Cellulose Acetate Membrane Electrophoresis in the Analysis of Urinary Proteins in Patients With Tubulointerstitial Nephritis

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Urinary proteins from 14 patients with tubulointerstitial nephritis were analyzed by cellulose acetate membrane electrophoresis. Urinary total protein concentrations were measured, and urinary 15 proteins (prealbumin, albumin, α_1 -microglobulin, α_1 -antitrypsin, α_2 -macroglobulin, haptoglobin, retinol binding protein, transferrin, β_2 -microglobulin, IgA, IgG, κ - and λ -light chains, cystatin C, and lysozyme) were identified by the use of a rapid and highly sensitive colloidal silver staining reagent suited for use with cellulose acetate membranes, as reported previously by Matsuda et al. (*J*

Clin Lab Anal 15:171–174, 2001; *Clin Chem* 47:763–766, 2001) and Hiratsuka et al. (*J Clin Lab Anal* 10:403–406, 1996). We also analyzed urinary total protein concentration and urinary protein fractions according to the presence of acute or nonacute interstitial nephritis. In addition, the relationship between urinary protein fraction and complications of interstitial nephritis was analyzed. The goal of this work was to find a useful index for the diagnosis of tubulointerstitial nephritis. *J. Clin. Lab. Anal.* 17:44–51, 2003. © 2003 Wiley-Liss, Inc.

Key words: tubulointerstitial nephritis; silver colloidal staining; urinary protein; cellulose acetate membrane electrophoresis

INTRODUCTION

Tubulointerstitial nephritis refers to a range of pathological processes that are responsible, at least in part, for the progression of renal disease of nearly all etiologies (4). Tubulointerstitial nephritis is frequently the most important pathological manifestation of progressive glomerulonephritis, obstructive uropathy, reflux nephropathy, and cystic disease, although it may also present as a primary disease process associated with infection, drug use, or other immunologically mediated disease (4). Tubulointerstitial nephritis can be classified as chronic or acute interstitial nephritis. Chronic interstitial nephritis is a disease process in which inflammation is accompanied by destructive tubulopathy and fibrogenesis. In acute interstitial nephritis there is a cessation and reversal of this process (4).

Despite the fact that many forms of tubulointerstitial nephritis are amenable to therapy once the diagnosis is made, there are few clinically useful markers of

tubulointerstitial nephritis available. Renal biopsies are performed when the data obtained from urinalysis are inconclusive, and the renal dysfunction is apparent.

In 1980 (5), four urinary protein indices were reported to be markers of tubulointerstitial nephritis: 1) ratio of clearance of lysozyme/clearance of albumin >20%; 2) ratio of clearance of IgG/clearance of albumin % >55%; 3) renal tubular epithelial antigen excretion >10 units/min; and 4) ratio of urinary albumin/urinary total protein concentration <50%. By combining these criteria into multiphasic testing profiles, 88% of patients can be categorized as having either tubulointerstitial

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nephritis or glomerulonephritis (5). There are few recent reports concerning specific urinary proteins and interstitial nephritis.

Urinary proteins are believed to be one of the best indices for determining renal injury. In particular, low-molecular-weight proteins (α_1 -microglobulin, retinol binding protein, and β_2 -microglobulin) appear in the urine of patients with tubular damage. In a previous study (6), samples from healthy children were analyzed by polyacrylamide gel electrophoresis and compared to samples from children with tubular damage. The results indicated that the determination of α_1 -microglobulin and β_2 -microglobulin, in contrast to the estimation of the retinol binding protein, is useful for the screening and diagnosis of tubular disorders.

It has also been reported that various urinary proteins (particularly Bence Jones proteins) can be identified by both electrophoresis and immunofixation of unconcentrated urines with the use of cellulose acetate membranes and colloidal gold solution (7).

In this study, a rapid and highly sensitive colloidal silver staining reagent suitable for use with cellulose acetate membranes, as reported by Matsuda et al. (1,2) and Hiratsuka et al. (3), was used. First, 15 urinary proteins in samples from patients with tubulointerstitial nephritis were identified, fractionated, and analyzed. We then analyzed urinary total protein concentrations and urinary protein fractions by classifying the samples according to the presence of acute or nonacute interstitial nephritis. The relationship between urinary protein fractions and complications of interstitial nephritis was also analyzed. Our goal was to find a useful index for the diagnosis of tubulointerstitial nephritis that can be used instead of renal biopsies, which can cause stress to the patient.

MATERIALS AND METHODS

Urine samples were obtained from 14 patients (22–84 years old) with tubulointerstitial nephritis who had been admitted to the Department of Nephrology, Sendai Shakaihoken Hospital. At their initial diagnosis, renal biopsies were performed because there was slight urinary protein excretion, and renal dysfunction was apparent. Informed consent was obtained from all patients.

Preparation of Colloidal Silver Staining Reagent

A 12 mL solution containing 0.25% Tween 20 (v/v) (Wako Pure Chemicals, Osaka, Japan) and 15 mL of 25 mmol/L $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (Wako Pure Chemicals) was added to a 50-mL conical tube (Becton Dickinson,

Franklin Lakes, NJ) and mixed. Next, 5 mL of 280 mmol/L $\text{Na}_3\text{-citrate}$ (Wako Pure Chemicals) were added and mixed. Then 1 mL of 350 mmol/L AgNO_3 (Wako Pure Chemicals) was added, and the mixture was shaken vigorously by hand to prevent premature flocculation of silver particles. Finally, 2 mL of 15% acetic acid (v/v) were added and mixed. This solution was prepared immediately before use.

Measurement of Urinary Total Protein Concentration by Silver Dot Blot Assay

A cellulose acetate membrane (70 × 100 mm, SeparaxSP; Fuji Photo Film, Tokyo, Japan) was used. Seven sets of 10 squares (10 × 10 mm) were drawn on the cellulose acetate membrane. Then 3- μ volumes of calibrator solution (FRACTOROL □ JOO KOO □, Jokoh, Tokyo, Japan) at 25, 50, 100, 200, 400, 700 mg/L in 9 g/L NaCl solution) were blotted along with urine samples onto the membrane with a Pipetman (Gilson Medical Electronics, Villiers-le-Bel, France). The proteins were then fixed in 0.4 mol/L trichloroacetic acid and 0.03 mol/L sulfosalicylic acid for 5 min, followed by immersion in 1% acetic acid (v/v) for 5 min, and staining with freshly prepared colloidal silver solution for 5 min with continuous shaking. The membranes were washed in distilled water and allowed to dry at room temperature. For semiquantitative analysis of total protein, the membrane was made transparent by immersion in decalin, and the absorbance was measured at 505 nm with a Shimadzu CS-9000 spectrophotometer (Shimadzu, Tokyo, Japan). A calibration curve was prepared according to the absorbance of the calibration spots, and the total protein concentration in the urine samples was calculated from the curve.

Cellulose Acetate Membrane Electrophoresis and Colloidal Silver Staining

A cellulose acetate membrane measuring 70 × 130 mm was prepared, and 3.2 μ L from the urine samples were applied to a 3-cm area at the tip of the membrane (cathode area). Electrophoresis was carried out at a constant current of 0.7 mA/cm per membrane for 25 min in barbital buffer (0.05 mol/L sodium barbital, pH 8.6). After electrophoresis, the urinary proteins were fixed in 0.4 mol/L trichloroacetic acid and 0.03 mol/L sulfosalicylic acid for 5 min, followed by immersion in 1% acetic acid (v/v) for 5 min, and staining in freshly prepared with colloidal silver solution for 20 min with continuous shaking. The membranes were washed in distilled water and allowed to dry at room temperature. Densitometry at 505 nm was carried out as described above.

TABLE 1. Patient profiles

No.	Sex	Age, years	Serum creatinine, (mg/dL)	Total protein, ·mg/L·	·Disease
1	F	66	1.2	698.5	IgA nephropathy + acute interstitial nephritis
2	F	63	5.8	220.7	Pyelonephritis + imminent interstitial nephritis
3	F	35	2.6	378.9	Interstitial nephritis (Chinese herbs nephritis)
4	M	72	4.9	155.1	Interstitial nephritis + acute renal failure
5	M	71	1.7	74.2	Interstitial nephritis
6	M	60	1.3	46.5	Sarcoidosis + interstitial nephritis
7	M	68	4.5	746.5	Acute interstitial nephritis, severe + rapidly progressive glomerulonephritis
8	M	22	2.0	319.5	Interstitial nephritis + hepatopathy
9	M	84	6.0	901.2	Acute interstitial nephritis + interstitial pneumonia
10	M	72	7.5	621.5	Acute interstitial nephritis + rapidly progressive glomerulonephritis
11	M	73	2.0	196.1	Interstitial nephritis
12	F	69	2.2	872.7	Acute interstitial nephritis + sarcoidosis
13	F	77	1.9	207.0	Interstitial nephritis
14	F	67	2.0	358.6	Interstitial nephritis + rheumatoid arthritis + chronic renal failure

Fig. 1. Electrophoretic patterns of urinary proteins from 14 patient samples. The numbers in the figure correlate with the patient numbers in Table 1.

Fig. 2. Average and standard deviation of each protein fraction percentage in nonacute interstitial nephritis with and without complications. * Significant difference ($P < 0.05$).

Immunofixation

After electrophoresis, each track was overlaid with cellulose acetate strips impregnated with specific antisera generated against prealbumin (preALB), albumin (ALB), α_1 -antitrypsin (α_1 -AT), α_1 -microglobulin (α_1 -m), α_2 -macroglobulin (α_2 -M), haptoglobin (Hp), retinol binding protein (RBP), transferrin (Tf), β_2 -microglobulin (β_2 -m), cystatin C (Cys-C), lysozyme (Lys), κ - and λ -light chains (κ , λ), IgG, or IgA (DAKO, Glostrup, Denmark) for 20 min. Immunofixed strips were washed in 0.9% (w/v) NaCl solution and stained with colloidal silver solution.

RESULTS

Urinary Total Protein Concentration

Urinary total protein concentrations of 14 samples were determined according to the calibration curve obtained by a silver dot blot assay, as described by Matsuda et al. (1,2) and Hiratsuka et al. (3). The values are shown in Table 1. The sample with the highest concentration (901.2 mg/L) was from patient 9, and the lowest (46.5 mg/L) was from patient 6. The average value was 414.1 ± 296.2 mg/L (mean $\pm$ 1 SD). The average urinary total protein concentration from these samples was approximately seven times greater than the normal values (60.0 ± 44.8 mg/L, mean $\pm$ 1 SD) obtained by this method.

Patients with strong cellular infiltration consisting mainly of monocytes were diagnosed as having acute interstitial nephritis, while other patients were considered to have nonacute interstitial nephritis (Table 1). When urinary total protein concentrations were classified according to the presence of acute ($n = 5$) or

TABLE 2. Average and standard deviation of each protein fraction % in acute and non-acute interstitial nephritis

	N	Fraction	Mean $\pm$ 1 SD (%)
Acute	5	ALB	24.5 $\pm$ 14.0
Non-acute	9		29.9 $\pm$ 17.7
Acute	5	α_1	8.56 $\pm$ 2.74
Non-acute	9		9.50 $\pm$ 3.77
Acute	5	α_2	12.9 $\pm$ 10.6
Non-acute	9		11.6 $\pm$ 6.50
Acute	5	β	21.5 $\pm$ 3.98
Non-acute	9		20.1 $\pm$ 8.55
Acute	5	γ	27.0 $\pm$ 9.97
Non-acute	9		24.1 $\pm$ 7.08

nonacute ($n = 9$) interstitial nephritis, the average value for acute interstitial nephritis was 768.1 ± 117.7 mg/L (mean $\pm$ 1 SD); for nonacute interstitial nephritis it was 217.4 ± 117.6 mg/L (mean $\pm$ 1 SD). This difference was significant ($P < 0.01$).

Urinary Protein Fractions

Urinary protein bands detected by colloidal silver staining after cellulose acetate membrane electrophoresis are shown in Fig. 1. Concentration of the sample was not necessary if there was a concentration of ≥ 5 mg/L in one band. The urinary electrophoretic patterns of the 14 samples show clear fractionation and various electrophoretic patterns.

The urinary protein fraction percentages were also obtained by densitometry. When samples from patients with nonacute interstitial nephritis were classified according to the presence or absence of complications (in this study, diseases other than interstitial nephritis

Sample 1

Sample 2

Sample 3

Sample 4

Fig. 3. Localization by immunofixation of some urinary proteins in four representative samples. Sample 1 is from a patient with IgA nephropathy; Tf, $\beta 2-m$, IgG, IgA, Hp, light chain (λ), and $\alpha 2-M$ are identified. Samples 2 and 3 are from patients with tubulointerstitial nephritis; preALB, ALB, RBP, $\beta 2-m$, Cys-C, Lys, $\alpha 1-m$, and light chain (κ) are identified. Sample 4 is from a patient with IgA nephropathy and tubulointerstitial nephritis; ALB, Tf, and $\alpha 1-AT$ are identified.

were assumed to be complications, as shown in Table 1), significant differences were obtained in the ALB fraction percentage ($P < 0.05$) (Fig. 2). The mean of each protein fraction percentage was then obtained according to the presence of acute or nonacute interstitial nephritis (Table 2). No significant difference was identified.

Urinary Proteins Identified by Immunofixation

Figure 3 shows the 15 proteins identified by immunofixation in four samples. A schematic representation of the typical banding pattern is shown in Fig. 4 (from the anode: prealbumin, albumin, α_1 -microglobulin, α_1 -antitrypsin, α_2 -macroglobulin, haptoglobin, retinol binding protein, transferrin, β_2 -microglobulin, IgA, IgG, κ - and λ -light chains, cystatin C, and lysozyme). The region enclosed by a square shows the γ -globulin area. Light chains appeared in various regions of the γ -globulin area depending on the sample. Although cellulose acetate membrane was used in the absence of electroosmosis, cystatin C and lysozyme clearly migrated cathodally to the application point.

Fig. 4. Schematic representation of the pattern of urinary proteins obtained by immunofixation.

Fig. 5. Distribution rate of urinary protein bands in acute and nonacute interstitial nephritis. The numbers in the figure correspond with the patient numbers in Table 1.

Distribution of Urinary Protein Bands

The percentages of the eight major identified urinary protein bands (preALB, ALB, α_1 -AT, RBP, Tf, β_2 -m, Cys-C, and Lys) were obtained by densitometry (Fig. 5).

Fig. 5. Continued.

No significant difference between acute and nonacute interstitial nephritis was identified.

DISCUSSION

Currently, renal biopsies are performed to definitively diagnose interstitial nephritis (8). However, this proce-

dure can cause stress to the patient. In addition, it has been reported that small amounts of urinary proteins are excreted in tubular interstitial nephritis (9), and a distinction between acute and chronic interstitial nephritis is not generally straightforward (10). In the present study, urinary total protein concentrations were obtained by silver dot blot assay (1–3). When urinary

total protein concentrations were classified according to the presence of acute or nonacute interstitial nephritis by this method, the values for acute interstitial nephritis were significantly higher ($P < 0.01$). The distinction between acute and nonacute interstitial nephritis is currently made by determining the severity of cellular infiltration on renal biopsy. The method presented herein facilitated diagnosis without renal biopsy. The urinary protein fraction percentage was also obtained with this method, and when nonacute interstitial nephritis samples were classified according to the presence or absence of complications, the ALB fraction percentage was significantly higher in the absence of complications ($P < 0.05$). Error in the electrophoretic pattern that occurs by protein degradation in concentrated samples was avoided, because it was not necessary to concentrate the sample. By the use of a rapid, simple, and highly sensitive colloidal silver staining reagent suitable for cellulose acetate membranes, urinary proteins in samples from patients with interstitial nephritis could be fractionated and then identified. Until now, there has been no index for diagnosis of interstitial nephritis with complications. In addition, urinary protein fractions are seldom run, and serum protein fractions are more often used in clinical laboratories; therefore, this rapid and simple method for the detection of the ALB fraction will be useful.

With this method, 15 urinary proteins (prealbumin, albumin, α_1 -microglobulin, α_1 -antitrypsin, α_2 -macroglobulin, haptoglobin, retinol binding protein, transferrin, β_2 -microglobulin, IgA, IgG, κ - and λ -light chains, cystatin C, and lysozyme) were identified clearly. We believe it will be useful to analyze the changes in each protein percentage over time as the disease progresses or remits.

Recently there have been reports that various medicines can induce interstitial nephritis (11–14), that urinary eosinophils increase in acute interstitial nephritis (15), and that increased urinary laminin and kallikrein in the rat are markers of tubulointerstitial damage, which is a symptom of tubulointerstitial nephritis (16). There have been few reports concerning specific urinary proteins and interstitial nephritis. By the current method, acute and nonacute interstitial nephritis can be distinguished, and the presence of complications can be determined. Rapid and simple diagnoses of tubu-

linterstitial nephritis by fractionating and measuring urinary proteins can now be achieved.

REFERENCES

1. Matsuda K, Hiratsuka N, Kurihara Y, Shiba K. 2001. Semiquantitative analysis of urinary low protein levels using silver dot blot assay. *J Clin Lab Anal* 15:171–174.
2. Matsuda K, Hiratsuka N, Koyama T, Kurihara Y, Hotta O, Itoh Y, Shiba K. 2001. Sensitive method for detection and semiquantification of Bence Jones protein by cellulose acetate membrane electrophoresis using colloidal silver staining. *Clin Chem* 47:763–766.
3. Hiratsuka N, Shiba K, Shinomura K, Hosaki S. 1996. Rapid and highly sensitive colloidal silver staining on cellulose acetate membrane for analysis of urinary proteins. *J Clin Lab Anal* 10:403–406.
4. Jones CL, Eddy AA. Tubulointerstitial nephritis. 1992. *Pediatr Nephrol* 6:572–586.
5. Zager RA. 1980. Urinary protein markers of tubulointerstitial nephritis. *Invest Urol* 18:197–202.
6. Grillenberger A, Weninger M, Lubec G. 1987. Determination of urinary low molecular weight proteins for the diagnosis of tubular damage. *Pediatr Pathol* 22:229–234.
7. Aguzzi F, Gasparro C, Bergami MR, Merlini M. 1993. High-sensitivity electrophoretic method for the detection of Bence Jones protein and for the study of proteinuria in unconcentrated urines. *Ann Clin Biochem* 30:287–292.
8. Kimura K. 1995. Renal biopsy for diagnosis of interstitial nephritis. *Nippon Rinsho* 53:1931–1938.
9. Heptinstall RH. 1992. Interstitial nephritis. In: Heptinstall RH, editor. *Diseases of the kidney*. 4th ed. Boston: Little-Brown. p 1315.
10. Lins RL, Verpooten GA, De Clerck DS, De Broe ME. 1986. Urinary indices in acute interstitial nephritis. *Clin Nephrol* 26:131–133.
11. Myers RP, McLaughlin K, Hollomby DJ. 2001. Acute interstitial nephritis due to omeprazole. *Am J Gastroenterol* 96:3428–3431.
12. Fisher AA, Le Couteur DG. 2001. Nephrotoxicity and hepatotoxicity of histamine H2 receptor antagonists. *Drug Safety* 24:39–57.
13. Ravnskov U. 1999. Glomerular, tubular and interstitial nephritis associated with non-steroidal antiinflammatory drugs. Evidence of a common mechanism. *Br J Clin Pharmacol* 47:203–210.
14. Schurman SJ, Alderman JM, Massanari M, Lacson AG, Perlman SA. 1998. Tubulointerstitial nephritis induced by the leukotriene receptor antagonist pranlukast. *Chest* 114:1220–1223.
15. Corwin HL, Haber MH. 1987. Award lecture and special reports: the clinical significance of eosinophiluria. *Am J Clin Pathol* 88:520–522.
16. Saw S, Aw TC, Sinniah R. 2001. Urine laminin and kallikrein, markers of tubulointerstitial damage in experimental protein overload on pre-existing renal damage. *Pathology* 33:37–43.