Development of a New Method for Measuring Total Urinary Protein Using Acid Violet 6B Pigment

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We have developed a new method for measuring total urinary protein using acid violet 6B (AV6B) pigment. Nine purified components of human urinary proteins and urine samples collected randomly from 123 diabetic outpatients were used. There were 62, 36, and 25 cases of preneuropathy, early neuropathy, and overt neuropathy, respectively. All samples were measured by Coomassie brilliant blue G 250 (CBB), pyrogallol red-molybdate (PRM), and AV6B methods using an optical photometer. In healthy subjects, the major components of urinary proteins, such as γ -globulins, IgG, IgA Tamm-Horsfall protein, and transferrin, the reactivity values of the AV6B and PRM methods were similar. The CBB method

was the least sensitive of the three methods. In the urine samples from diabetic patients, the urinary protein values measured by the AV6B method were higher than those measured by the CBB method in the preneuropathy stage. The values obtained by the AV6B method (y) correlated well with those from the CBB method (x) ($y = 1.243x + 3.61$, $r = 0.904$). When the values from the AV6B method (y) were compared to those from the PRM method (x), correlation was low ($y = 1.406x - 29.15$, $r = 0.786$). In conclusion, the AV6B method was more useful than the CBB and PRM methods for low levels of urinary protein. *J. Clin. Lab. Anal.* 17: 147–154, 2003. © 2003 Wiley-Liss, Inc.

Key words: Coomassie brilliant blue G 250; pyrogallol red-molybdate; diabetics; neuropathy

INTRODUCTION

The determination of urinary total protein is one of the key tests in screening for physiologic and pathophysiologic changes, and the detection of a small increase in the lower range of protein levels in proteinuria may provide important clinical information for the early diagnosis of neuropathy. Because major protein components in healthy subjects may increase in the early stages of neuropathy, a method for urinary protein analysis, that can detect normal protein levels in urine, and that has equal reactivity toward albumin and globulins is desirable.

In 1981, we adapted the CBB method to the AutoanalyzerTM (1). The CBB method is simple and highly sensitive (2); however, it has the disadvantage, that CBB-protein complexes readily adhere to the

surface of optical cells and test tubes, leading to the underestimation of results. Another disadvantage of the CBB method is that it suffers from color variations, due to different binding affinity towards various proteins. An improved method using PRM was reported by Watanabe et al. (3) in 1986. With their method, linearity was maintained until 5,000 mg/L, but reproducibility in

Abbreviations: CBB, Coomassie brilliant blue G 250; AV6B, acid violet 6B; PRM, pyrogallol red-molybdate.

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the range of low protein concentrations was not good because the optical density measured was low.

Recently we reported that in healthy subjects the determination of urinary protein fractions separated by cellulose acetate membrane electrophoresis was useful for screening subjects who were probably in subclinical stages of renal disease of glomerular or tubular origin (4). In that report we stressed that urinary protein measurements were more useful in specimens with a low range of proteinuria concentration than in those with gross proteinuria. It has been reported (5–8) that the urine of healthy subjects has a higher concentration of Tamm-Horsfall protein (50% of total protein) than albumin.

In the present study, we developed a new method of urinary protein measurement AV6B, which was previously used for cell staining (9) and thin gel staining (10), and compared the AV6B method to the CBB and PRM methods.

MATERIALS AND METHODS

Subjects

Normal spot urine was collected from 57 healthy staff members of the Tokyo Medical and Dental University Hospital. Urine samples from diabetics were collected randomly from 123 outpatients (67 men, 28–89 years old, and 56 women, 27–84 years old) at Tokyo Metropolitan Fuchu Hospital. We divided the diabetic nephropathy cases into three classes according to albumin values (mg/g·Cre.), as proposed by Shigeta (11), as follows: 1) urinary albumin values <30 mg/g·Cre. (normo-albuminuria), 2) values of 30–300 mg/g·Cre. (micro-albuminuria), and 3) values >300 mg/g·Cre. (macro-albuminuria). Classes 1, 2, and 3 were termed pre-nephropathy, early nephropathy, and overt nephropathy, respectively. There were 62, 36, and 25 cases of pre-nephropathy, early nephropathy, and overt nephropathy, respectively.

Reagents

Human serum albumin, γ -globulins, and β_2 -microglobulin were purchased from Sigma Chemical, (St. Louis, MO). IgA from human serum, and IgG from human serum were purchased from Oriental Yeast (Tokyo, Japan). Human Tamm-Horsfall glycoprotein and human transferrin were purchased from Chemicon International Inc. (Temecula, CA). Bence Jones κ -chain and Bence Jones λ -chain were from Gelco Diagnostics (Shreveport, LA). α_1 -Acid glycoprotein was from COSMO BIO (Tokyo, Japan). AV6B (Serva Violet 49) was from Serva Electrophoresis GmbH, (Heidelberg, Germany). The protein assay kit for the CBB method

was from Bio-Rad Laboratories (Hercules, CA). The micro TP-test Wako for the PRM method, and other chemicals of reagent grade were purchased from Wako Pure Chemicals (Osaka, Japan).

Preparation for the Assay

AV6B reagent: 500 mg of AV6B was dissolved in 200 mL of distilled water and stirred for 30 min. Then 4.3 g of sodium chloride (final concentration 0.075 mol/L) and 127.4 g of phosphoric acid (final concentration 1.3 mol/L) were added to the solution, which was adjusted to 1,000 mL with distilled water after it was left to stand overnight at room temperature. This solution was then filtered with a 0.22- μ m microfilter (Toyo Roshi Kaisha, Tokyo, Japan), and the resulting solution was used as the AV6B reagent. Protein standard solution: 100 mg of human serum albumin was dissolved in 100 mL of 0.9% sodium chloride solution. This was used as the standard solution (final concentration 1,000 mg/L).

Procedures

The AV6B method was performed as follows: 0.1 mL of urine sample or 1,000 mg/L albumin solution was mixed with 3 mL of AV6B reagent, and the solution was allowed to stand for 120 min at room temperature. The absorbance of each specimen was measured at 546 nm using a UV-1200 spectrophotometer (Shimadzu, Kyoto, Japan) or a U-3210 spectrophotometer (Hitachi, Tokyo, Japan). The CBB and PRM methods were performed according to the manufacturers' protocols.

RESULTS

Absorption Spectra of the AV6B Reagent

The basic structure of AV6B is similar to that of CBB, with a color index of 42,640. Figure 1 shows the absorption spectra of the reagent with and without albumin. The absorption peak for the reagent was at about 630 nm. After it was bound to albumin, a new peak appeared at 546 nm.

Pigment Adherence to Optical Cells

After a 1,000 mg/L albumin solution was assayed using the CBB method and the AV6B method, the pigment solution in each optical cell was removed, and optical cells were washed three times with distilled water. Then the residual pigment on each optical cell was determined by adding distilled water into the cell. Optical density using the CBB and AV6B methods was 0.031 and 0.003, respectively. Unlike the CBB method, nonspecific adherence of pigment to optical cells was negligible with the AV6B method.

Fig. 1. Absorption spectra of AV6B reagent with or without albumin. The broken line shows the absorption spectrum of AV6B reagent plus 0.9% NaCl vs. distilled water, and the solid line shows AV6B reagent with 1,000 mg/L albumin solution vs. distilled water.

Fig 2. Change of optical density with incubation time in albumin solution and γ -globulins solution with AV6B reagent. Closed circles and closed squares show the 1,000 mg/L albumin and 1,000 mg/L γ -globulins, respectively.

Incubation Time

A solution of 1,000 mg/L albumin or 1,000 mg/L γ -globulins was mixed with the AV6B reagent according to the procedure described above. We immediately started to scan absorbance at 546 nm and continued to scan for 180 min. As shown in Fig. 2, the absorbance increased for 50 min after the start of scanning. Although the absorbance of albumin differed from that of γ -globulins at starting time, there was no difference between them as regards the changing rate of absorbance for about 10 min. The absorbance of albumin was constant from 100 min to 130 min, while the absorbance of γ -globulins slightly increased from 90 min to 120 min. Therefore, an incubation time of 120 min was determined for the end-point assay.

Reproducibility

Urine samples with three different protein concentrations (113.6, 526.1, and 987.7 mg/L) were used to study the reproducibility of the AV6B method. The within-run

coefficients of variation (CVs) of the three samples were 4.68%, 1.40%, and 0.47%, respectively for ten times of a single run. The day-to-day CVs of three samples for five independent assays were 13.10%, 5.51%, and 4.03%, respectively.

Recovery Test

We mixed 1 mL of a 2,000–7,000 mg/L solution of human albumin or γ -globulins with 9 mL urine sample per tube (protein concentration: 36 mg/L), and then assayed the mixture with the AV6B reagent. The recovered values were 95–104% of the added values for albumin and 97–115% for γ -globulins, respectively.

Linearity and Sensitivity

Using human serum albumin, we examined the linearity and sensitivity of the CBB, PRM, and AV6B methods in the range of 0–2,000 mg/L of albumin (Fig. 3). The upper limit of linearity was found to be 1,000 mg/L of albumin in solution using the AV6B

method. However, it was 400 mg/L with albumin using the CBB method. The AV6B method could thus cover wider ranges than the CBB method. Although linearity continued until 2,000 mg/L using the PRM method, optical intensity at low albumin levels was small. The minimum sensitivity was 5.9 mg/L for albumin solution using the AV6B method.

Reactivity With Purified Proteins

Reactivities with various purified proteins (albumin, transferrin, γ -globulins, IgG, IgA, Bence Jones κ -chain, Bence Jones λ -chain, α_1 -acid glycoprotein, β_2 -microglobulin, and Tamm-Horsfall protein) were compared using the AV6B, CBB, and PRM methods (Table 1). We prepared 500 mg/L solutions of the proteins according to the values quantified by each supplier. The concentration of the 500 mg/L albumin solution was taken as a standard and used to determine the concentrations of various protein solutions measured by the CBB, PRM, and AV6B methods. The values of γ -globulins, IgG, IgA, Tamm-Horsfall protein, and transferrin obtained with the AV6B and PRM methods were about equal, but the values obtained with the CBB method were the lowest of the three methods. However, the values of Bence Jones κ -chain, Bence Jones λ -chain, and α_1 -acid glycoprotein measured using the AV6B method were lower than the corresponding values measured using the other methods. γ -Globulins and Tamm-Horsfall protein measured by the AV6B method had almost the same values as albumin. The reactivity of these purified proteins in the AV6B and PRM methods were about the same, but reactivity in the CBB method was slightly lower. The reactivity of Tamm-Horsfall protein were especially weak using the CBB method.

Fig. 3. Linearity of albumin measurement using the CBB (closed triangles), PRM (closed squares), and AV6B (closed circles) methods.

Comparison of the AV6B, CBB, and PRM Methods in the Urine of Healthy Subjects

Fifty-seven urine samples from healthy subjects were assayed using the CBB, PRM, and AV6B methods (Fig. 4). The values determined by the AV6B method (y) correlated with those determined by the CBB method (x) ($y = 0.782x + 53.5$, $r = 0.778$). The values determined by the AV6B method (y) correlated with those determined by the PRM method (x) ($y = 1.407x + 4.4$, $r = 0.851$). When total urinary protein levels in 57 cases were compared, the values obtained by the AV6B method were higher than those from the CBB and PRM methods, except for seven cases with the CBB method and five cases with the PRM method.

Comparison of the AV6B, PRM, and CBB Methods in the Urine of Diabetics

We assayed 123 urine samples from diabetes mellitus patients using the AV6B, PRM, and CBB methods. According to the classification of the nephropathy stage, the calculated regression line and coefficients of correlation are shown in Table 2. The relationships among three methods are shown in Figs. 5 and 6. In the correlation between the CBB and AV6B methods, the inclination of the regression line became smaller in the order of the pre-nephropathy stage, early nephropathy stage, and overt nephropathy stage. However, the intercept of each regression line was different. In cases of pre-nephropathy, with many samples having < 500 mg/L protein levels, the values measured using the AV6B method (y) were higher than those using the CBB method (x), and they correlated well ($y = 1.243x + 3.61$, $r = 0.904$). In cases of early nephropathy, the values measured using the AV6B method were almost

TABLE 1. Reactivity of various purified proteins using the AV6B, CBB, and PRM methods

Protein	AV6B method ^a (mg/L)	CBB method ^b (mg/L)	PRM method ^c (mg/L)
Albumin	500.0	500.0	500.0
γ -globulins	473.3	337.3	417.4
Bence Jones κ -chain	242.4	295.7	322.9
Bence Jones λ -chain	227.6	341.8	318.6
IgG	633.8	450.8	575.2
IgA	418.9	299.2	327.4
α_1 -acid glycoprotein	127.1	182.3	169.9
β_2 -microglobulin	721.1	638.4	757.0
Tamm-Horsfall protein	468.5	297.5	402.3
Transferrin	760.5	511.9	678.2

^aAV6B, acid violet 6B.

^bCBB, Coomassie brilliant blue G 250.

^cPRM, pyrogallol red-molybdate.

Fig. 4. Comparison of the three methods in the urine of healthy subjects. Figures of correlation show a comparison of (a) the AV6B and CBB methods, and (b) the AV6B and PRM methods.

the same as those measured using the CBB method. In many cases of overt nephropathy, the values measured using the AV6B method were lower than those measured using the CBB method. Overall, the values determined by the AV6B method (y) correlated well with those determined by the CBB method (x) ($y = 0.819x + 52.87$, $r = 0.975$).

In the correlation between the PRM method and the AV6B method, the inclination of the regression line became smaller in the order of the preneuropathy

stage, early nephropathy stage, and overt nephropathy stage, as described above. The values measured by the AV6B method (y) were higher than those measured by the PRM method (x) for preneuropathy subjects ($y = 1.406x - 29.15$), but their coefficient of correlation was low (0.786) due to some samples with low protein level that the values obtained using the PRM method were lesser than those using the AV6B method. The good coefficient of correlations obtained in early nephropathy stage and overt nephropathy stage. For all diabetic subjects, the values measured by the AV6B method (y) correlated well with those measured by the PRM method (x) for all diabetic subjects ($y = 1.087x + 10.88$, $r = 0.978$).

DISCUSSION

In clinical laboratories, turbidimetric methods such as the benzethonium chloride method (12), and dye-binding methods utilizing pigments such as CBB and PRM (2,3) are commonly used for measurement of urinary total protein. Because pigment methods have a higher sensitivity than turbidimetric methods (13), and the AV6B method is a pigment method, we compared the AV6B method with two other pigment methods, the CBB and PRM methods.

The CBB and PRM methods have been widely used to measure urinary total protein in clinical laboratories. They are both simple and easy to use. However, a disadvantage of the CBB method is, that CBB-protein complexes readily adhere to the surface of optical cells and test tubes, leading to the underestimation of results. Furthermore, the use of surface detergents such as SDS and Triton X improved the reactivity of each protein toward CBB pigment, but decreased its sensitivity (14–16). We developed a new method for measuring total urinary protein using AV6B pigment. Although the structure of AV6B pigment is similar to that of CBB pigment, AV6B-protein complexes do not adhere to optical cells.

In this study, we used a long incubation time of 120 min for the end-point assay for manual measurement. Because changing rates of absorbance in γ -globulins and albumin were similar for about 10 min after the start of scanning (Fig. 2), the incubation time could be shortened to around 10 min and our method could be applied as a rate assay in clinical laboratories.

It has been reported that in the urine of healthy subjects there is a higher concentration of Tamm-Horsfall protein than albumin, and that Tamm-Horsfall protein comprises 50% of the total protein concentration (5–8). When glomerular dysfunction progresses in diabetic mellitus, albumin becomes a major protein in urine. However, in the case of diabetic early nephropathy, transferrin, which has higher molecular weight

TABLE 2. Comparison of the AV6B and CBB methods, and the AV6B and PRM methods in each nephropathy stage for regression lines and coefficient of correlation

		Pre-nephropathy (n = 62)	Early nephropathy (n = 36)	Overt nephropathy (n = 25)	Total (n = 123)
AV6B ^a vs. CBB ^b method	Regression line	$y = 1.243x + 3.61$	$y = 1.113x - 31.49$	$y = 0.788x + 125.8$	$y = 0.819x + 52.87$
	r	0.904	0.974	0.959	0.975
AV6B vs. PRM ^c method	Regression line	$y = 1.406x - 29.15$	$y = 1.199x + 11.09$	$y = 1.091x - 13.08$	$y = 1.087x + 10.88$
	r	0.786	0.933	0.966	0.978

^aAV6B, acid violet 6B;

^bCBB, Coomassie brilliant blue G 250;

^cPRM, pyrogallol red-molybdate.

(a)

(b)

Fig. 5. Comparison of the AV6B and CBB methods in the urine of diabetics. (a) All samples measured were plotted, and (b) the range under 500 mg/L protein levels was magnified. Closed circles, closed triangles, and closed squares show the pre-nephropathy stage, early nephropathy stage, and overt nephropathy stage, respectively.

(a)

(b)

Fig. 6. Comparison of the AV6B and PRM methods in the urine of diabetics. (a) All samples measured were plotted, and (b) the range under 500 mg/L protein levels was magnified. Closed circles, closed triangles, and closed squares show the pre-nephropathy stage, early nephropathy stage, and overt nephropathy stage, respectively.

than albumin preceded in urine to albumin because transferrin has higher isoelectric point than albumin, and could pass through the glomerular basement membrane with slightly low negative charge due to diabetic (17–19). It is known that excretion of β_2 -microglobulin and Tamm-Horsfall protein increases in cases of renal tubular dysfunction (5,20). Thus, detection of these proteins may be useful for early diagnosis of nephropathy, and the development of methods having equal reactivity with these proteins is desirable.

γ -Globulins, IgG, IgA, Tamm-Horsfall protein, and transferrin reactivity were about equal in the AV6B and the PRM methods. The CBB method was the least sensitive of three methods. Bence Jones κ -chain, Bence Jones λ -chain, and α_1 -acid glycoprotein showed lower reactivity in the AV6B method than in the CBB and PRM methods. However, Bence Jones κ -chain and Bence Jones λ -chain are minor components of urine except under special pathological conditions, such as multiple myeloma, so their influence can usually be neglected. The measured value of the α_1 -acid glycoprotein level was low in all methods. One reason for this may be that we purchased α_1 -acid glycoprotein as a solution, and diluted it to 500 mg/L according the concentration in the label. Furthermore, in the AV6B method, the poor reactivity of α_1 -acid glycoprotein, in which sugar is abundant, would affect the measurement value. We are in the process of modifying this method to obtain higher sensitivity toward glycoproteins.

In comparison of three methods of urinary protein for samples with low protein concentration, that is, samples in healthy subjects and prenephropathy patients, the protein level obtained by the AV6B method was higher than that obtained by the CBB method in most cases. This was because Tamm-Horsfall protein comprised 50% of the total protein level in healthy subjects, and there was lower reactivity of the CBB pigment with Tamm-Horsfall protein. In the PRM method, absorbance of reactant from the same sample was lower than that in the AV6B method. Thus small changes of absorbance due to factors other than protein could result in large changes of measurement value, especially in the lower range of protein concentrations. Therefore, the PRM method did not correlate well with the AV6B method in the prenephropathy stage (Table 2).

Recently we reported that the measurement of urinary protein in specimens with a low level of proteinuria provided important clinical information for the diagnosis of subclinical stages of tubular diseases (4). In urine specimens with proteinuria <750 mg/L, the fraction of globulins generally increases. In the measurement of low levels of total protein in urine, one of the most desirable characteristics for a dye reagent is equal affinity for all urinary proteins. In this respect, the

AV6B method appears to be the best of the three methods described here, because it has a higher reactivity to globulins than the CBB method and provides more accurate values in the lower range of protein concentrations than the PRM method. At present, the ideal dye reagent has yet to be found; however, our new method is more useful than the CBB and PRM methods, especially for samples with urinary protein concentrations of <1,000 mg/L.

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