Presence of Immunounreactive Albumin in the Urine of Diabetic Patients

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Recent studies have demonstrated that conventional immunochemical assays underestimate urinary albumin concentration because of the presence of immunounreactive albumin. It has been reported that intact urinary albumin in 24-hr diabetic urine samples could be detected as total concentration (immunoreactive+immunounreactive) by an HPLC method based on size exclusion chromatography. The aim of this study was to investigate urinary albumin concentration in diabetic spot urine samples by comparing the HPLC method with several other methods. The albumin concentrations on 80 diabetic spot urine specimens were measured by turbidimetric immunoassay (TIA), high performance liquid chromatography (HPLC), and a dipstick method.

Key words: diabetes; intact albumin; immunounreactive; TIA method; HPLC method; SDS PAGE; Native PAGE

In addition, they were also analyzed by reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) and native polyacrylamide gel electrophoresis (Native PAGE). The albumin concentrations derived from diabetic spot urine samples measured by the HPLC method were higher than those of the other methods except for five of 80 samples. Furthermore, the albumin concentrations analyzed by Native PAGE were higher than SDS PAGE in 61 (76.2%) of 80 samples. This study suggests the need for evaluating diabetes not only by HPLC, but also by combining it with another method. J. Clin. Lab. Anal. 20:29–36, 2006. © 2006 Wiley-Liss, Inc.

INTRODUCTION

Microalbuminuria defined as 30–300 mg/gCr in spot urine samples is routinely detected by immunochemical methods for type 1 and 2 diabetic patients as a test of an earlier diagnosis of diabetic nephropathy [1]. Current data have shown that urinary albumin represents endothelial dysfunction and its excretion is increased in patients with atherosclerosis or hypertension without diabetes [2–5].

Glomerular filtered albumin may be considerably modified by lysosomal proteases in tubular cells before excretion and excreted as <1% intact albumin and >99% albumin-derived fragments [6–10]. These fragments are thought to be undetected by conven-

tional immunochemical methods or urinary dipstick methods [8–10].

Greive et al. reported that immunounreactive albumin increased in diabetic rats, implying that conventional immunochemical assays underestimate urinary albumin concentration since 2001 [11]. This was also found in 24-hr urine samples of diabetic

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Received 6 July 2005; Accepted 6 October 2005

DOI 10.1002/jcla.20100

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

patients, and intact (monomer) total urinary albumin, i.e., immunoreactive albumin plus immunounreactive albumin is thought to be detectable by a high performance liquid chromatography (HPLC) method using a size exclusion column [12]. Furthermore, this method may predict microalbuminuria earlier than conventional immunochemical assays [13–15].

Comper et al. measured the urinary albumin concentration of diabetic patients by an HPLC method, radioimmunoassay (RIA), reducing dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE), and native polyacrylamide gel electrophoresis (Native PAGE). As a result, there were good correlations between the HPLC method and Native PAGE, and the RIA method and SDS PAGE. Purified immunounreactive intact albumin was also analyzed by SDS PAGE, and was all fragmented into smaller bands than the molecular weight of the intact albumin. Therefore, this indicated that immunounreactive intact albumin had structural characteristics of fragmentation due to a reducing process [12].

Recently, a new HPLC system that can detect total intact urinary albumin has been developed. We analyzed the urinary albumin concentration of diabetic spot urine samples and compared measurements of a turbidimetric immunoassay (TIA), which is the standard method in Japan, with the new HPLC method based on the study of Osieka et al [13]. We also investigated a novel dipstick method.

MATERIALS AND METHODS

Subjects

Analysis was performed on 80 spot urine samples from patients with diabetes mellitus (25–89 years old) who consulted Nakano General Hospital (Nakano Ward, Tokyo, Japan), and 50 healthy control subjects. Informed consent was obtained from all subjects. All urine samples were stored at -80°C before analyses.

Urinary Albumin Assays

Urinary albumin concentration was measured with an HITACHI7070 Automatic Analyzer (Hitachi, Tokyo, Japan) using commercial immunoturbidimetry reagents kit (N-assay TIA micro Alb; Nittobo, Tokyo, Japan). The detection limit was 5 mg/L.

HPLC assays were carried out with Accumin^{TM} (Total Intact Albumin Assay^{TM}; AusAm Biotechnologies, Inc., New York, NY). The detection limit was 2 mg/L.

We used an Aution Screen Microalbumin/Creatinine (ARKRAY, Inc., Kyoto, Japan) for the dipstick method. It can detect both urinary albumin and

creatinine, and the concentrations were examined by Aution Max AX-4280 (ARKRAY, Inc., Kyoto, Japan) [16].

Total Urinary Protein and Creatinine Assays

The semiquantitative analysis of total urinary protein was determined by a Silver Dot Blot assay [17]. Urinary creatinine concentration was also measured with an HITACHI7070 Automatic Analyzer using commercial enzyme reagents (N-assay CRE-E; Nittobo).

Electrophoresis

We analyzed 80 diabetic samples with 12.5% uniform polyacrylamide gels (PAG Mini “DAIICHI” 12.5; Daiichi Pure Chemicals, Tokyo, Japan). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) was followed the Laemmli method [18]. Samples were diluted with physiological saline, and approximately 0.6 μg protein was mixed with the sample treatment solution (SDS β ME sample treatment solution, Daiichi pure chemicals) at an equal volume. Then, the sample was denatured for 5 min at 100°C . The running buffer was SDS-Tris-glycine running buffer (Daiichi Pure Chemicals). Polyacrylamide gel electrophoresis (Native PAGE) was followed the Davis procedure [19]. Approximately 0.6 μg protein was also mixed with the sample treatment solution (Tris HCl sample treatment solution; Daiichi Pure Chemicals) at an equal volume. The running buffer was Tris-glycine running buffer (Daiichi pure chemicals). Then, the gels were stained with silver staining kit (Silver stain II kit WAKO; Wako Pure Chemical Industries, Ltd., Osaka, Japan). The albumin band after each electrophoresis procedure was quantified by PC densitometric analysis (Scion Image Beta 4.02 Win; Scion Corporation, Frederick, MD). The concentration was correlated by a 100 mg/L human serum albumin (HSA) standard run on each gel. In this method, densitometry was quantified linearly for 6.25–100 mg/L HSA. HSA was purchased from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan) as the standard for calibration and the linearity for 6.25–100 mg/L HSA was obtained from densitometry.

Western Blotting

The gels were transferred PVDF membranes (Immobilon-P; Millipore). We chose the following antibodies: rabbit anti-human albumin (DAKO, Glostrup, Denmark), peroxidase-conjugated swine anti-rabbit immunoglobulins (DAKO), monoclonal anti-human serum albumin clone HAS-11 (Sigma), and goat anti-mouse IgG (whole molecule) Peroxidase Conjugate (Sigma).

After antibody reaction, the albumin bands were detected using Tris-HCl buffer containing 0.02% 3-3' Diaminobenzidine 4HCl (Wako, Osaka, Japan) and 0.02% H₂O₂.

RESULTS

Comparison of Each Assay

Urinary albumin concentration of diabetic patients measured by TIA was 0.0–736.0 mg/L (mean $\pm$ 2 standard deviation [SD] = 117.7 $\pm$ 367.3 mg/L), and HPLC was 7.0–3877.0 mg/L (mean $\pm$ 2 SD = 308.0 $\pm$ 1294.7 mg/L). It was 2.3–671.8 mg/L (mean $\pm$ 2 SD = 111.4 $\pm$ 342.3 mg/L) by the dipstick method. Figure 1. shows the differences among these three methods. The correlation coefficient between the TIA method and

dipstick method was 0.971 ($y = 0.908x + 0.581$, $P < 0.01$), 0.934 ($y = 0.418x - 0.429$, $P < 0.01$) between the HPLC method and dipstick method, and 0.953 ($y = 0.49x - 0.637$, $P < 0.019$) between the TIA method and HPLC method.

In the HPLC method, albumin concentrations of 75 of 80 samples were higher than those of the other two assays. The measurements by the TIA method were higher than those by the dipstick method in 43 samples. Albumin concentrations of all the samples analyzed by the HPLC method were higher than those of the TIA method. The assay of albumin by the HPLC method was more sensitive than the TIA or dipstick methods (Table 1).

Diabetic samples were expediently divided into three groups according to the albumin concentration detected by the HPLC method: <30 mg/L (25 samples), 30–100 mg/L (23 samples), >100 mg/L (32 samples). A comparison of the TIA method and HPLC method for albumin concentration analyzed in each group is shown in Fig. 2. The coefficient of correlation in the <30 mg/L group was 0.589 ($y = 2.43x - 1.04$, $P < 0.01$), that of the 30–100 mg/L group was 0.681 ($y = 1.91x + 8.97$, $P < 0.01$), and the >100 mg/L group was 0.951 ($y = 1.85x + 19.8$, $P < 0.01$).

We also showed in the 46 samples (group A) that albumin concentration detected by the HPLC method was twice as high as with the TIA method. Albumin concentrations of these 46 samples analyzed by the TIA and HPLC methods gave good correlation $r = 0.978$ ($y = 2.72x + 0.552$, $P < 0.01$) (Table 2). The other 34 samples (group B) also showed a good correlation coefficient of 0.982 ($y = 1.68x - 0.764$, $P < 0.01$).

The albumin band widths for on each electrophoresis procedure for group A and B were compared (Table 2). The band width on the SDS PAGE of group A was 04.0 mm (mean $\pm$ 2 SD = 2.18 $\pm$ 1.54 mm) and that on Native PAGE was 0–6.0 mm (mean $\pm$ 2 SD = 4.00 $\pm$ 3.09 mm). There was no significant difference. On the other hand, the band width on SDS PAGE of group B was 1–3.0 mm (mean $\pm$ 2 SD = 2.28 $\pm$ 1.09 mm) and that on Native PAGE of group B was 1–8.0 mm (mean $\pm$ 2 SD = 4.12 $\pm$ 3.09 mm). There was a significant difference.

Fig. 1. Comparison of albumin concentrations detected by each method: (A) TIA method and dipstick method, (B) HPLC method and dipstick method, and (C) TIA method and HPLC method.

TABLE 1. Ratio of urinary albumin concentration determined by the three methods

	Albumin concentration	Creatinin Compensation value
HPLC	1.00	1.00
TIA	0.38	0.39
Dipstick	0.36	0.32

Fig. 2. Comparison of albumin concentrations detected by the HPLC and TIA methods more closely correlated. The three groups were classified according to albumin concentration measured by the HPLC method: (A) <30 mg/L, (B) 30–100 mg/L, and (C) >100 mg/L.

TABLE 2. Comparison with the group that albumin concentration detected HPLC method was twice as high as that of TIA method and the other subjects group

	HPLC > 2 × TIA group	The others
	Mean ± 2 SD	Mean ± 2 SD
TIA (mg/L)	96.70 ± 344.5	146.2 ± 388.9
HPLC (mg/L)	366.2 ± 1662	229.9 ± 583.6
SDS PAGE (mg/L)	243.0 ± 1160	159.4 ± 347.6
Native PAGE (mg/L)	402.2 ± 1746	218.8 ± 530.7
SDS PAGE albumin band width	2.180 ± 1.540	2.280 ± 1.090
Native PAGE albumin band width	4.000 ± 3.090	4.120 ± 3.090

Diabetic urine samples were divided into three groups according to the creatinine value of albumin concentration measured by the TIA method, HPLC method, and dipstick method: normal (<30 mg/gCr), microalbuminuria (30–300 mg/gCr), macroalbuminuria (>300 mg/gCr).

The diagnosis results are shown in Table 3. In 51 (63.8%) of 80 samples, the diagnosis by each of the three methods was the same. Between the dipstick method and TIA method there were 64 (80%) samples, between the dipstick method and HPLC method there were 53 (66.3%) samples, and between the TIA method and HPLC method there were 64 (80%) samples. The 26, 23, and 27 (Table 3A, B, and C, respectively) samples corresponding to microalbuminuria using the two methods were included in the same 22 samples.

There were seven (8.8%) of 80 samples diagnosed as a normal albumin concentration by the TIA and dipstick methods, these were classified as microalbuminuria by the HPLC method. While eight (10%) of 80 samples were diagnosed as normal by the dipstick method were classified as microalbuminuria by the TIA method, and three (3.8%) samples were contrary (Table 3A). Thirteen (16.3%) samples classified as normal albumin concentration by the dipstick method were classified as microalbuminuria by the HPLC method, and three (3.8%) samples were contrary (Table 3B). Between the TIA method and HPLC method, 8 (10%) samples diagnosed as normal albumin concentration by the TIA method were classified as microalbuminuria by the HPLC method (Table 3C). These eight samples were included in 13 samples classified as normal by the dipstick method, but as microalbuminuria by the HPLC method.

We also compared albumin measurements of the TIA and HPLC methods in 55 healthy control samples. Albumin concentration detected by the TIA method was 1.1–16.9 mg/L (mean ± 2 SD = 6.2 ± 8.2 mg/L), but was 4–33 mg/L (mean ± 2 SD = 14.0 ± 14.5 mg/L) by the HPLC method. Except for two samples, albumin concentrations by the HPLC method were higher than the TIA method ($y = 1.82x + 2.91$, $r = 0.649$, $P < 0.01$). Fifty-five samples were also divided into three groups the same as the diabetic samples, and 45 (81%) of 55 samples were classified as normal albumin concentration. However, the remaining 10 (18.2%) samples diagnosed as normal by the TIA method were classified as microalbuminuria by the HPLC method.

Comparison of SDS PAGE and Native PAGE

Typical electrophoresis patterns are shown in Fig. 3. Each albumin band of 80 diabetic urine samples was measured with slide calipers. As the results, albumin band widths on SDS PAGE were 0.0–4.0 mm (mean ± 2 SD = 2.2 ± 1.4 mm) and on Native PAGE were 0.0–8.0 mm (mean ± 2 SD = 4.05 ± 3.1 mm). Albumin bandwidths on SDS PAGE were larger than Native PAGE in nine of 80 samples, while three samples were the same width. The other 68 samples, and albumin

TABLE 3. The differences in diagnoses among the three methods

A.			
	TIA		
	Normal	Microalbuminuria	Macroalbuminuria
	(n = 32)	(n = 34)	(n = 14)
Dipstick			
Normal (n = 37)	29	8	0
Microalbuminuria (n = 34)	3	26	5
Macroalbuminuria (n = 9)	0	0	9
B.			
	HPLC		
	Normal	Microalbuminuria	Macroalbuminuria
	(n = 24)	(n = 36)	(n = 20)
Dipstick			
Normal (n = 37)	21	13	3
Microalbuminuria (n = 34)	3	23	8
Macroalbuminuria (n = 9)	0	0	9
C.			
	HPLC		
	Normal	Microalbuminuria	Macroalbuminuria
	(n = 24)	(n = 36)	(n = 20)
TIA			
Normal (n = 32)	24	8	0
Microalbuminuria (n = 34)	0	27	7
Macroalbuminuria (n = 14)	0	1	13

band width Native PAGE samples were larger than those of SDS PAGE.

Urine albumin concentrations of diabetic samples analyzed by SDS PAGE were $0.86\text{--}2444.4\text{ mg/L}$ (mean $\pm 2\text{ SD} = 207.0 \pm 912.6\text{ mg/L}$), and the concentrations by Native PAGE were $1.16\text{--}3451.8\text{ mg/L}$ (mean $\pm 2\text{ SD} = 324.2 \pm 1380.6\text{ mg/L}$). Analysis by these two electrophoresis techniques showed a good correlation ($y = 0.554x + 10.02$, $r = 0.903$, $P < 0.01$). In 61 of 80 samples, urinary albumin concentrations calculated from Native PAGE were higher than those of SDS PAGE.

Comparison of the Three Albumin Assays and Two Electrophoretic Profiles

We also compared the differences among the three albumin assays and two electrophoretic profiles in order to investigate in more detail. There was a statistically

good correlation among the TIA method and SDS PAGE ($y = 0.803x - 11.1$, $r = 0.910$, $P < 0.01$), and the TIA method and Native PAGE ($y = 0.434x - 2.58$, $r = 0.969$, $P < 0.01$). Between the dipstick method and SDS PAGE was 0.910 ($y = 1.25x - 14.1$, $P < 0.01$), while the dipstick method and Native PAGE was 0.942 ($y = 2.3x + 6.36$, $P < 0.01$). There was also a good correlation between the HPLC method and SDS PAGE ($y = 1.67x - 18.1$, $r = 0.910$, $P < 0.01$), and HPLC method and Native PAGE ($y = 0.951x - 4.38$, $r = 0.956$, $P < 0.01$). These results showed that the three measurements were correlated with Native PAGE in particular; however, on the whole, they correlated well with each other.

Next, we investigated the eight samples classed as microalbuminuria in the HPLC method, although diagnosed as normal by the TIA method. Two electrophoretic samples from eight are shown in Fig. 4. The albumin concentrations calculated from SDS PAGE

Sample No	1	2	3	4
TIA(mg/L)	301.0	207.0	89.0	102.0
HPLC(mg/L)	545.0	406.0	185.0	175.0

Fig. 3. Common electrophoretic figures. (A) is SDS PAGE, and (B) is Native PAGE of the same samples. The main protein is albumin in Native PAGE; however, some low molecular weight protein bands were increased in SDS PAGE.

were 0.86–61.6 mg/L (mean ± 2 SD = 21.2 ± 32.6 mg/L), and from Native PAGE were 1.2–33.6 mg/L (mean ± 2 SD = 19.5 ± 33.2 mg/L). The albumin bandwidths of SDS PAGE were 0.0–2.5 mm (mean ± 2 SD = 1.9 ± 1.9 mg/L), and of Native PAGE were 0.0–4.5 mm (mean ± 2 SD = 2.1 ± 2.7 mg/L).

Furthermore, we investigated the 27 samples diagnosed as microalbuminuria by both the TIA and HPLC methods. The albumin concentrations calculated from SDS PAGE were 22.7–2,072.4 mg/L (mean ± 2 SD = 176.0 ± 768.1 mg/L), and from Native PAGE were 25.8–2,576.7 mg/L (mean ± 2 SD = 264.6 ± 972.1 mg/L). The albumin band widths of SDS PAGE were 1.0–4.0 (mean ± 2 SD = 2.2 ± 1.2 mg/L), and of Native PAGE were 2.5–6.0 mm (mean ± 2 SD = 4.6 ± 2.2 mg/L).

DISCUSSION

Serum albumin is produced in the liver and modified during renal passage before being excreted in urine.

Albumin molecules are thought to be fragmented during renal passage in healthy samples [20–24]. The TIA method is commonly used for a urinary albumin diagnosis in Japan.

We compared the albumin concentrations in diabetic urine samples measured by the TIA, HPLC, and dipstick methods. In this study, the albumin concentrations measured by the HPLC method were higher than those of the other methods in all but five of 80 samples. As compared with the TIA method, the albumin concentrations by the HPLC method were more than twice as high in 46 of 80 samples. In addition, as compared with the dipstick method, the albumin concentrations by the HPLC method accounted for 55 of 80 samples. These results suggest that the TIA or dipstick methods underestimate urinary albumin concentration more than the HPLC method. There may be intact albumin detected by the HPLC method because of intact molecular weight, but that did not react with the anti human albumin antibody or bound the pigments.

(A) is SDS PAGE, and (B) is Native PAGE of the same samples.

There are two types of albumin, a monomer and polymer [12,25]. The polymer albumin exists in protein urea. This means that both monomer albumin and polymer albumin can be detected by the TIA method. Essentially, concentrations of immunoreactive polymer albumin must be subtracted from total immunoreactive albumin concentrations measured by the TIA method when compared with the HPLC method, as the HPLC method can measure only intact (66 kD) monomer albumin (including immunoreactive+immunounreactive albumin). We did not analyze immunoreactive polymer albumin only; however, we confirmed that albumin concentrations detected by the HPLC method were higher than those by the TIA method, as reported by Comper et al. [26].

The classification of normal, microalbuminuria, and albuminuria by each assay was not consistent with the current diagnostic criteria by the immunochemical method. Especially, we would like to point out the discrepancy in the microalbuminuria diagnosis. While eight (10%) of 80 samples were classed as normal by the dipstick method, they were classified as microalbumi-

nuria by the TIA method, and three (3.8%) samples were contrary. Thirteen (16.3%) samples classed as normal albumin concentrations by the dipstick method were classified as microalbuminuria by the HPLC method, and 3 (3.8%) samples were contrary. Between the TIA and HPLC methods, eight (10%) samples diagnosed as normal albumin concentrations by the TIA method were classified as microalbuminuria by the HPLC method. These data suggest that measurement of urinary albumin by the HPLC method is likely to cover all intact albumin molecules; however, we need to diagnose combining other applications.

We also compared albumin concentration analyzed by the HPLC and TIA methods in healthy controls. There were 10 (18.2%) of 55 samples classified as microalbuminuria by the HPLC method, but diagnosed as normal by the TIA method. We need to observe these healthy controls from now on and see whether these 10 samples develop diabetes mellitus or endothelial dysfunction. On the other hand, in the case of false positives, the original reference interval for the HPLC method may be required.

Our results indicated that albumin concentration seemed not to correlate with the width of the albumin band in the two electrophoresis method. Urinary albumin concentration in diabetic samples analyzed by SDS PAGE, Native PAGE, the TIA method, HPLC method and dipstick method correlated well with each other; in particular, these three analyses were well correlated with Native PAGE. These results suggest that the TIA and HPLC methods do not necessarily reflect SDS PAGE or Native PAGE specifically. Our results were not consistent with those of Osieka et al [12]. They reported that albumin concentrations in diabetic urine by the TIA method were correlated with SDS PAGE, and albumin concentrations by the HPLC method were correlated with Native PAGE. As a result, they suggested the TIA method and SDS PAGE reflected immunoreactive albumin, and while the HPLC and Native PAGE methods reflected immunounreactive albumin [12].

They found purified immunounreactive albumin molecules from diabetic samples were degraded into smaller molecular weight fragments than intact albumin (66 kD) on SDS PAGE. We did not purify the samples: SDS PAGE of 61 (76.2%) of 80 samples had lower albumin concentrations than on Native PAGE. This may indicate that decomposition of immunounreactive albumin, as reported by Osieka et al. occurred in our 61 samples. On the other hand, albumin measurements from Native PAGE were lower than SDS PAGE in the remaining 19 samples. This makes it hard to say that decomposition occurred. These results suggest that immunounreactive albumin molecules are not decomposed in all diabetic samples by reducing SDS PAGE and that there are individual differences. We will further investigate urinary albumin molecules in diabetes mellitus and healthy volunteers.

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