

Original article

## A rapid pretreatment method for the determination of pentosidine in human plasma by high performance liquid chromatography

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### Abstract

**Purpose:** In HPLC-fluorescence measurement of pentosidine in blood, multiple-specimen measurement has problems such as complexity of sample pretreatment, and measurement time. In this study, a sample pretreatment method using a spin column was verified for the purpose of simplifying sample processing.

**Methods:** The blood sample (specimen) used was plasma provided by healthy men and women between the age of 20 to 100 years. For verification of the pretreatment method of the sample, plasma provided by 40 men and women in their 50s to 80s was mixed and used after homogenization. The sample was hydrolyzed under 6 N hydrochloric acid, added to a spin column (Monospin AG) having a mixed group consisting of a cation exchange group and a hydrophobic group as a carrier, followed by elution. Pentosidine was fluorescently detected by HPLC using a reverse phase column. Targeting 72 subjects aged between 20 and 80, a correlation analysis of plasma pentosidine was performed with subject age and age-related physical markers. This study was carried out under the deliberation and approval of the Ethics Review Committee of Doshisha University and the Society for Glycative Stress Research.

**Results:** The recovery rate of pentosidine in the sample was 116% on average by this pretreatment method. The time required for pretreatment after hydrolysis was within 10 minutes. There was a positive correlation between the measured plasma pentosidine levels and age, and a weak positive correlation with HbA1c. Regarding the value of skin autofluorescence (AF) resulting from AGEs and the plasma pentosidine level, the value measured by AGE Reader mu was positively correlated, while by AGEs Sensor was weakly positively correlated.

**Conclusion:** The pretreatment time of the blood sample was shortened from about 12 hours in the ion pair-HPLC method to approximately 10 minutes by using the spin column. Furthermore, this pretreatment could reduce contaminants, and the HPLC measurement time could be shortened to one-third that of the citric acid-HPLC method. It was verified that this method is useful as a multi-specimen assay for pentosidine in blood.

**KEY WORDS:** human plasma pentosidine, high performance liquid chromatography (HPLC), glycative stress

### Introduction

In the process of non-enzymatical reaction with the proteins in the body and reducing sugars, *i.e.* glucose, advanced glycation end products (AGEs) are produced in the tissue<sup>1)</sup>. Accumulation of AGEs in the body is involved in the onset and development of a variety of regenerative diseases, *i.e.*, diabetic complications, arteriosclerosis, and osteoporosis. The

physical effect of such glycation is called glycative stress<sup>2)</sup>.

Pentosidine, a type of AGEs, was isolated from collagen in the human dura matter in 1989<sup>3)</sup>. The pentosidine molecule has an imidazopyridinium ring in which pentose crosslinks between the amino side chains of lysine and arginine, thus showing properties of fluorescence and protein crosslinkable actions. Pentosidine is present in human synovium,

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Glycative Stress Research 2020; 7(3): 211-219  
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cartilage<sup>4,5</sup>, skin collagen<sup>6,7</sup> or other tissue, in which the accumulated amount is known to progress with aging.

Pentosidine accumulation in bone collagen deteriorates bone quality and is associated with fracture risk<sup>8</sup>. High concentrations of blood pentosidine lead to the onset and progression of coronary artery disease<sup>9</sup>. Besides, it has been reported that blood pentosidine levels are high in the patients with diabetes<sup>10</sup>, chronic renal failure<sup>11,12</sup>, rheumatoid arthritis<sup>13,14</sup>, and schizophrenia<sup>15</sup>. Thus, the amount of blood pentosidine may be a biomarker for various diseases.

Methods for measuring blood pentosidine are classified enzyme-linked immunosorbent assay (ELISA)<sup>16</sup> and HPLC-fluorescence method including ion-pair-HPLC method<sup>17-19</sup> and citric acid-HPLC method<sup>20,21</sup>. The conventional HPLC methods for pentosidine have problems in application to multi-specimen measurement. The complicated pretreatment operations such as, after hydrolysis of blood sample with hydrochloric acid, adsorption/elution by column chromatography and dryness of the sample, and also long measurement time by HPLC had problems. The spin column is a solid-phase extraction column for pretreatment of a small amount of biological samples, which enables rapid concentration/purification by centrifugation without performing concentration/drying operation, e.g., evaporation.

The purpose of this study was to simplify the pretreatment method for HPLC measurement of blood pentosidine. For this purpose, the applicability of the pretreatment method for blood samples, which used a spin column with a cation exchange group and a hydrophobic group (octadecyl group) as the functional group of the carrier, was verified.

## Methods

### Reagents

As a reagent for plasma hydrolysis, 35% iron-free hydrochloric acid (Fe free; Nacalai Tesque, Kyoto, Japan) was used. Acetonitrile for HPLC grade was used (ACN: Fuji Film Wako Pure Chemical, Osaka, Japan). Other reagents with a special grade were purchased from Fuji Film Wako Pure Chemical or Sigma-Aldrich Japan (Meguro-ku, Tokyo, Japan).

### Apparatus

The measurement was performed with the HPLC system (3-line degasser: DG-980-50, Ternary gradient unit: LG-1580-02, Intelligent HPLC pump: PU-980, Intelligent sampler: AS-2057-Plus, Fluorescence detector: FP-4020, Column oven: CO-4020) and ChromNAV data analysis system (JASCO, Hachioji, Tokyo). A Varioscan flash microplate reader (Thermo Scientific, Minato-ku, Tokyo) was used for fluorescence measurement.

### A pentosidine standard product

As the standard pentosidine, pentosidine-trifluoroacetic acid salt (pentosidine-TFA salt) from Peptide Research Institute (Ibaraki, Osaka) was purchased. Pentosidine-TFA salt was dissolved in an appropriate amount of distilled water, and the pentosidine concentration (pmol/mL) was

calculated based on the content information described in the attached document (molecular weight: 378.43). The pentosidine solution was divided into aliquotes and stored at  $-20^{\circ}\text{C}$  until using.

### Preparation of plasma hydrolysis solution

Plasma samples were reduced in advance with sodium borohydride solution to prevent pentosidine formation during the hydrolysis process<sup>21</sup>. To 50  $\mu\text{L}$  of plasma, 50  $\mu\text{L}$  of 200 mmol/L sodium borohydride solution (pH 9.2) was added, and the mixture was allowed to stand at room temperature for 30 minutes or more. After the reduction, 100  $\mu\text{L}$  of 6 N iron-free hydrochloric acid was added, and the mixture was heated at  $105^{\circ}\text{C}$  for 18 hours in a block incubator for hydrolysis. After hydrolysis, 200  $\mu\text{L}$  of 1.5 mol/L Tris (hydroxymethyl) amino-methane (Tris) solution was added to dilute the hydrochloric acid in the solution.

The precipitate in the hydrolyzed solution was removed by centrifugation (5,000 x g, 1 minute,  $4^{\circ}\text{C}$ ) using a centrifugal filtration filter with PTFE membrane pore size 0.45  $\mu\text{m}$  (Centricut ultra mini W-MR, Kurabo, Chuo-ku, Osaka).

In the recovery test of pentosidine to plasma, 10  $\mu\text{L}$  of pentosidine standard solution was added to the diluted hydrochloric acid solution (400  $\mu\text{L}$ ) after hydrolysis.

### Pretreatment for pentosidine measurement using spin column for HPLC

Pretreatment for pentosidine measurement by spin column was performed using Monospin AG column (GL Sciences, Shinjuku-ku, Tokyo). The Monospin AG column is characterized by having a carrier in which a mixed group of a cation exchange group and a hydrophobic group (octadecyl group) is bonded to monolithic silica which is a high-purity silica gel with a large surface area with pores in the silica skeleton. In each step of the pretreatment, the solution was added to the Monospin AG column, and the solution was passed through centrifugation (5,000 x g, 1 minute,  $4^{\circ}\text{C}$ ). The solution conditions were the following six steps; (1) Conditioning (0.1 mol/L citric acid/ACN (990/10) solution 200  $\mu\text{L}$ ), (2) Equilibration (400  $\mu\text{L}$  of 0.1 mol/L citric acid), (3) Add sample (150  $\mu\text{L}$ ), (4) Wash (500  $\mu\text{L}$  of 0.1 mol/L citric acid), (5) Elution 1 (75  $\mu\text{L}$  of 1 mol/L ammonium formate solution), (6) Elution 2 (75  $\mu\text{L}$  of 1 mol/L ammonium formate/ACN (50/50) solution). The eluates obtained in steps (5) and (6) were uniformly mixed to use a measurement sample.

### Measurement conditions by HPLC

A reverse phase silica gel column Inert Sustain AG (3  $\mu\text{m}$ , 100 mm x 4.5 mm ID; GL Sciences) was used for the HPLC measurement. The measurement conditions were as follows: column temperature,  $20^{\circ}\text{C}$ ; eluent, A solution: 0.1% (v/v) formic acid aqueous solution, B solution: 100% ACN; elution conditions: 0%B (0-10 min), 50%B (10-12 min), 0%B (12-18 min); flow rate, 1.0 mL/min; detection, fluorescence (excitation [Ex] wavelength 325 nm, emission [Em] wavelength 385 nm); injection volume, 20  $\mu\text{L}$ .

## Subjects

The samples used in this study were plasma provided by healthy men and women aged 20 to under 100 years. The subjects were persons involved in the research at the Anti-Aging Research Center/Glycative Stress Research Center, Graduate School of Life and Medical Sciences, Doshisha University, who participated in a briefing session that was held in advance followed by agreement in writing to participate in this test.

In the verification of the pretreatment method of blood pentosidine, 1 mL each of plasma provided by 40 men and women in their 50s to 80s was mixed and homogenized before use. In the verification of correlation between blood pentosidine levels and glycative stress index, the subjects of 72 men and women (13 men and 59 women) were in their 20s to 80s with the age of  $48.3 \pm 18.6$  (mean  $\pm$  SD).

## Blood chemistry

A blood biochemistry test was performed using a blood sample obtained intravenously from subjects. Examination items were as follows: white blood cell count (WBC), red blood cell count (RBC), hemoglobin (Hb), hematocrit (Ht), average red blood cell volume (MCV), average red blood cell hemoglobin amount (MCH), average red blood cell hemoglobin concentration (MCHC), platelets (TP), peripheral hemogram (neutrophil: Neutro, lymphocyte: Lympho, monocyte: Mono, eosinophil: Eosino, basophil: Baso), total protein (TP), total cholesterol (TC), high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), serum urea nitrogen (BUN), creatinine (CRE), uric acid (UA), and hemoglobin A1c (HbA1c [NGSP: national glycohemoglobin standardization program]). Blood glucose and insulin measurements were excluded from the results because casual blood was collected. The blood biochemical test was performed by requesting LSI Medience (Chiyoda-ku, Tokyo).

## Measurement of skin AGEs content by autofluorescence (AF) resulting from AGEs

Skin AGEs content was measured non-invasively, using two pieces of equipment: AGE Reader mu (DiagnOptics, Groningen, Nederland) and AGEs Sensor (Sharp, Osaka).

Measurement sites were on the right forearm by AGE Reader mu and on the left middle finger by AGEs Sensor.

## Statistical analysis

Fundamental statistics (average value, standard deviation) were calculated for each data point. The data were analyzed with the statistical analysis software BellCurve for Excel (Social Information Service, Shinjuku-ku, Tokyo). The correlation between plasma pentosidine and SAF was evaluated using the Pearson product-moment correlation coefficient ( $r$ ), values of which were defined  $0.4 < |r| \leq 1.0$  as correlated and  $0.2 < |r| \leq 0.4$  as weak correlation. Statistical analysis results defined the risk rate of less than 5% as significant and less than 10% as a tendency.

## Ethical standards

This study was conducted in compliance with the Declaration of Helsinki (revised at the 2013 WMA Fortaleza General Assembly) and the ethical guidelines for human-based medical research (notification by Ministry of Education, Culture, Sports, Science and Technology [MEXT] and Ministry of Health, Labour and Welfare [MHLW]). This research has obtained the approval of the Ethical Committee of Doshisha University (#180003) and the Society for Glycative Stress Research (GSE 2018-003), which has deliberated the ethics and relevance of the study.

## Results

### Validation of pretreatment for plasma pentosidine HPLC measurement

The recovery of pentosidine from the spin column (Monospin AG) was determined using a 26.4 pmol/mL pentosidine solution prepared with 0.1 mol/L citric acid solution. The recovery rate of pentosidine was calculated by multiplying the ratio of the fluorescence intensity (Ex/Em wavelength: 325/385 nm) of the column eluate to the added solution by 100. The recovery rate was 97%.

**Table 1** shows peak area values, signal/noise (S/N) ratio, and retention time at pentosidine concentrations of 0.08, 0.26, 0.79, 2.64, and 7.93 pmol/mL. The detection

**Table 1. Pentosidine peak parameters measured by HPLC.**

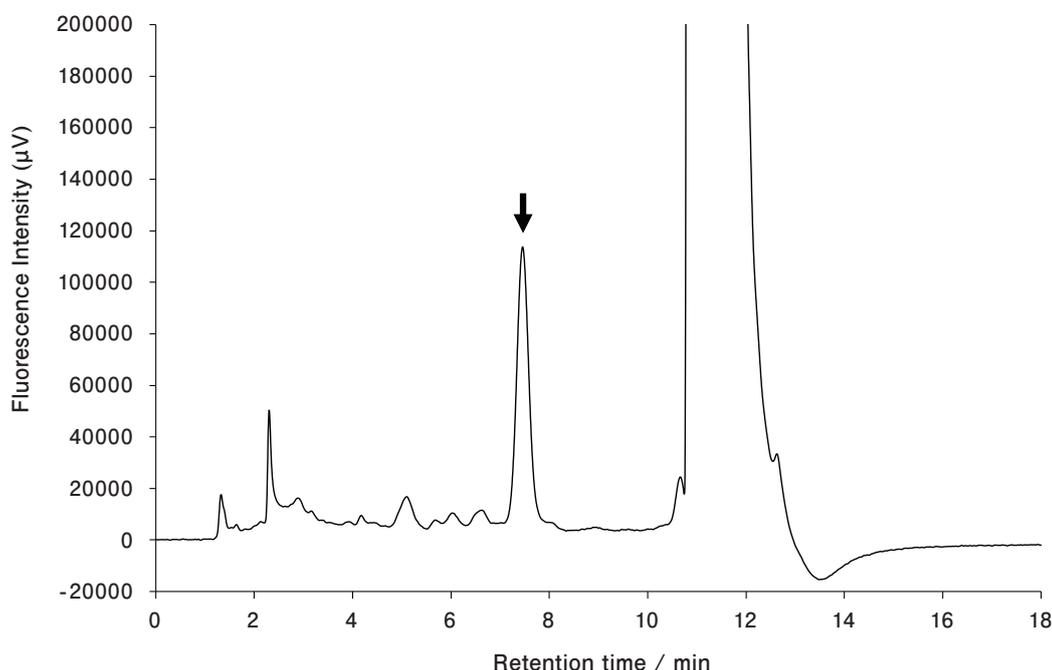
Concentration (pmol/mL)	Peak area ( $\mu\text{V} \cdot \text{sec}$ )	CV (%)	S/N	Retention time (min)
7.93	3,394,888 $\pm$ 194,212	5.7	768.8 $\pm$ 71.0	7.29 $\pm$ 0.19
2.64	1,217,602 $\pm$ 48,717	4.0	271.1 $\pm$ 19.7	7.29 $\pm$ 0.16
0.79	403,746 $\pm$ 15,842	3.9	87.7 $\pm$ 4.2	7.28 $\pm$ 0.19
0.26	154,947 $\pm$ 10,491	6.8	32.0 $\pm$ 1.8	7.25 $\pm$ 0.17
0.08	61,954 $\pm$ 7,282	11.8	11.7 $\pm$ 0.9	7.27 $\pm$ 0.14

Parameters are expressed as mean  $\pm$  standard deviation,  $n = 4$ . HPLC was performed as follows: column, InertSustain AG (3  $\mu\text{m}$ , 100  $\times$  4.6 mm I.D.); eluent, A solution : 0.1% (v/v) formic acid, B solution: 100% ACN; elution condition, 0%B (0-10 min), 50%B (10-12 min), 0%B (12-18 min); column temperature, 20  $^{\circ}\text{C}$ ; flow rate, 1.0 mL/min; detection, Ex 325 nm/Em 385 nm; injection volume, 20  $\mu\text{L}$ . ACN, acetonitrile; CV, coefficient of variation; S/N, signal to noise ratio; HPLC, high performance liquid chromatography; Ex, excitation wavelength; Em, emission wavelength.

limit of pentosidine (S/N ratio: 3) estimated from the S/N ratio of 0.08 pmol/mL (11.7) was 0.02 pmol/mL. The coefficient of variation (CV) of the peak area value was 10% or less at 0.26 to 7.93 pmol/mL and 11.8% at 0.08 pmol/mL. The peak area value and concentration of pentosidine showed good linearity ( $y = 423,054 x + 55,131$ ,  $r = 0.9998$ ,  $p < 0.001$ ) in the concentration range of 0.08 to 7.93 pmol/mL. The area value of 0.08 pmol/mL (61,954  $\mu\text{V}\cdot\text{sec}$ ) was higher than the area value of y-intercept (56,150  $\mu\text{V}\cdot\text{sec}$ ), and the lower limit of quantification was 0.08 pmol/mL. The average retention time in each concentration four times measurement was  $7.28 \pm 0.15$  minutes (mean  $\pm$  standard deviation), and CV was 2.1%.

The sample was made by mixing and homogenizing plasma provided by 40 men and women in their 50s to 80s. **Figure 1** shows the chromatograph of pretreated plasma. The pretreatment reduced contaminants and the pentosidine peak was well separated.

The addition recovery rate of pentosidine in the sample was determined by this pretreatment method (**Table 2**). The recoveries were 113%, 105%, and 131% for the pentosidine additions of 19.8, 39.6, and 79.3 fmol per HPLC analysis sample injection amount (20  $\mu\text{L}$ ), and the average value was 116%.



**Fig. 1.** The chromatograph of human plasma pretreated by spin column (Monospin AG).

50  $\mu\text{L}$  plasma was hydrolyzed and pretreated by spin column (Monospin AG). The arrow shows the pentosidine peak. HPLC was performed as follows: column, InertSustain AG (3  $\mu\text{m}$ , 100 mm  $\times$  4.6 mm ID); flow rate, 1.0 mL/min; detection, Ex 325 nm/Em 385 nm; eluent, A solution : 0.1% (v/v) formic acid, B solution: 100% ACN; elution condition, 0%B (0-10 min), 50%B (10-12 min), 0%B (12-18 min); column temperature, 20°C; injection volume 20  $\mu\text{L}$ . ACN, acetonitrile; HPLC, high performance liquid chromatography; Ex, excitation wavelength; Em, emission wavelength.

**Table 2.** Recovery of pentosidine in plasma sample.

Added amount per injection volume (fmol)	Measured amount per injection volume (fmol)	Recovery (%)
0.0	116 $\pm$ 9	–
19.8	139 $\pm$ 1	113 $\pm$ 5
39.6	158 $\pm$ 12	105 $\pm$ 30
79.3	220 $\pm$ 84	131 $\pm$ 8

The values are expressed as mean  $\pm$  standard deviation,  $n = 3$ . The added volume of pentosidine was 10  $\mu\text{L}$ . HPLC was performed as follows: column, InertSustain AG (3  $\mu\text{m}$ , 100  $\times$  4.6 mm I.D.); eluent, A solution : 0.1% (v/v) formic acid, B solution: 100% ACN; elution condition, 0%B (0-10 min), 50%B (10-12 min), 0%B (12-18 min); column temperature, 20°C; flow rate, 1.0 mL/min; detection, Ex 325 nm/Em 385 nm; injection volume, 20  $\mu\text{L}$ . ACN, acetonitrile; HPLC, high performance liquid chromatography; Ex, excitation wavelength; Em, emission wavelength.

*Relationship between glycative stress index, age and blood pentosidine concentration by the pretreatment method*

The relationship between blood pentosidine level (pmol/mL), age, HbA1c, and skin AGEs content (glycosylation index) was examined using this method.

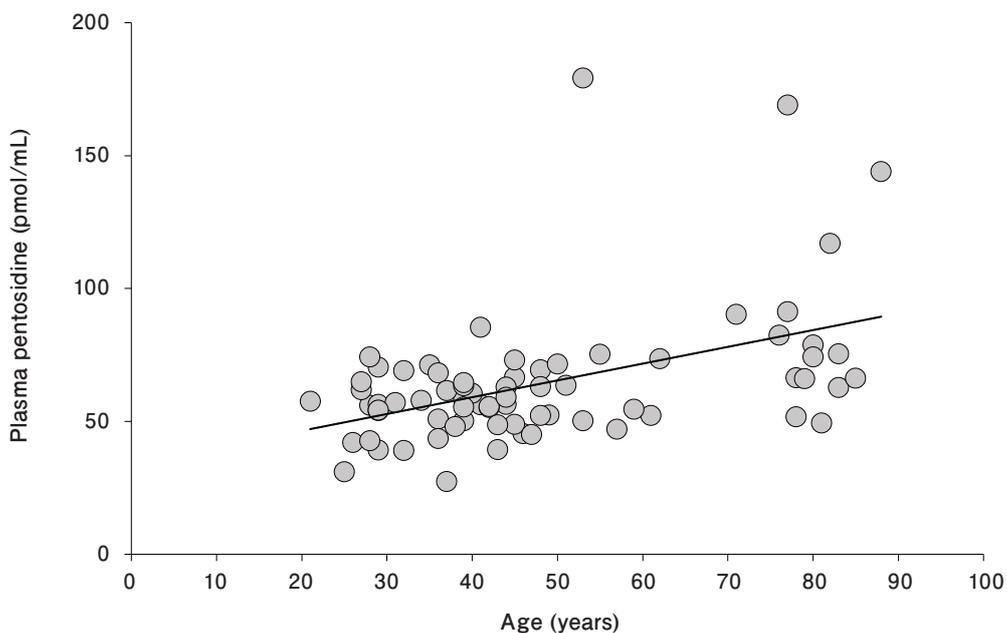
The subjects were all healthy, in whom all test items in the blood chemistry tests did not fall under the common standard range of the Japanese Society of Clinical Laboratory Medicine<sup>22)</sup> or the Ningen Dock Society Judgment Category D (requires treatment) (**Table 3**). The blood pentosidine level

was positively correlated with age ( $y = 0.631x + 33.851$ ,  $r = 0.458$ ,  $p < 0.01$ , **Fig. 2**). It had a weak positive correlation with HbA1c ( $y = 15.250x - 20.971$ ,  $r = 0.330$ ,  $p < 0.01$ , **Fig. 3**). Blood pentosidine level and skin AGEs content were positively correlated with the measured value of AGE Reader mu ( $y = 23.586x + 10.095$ ,  $r = 0.435$ ,  $p < 0.01$ , **Fig. 4**), while a weak positive correlation ( $y = 67.639x + 31.753$ ,  $r = 0.254$ ,  $p < 0.05$ ) was observed with measurements by the AGEs Sensor (**Fig. 5**). No correlation was observed between the values measured by the AGE Reader mu and the AGEs Sensor.

**Table 3. Results of blood biochemistry and hemogram.**

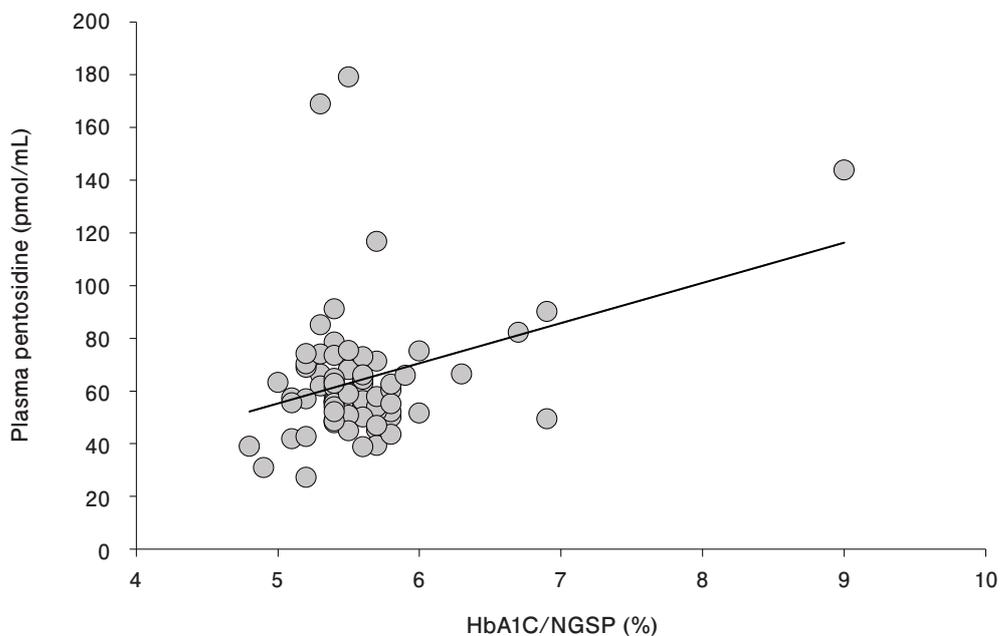
Items	Unit	Reference interval <sup>1)</sup>	Mean ± SD
TP	g/dL	6.6 - 8.1	7.3 ± 0.4
BUN	mg/dL	8 - 20	14.0 ± 3.9
CRE	mg/dL	male: 0.65 - 1.07 female: 0.46 - 0.79	0.85 ± 0.22 0.63 ± 0.09
UA	mg/dL	male: 3.7 - 7.8 female: 2.6 - 5.5	5.2 ± 0.9 4.2 ± 0.8
HbA1c [NGSP]	%	4.9 - 6.0	5.6 ± 0.6
TC	mg/dL	142 - 248	202 ± 38
TG	mg/dL	male: 40 - 234 female: 30 - 117	107 ± 69 75 ± 35
HDL-C	mg/dL	male: 38 - 90 female: 48 - 103	72 ± 24 80 ± 15
LDL-C	mg/dL	65 - 143	103 ± 30
RBC	10 <sup>6</sup> /μL	male: 4.35 - 5.55 female: 3.86 - 4.92	4.54 ± 0.41 4.41 ± 0.36
MCV	fL	83.6 - 98.2	91.0 ± 6.7
MCH	pg	27.5 - 33.2	29.8 ± 2.3
MCHC	%	31.7 - 35.3	32.8 ± 1.4
Hb	g/dL	male: 13.7 - 16.8 female: 11.6 - 14.8	14.2 ± 1.7 13.0 ± 1.3
Ht	%	male: 40.7 - 50.1 female: 35.1 - 44.4	44.0 ± 3.3 39.4 ± 3.4
WBC	10 <sup>3</sup> /μL	3.3 - 8.6	5.1 ± 1.6
Neutro	%	—	58.9 ± 8.5
Lympho	%	—	32.3 ± 7.9
Mono	%	—	5.2 ± 1.6
Eosino	%	—	2.9 ± 2.0
Baso	%	—	0.7 ± 0.5
PLT	10 <sup>4</sup> /μL	15.8 - 34.8	26.0 ± 5.9

1) Guideline JSLM2018 of a clinical laboratory test. See text for abbreviations.



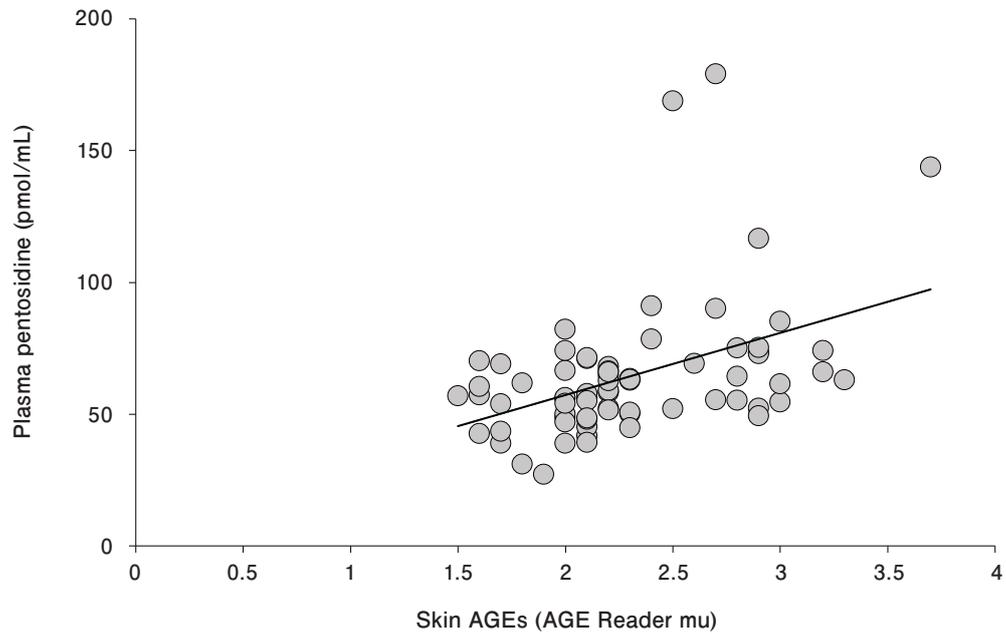
**Fig. 2. Correlation between age and the plasma pentosidine concentration.**

Regression line:  $y = 0.631x + 33.851$ ,  $r = 0.458$ ,  $n = 72$ ,  $p < 0.01$ . Pearson's product moment correlation coefficient was used for statistical analysis.



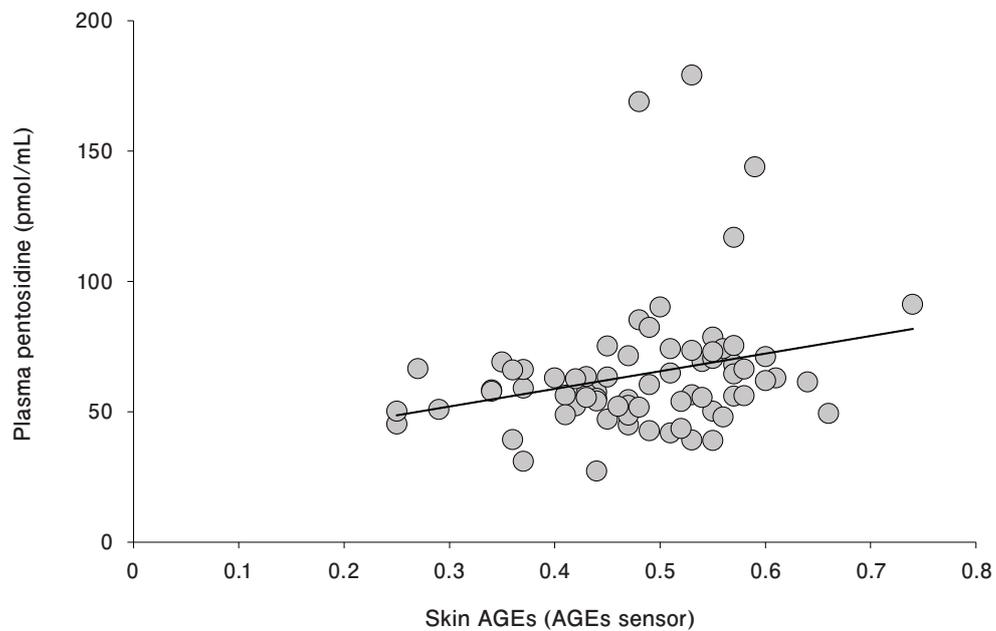
**Fig. 3. Correlation between HbA1c and the plasma pentosidine concentration.**

Regression line:  $y = 15.250x - 20.971$ ,  $r = 0.330$ ,  $n = 72$ ,  $p < 0.01$ . Pearson's product moment correlation coefficient was used for statistical analysis.



**Fig. 4. Correlation between skin AGEs content measured by AGE Reader mu and the plasma pentosidine concentration.**

Regression line:  $y = 23.586 x + 10.095$ ,  $r = 0.435$ ,  $n = 72$ ,  $p < 0.01$ . Pearson's product moment correlation coefficient was used for statistical analysis. AGEs, advanced glycation end products.



**Fig. 5. Correlation between skin AGEs content measured by AGEs Sensor and the plasma pentosidine concentration.**

Regression line:  $y = 67.639 x + 31.753$ ,  $r = 0.254$ ,  $n = 72$ ,  $p < 0.05$ . Pearson's product moment correlation coefficient was used for statistical analysis. AGEs, advanced glycation end products.

## Discussion

### *Applicability of pretreatment using Monospin AG column for pentosidine HPLC measurement*

The recovery rate was 97% when 150  $\mu\text{L}$  (3.96 pmol) of 26.4 pmol/mL pentosidine standard solution was added to the Monospin AG column used for pretreatment in this method. Here, 50  $\mu\text{L}$  of plasma was diluted (8-fold dilution) during the hydrolysis process, and the plasma volume in the added amount (150  $\mu\text{L}$ ) of the Monospin AG column was 18.75  $\mu\text{L}$ . The standard value for blood pentosidine is indicated by the ELISA method as 0.00915 to 0.0431  $\mu\text{g/mL}$  (24.2 to 113.9 pmol/mL). Therefore, this method is considered to be sufficient to treat the upper limit of the standard value (2.13 pmol). In this method, 1 mol/L ammonium formate solution was eluted with 75  $\mu\text{L}$ , followed by the elution with 75  $\mu\text{L}$  of 1 mol/L ammonium formate/ACN (50/50) solution, and both eluates were mixed, resulting in obtaining a good recovery rate. Also, in the addition recovery test for plasma pentosidine, the average addition recovery rate was as high as 116%. In contrast, the recovery rate was higher (131%) than other concentrations under the condition where 79.3 fmol of pentosidine was added. It is considered that the slight peaks of impurities near the pentosidine peak might have affected the peak area value of pentosidine. There still remains room for further optimization of the pentosidine separation conditions in the HPLC measurement.

### *Process time of pretreatment for pentosidine HPLC measurement*

In this method, after hydrochloric acid hydrolysis, hydrochloric acid was not removed using a centrifugal concentrator, which requires time, and the pretreatment process by a spin column was performed simply by diluting hydrochloric acid by adding 1.5 mol/L Tris solution. Therefore, the time required for pretreatment could be significantly reduced. No special equipment for removing hydrochloric acid is required. Furthermore, the Monospin AG column allows the solution to be passed through the centrifugation operation for one minute per step. The procedure from the conditioning of the spin column to the pentosidine elution was six steps.

Thus far, ion pair-HPLC pretreatment requires about 12 hours due to operations such as elution of the sample by column chromatography and drying<sup>18)</sup>. The citric acid-HPLC method does not require pretreatment, but it takes about 4 hours to remove hydrochloric acid after hydrolysis<sup>21)</sup>. In the citric acid-HPLC method, the HPLC run time requires 60 minutes per sample for sample elution, column washing and equilibration. In contrast, this procedure, from the end of the hydrochloric acid hydrolysis of plasma to the preparation of the HPLC measurement sample, was completed within 10 minutes. Since this method is simple in operation, 24 samples could be processed simultaneously. The pentosidine measurement time by HPLC was 18 minutes per sample, and it was possible to measure about 80 samples per day (24 hours). This method has made it possible to considerably simplify the pretreatment operation of plasma pentosidine HPLC measurement and to shorten the operation time.

### *Relationship between blood pentosidine levels and age-related changes*

It has been reported so far that blood pentosidine levels correlate with age, skin AGEs content, which is an index of glycation stress, and HbA1c level, which is an index of glucose metabolism<sup>21)</sup>. In this verification of 72 cases regarding the correlation between plasma pentosidine level measured by this method and physical information, plasma pentosidine levels were positively correlated with age and skin AGEs content by AGE Reader mu, and weakly positively correlated with HbA1c levels. However, plasma pentosidine levels were found to deviate from the regression line of each item in four subjects aged 53 to 88 years. Although the reason for this has not been clarified by the blood biochemical test this time, it has been reported that blood glucose is greatly affected and changed by lifestyle<sup>2)</sup>. These may have contributed to the increase in plasma pentosidine levels.

The correlation between the plasma pentosidine level and the skin AGEs content was verified using the measured values of two models. A positive correlation was observed with the measured value of AGE Reader mu, while a weak positive correlation with AGEs Sensor. A previous report shows that there is no correlation between the measured values of AGE Reader mu and the AGEs Sensor, thus it is considered that the significance of measurement is different<sup>24)</sup>. Although the significance of measurement could not be clarified in this study, blood pentosidine may correlate with the significance of measurement in both models.

### *Effect of trifluoroacetic acid (TFA) content in pentosidine standard*

The elemental analysis results of the pentosidine standard used in this study were carbon (C), hydrogen (H), and nitrogen (N) ratios of 38.47%, 4.10%, and 12.00% according to the attached document. Since pentosidine is purified as TFA salt and then dried, the solid standard product contains pentosidine and TFA. As a result of calculation from the molar ratio of C, H, and N of the elemental analysis and the molecular formula of pentosidine, the pentosidine used in this study contains 2.7 molecules of TFA. The molecular weight of pentosidine is 378.43, but the molecular weight of TFA salt in which 2.7 molecules of TFA are bound is 686.28, thus showing that pentosidine content is 55.1%. In this study, the entire content of one vial was dissolved, and the pentosidine concentration was determined based on this value. At present, the pentosidine concentrations, *e.g.*, substance concentrations and molar concentrations, vary depending on the report, however, when comparing the measured values, it is necessary to verify the difference in the number of TFA molecules bound to pentosidine used as a standard.

## Conclusion

The pretreatment method using a spin column having a cation exchange group and a hydrophobic group shortened the pretreatment and measurement time in blood pentosidine

HPLC measurement, and expectedly enabled the simplification of the operation and the time reduction in the measurement of multiple samples.

### Conflict of Interest Statement

The present study was supported by GL Sciences Inc.

### Acknowledgement

This study was presented at the 18th Meeting of Society for Glycative Stress Research on August 31, 2019, Kyoto, and 20th Scientific Meeting of the Japanese Society of Anti-Aging Medicine on September 25-27, 2020, Tokyo.

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