Urinary Protein Fraction in Healthy Subjects Using Cellulose Acetate Membrane Electrophoresis Followed by Colloidal Silver Staining

Ryoko Machii,¹ Ryo Kubota,² Nobuo Hiratsuka,¹ Kayo Sugimoto,¹ Rie Masudo,¹ Yuriko Kurihara,¹ Shizuko Kobayashi,² and Kiyoko Shiba^{1*}

¹Analytical Laboratory Chemistry, Graduate School of Allied Health Sciences, Tokyo Medical and Dental University, Tokyo, Japan

²Department of Molecular Physiology, Kyoritsu College of Pharmacy, Tokyo, Japan

We previously reported a rapid and highly sensitive colloidal silver staining solution suitable for the cellulose acetate membrane. This method was useful for detecting even very small amounts of urinary protein. In the present study, we examined urinary protein fractions in healthy subjects, using cellulose acetate membrane electrophoresis (CAE) with a highly sensitive colloidal silver staining, in an attempt to determine the clinical relevance of urinary protein fractions. Sixty unconcentrated spot urine specimens were analyzed by CAE and calculated by densitometry. All of the samples were separated into five fractions by CAE. The mean $\pm$ 1 SD of the per-

centage of five fractions was 28.37 ± 8.51 in albumin, 4.30 ± 4.19 in α_1 -globulin, 14.41 ± 6.14 in α_2 -globulin, 19.45 ± 7.10 in β -globulin, and 33.46 ± 8.24 in γ -globulin. The albumin/globulin (A/G) ratio was 0.41 ± 0.17 . These six items and the concentrations of total protein, albumin, and β -N-acetyl-D-glucosaminidase (NAG) did not significantly differ between males and females. NAG is the marker of tubulointerstitial nephropathy. The results suggest that there are no gender-dependent differences in the urinary protein fractions of healthy subjects. *J. Clin. Lab. Anal.* 18:231–236, 2004. © 2004 Wiley-Liss, Inc.

Key words: cellulose acetate membrane electrophoresis; healthy subjects; colloidal silver staining; urinary protein

INTRODUCTION

The analysis of urinary protein provides useful information not only for screening kidney substances or urinary tract disease, but also for evaluating the disease stage and effect of medical treatment. Various analytical methods are used to fractionate urinary protein, including cellulose acetate membrane electrophoresis (CAE), sodium dodecyl sulfate polyacrylamide gel electrophoresis, capillary electrophoresis, and two-dimensional electrophoresis. We analyzed urinary protein using CAE because of its reproducibility, technical simplicity, and rapid analytical results. CAE is used in many clinical laboratories to determine serum protein fractions. To analyze urinary protein, after CAE is performed, the membrane is stained with a staining solution, such as acid-violet 17 (1) and Coomassie brilliant blue (2). However, it is often necessary to concentrate the urine before electrophoresis because of the low urinary protein level, and this is a time-consuming process. We previously reported a rapid

and highly sensitive colloidal silver staining solution suitable for the cellulose acetate membrane. With this method, it is possible to detect specific abnormalities in urinary proteins, such as abnormalities in the pattern of urinary protein fractions on CAE. In previous studies, we found that some specific patterns of urinary protein fractions appear in various renal disorders (3–5). The detection of Bence-Jones protein (BJP), which appears in the urine of patients with multiple myeloma, malignant monoclonal gammopathy, and lymphoproliferative disease, is particularly useful clinically.

We previously compared our method (using unconcentrated urine) and the kit for immunity electrophoresis

*Correspondence to: Kiyoko Shiba, Analytical Laboratory Chemistry, Graduate School of Allied Health Sciences, Tokyo Medical and Dental University, Yushima 1-5-45, Bunkyo-ku, Tokyo 113-8519, Japan. E-mail: k.shiba.mtec@tmd.ac.jp

Received 2 December 2003; Accepted 3 February 2004

DOI 10.1002/jcla.20028

Published online in Wiley InterScience (www.interscience.wiley.com)

produced by Helena Institute in Texas (using concentrated urine) for detection sensitivity for BJP. We found that our method had a higher sensitivity of detecting BJP in low protein concentrations than the other method (3). Furthermore, we identified 15 urinary proteins (pre-albumin, 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), and analyzed the relation between urinary protein fractions and complications of interstitial nephritis (4). Retinol binding protein, β_2 -microglobulin, cystatin C, and lysozyme were found in eight, seven, six, and 10 patients, respectively, out of 14 patients with interstitial nephritis (4). Analysis of these single proteins is useful for diagnosing glomerulopathy and tubulo-interstitial nephropathy (6–9). Glomerulopathy and tubulo-interstitial nephropathy can be distinguished by urinalysis when a tubular marker (e.g., α_1 -microglobulin, retinol-binding protein, or β_2 -microglobulin) is considered in addition to albumin. With CAE using our method, the pattern of typical urinary protein fractions with albumin, α_1 -antitrypsin, transferrin, and IgG suggests glomerulonephropathy, and the pattern of urinary protein fractions with α_1 -microglobulin, retinol-binding protein, and β_2 -microglobulin suggests tubulo-interstitial nephropathy. Three selected protein electrophoretic patterns with IgA nephropathy, interstitial nephritis, and primary amyloidosis are shown in Fig. 1. Albumin, α_1 -antitrypsin, transferrin, and IgG are identified as typical protein bands in IgA nephropathy. Retinol-binding protein, α_1 -microglobulin, and β_2 -microglobulin are identified as typical protein bands in interstitial nephritis. BJP is identified as a typical protein band in primary amyloidosis. In previous studies (8,9), the Urinary Protein Expert System (UPES, Institute of Clinical Chemistry, Municipal Hospital München-Bogenhausen, München, Germany) was established by carrying out the dipstick test, and the quantity of some these characteristic single urinary proteins, and it was used as a method of judging glomerulopathy and tubulointerstitial nephropathy. However, in the current study we attempted to develop image analysis software based on a visual judgment of characteristic patterns of urinary protein fractions on a single CAE, and to semiquantify these single proteins by densitometry. To determine the patterns of urinary protein fractions in healthy subjects in this study, we examined the protein fractions of healthy subjects, using CAE with a highly sensitive colloidal silver staining. We fractionated the urinary proteins of 60 healthy subjects in their 20s, and assayed their urinary total protein, urinary albumin, urinary β -N-acetyl-D-glucosaminidase (NAG), and urinary creatinine.

Fig. 1. Three selected protein electrophoretic patterns with glomerulopathy and tubulointerstitial nephropathy and identified urinary protein bands. Each urinary protein band was identified by immunofixation. **A:** IgA nephropathy. **B:** Interstitial nephritis. **C:** Primary amyloidosis. 1) Albumin. 2) α_1 -antitrypsin. 3) α_1 -microglobulin. 4) Retinol binding protein. 5) Transferrin. 6) β_2 -microglobulin. 7) IgG. 8) BJP.

MATERIALS AND METHODS

Subjects

Sixty spot urine specimens were collected from 60 healthy hospital staff members in their 20s at Tokyo Medical and Dental University. The average ± 1 SD age of the 30 males and 30 females was 24.50 ± 2.16 and 21.57 ± 1.74 , respectively. All of the samples were tested by means of the dipstick test, and the protein concentrations of all of the samples were negative (< 300 mg/L). The samples were stored at -80°C before they were analyzed. Informed consent was obtained from all of the subjects.

Sample Preparation

Unconcentrated urine was used for all of the assays. The semiquantitative analysis of the total protein in urine was performed with the use of our silver dot blot assay (10). Urinary Tamm-Horsfall protein (THP) was purified by the Tamm and Horsfall method (11).

Electrophoresis

The cellulose acetate membranes used in the electrophoresis were purchased from Fuji Photo Film Co., (Tokyo, Japan). With the use of the Sample Applicator 10 (Joko, Tokyo, Japan), the samples that contained < 30 mg/L protein concentration were applied at $1.6 \mu\text{L}$, and the samples that contained > 30 mg/L protein were applied at $0.8 \mu\text{L}$. The samples were electrophoresed at a constant current of 0.7 mA/cm per membrane for 25 min in veronal buffer (60 mmol/L , pH

8.6). Silver staining was carried out using our method as previously described (3,6). The membranes were then treated with decaline, and densitometric analysis was performed with the use of Cliniscan (Helena Institute, TX). The albumin/globulin (A/G) ratio was calculated from the area under the curve (AUC) of albumin, and the globulin fractions were determined by densitometry.

Determination of Total Protein, Albumin, Creatinine, and NAG Concentrations in Urine

The semiquantitative analysis of total protein in urine was performed by means of the silver dot blot assay (6). The albumin concentration was determined by the immunoturbidimetric method with the N Assay TIA Micro Alb (Nittobo, Tokyo, Japan). The creatinine concentration in urine was determined enzymatically with the N Assay CRE-L (Nittobo). The NAG concentration was determined by the NAG Rate Shionogi (Shionogi, Tokyo, Japan). An Hitachi 7070 automated analyzer (Hitachi, Tokyo, Japan) was used for the three measurements.

Removal of Salt From the Urine

Six urine samples were dialyzed against deionized water with the use of seamless cellulose tubing (Sanko Junyaku, Tokyo, Japan) for 18 hr at room temperature. The dialyzed samples were then electrophoresed under the same conditions as described above.

Statistical Analysis

SPSS for Windows (11.0 J) software was used for the statistical analysis. The Mann-Whitney U-test was used to compare variables between males and females. Spearman's rank correlation test was used to investigate correlations. To compare different methods, we performed a regression analysis as described by Passing and Bablok (12). All of the tests were two-tailed, and differences were considered statistically significant at $P < 0.05$ for the null hypothesis.

RESULTS

Classification of Urinary Protein Fractions in Healthy Subjects

All of the samples were separated into five fractions. Thus, they had an albumin peak and four globulin peaks (α_1 -globulin (α_1 -G), α_2 -globulin (α_2 -G), β -globulin (β -G), and γ -globulin (γ -G)), as shown in Fig. 2B. The serum protein in healthy subjects was also separated into five fractions, as shown in Fig. 2A.

Fig. 2. Two selected protein electrophoretic patterns obtained with a colloidal silver staining solution, and their densitometric patterns. **A:** Serum protein pattern. **B:** Urinary protein pattern.

Percentages of Urinary Protein Fractions, and the A/G Ratio

The mean ± 1 SD% of each fraction, and the A/G ratio in the urinary protein of 60 samples are shown in Table 1. There were no significant differences between males and females for six items.

Influence of Fractions on Other Factors

We sought to determine whether daily exercise affects the protein fraction in urine. Eight males and nine females exercised daily. Each daily exercise session was about 1 hr at maximum. There was no significant difference in the fraction pattern, the percentage of each fraction, or the component in urine with daily exercise. We also examined whether anamnesis affects the protein fraction in urine. The study group included one case of acute glomerulonephritis (the patient had a history of acute glomerulonephritis at 11 years of age, but was healthy at the age of 23 years), one case of Kawasaki disease, one case of urinary calculus, one case of peritonitis, and one case of ulcerative enteritis. The percentages of the five urinary protein fractions in these samples were within the mean ± 1 SD of the 60 sample urinary protein fraction percentages. Data from the subject with anamnesis were excluded. The results

suggest that there were no individual differences in urinary protein patterns.

Concentrations of Total Protein, Albumin, NAG, and Creatinine, and Their Creatinine Index in Urine

The mean ± 1 SD of the concentrations of total protein, albumin, NAG, and creatinine, and their creatinine index (rectified by creatinine) in urine are shown in Table 2. NAG is located in the lysosomes of proximal tubule cells, and indicates a hypertrophy of tubular epithelial cells when its activity is elevated in urine. High activity of NAG indicates tubulointerstitial nephropathy, and acute and chronic renal failure. There were significant differences between males and females in the creatinine index of albumin and NAG ($P < 0.05$). For the other factors, there were no significant differences between males and females.

Correlation Between the Concentrations of Albumin in Urine Determined by the Hitachi 7070 and the AUC of the Albumin Fraction

A comparison of two methods (the Hitachi 7070 and the AUC of the albumin fraction) by Passing-Bablok regression analysis demonstrated the following: $AUC = 1.288$ (Hitachi 7070) $+ 0.061$ mg/L ($r_s = 0.862$, $P < 0.01$). The standard error of estimates $S_{y/x} = 3.967$. The 95% confidence intervals for the slope and intercept were 1.075–1.454 and -1.026 to 1.209, respectively.

Electrophoretic Pattern of Purified THP

Purified THP was electrophoresed as a broad band from α_1 -G to the original fraction area (Fig. 3).

Effect of Dialysis on Each Protein Fraction

The AUCs of each urinary protein fraction estimated by densitometry before and after dialysis were compared. As shown in Fig. 4, although there was no change in the AUC of the albumin fraction by dialysis, the

Fig. 3. The electrophoretic pattern of urinary protein (A), and the purified THP electrophoretic pattern (B). The THP concentration was 250 mg/L.

TABLE 1. The percentage of urinary protein fractions and A/G ratio in 60 samples

Group	Case	Albumin (%)	Globulin (%)				A/G ratio
			α_1 -G	α_2 -G	β -G	γ -G	
Total	60	28.37 ± 8.51	4.30 ± 4.19	14.41 ± 6.14	19.45 ± 7.10	33.46 ± 8.24	0.41 ± 0.17
Male	30	27.09 ± 9.45	4.95 ± 4.19	15.00 ± 6.06	19.37 ± 5.20	33.69 ± 7.72	0.39 ± 0.19
Female	30	29.67 ± 7.39	3.66 ± 4.15	13.82 ± 6.27	19.52 ± 8.70	33.33 ± 8.86	0.44 ± 0.15
p		ns	ns	ns	ns	ns	ns

mean ± 1 SD; p, Male vs Female; ns, not significant.

TABLE 2. The concentrations of total protein, albumin, NAG, and creatinine and their creatinine indexes in urine

Case	Total 60	Male 30	Female 30	p
Total protein (mg/L)	31.03 ± 19.41	35.01 ± 20.93	27.04 ± 17.19	ns
Albumin (mg/L)	6.93 ± 5.87	7.37 ± 7.19	6.50 ± 4.24	ns
NAG (IU/L)	2.35 ± 1.99	2.24 ± 1.94	2.45 ± 2.06	ns
Creatinine (mg/dL)	131.96 ± 76.53	151.10 ± 88.18	112.82 ± 58.16	ns
Total protein (mg/g.CRE)	25.41 ± 15.16	26.65 ± 18.46	24.16 ± 11.11	ns
Albumin (mg/g.CRE)	5.20 ± 2.86	4.44 ± 2.46	5.96 ± 3.06	$p < 0.05$
NAG (IU/g.CRE)	1.41 ± 1.27	1.41 ± 1.27	2.15 ± 1.30	$p < 0.05$

mean $\pm$ SD.

p, Male vs. Female; ns, not significant.

Fig. 4. Effect of dialysis on each protein fraction estimated by densitometry. 1) Comparison of the AUCs for albumin and globulin before and after dialysis. 2) Comparison of the AUCs before and after dialysis for all globulin fractions. 3) Comparison of the A/G ratios before and after dialysis. 4) The densitometric urinary protein patterns before (A) and after (B) dialysis of urine. * $P < 0.05$, ** $P < 0.01$.

AUC of each globulin fraction (α_1 -G + α_2 -G + β -G + γ -G) was significantly decreased by dialysis ($P < 0.05$). The AUC of each globulin fraction (α_1 -G, α_2 -G, β -G, and γ -G) was compared before and after dialysis. Although there was no change in the AUC of α_2 -G and β -G by dialysis, the AUC of α_1 -G and γ -G was decreased significantly by dialysis ($P < 0.05$ and $P < 0.01$, respectively). The AUC of γ -G decreased about 50% after dialysis. Furthermore, the A/G ratio was significantly increased after dialysis ($P < 0.05$).

DISCUSSION

We previously assessed urinary protein fractions in healthy subjects, using CAE followed by staining with acid violet 17 (1). In the previous report, the urinary protein fractions were classified into four groups. Each group had an albumin peak and one or several other globulin peaks, and various electrophoretic patterns, possibly due to denaturation of the protein by concentration and excessive application. In practice, when 2.5–5 times the conventional amount of urine was applied, the urinary fraction of α_1 -G to γ -G and α_2 -G to γ -G changed to a broad pattern. Consequently, the separation ability of β -G was decreased. Therefore, it appears that urinary protein fractions should be classified into five groups. We solved this problem with the use of highly sensitive silver staining. We were able to divide the urinary protein into five fractions using only 0.8 or 1.6 μ L of the samples.

There were no significant differences between males and females in the percentage of the urinary protein fraction or the A/G ratio. The significant differences between males and females in the creatinine index of albumin and NAG probably reflect the fact that creatinine excretion is slightly higher in males, who have greater muscle mass than females. In a comparison of the albumin concentration according to the AUC (determined by densitometry) and the Hitachi 7070, a good correlation coefficient and slope were obtained.

In the glomerulus, albumin is filtered to only 0.5–0.6% in serum, but almost all of the albumin is reabsorbed with a uriniferous tubule. This suggests that the A/G ratio of urinary protein is lower than that of serum. The interpretation of the fraction pattern of urine differs from that of serum. In urine, there are two types of protein that originate from plasma and the kidney and the lower urinary tract. The major proteins in urine are THP (50%), albumin (20%), and immunoglobulin (5%). THP is the most abundant protein in normal urine, and is electrophoresed broadly from origin to α_1 -G by CAE. The high A/G ratio of urinary protein appears to be related to THP. THP forms complexes with various substances (such as IgG light chains and albumin (13–15)) under high salt concentrations (such as in urine). To determine the influence of salt concentration on the urinary protein fraction, we dialyzed the urine to decrease the salt concentration. Consequently, the AUC of γ -G was decreased about 50% by dialysis. There are two possible explanations for this phenomenon: 1) decreasing the salt concentration

caused the THP to be dissociated from substances that exist in the γ -G fraction, or 2) dissociated THP is difficult to detect with highly sensitive colloidal silver staining because an acidic solution is used as a fixative. THP is apt to dissolve under acidity, and we use an acidic solution (0.4 mol/L trichloroacetic acid-0.03 mol/L sulfosalicylic acid) as a fixative before the silver staining. THP in urine can be detected without dissolving in an acidic solution because it is protected by forming complexes with various substances (e.g., IgG light chains) under high salt concentrations. However, when THP is dissociated from such substances by dialysis, it becomes difficult to detect and the γ -G fraction is decreased.

For clinical diagnoses and assessments of medical treatment in glomerulopathy or tubulointerstitial nephropathy, it is very effective to evaluate the features of urinary protein. Ivandic et al. (9) classified urinary proteins into four patterns in UPES (interstitial nephropathies, primary glomerulopathies, secondary glomerulopathies, and renal dysfunction) by quantifying albumin and α_1 -microglobulin in urine. We attempted to classify glomerulopathy and tubulointerstitial nephropathy according to the detection of specific protein bands by CAE and highly sensitive colloidal silver staining. This method can be used to analyze urine with a low protein concentration (negative by the dipstick test) because the minimum detection sensitivity is 2.5 mg/L, and can detect certain kinds of protein markers. A prospective target is to establish image analysis software to support clinical diagnoses by simultaneously detecting an abnormal pattern of urinary protein fractions (qualitative abnormalities) and abnormal concentrations of single proteins (quantitative abnormalities) in urine with only one performance of electrophoresis.

Our highly sensitive silver staining can only be applied to the cellulose acetate membrane. It is not suitable for agarose gel. We attempted to perform a similar analysis using the Titan Gel Silver Stain Kit (Helena Laboratories, Beaumont, TX); however, since the minimum detection sensitivity of the silver staining solution is 300–500 mg/L, urinary protein in healthy subjects was not detected. Thus, for detection of urinary protein in healthy subjects, urine needs to be concentrated about 10 times. Furthermore, the pattern of the urinary fraction will change after concentration procedure is conducted. Future studies should include subjects of all ages. By establishing a normal urine pattern with a

combination of the cellulose acetate membrane and highly sensitive silver staining, qualitative abnormalities could be judged according to an electrophoretic pattern, resulting in an effective method for pathological analyses.

REFERENCES

1. Hiratsuka N, Shiba K, Shinomura K, et al. Urinary protein fractions in healthy subjects using CAE followed by staining with acid violet 17. *Biol Pharm Bull* 1994;17:1355–1357.
2. Shiba KS, Kanamori K, Harada T, et al. A cause of discrepancy between values for urinary protein as assayed by the Coomassie brilliant blue G-250 method and the sulfosalicylic acid method. *Clin Chem* 1985;31:1215–1218.
3. Matsuda K, Hiratsuka N, Shiba K. Sensitive method for detection and semiquantification of Bence Jones protein by cellulose acetate membrane electrophoresis using colloidal silver staining. *Clin Chem* 2001;47:763–766.
4. Kubota R, Machii R, Hiratsuka N, Shiba K. Cellulose acetate membrane electrophoresis in the analysis of urinary proteins in patients with tubulointerstitial nephritis. *J Clin Lab Anal* 2003;17:44–51.
5. Machii R, Matsuda K, Hiratsuka N, Sugimoto K, Shiba K. Analysis of an expanded width of albumin fraction by cellulose acetate membrane electrophoresis in IgA nephropathy urine before treatment. *J Clin Lab Anal* 2003;17:37–43.
6. Zager RA. Urinary protein markers of tubulointerstitial nephritis. *Invest Urol* 1980;18:197–202.
7. Guder WG, Ivandic M, Hofmann W. Physiopathology of proteinuria and laboratory diagnostic strategy based on single protein analysis. *Clin Chem Lab Med* 1998;36:935–939.
8. Ivandic M, Hofmann W, Guder WG. The use of knowledge-based systems to improve medical knowledge about urine analysis. *Clin Chim Acta* 2000;297:251–260.
9. Ivandic M, Hofmann W, Guder WG. Urinary Protein Expert System (UPES) Homepage. München, Germany: Institute of Clinical Chemistry, Municipal Hospital München-Bogenhausen. <http://wilas.zait.uni-bremen.de/upes/main.htm>.
10. Matsuda K, Hiratsuka N, Shiba K. Semiquantitative analysis of urinary low protein levels using silver dot blot assay. *J Clin Lab Anal* 2001;15:171–174.
11. Tamm I, Horsfall Jr FL. Characterization and separation of an inhibitor of viral hemagglutination present in urine. *Proc Soc Exp Med* 1950;74:108–114.
12. Passing H, Bablok W. A new biometrical procedure for testing the equality of measurements from two different analytical methods. *J Clin Chem Clin Biochem* 1983;21:709–720.
13. Huang Z-Q, Sanders PW. Biochemical interaction between Tamm-Horsfall glycoprotein and Ig light chains in the pathogenesis of cast nephropathy. *Lab Invest* 1995;73:810–817.
14. Rodhes DCJ, Hinsman EJ, Rodhes JA. Tamm-Horsfall glycoprotein binds IgG with high affinity. *Kidney Int* 1993;44:1014–1021.
15. Huang Z-Q, Sanders PW. Localization of a single binding site for immunoglobulin light chains on human Tamm-Horsfall glycoprotein. *J Clin Invest* 1997;99:732–736.