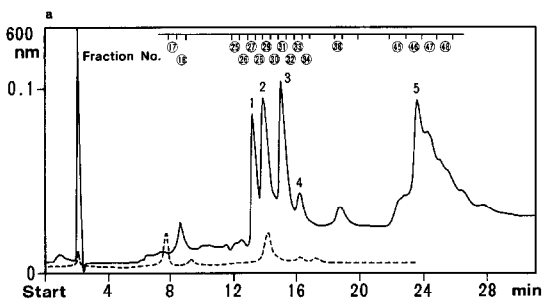
Analysis of urine samples

Chromatogram of urine from a patient with a renal tumour. A chromatogram of urine from a patient with a renal tumour is shown in Fig. 5a. Each fraction collected by the fraction collector was characterized by SDS-PAGE and enzymatic immunoblotting. As shown in Fig. 5b, most peaks showed a main protein band and a few faint protein bands. The main bands of peaks 1–5 were proved to be β_2M, RBP, RBP, α_1M and Alb. Peaks 2 and 3 were both identified as RBP. In



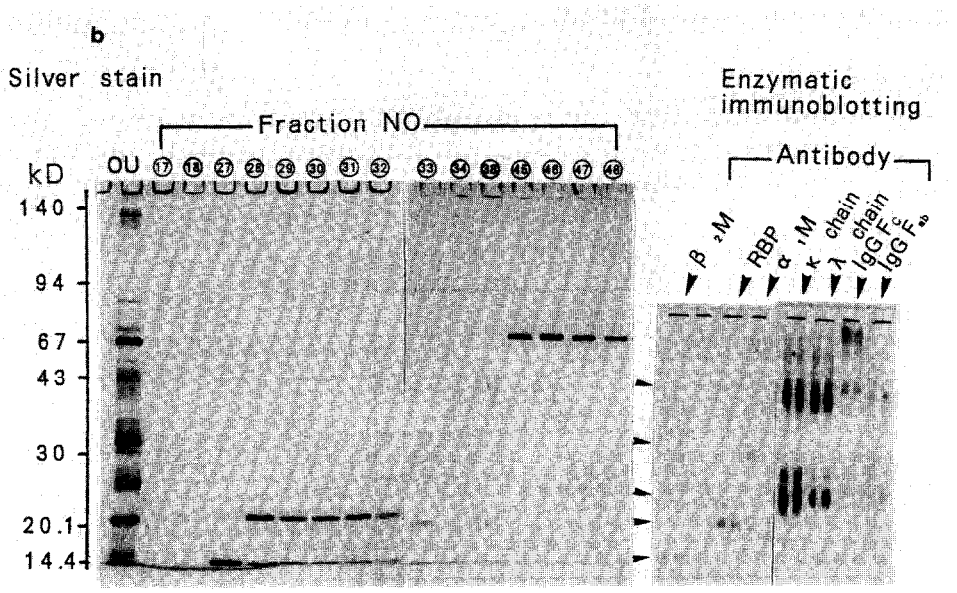


Fig. 5. (a) Chromatogram of urine (TP = 123 mg/dl) from a patient with a renal tumour. The dashed line is the trace for desalted urine detected at 330 nm using distilled water in place of PR reagent. Peaks: 1 = β_2M; 2 = holo-RBP; 3 = apo-RBP; 4 = α_1M; 5 = Alb. (b) SDS-PAGE (10–20%T) of each fraction. OU = original urine.

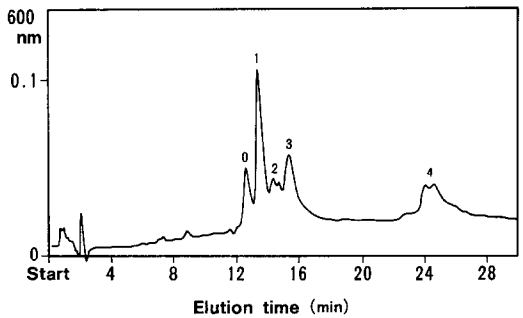


Fig. 6. Chromatogram of urine (TP = 48 mg/dl) from a patient with immunodeficiency syndrome. Peaks: 0 = modified β_2M; 1 = β_2M; 2 = holo-RBP; 3 = apo-RBP; 4 = Alb.

order to establish which was the holo-RBP peak, we measured the absorbance of vitamin A at 330 nm in the eluate of the desalted urine [11] using distilled water in place of PR reagent. As shown by the dashed line in Fig. 5a, the absorption at 330 nm was observed only at the same elution time in peak 2. This result suggested that the main protein of peak 2 was holo-RBP and that of peak 3 was apo-RBP.

Chromatogram of urine from a patient with immunodeficiency syndrome. A chromatogram of urine from a patient with immunodeficiency syndrome, which had been stored at −20°C for one month, is shown in Fig. 6. Each peak was characterized by SDS-PAGE and enzymatic immunoblotting. The main protein of peaks 0–4 were identified as β_2M, β_2M, holo-RBP, apo-RBP and Alb. However, when we analysed fresh urine obtained from the same patient about two months later, we could not detect peak 0. We therefore studied whether peak 0 was produced during storage. As shown in Fig. 7, when the urine was incubated

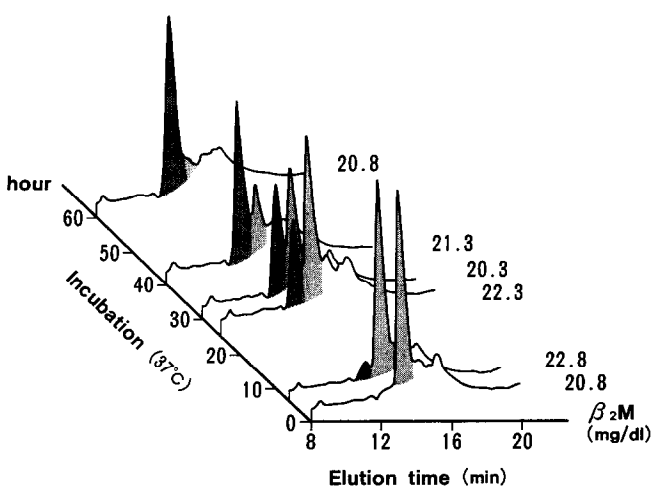


Fig. 7. Modification of β_2M.

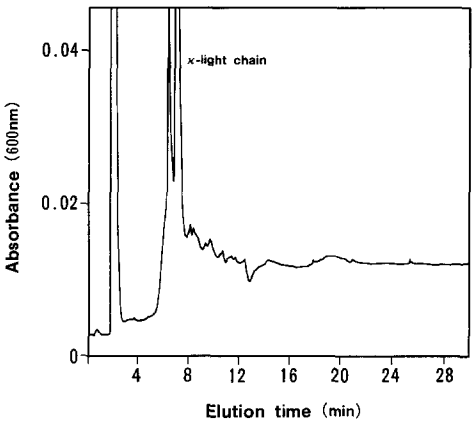


Fig. 8. Isolation of κ-light chain in urine from a patient with multiple myeloma.

at 37°C, peak 0 appeared and increased as the incubation time increased, and peak 1 decreased concomitantly. This result suggested that peak 0 was derived from peak 1 (β_2M) during storage.

Chromatogram of urine from a patient with multiple myeloma. A chromatogram of urine from a patient with multiple myeloma is shown in Fig. 8. Two peaks of κ-light chain were detected.

DISCUSSION

Cooper *et al.* [3] separated urinary proteins on an anion-exchange column using gradient elution with increasing NaCl concentration and pH. In this study, we also used gradient elution with increasing NaCl concentration but decreasing pH in order to minimize baseline shifts. By this method, LMP as β_2M and RBP were better separated with respect to the previously reported results [3–5].

The proposed method has the advantage that it does not require desalting of urine and offers excellent reproducibility and resolution. However, protein concentrations determined with the PR reagent were variable for different kinds of proteins. Recently, Orsonneau *et al.* [12] showed that the reactivity of Alb and γ-globulins towards PR became the same when SDS was added to PR reagent. However, when SDS was added to PR reagent, the peaks became broad and the sensitivity was lowered. Consequently, addition of SDS to PR reagent is not useful in this method.

Urinary proteins in cases of glomerular damage consisted mainly of high-molecular-mass proteins (HMP) and those in tubular damage consisted of LMP. The chromatogram obtained by this HPLC system can easily discriminate between urine samples consisting mainly of HMP (Fig. 3) and those of LMP (Figs. 4–6). In addition, this method is efficient for the separation of LMP and the sensitivity is good.

Hall *et al.* [13] showed the existence of differently charged forms of β_2M in serum and urine analysed by isoelectric focusing. Nissen *et al.* [14] reported the modification β_2M *in vitro* during storage at 20°C of a sample from a patient with a small cell lung cancer. It was not clear whether β_2M of peak 0 in Fig. 7 was the same or not as those recognized by Hall *et al.* [13] or Nissen *et al.* [14].

In conclusion, the proposed method for urinary protein determination appears useful particularly for studies of renal diseases and seems promising for application in clinical practice.

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