Determination of Urinary and Serum Pentosidine and Its Application to Elder Patients

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Pentosidine, a fluorescent cross-linking compound, accumulates in extracellular matrix, especially in the collagen, and is formed by the nonenzymatic process of advanced Maillard reaction. We developed a method of determination of pentosidine and tried to examine its level in urine and serum of elderly patients. The method, which involves the hydrolysis of samples, pretreatment using a CF-11 cellulose column and HPLC quantification, resulted in a high recovery (94.3%) of pentosidine with low coefficient of variation (8%–10%) of total analysis. Serum and urinary levels of pentosidine in control subjects gradually increased with age. Elder patients with cerebral infarction showed higher levels of serum and urinary pentosidine as compared with those with senile dementia and other geriatric disorders. These results suggest that serious damage to systemic vascular tissues has already occurred in these patients due to glycation.

Key words pentosidine; urinary excretion level; serum; biomedical marker; HPLC; cerebral infarction

Pentosidine, a fluorescent cross-linking compound that accumulates in human tissues, especially in extracellular matrix, was identified and characterized by Sell and Monnier.1) The compound, composed of an imidazo[4,5-b]pyridinium ring that consists of lysine and arginine residues cross-linked by a reducing sugar, is formed by the nonenzymatic process of advanced Maillard reaction. Age-related accumulation of pentosidine in human dura mater collagen has been demonstrated in recent years,1) and it is therefore believed that the amount of pentosidine in a tissue indicates the degree of senescence. The amount of pentosidine in tissues, blood, and urine has provided instructive information in cases of uremia2) and diabetes,3) and thus, determination of the compound aids in understanding the anomalous behavior of extracellular matrix.

Rosenberg et al.3) reported that non-enzymatic glycation of arterial collagen occurs in diabetes, a typical geriatric disorder. Odetti et al. also described the increase of serum pentosidine level in diabetic patients.4)

We were interested in studying the biological relationship between pentosidine and diseases, especially geriatric disorders. Cerebral infarction, a typical geriatric disorder, is common among the aged in Japan. This disease is caused by a localized obstruction of arterial bloodstream in the brain, and results in a partial dysfunction of the brain. Therefore, we conducted a study to determine the serum and urinary pentosidine levels mainly in cases of cerebral infarction, one of the cerebrovascular diseases.

Determination of pentosidine in biological samples has been carried out recently by HPLC.4) However, there are few procedures to determine pentosidine in urine, serum, and tissues by same way. Most pentosidine in biological fluid also contains peptides,5) and thus requires acid hydrolysis; the hydrolysate would also require pretreatment prior to chromatographic analysis.

In the present study, we developed a method of determination of pentosidine using a pretreatment column prior to analytical HPLC, and applied this method to analyze samples from elderly patients.

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MATERIALS AND METHODS

Chemicals tert-Butoxy carbonyl (BOC)-lysine and BOC-arginine were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Acetonitrile (HPLC grade) and heptfluorobutyric acid (for amino acid sequencing analysis; HFBA) were obtained from Wako Pure Chemical Ind., Ltd.,(Tokyo, Japan) and were used without further purification. CF-11 cellulose powder was obtained from Whatman Biochemicals. Other reagents obtained from commercial suppliers were of special-reagent grade and/or analytical grade.

Control Subjects Control serum and urine samples were obtained from healthy volunteers aged 22–70 years. None of the subjects was taking any medication or had any recognizable disease or history of renal disease or diabetes mellitus. Spot urine and serum samples were collected between 9 and 10 a.m. with no dietary restrictions and were stored at −20°C until their analysis.

Patients Serum and urine samples of 42 patients aged 50–57 years were collected from in-patients of the local medical facility located in Sagamihara, Kanagawa, Japan, which is directed by the Department of Third Internal Medicine, School of Medicine. St. Marianna University. They were divided into three groups according to their diagnosed diseases: senile dementia, cerebral infarction, and others, like hypertension, bone fracture, and Parkinson disease. They had no episodes of diabetes or renal pathology, or abnormal data for creatinine clearance. All these in-patients were in good health except for their diagnosed disease and able to walk alone. Only one individual with hyperglycemia, an important factor which can escalate the pentosidine level, was included in the "others" group. Spot urine samples were collected as described above.

Preparation of Pentosidine Standard Standard pentosidine was synthesized and purified, with some modifications, according to the method of Grandhee and Monnier.5) D-Ribose (6.60 g), BOC-lysine (10.0 g) and BOC-arginine (13.66 g) were dissolved in 100 ml of 0.5 M sodium phosphate buffer (pH 8.5). The mixture was heated at 80°C for
6d, and then cooled. The cooled reactant was loaded onto a gel-filtration column (30 mm x 1000 mm, Bio Gel P-2, 45-90 µm, Bio-Rad Labs., CA, U.S.A.) with 10% (v/v) acetic acid as an elution solvent at a flow rate of 36 ml/h. Fluorescence intensity of each fraction (16 ml/tube) was determined by \( E_{\lambda} \): 335 nm, and \( E_{\text{em}} \): 385 nm. The fractions which contained pentosidine were mixed together and evaporated to a greasy substance, then HPLC eluent was added and the preparative HPLC procedure was performed. Briefly, a preparative ODS column (Wakosil-II SC18 AR, 10 mm x 250 mm, Wako Pure Chemical Ind., Ltd.) was used, with the flow rate maintained at 4.5 ml/min. Other conditions were the same as in the analytical method described below. The standard pentosidine obtained was analyzed by mass spectrometry and NMR spectrometry to confirm its structure.

**Pretreatment of Samples** The pretreatment column was prepared as follows. The washing solvent was made up of a mixture of \( n \)-butanol, acetic acid, and water (8 : 1 : 1, v/v). CF-11 slurry was prepared by making a 5% (w/v) suspension of CF-11 cellulose powder in the washing solvent. The pretreatment column was prepared by adding 15 ml of CF-11 slurry to a glass column (8 mm i.d. x 240 mm) stopped with fiberglass.

One milliliter of urine or serum sample was hydrolyzed with an equal volume of concentrated hydrochloric acid (HCl), in a screw-capped Pyrex tube sealed with a Teflon liner, at 108 °C for 18 h to determine the total pentosidine. Thermal stability of pentosidine was already confirmed. An aliquot of hydrolysate (500 µl) was mixed with 500 µl of CF-11 slurry, acetic acid, and 4 ml of \( n \)-butanol, then loaded to the pretreatment column. After washing the column with 30 ml of the washing solvent, pentosidine was eluted from the column with 20 ml of 0.05 M HCl and evaporated to dryness under reduced pressure. The dry residue was then dissolved in 500 µl of HPLC eluent described below and an aliquot (50 µl) of each sample was applied to analytical HPLC.

**Chromatographic Procedure and Instruments** Pentosidine was separated and determined chromatographically by HPLC. The HPLC system was equipped with a L-6200 solvent delivery pump (HITACHI Co., Japan), a model F-1050 fluorescence detector (HITACHI Co.), set at an excitation wavelength of 335 nm and an emission wavelength of 385 nm, and Wakopak column (Wakosil-II SC18 AR, 4.6 x 250 mm, Wako Pure Chemical Ind., Ltd.). The HPLC eluent consisted of 12% acetonitrile (v/v) containing 2.5 mM of HFBA. The flow rate was maintained at 1.0 ml/min and the column was kept at 40 °C. Total cycle time between two injections was 50 min including column washing.

**Statistical Analysis** Serum pentosidine levels were expressed as nm, and urinary levels were nmol/mmol creatinine, which is correct for urine dilution and body mass. The results were expressed as the mean ± standard deviation (S.D.). Comparisons for significance were made using the Student’s t-test, and all p values of less than 0.05 were regarded as statistically significant.

**RESULTS AND DISCUSSION**

**Suitable Pretreatment Conditions** The pretreatment procedure should have the ability to eliminate interfering

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**Fig. 1. Relationship between Butanol Content of Washing Solvent and Recovery of Standard Pentosidine**

Standard pentosidine was added to the top of the bed of the pretreatment column and 30 ml of each component of washing solvent was passed through the column. Volume of pentosidine eluted by 20 ml of 0.05 M HCl was calculated and plotted. The parentheses in the figure show the component ratio of the washing solvent by volume of \( n \)-butanol-acetic acid-water in each point made up.

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**Fig. 2. Relationship between HCl Concentration of Eluant and Recovery of Standard Pentosidine**

Standard pentosidine was added to the top of the bed of pretreatment column and 30 ml of each component of washing solvent was passed through the column. Pentosidine eluted with 20 ml of each concentration of HCl was collected, determined and plotted.

Substances from hydrolysate with a high yield of pentosidine before chromatographic analysis. Cellulose is commonly used as an adsorbent in the determination of pyridinoline. Pentosidine has a similar structure to pyridinoline, hence we chose cellulose as the carrier of pentosidine in the pretreatment column. To determine a suitable composition of washing solvent, the relationship between the proportion of \( n \)-butanol in the solvent and the amount of pentosidine recovered was then investigated (Fig. 1). Recovery rate of pentosidine from the pretreatment column increased with increasing proportion of \( n \)-butanol, and the maximum recovery rate was achieved at the 8 : 1 : 1 ratio of \( n \)-butanol-acetic acid-water.

The eluant of pentosidine was also evaluated (Fig. 2). Water (0 M HCl) recovered 84% of pentosidine from the pretreatment column. Remarkable recovery was observed when acidic eluants were used: 0.05 M HCl recovered 95% of pentosidine while 0.1 M HCl recovered 86%. Therefore, 0.05 M HCl was used as the eluant for the pretreatment procedure in this study.

A pretreatment procedure should be effective in excluding interference from hydrolysate and should give a clear chromatogram of pentosidine. The pretreatment procedure reported earlier, however, was complicated and the rate of re-
covery of pentosidine was not satisfactory. Our present method achieved higher recovery with lower variation, and showed high reproducibility.

Chromatogram of pentosidine The HPLC chromatogram of pentosidine standard was compared with pentosidine in urine and serum sample from healthy volunteers. Pentosidine standard showed a single and sharp peak with retention time of about 16 min (Fig. 3), while that from urine and serum samples gave an irregular chromatogram pattern; a sharp peak with the same retention time as pentosidine standard was observed however, the mass spectrum (Fig. 3 inset) of pentosidine in HPLC analysis of urine samples collected showed the same spectrum as the standard, confirming the substance as pentosidine and the absence of contaminants. Intra-assay coefficients of variation (C.V.) for serum and urine pentosidine measurements were 4.38% \( (n=5) \) and 5.89% \( (n=5) \), respectively. The efficiency of the CF-11 cellulose column procedure for the pretreatment of urine hydrolysate was determined by measuring the recovery of pentosidine standard added to urine before hydrolysis. Determined from 10 separate analyses, recovery \( \pm S.D. \) of pentosidine was 94.3 \( \pm 4.1\% \). The overall reproducibility of the whole analysis, including the pretreatment step, calculated as the C.V. was found to be 8—10%.

Serum and Urinary Pentosidine in Control Subjects Figure 4 shows the results of determination of serum pentosidine in control subjects; the concentration gradually increased with their age. In urinary pentosidine (Fig. 5), a positive correlation \( (r=0.802) \) between pentosidine level and age was also observed. Since pentosidine is known to be formed in the course of senescence in tissues, these data probably represent pentosidine levels due to normal aging. These results from control subjects were used as normal values against the results obtained below.

Serum and Urinary Level of Pentosidine in Elder Patients The pretreatment method proposed here was applied to the samples taken from 32 elderly patients 50—77 years old. Results obtained from control subjects over 50 years of age were used as the reference range and were compared with the results obtained from patients. Serum and urinary pentosidine levels of the control were 434 \( \pm 79 \) nmol/mmol cre. (50—77 years old, \( n=37 \)), respectively. These controls showed no statistical significance to their age compared to the three patient groups. Figure 6 shows high levels of serum pentosidine in case of cerebral infarction and are significantly higher than those in control, while values from the senile dementia and others groups were similar to those of control. The concentration of pentosidine in the circulatory system increased in parallel with the extent of renal failure, but all the patients with cerebral infarction and the other two patient groups main-
tained normal kidney function and creatinine clearance. Similar results were also observed in the determination of urinary excretion levels of pentosidine (Fig. 7). Urinary pentosidine level of the cerebral infarction group is statistically higher than those of control. In the other two groups, senile dementia and others, their urinary pentosidine levels are slightly higher but not statistically significant compared with the control. Diabetic patients also showed a high level of pentosidine whereas there were no patients with hyperglycemia and diabetes in this study. These results, therefore, suggest that the high pentosidine level in the cerebral infarction group may be due to their pathogenesis including their past lifestyle.

We were interested in studying the relationship between serum and urinary levels of pentosidine. Twenty-six serum samples were collected from patients on the same day and at the same time as the urine samples were obtained, and then analyzed. Urinary pentosidine level, as shown in Fig. 8, was correlated with serum pentosidine level, indicating that pentosidine in the circulatory system may be duly excreted into the urine according to its serum concentration.

The relationships between age-related changes of human tissues and geriatric disorders have been studied in recent years. Non-enzymatic glycation is believed to gradually increase with normal aging, that it may cause insolubilization, hardening, and increased resistance against enzymatic digestion, and it is presumed that this reaction is responsible for senescence and geriatric disorders. Results of our study support the findings of the previous reports. High levels of urinary and serum pentosidine in cases of cerebral infarction were confirmed in this preliminary study. A Recent simultaneous study of ours shows that hyperglycemia is not only a factor in pentosidine formation but that oxidative stress also causes accumulation of pentosidine in rheumatoid arthritis with normoglycemia.

It is possible that the systemic aorta collagen has already glycated, formed pentosidine, and made the vessels fragile. In cases of other diseases that involve damage to the vascular vessels, the serum and urinary pentosidine levels of patients should be determined and compared with the present data and recommendations made on their treatment.

REFERENCES