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Direct quantification of pentosidine in urine and serum by HPLC with column switching

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Concentrations of pentosidine, an advanced glycation end product, are increased in aging, diabetes mellitus, and uremia. Using HPLC with column switching, we developed a direct method of measuring pentosidine in urine and serum. We inject the sample directly onto a gel-filtration precolumn, select ("heart-cut") the eluate fraction containing pentosidine, and introduce this fraction into a reversed-phased column by use of a switching valve. The recovery rate of the complete method was 97.7-99.9%. The intraassay CV was 5.7%, and the interassay CV was 5.8%. The calibration curve showed significant linearity (r = 0.998, P = 0.0001). We examined urinary concentrations of pentosidine in 12 diabetic patients (mean ± SD, 8.7 ± 2.3 μmol/mol of creatinine), 32 patients with chronic renal failure (CRF; 36.1 ± 39.0), 19 osteoporotic patients (7.9 ± 5.3), and 29 healthy control subjects (5.2 ± 2.3). In CRF, urinary pentosidine in the patients undergoing hemodialysis was significantly higher than in CRF patients not being treated by hemodialysis (mean, 58.1 vs 18.2; P <0.001). Also, concentrations of urinary and serum pentosidine were significantly correlated (r = 0.797, P = 0.0011). Because this method does not require pretreatment of samples, it is convenient and useful for measuring urinary and serum pentosidine.

INDEXING TERMS: chromatography, reversed-phase • chromatography, gel-filtration • sample treatment • advanced glycation end product • diabetes mellitus • chronic renal failure • hemodialysis • osteoporosis

Proteins in long-lived tissues are modified posttranscriptionally by reducing sugars. Chronic hyperglycemia leads to the accumulation of nonenzymatically derived glycation products on proteins. The early glycation products undergo a slow rearrangement to form irreversible advanced glycation end products (AGEs).[1] AGEs are believed to contribute to the chronic complications of diabetes mellitus (DM). Pentosidine, one of those AGEs, is a bifunctional condensation cross-link product of arginine, lysine, and ribose and is formed through the Maillard reaction *[1]*. Lyons et al. *[2]* and Sell and Monnier *[3]* reported that pentosidine accumulates in human tissues with age and that its formation is affected by the acceleration of this process in diabetic and uremic conditions. Pentosidine concentrations are also increased in serum *[4, 5]* and urine *[6]* under diabetic and uremic conditions. Hricik et al. reported that kidney and kidney–pancreas transplantations were accompanied by greatly decreased concentrations of serum pentosidine in patients with end-stage renal disease *[7]*. Therefore, pentosidine may be useful as a biochemical marker for examining the status and complications of DM and uremia and also for monitoring the effects of treatment for these disorders.

Column switching has been used with direct injection of biological fluids into HPLC to exclude interferences from endogenous substances in the biological fluids *[8]*. The column-switching technique was developed to determine the urinary excretion of pyridinoline, a trifunctional intermolecular fluorescent cross-link in collagen *[9]*. In a previous study, we developed a method to quantify both cross-links, pyridinoline and pentosidine, in hydrolysates of human urine *[10]* and serum *[5]*, using SP-Sephadex C-25 to pretreat the samples for HPLC. However, this method requiring pretreatment of samples is long and tedious. Having previously observed that 80-90% of pentosidine was present as its free (noncomplexed) form in urine from healthy subjects and diabetic patients *[6]*, we no longer hydrolyze urine samples to measure the urinary excretion of pentosidine. Here we report our development of a direct method for measuring pentosidine in urine and serum without pretreating samples.

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[1] Nonstandard abbreviations: AGEs, advanced glycation end products; HFBA, *n*-heptafluorobutyric acid; DM, diabetes mellitus; CRF, chronic renal failure; HD, hemodialysis; and OP, osteoporosis.

Materials and Methods

REAGENTS

HPLC-grade acetonitrile and sequenation-grade *n*-heptafluorobutyric acid (HFBA) were obtained from Wako Pure Chemical Industries, Osaka, Japan. All water used was purified with a MilliQ Water Purification System (Millipore Corp., Bedford, MA).

We prepared calibrators from pentosidine isolated from human articular cartilage. Methods of purification and characterization were described previously *[11]*.

SUBJECTS

We studied 15 diabetic patients, ages 22-74 years (mean ± SD, 55.5 ± 12.1), who all had type 2 DM and were currently being treated with insulin.

Of 29 patients with chronic renal failure (CRF), ages 43-83 years (mean ± SD, 64.2 ± 10.2), 13 were end-stage renal failure patients undergoing hemodialysis [HD group; ages 48-69 years (mean ± SD, 61.0 ± 6.6)]. The other 16 patients [ages 43-83 (mean ± SD, 66.8 ± 12.0)] were not treated with hemodialysis (non-HD group); their glomerular filtration rates of creatinine clearances were <30 mL/min.

We also studied 19 osteoporotic (OP) patients, ages 71-92 years (mean ± SD, 81.5 ± 6.0), who had low-energy hip fracture. Patients with renal dysfunction were excluded from the DM and OP groups.

The control group consisted of 29 healthy volunteers, ages 47-60 years (55.3 ± 3.2), with no history of DM, no renal dysfunction, and no current known disease.

SAMPLE COLLECTION AND TREATMENT

We collected urine samples from all participants and stored them at −30 °C until analysis. Blood was collected from the 13 HD patients (with CRF) and from 4 healthy normal subjects (ages 28-31 years) who were not included in the control group. All procedures were in accordance with the principles of the Declaration of Helsinki in 1975, as revised in 1983.

We centrifuged urine and blood samples at 1000*g* for 15 min to exclude some contamination. For comparison studies, we hydrolyzed portions of the samples with acid, mixing 1 mL of sample with 1 mL of concentrated HCl and heating at 110 °C for 20 h in a sealed glass tube. After filtering the hydrolysate through a 0.45-μm pore-size membrane filter (DISMIC-25cs; Toyo Roshi, Tokyo, Japan), we mixed the filtrate (125 μL) with 5 mL of water, evaporated the mixture under reduced pressure with a TC-8 concentrator (Taitec, Tokyo, Japan), and dissolved the residue in 200 μL of 10 mL/L HFBA.

Because the concentrations of pentosidine in urine were expressed as μmol/mol creatinine, we determined the creatinine content of aliquots of the urine samples enzymatically, using a Shimadzu CL-20 clinical chemistry analyzer (Shimadzu, Kyoto, Japan).

HPLC INSTRUMENTATION AND PROCEDURE

The HPLC system consisted of a Model CCPM-II pump, which has two pumps, a Model FS-8010 spectrofluorometer, a Model UV-8010C ultraviolet absorbance detector, a Model VC-8020 six-port switching valve, a Model SD-8022 air vacuum pump, a Model AS-8020 autosampler, and a Model SC-8020 super system controller (all from Tosoh, Tokyo, Japan). A gel-filtration column, TSK precolumn PW (4.6 mm × 3.5 cm), was used as column 1, and an octadecylsilyl column, TSK-GEL ODS-80T (4.6 mm × 15 cm), was used as column 2 (both columns from Tosoh). The mobile phase for column 1 was 50 mL/L acetonitrile containing 30 mmol/L HFBA; the mobile phase for column 2 was 200 mL/L acetonitrile containing 30 mmol/L HFBA. The flow rate was 1.0 mL/min. Detector 1, which detected absorbance at 297 nm, monitored the separation of analyte on column 1. Detector 2, a spectrofluorometer, detected pentosidine eluted from column 2 (emission 385 nm, excitation 335 nm).

A schematic diagram of the HPLC system with the column-switching valve is shown in Fig. 1. At time zero, we injected sample onto column 1 and eluted with mobile phase 1, while column 2 was eluted with mobile phase 2 (valve position A). Just before the pentosidine was eluted from column 1, the valve was switched to position B and the eluate fraction containing pentosidine was introduced into column 2. After the transfer of pentosidine to column 2, the valve was switched back to position A. Then, the pentosidine-containing eluate from column 1 was further separated by column 2 and detected by detector 2; during this time, column 1 was washed and conditioned with mobile phase 1 until the next injection of sample. Thus, at 2.8 min after sample injection, the valve was switched to position B; at 4.2 min, it was switched back to position A. All operations were performed automatically and sequentially by the autosampler and the system controller.

Sample injection volumes were 5-160 μL, depending on the experimental conditions. For routine analysis of samples without hydrolysis, 60 μL of urine or 5 μL of serum was injected into HPLC. For the hydrolyzed samples, 120 μL of urine hydrolysate or 10 μL of serum hydrolysate (prepared as described above) was injected into the HPLC. Under these experimental conditions, the minimum amount of pentosidine detectable (signal-to-noise ratio = 4) was ~0.33 pmol/injection.

STATISTICAL ANALYSIS

Statistical significance was determined nonparametrically by Mann–Whitney *U*-tests between two groups and by Kruskall–Wallis tests among multiple groups. After simple regression analysis, the statistical significance of the correlation was deter-

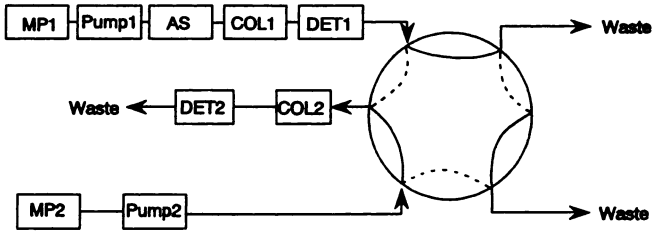

Fig. 1. HPLC system with a column-switching valve.

AS, autosampler; *DET*, detector; *COL*, column; *MP*, mobile phase. *Solid lines*, valve position A; *dotted lines*, valve position B.

mined by the *F*-test. The analyses were performed with StatView II software (Abacus Concepts, Berkeley, CA) on a Macintosh computer (Apple Computer, Cupertino, CA). $P < 0.05$ was considered significant.

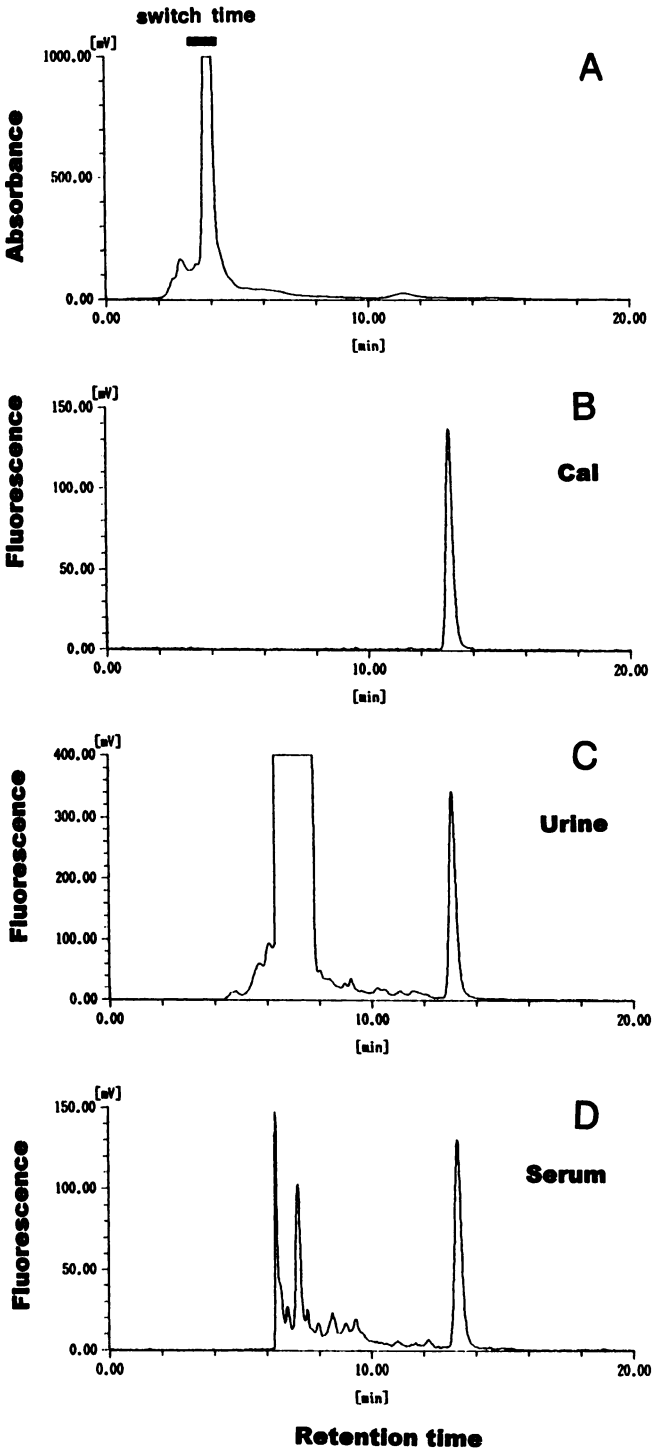

Fig. 2. Chromatograms of a calibrator, urine, and serum.

(*A*) Unhydrolyzed urine sample from a HD patient, monitored with an ultraviolet absorbance detector; (*B*) pentosidine calibrator, monitored with a fluorescent detector; (*C*) unhydrolyzed urine sample from a HD patient; (*D*) hydrolyzed serum sample from a HD patient. *Bar* in *panel A* indicates the duration of collection of fraction ("heart cutting") to be switched onto column 2.

Results

Figure 2A shows a typical chromatogram of a urine sample eluted from column 1 and monitored for ultraviolet absorbance with detector 1; at the zone marked in the chromatogram, the effluent flow is switched into column 2. The other panels in Fig. 2 show typical chromatograms of a pentosidine calibrator (B), a urine sample without hydrolysis (C), and a hydrolyzed serum sample (D) from an HD patient, after passage through column 2 as monitored with a fluorescence detector (detector 2).

We generated a calibration curve by assaying seven urinary calibrations, prepared by adding 40 pmol to 4 nmol of pentosidine to 0.5 mL of urine from a healthy subject and injecting 70 μL of this onto the HPLC. Linear regression analysis showed significant linearity of the curve (r = 0.998, slope = 6.149, intercept = −0.292, SE = 0.17, P = 0.0001) (Fig. 3). To calculate the recovery rate of pentosidine, we compared the results of direct injection onto column 2 with the results of the column-switching method for 10 μL of a calibrator solution added to a urine sample. The recovery rate was 99.9% for a 377 nmol/L calibrator added to the urine and 97.7% for a 754 nmol/L calibrator. At 337 nmol/L pentosidine, the intraassay CV was 5.7% (n = 25), and the interassay CV was 5.8% (n = 25).

We detected pentosidine in both hydrolyzed and unhydrolyzed urine samples from control subjects and HD patients but not in unhydrolyzed serum from control subjects. Pentosidine was detected in unhydrolyzed serum from HD patients, even though the concentration was much lower than in hydrolyzed serum. Pentosidine exists in body fluids both free and peptide-bound. In unhydrolyzed samples, free pentosidine is measured, whereas in hydrolyzed samples, the total for free and peptide-bound pentosidine is measured. Table 1 shows the percentages of total pentosidine present as free pentosidine in urine and serum from HD patients and control subjects. In urine, the

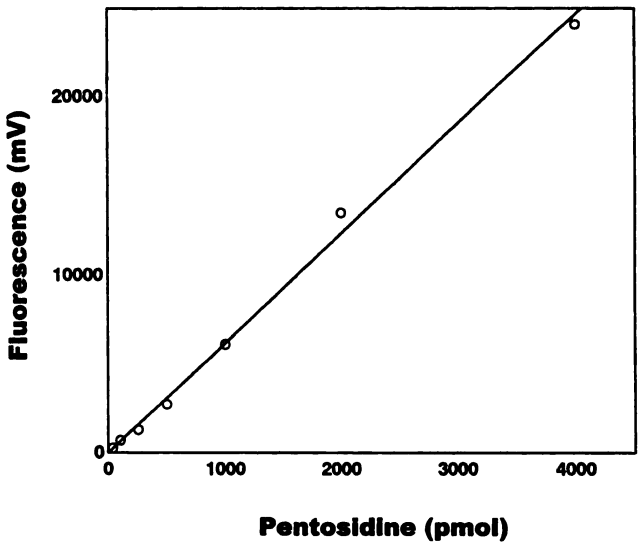

Fig. 3. Linear regression analysis of a calibration curve generated with seven calibrators prepared by adding pentosidine (40 pmol to 4 nmol) to 0.5 mL of urine.

Linear regression equation: $y = 6.149x - 0.292$ (r = 0.998, P = 0.0001).

Table 1. Percentages of free pentosidine in urine and serum from patients undergoing HD and control subjects.

	% of total pentosidine, mean ± SD[a]	
Sample	HD (n = 13)	Control (n = 4)
Urine	95.3 ± 8.4	95.7 ± 7.9
Serum	4.3 ± 2.6	0[b]

[a] [Free (concentration in sample without hydrolysis)/total (concentration in hydrolyzed sample)] × 100.

[b] Pentosidine was not detected without hydrolysis in serum from control subjects.

percentage of free pentosidine was 95.3% in HD patients and 95.7% in control subjects; in serum, however, the percentage of free pentosidine was only 4.3% in HD patients and undetectable in the control subjects.

The mean ± SD concentrations of pentosidine in urine from control subjects and from DM, CRF, and OP patients (Fig. 4) were 5.2 ± 2.3, 8.7 ± 2.3, 36.1 ± 39.0, and 7.9 ± 5.3 μmol/mol creatinine, respectively. These means differed significantly between groups ($P = 0.0001$). In CRF, the mean concentration of urinary pentosidine in HD patients was significantly higher than in non-HD patients (58.1 ± 47.8 vs 18.2 ± 16.1 μmol/mol creatinine, $P < 0.001$). There was also a significant correlation between urinary pentosidine and serum pentosidine in HD patients ($r = 0.797$, $P = 0.0011$) (Fig. 5) and between urinary pentosidine and the duration of CRF in HD patients ($r = 0.680$, $P = 0.0106$) (Fig. 6A) but not between urinary pentosidine and the duration of CRF in non-HD CRF patients ($r = 0.173$, $P = 0.5221$) (Fig. 6B). The concentration of serum pentosidine was significantly correlated with the duration of CRF ($r = 0.693$, $P = 0.0087$) and HD ($r = 0.718$, $P = 0.0057$) (Fig. 6, C and D).

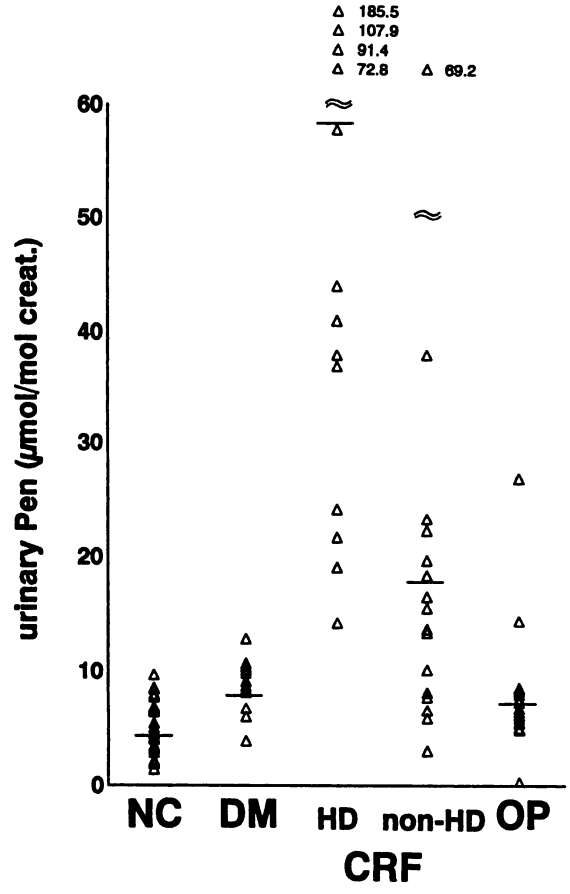

Fig. 4. Concentrations of urinary pentosidine in the control subjects (NC) and in the patients with DM, the patients with CRF undergoing HD or not (non-HD), and the OP patients.

Bars indicate means, which were significantly different ($P = 0.0001$) among groups.

Discussion

Pentosidine has been measured by HPLC in dura mater *[1]*, lens *[2]*, skin *[3]*, cartilage *[12]*, and glomerular basement membrane *[13]* under various conditions. However, the reports on measurements of pentosidine in body fluids have been few. Odetti et al. developed a combined reversed-phase ion-exchange HPLC method to measure pentosidine in serum hydrolysate *[4]*. In their method, reversed-phase HPLC fractions containing a pentosidine peak were collected and then rechromatographed on another cation-exchange HPLC. We previously reported the method in which SP-Sephadex C25 is the pretreatment before reversed-phase HPLC to measure pentosidine in hydrolysates of serum and urine *[5, 6, 10]*. By requiring two steps for detecting pentosidine in body fluids, these HPLC procedures are relatively tedious. The column-switching method described here does not require pre- or postchromatographic steps. Pentosidine in body fluids is measured by a single injection into a gel-filtration precolumn, which is then switched into a reversed-phase column. The mean serum pentosidine concentrations measured in 13 end-stage renal failure patients by this method

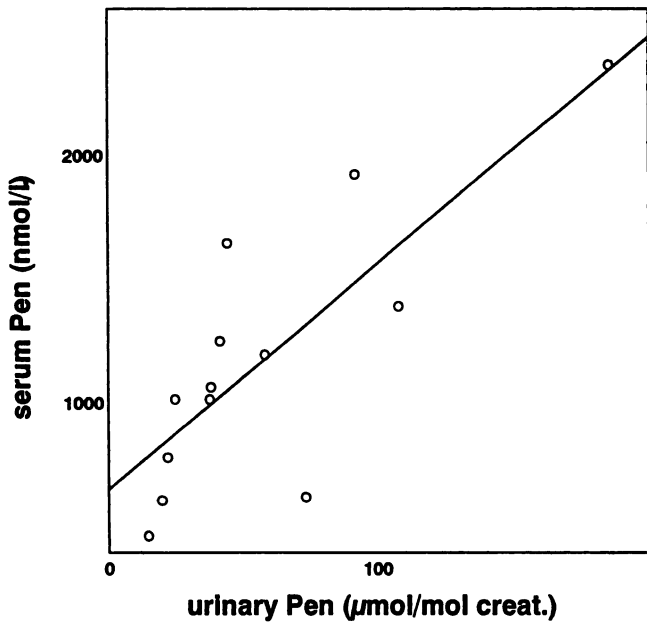

Fig. 5. Correlation between serum pentosidine and urinary pentosidine in HD patients.

Linear regression equation: Serum pentosidine (nmol/L) = 9.177 × urinary pentosidine (μmol/mol creatinine) + 649.5. $r = 0.797$, $P = 0.0011$.

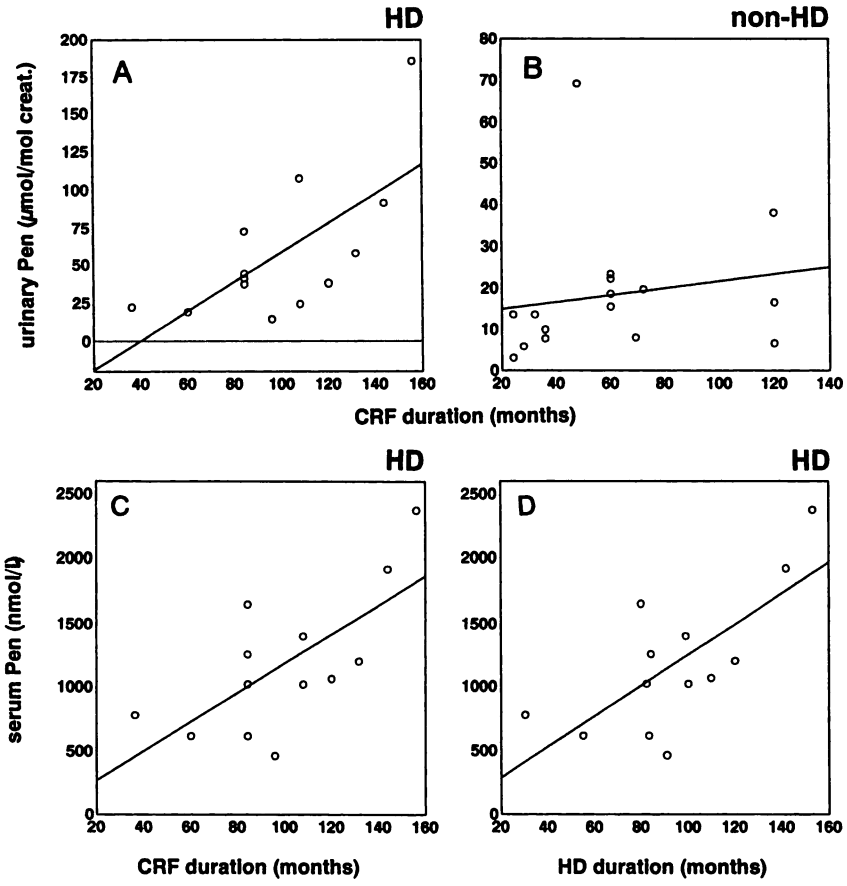

Fig. 6. Relationships between the concentrations of urinary pentosidine and the duration of CRF in HD patients (*A*) and non-HD patients (*B*), and between the concentrations of serum pentosidine and the duration of chronic renal failure (*C*) and hemodialysis (*D*) in HD patients.

Linear regression equation:
A, $y = 0.973\,x - 38.9$, $r + 0.680$, $P = 0.0106$.
B, $y = 0.083\,x + 13.148$, $r = 0.173$, $P = 0.5221$ (not significant).
C, $y = 11.422\,x + 43.722$, $r = 0.693$, $P = 0.0087$.
D, $y = 12.018\,x + 46.159$, $r = 0.718$, $P = 0.0057$.

and by our previous method *[5]* were 1182 ± 550 and 1238.7 ± 621 nmol/L, respectively, significantly correlated ($r = 0.914$, $P = 0.0001$). Therefore, the pentosidine concentrations measured with this direct method are compatible with those of the previous method, but are obtained more conveniently.

A recent immunohistochemical study, using antibodies against pentosidine, demonstrated pentosidine in brain tissue *[14]*. Another study, involving an immunoassay (ELISA), measured pentosidine in serum and tissue hydrolysates *[15]*; we have previously reported measuring pentosidine in urine *[6, 10]*. Although most pentosidine reportedly exists in protein-bound form in blood *[5]*, the free form predominates in urine *[6]*. In the present study, in which we measured pentosidine in urine and serum without and with hydrolysis by using column switching, we found, in accordance with the previous studies, that pentosidine circulates as the protein-bound form in blood and may be catabolized to its free form and excreted into urine. Thus, acid hydrolysis or digestion of the urine samples is not necessary to measure urinary excretion of pentosidine.

The concentrations of urinary pentosidine we measured in the patients with DM correspond to those determined in our previous study *[6]*. The mean urinary pentosidine concentration in OP patients was slightly higher than in control subjects; however, OP patients were older than control subjects (mean ages, 81.5 vs 55.3 years) and pentosidine is reported to accumulate in tissues during aging *[3, 13]*, which means that urinary pentosidine concentrations should increase with age *[10]*.

Pentosidine concentrations in CRF were higher than those in DM and OP. We previously suggested that pentosidine was produced in the body and excreted into urine to prevent its accumulation in tissues *[6]*. The fact that pentosidine is very high in collagen *[1]* and erythrocytes *[4]* from uremic patients suggests that pentosidine precursor sugars are retained by the diseased kidney and that the accumulation of pentosidine precursors may cause the increased synthesis of pentosidine in serum from uremic patients. Accordingly, the increased synthesis of pentosidine may lead to increased urinary excretion of pentosidine in CRF. However, the increased synthesis of pentosidine alone is not enough to explain the extreme increase of pentosidine in uremic serum. Because serum pentosidine concentrations did not change after HD in end-stage renal failure, HD could not eliminate pentosidine from serum *[16]*. Pentosidine may be mostly eliminated or catabolized through the kidney, and a diseased kidney may mean that pentosidine is retained in the blood circulation.

In non-HD patients with CRF, high concentrations of urinary pentosidine may be mainly caused by increased synthesis of pentosidine. The absence of correlation of urinary pentosidine with CRF duration in non-HD patients suggests that most of pentosidine can be excreted into urine; therefore, serum concentrations are below the excretion threshold in such patients. On the other hand, urinary concentrations of pentosidine in HD patients did correlate with the duration of CRF and HD, the urinary pentosidine concentrations in HD patients being

(surprisingly) threefold higher than in non-HD patients. Pentosidine could not be fully excreted by the diseased kidney in HD patients. The significant correlation between urinary pentosidine and serum pentosidine thus indicates that the accumulated pentosidine in serum is directly responsible for the increased excretion.

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