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Original article

Measurement of pentosidine in human plasma by the high performance liquid chromatography

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Abstract

***Objectives*:** There are no blood component measurement methods that are both accurate and have good operability in assessing glycative stress. The objective of this study was to optimize the reverse phase high performance liquid chromatography using citric acid eluent (citric acid HPLC) to measure plasma pentosidine, one of the advanced glycation end products (AGEs).

***Methods*:** This study employed the reverse phase HPLC and examined column temperature conditions for the improvement of chromatographic separation, pretreatment conditions of samples, and additional recovery. Subsequently, the optimized method system was conducted to measure the plasma pentosidine of 83 subjects, each being a medical examiner between 20 and 99 years of age, for an Anti-Aging Medical Checkup. The effectiveness of this measuring method was examined with relations between age and each evaluation marker.

***Results*:** The optimal conditions of pretreatment were as follows: the plasma was processed with sodium borohydride, the precipitation of protein using trichloroacetic acid (TCA) was performed, and hydrolysis was performed, using hydrochloric acid, Fe free. When the column temperature of HPLC is at 20°C, pentosidine was separated from minor peaks in plasma. Additional recovery of pentosidine was satisfactory between 91.8 and 109.7% with the optimized citric acid HPLC method. Measurements of the Anti-Aging Medical Checkup examinees recognized positive correlations of plasma pentosidine concentration with age, HbAlc and skin AGEs, and negative correlations of plasma pentosidine with DHEA-s, and calcaneal stiffness value measured by an ultrasonic bone density measuring apparatus.

***Conclusion*:** The measurement method of plasma pentosidine was optimized in citric acid HPLC. It was recognized that the measurement value of plasma pentosidine can be an index of aging and glycative stress.

***KEY WORDS*:** evaluation for glycative stress, advanced glycation end product (AGEs), pentosidine, high performance liquid chromatography (HPLC)

Introduction

Advanced glycation end products (AGEs) are formed and accumulated with non-enzymatic reactions between proteins and glucose in tissues of living organisms [1, 2]. The accumulation of AGEs in organisms are involved in the onset and progression of diabetes mellitus, inflammation, and arteriosclerosis. Therefore, influences of AGEs in organisms are referred to as glycative stress [3]. AGEs in organisms exist in various compounds. Pentosidine, one of the AGEs, is a molecule with a molecular weight of 379, and a cross-link structure between lysine and arginine residue in the imidazopyridinium ring. Moreover, pentosidine has fluorescence (excitation wavelength: 335 nm, fluorescent wavelength: 385 nm) [4].

Pentosidine exists in synovial membranes, cartilages [5], and skin collagen [3, 5, 6] of humans. It is well known that pentosidine accumulates in tissues as age progresses. It is also reported that blood pentosidine concentration is high in patients with diabetes [7], chronic renal failure [3, 6, 8] and rheumatoid arthritis [1, 6]. A high blood pentosidine concentration level leads to the onset and progression of coronary artery disease [9], as has been recognized. The accumulation of pentosidine in bone collagen is associated with the reduction of bone quality and is a risk factor of bone fracture [10]. Therefore, researches on pentosidine are being conducted for applications as a biomarker, which assesses diabetic

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complications and renal failure [2, 7, 11]. With a goal of the evaluation of glycative stress on organisms, it is meaningful in determining the quantity of blood pentosidine.

Measurement accuracy and operability in preparation are required to assess glycative stress of organisms. At present, there are several methods to measure blood pentosidine level; although, the methods have various problems. For example, a measurement kit sold as enzyme-linked immuno sorbent assay (ELISA) [12] has a heating process in pretreatment to measure human blood pentosidine. This pentosidine, which is artificially produced by heat treatment, has a possibility of increasing measurement levels [13, 14]. Moreover, the ion-pair HPLC method [15,16, 17] requires burdensome experimental operations and preparation of samples for a long period of time, hydrolyzing blood samples and removing contaminants with ion exchange columns. In addition, measurement values are likely to be affected by the rates of sample recovery. Contrarily, the citric acid HPLC method [18], employing reverse-phase HPLC methods as a citric acid of eluate, has a possibility to advance in analytical precision with shorter measurement time and stabilized sample recovery rate, and hydrolyzing blood samples, without column refining in pretreatment.

The aim of this study was to optimize the measurement method of plasma pentosidine levels by the citric acid HPLC method in order to develop an assessment tool for glycative stress on organisms, examining sample pretreatments, improvement in separation by column temperature and additional recovery. Furthermore, the optimized measurement system was performed to measure the plasma pentosidine concentration of anti-aging medical check-up examinees between 20 and 99 years of age. The effectiveness of measurement was examined by correlating age with each of the assessment evaluation markers.

Methods

Reagents

Pentosidine-TFA salt (Polypeptide Laboratories France, Strasbourg, France) was used as the standard reference material. Heptafluorobutyric acid (HFBA; Sigma-Aldrich Japan, Meguro-ku, Tokyo, Japan) was used as the ion-pair reagent for the ion-pair HPLC method. For hydrolysis of each blood sample, hydrochloric acid (35%) (Wako Pure Chemical Industries, Osaka, Japan) and Fe-free hydrochloric acid (35%) (Nacalai Tesque, Kyoto, Japan) were used. Acetonitrile (ACN) and methanol (MeOH) for the HPLC method were used. Human serum albumins (HSA), lyophilized powder, and ≥ 96% agarose gel electrophoresis (Sigma-Aldrich Japan, Meguro-ku, Tokyo, Japan) were used for the production of *in vitro* glycated protein. Other reagents of Special Grade were purchased from Wako Pure Chemical Industries.

Equipment

This study employed HPLC Prominence system (on-line degassing unit; DGU20A3, pump unit with a low-pressure gradient unit; LC20AT, auto-sampler; SIL20AC, column oven; CTO20AC, fluorescence detector; RF20Axs; Shimadzu, Kyoto, Japan) and data analysis system (LC solution; Shimadzu). For the evaporation of reagents, a centrifugal evaporator (CC-105; Tommy Seiko, Nerima-ku, Tokyo, Japan) was employed. A block incubator (BI-516S; Astec, Kasuya-gun, Fukuoka, Japan) was employed to heat samples in hydrolysis. For the dissolution, dispersion and suspension of reagents, ultrasonic cleaning machine (VS-150; As one, Osaka, Japan) was employed.

Column

Cadenza CD-C18 (3 μm, 75×4.6 mm I.D) or Unison US-C18 (5 μm, 150×4.6 mm I.D) (Imtakt, Kyoto, Japan) were used as columns for HPLC measurement.

In vitro glycated HSA

In vitro glycated HSA (G-HSA) was produced, as previously reported [19], the mixture of 40 mg/mL of HSA, 2.0 mol/L of glucose, and 100 mmol/L of phosphate buffer solution (PBS) (pH 7.4) at the ratio of 2 : 1 : 7 was reacted at 60 °C for 40 hours.

Human plasma sample

Potential subjects of this study were examinees of an Anti-Aging Medical Checkup, which was conducted by the Anti-Aging Medical Research Center, Doshisha University. Examinees were fully informed of this study. Among them, 83 males and females (37 males and 46 females, from 20 to 99, 59.9 ± 21.4 years of age) agreed to participate in the study, and blood samples were collected from their antecubital veins for plasma samples.

Measurement conditions of pentosidine by ion-pair HPLC method

The measurement conditions of pentosidine by the ion-pair HPLC method, as was previously reported [16], column: Cadenza CD-C18, eluent: ACN / MeOH / water / HFBA (16 / 4 / 76 / 0.2), flow rate: 1.0 mL/min, column temperature: 30 °C, fluorescence detection: excitation wavelength (ex) 335 nm, emission wavelength; (em) 385 nm, sample injection volume: 20 μL. LSI Medience (Chiyoda-ku, Tokyo, Japan) was used to perform the plasma pentosidine measurement by the ion-pair HPLC method.

Measurement conditions of pentosidine by the citric acid HPLC method

For the optimization of pentosidine measurement conditions in the reverse-phase HPLC method using citric acid of eluent, column temperature and conditions of sample pretreatment were examined. Two types of columns, Cadenza CD-C18 and Unison US-C18 were used for HPLC column. Other measurement conditions on HPLC method were as follows: eluent, 100 mmol/L citric acid / ACN (99.5 / 0.5); flow rate, 1.0 mL/min; fluorescence detection; excitation wavelength, (ex) 335 nm; emission wavelength, (em) 385 nm; and sample injection volume, 20 μL.

Four pretreatment conditions were examined: Preparation A [20], processing to prevent the elevation of level during preparation of blood samples with sodium borohydride; Preparation B, protein precipitation with trichloroacetic acid (TCA); Preparation C, hydrolysis with hydrochloric acid; and Preparation D, hydrolysis with hydrochloric acid Fe-free were performed. The details are as below.

Preparation A: Sample 50 μL, distilled water 25 μL, and sodium borohydride solution (pH 9.2) 250 μL were mixed and kept still at room temperature for 30 minutes.
Preparation B: Sample 50 μL and distilled water 25 μL were added, and 1.0 mL of 20% TCA was mixed. After being sufficiently stirred, the mixture was cooled with ice for 15 minutes and then centrifuged (4,500×g, 10 minutes, 4 °C). Subsequently, supernatant liquid was removed, and the remaining precipitate was added to 1.0 mL of 5% TCA, which was centrifuged. After the supernatant liquid was removed, 100 μL of distilled water was added to the remaining precipitate, which was in the ultrasonic cleaning machine for one minute for the dispersion and suspension of solid matter.
Preparation C: Sample 50 μL and distilled water 50 μL were mixed and 100 μL of 6N hydrochloric acid was added.
Preparation D: Sample 50 μL and distilled water 50 μL were mixed and 100 μL of 6N Fe-free hydrochloric acid was added.

To sample solutions with Preparation A and B, 100 μL of 6N hydrochloric acid was added. Nothing was added to sample solutions with Preparation C and D. Sample solutions were heated at 105 °C by the block incubator for 18 hours to be hydrolyzed. After being hydrolyzed, samples were evaporated and dried by the centrifugal evaporator for four hours without heating. The dried and solid material was dispersed and suspended by 400 μL of eluent in citric acid HPLC method and then centrifuged (13,200×g, 10 minutes, 4 °C). The supernatant liquid was used as a sample for measurement of the HPLC method.

Statistical analysis

Results of pentosidine measurements were expressed as mean ± standard deviation. For examinations of comparison between groups, comparison between two groups and three or more groups employed Mann-Whitney U test and Tukey's test, respectively. For correlation analysis between measurement values, Pearson product-moment correlation coefficient was employed. Presence of correlation was $0.4 < |r| \leqq 1.0$ and the weak presence of correlation was $0.2 < |r| \leqq 0.4$. A hazard rate of less than 5% was considered to be statistically significant in examining results of the statistical analysis.

Ethical consideration

This study was implemented in compliance with Helsinki Declaration (Note of Clarification added by the WMA General Assembly, Tokyo 2004), abiding by ethical principles and personal information protection law and referring the Ministerial Ordinance on Good Clinical Practice (GCP) for Drug (Ordinance of Ministry of Health and Welfare No. 28 of March 27, 1997) and the Ethical Guidelines for Epidemiological Research established by Japan's Ministry of Health, Labour and Welfare, and Ministry of Education, Culture, Sports, Science and Technology. Further, the Doshisha University Ethics Review Committee on Research with Human Subjects was held to deliberate ethics and validity for this study. Under approval, this research was conducted with advanced registration (application number #14089).

Results

Comparison in measurement of pentosidine standard material by between ion-pair and the citric acid HPLC method

Peak area values of pentosidine standard solution were measured both in ion-pair and the citric acid HPLC method. In both methods, Cadenza CD-C18 was used for HPLC column for measurement. Peak area values were measured with nine concentrations of pentosidine standard solution (0.00625 ng/mL, 0.0125 ng/mL, 0.025 ng/mL, 0.05 ng/mL, 0.1 ng/mL, 0.25 ng/mL, 0.5 ng/mL, 1.0 ng/mL, 2.0 ng/mL). The sample injection volume was 20 μL and measurement was performed three times for every concentration level. The column temperature in the citric acid HPLC method was 20 °C.

Coefficient of variation (CV) values of peak area values for each concentration of pentosidine standard solution showed less than 5% between 0.0125 and 2.0 ng/mL in the measurements by ion-pair HPLC method. However, in a standard solution of 0.00625 ng/mL concentration, CV value increased to 25% or more. Signal/noise (S/N) ratio of peak height of pentosidine was 7.4 with 0.00125 ng/mL and 3.0 with 0.00625 ng/mL, which was lowered 2.5 times. Peak area value and concentration of pentosidine showed a satisfactory linearity with concentration between 0.00125 and 2.0 ng/mL ($y = 13771x + 282.23$, $r = 0.999$, $p < 0.001$). At this point, the range of the quantitative calibration curve was between 0.0125 and 2.0 ng/mL.

By the citric acid HPLC method, CV values were less than 5% with pentosidine concentration between 0.0125 and 2.0 ng/mL. However, S/N ratio was 2.2 with 0.00625 ng/mL of concentration, which showed the lower detection limit. S/N ratio was 5.2 with 0.0125 ng/mL of concentration. Peak area value and concentration of pentosidine showed a satisfactory linearity with concentration between 0.0125 and 2.0 ng/mL ($y = 13150x + 918.87$, $r = 1.000$, $p < 0.001$). At this point, the range of quantitative calibration curve was between 0.0125 and 2.0 ng/mL.

Peak area values measured by ion-pair and citric acid HPLC method in pentosidine standard solution with nine concentration showed satisfactory linear correlation ($y = 1.047x - 585.35$, $r = 0.999$, $p < 0.01$) in ***Fig. 1***. Further, the approximation formula for linear correlation showed that gradient 1.047 and intercept 585.35 and were smaller than the peak area of the quantitative lower limit of 0.0125 ng/mL in citric acid HPLC method (3397).

As a result, it was confirmed that pentosidine at the same concentration was able to be measured as almost the same area value by both ion-pair and citric acid HPLC method.

Optimization of plasma pentosidine measurement conditions by citric acid HPLC method

1) Examination of sample pretreatment method

A plasma sample from a healthy woman in her twenties was used. Sample treatment conditions were examined, comparing processing to prevent the elevation of levels during preparation of blood samples with sodium borohydride,

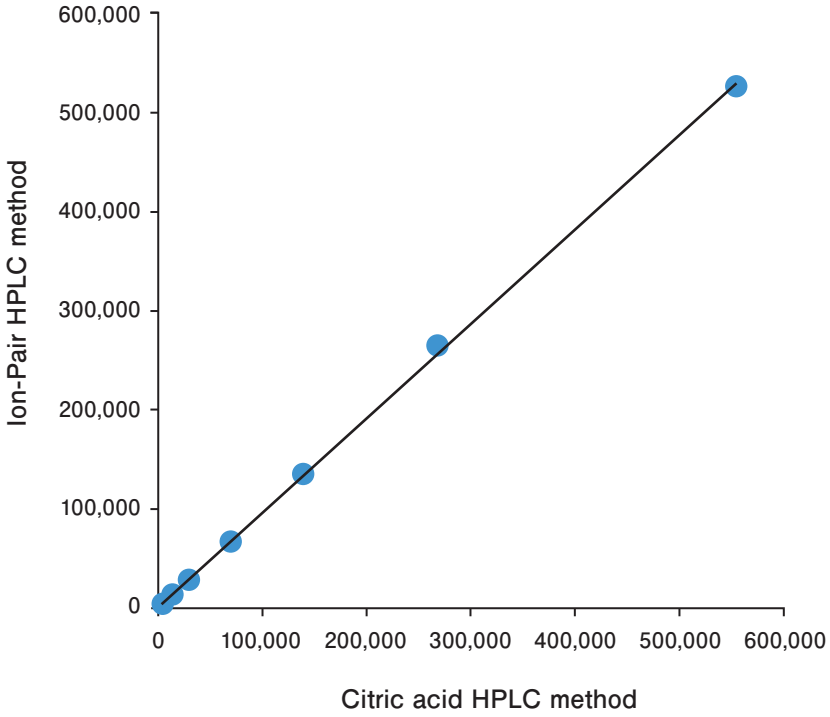

Fig. 1. **Correlation of the two methods in measurement of a pentosidine standard.**

Regression line: y = 0.9548x + 602.71, r = 0.999, p < 0.01. Injection volume; 0.25, 0.5, 1.0, 2.0, 5.0, 10, 20, 40 pg/20 µL. Method: column; Cadenza CD-C18 (3 µm, 75×4.6 mm I.D), flow rate; 1.0 mL/min, detection; ex 335 nm / em 385 nm. Ion-pair HPLC method: eluent; ACN / MeOH / water / HFBA (16/4/76/0.2), column temperature; 30°C. Citric acid HPLC method: eluent; 100 mmol/L citric acid / ACN (99.5/0.5), column temperature; 20°C. ACN, acetonitrile; MeOH, methanol; HFBA, heptafluorobutyric acid; HPLC, high performance liquid chromatography; ex, excitation wavelength; em, emission wavelength.

Table 1. The preparation method comparison to plasma sample.

Preparation method	Pentosidine peak area	CV (%)
A	72,601 ± 1,885	2.6
B	58,061 ± 2,442**	4.2
C	64,212 ± 6,876	10.7
D	59,726 ± 2,752*	4.6

Results are expressed as mean ± standard deviation, n = 3, ** p < 0.01 vs A, * p < 0.05 vs A by Tukey' s test. Plasma sample: 50µL (collected from healthy 20 years-old woman). Preparation method A-C; The details of the method were indicated in the text, measured by citric acid HPLC method: column; Unison US-C18 (5 µm, 150×4.6 mm I.D), eluent; 100 mmol/L citric acid/ACN (99.5/0.5), column temperature; 20 °C, flow rate; 1.0 mL/min, detection; ex 335 nm/em 385 nm, injection volume; 20 µL. CV, coefficient of variation; ACN, acetonitrile; HPLC, high performance liquid chromatography; ex, excitation wavelength; em, emission wavelength.

protein precipitation with trichloroacetic acid (TCA), hydrolysis with hydrochloric acid, and hydrolysis with Fe-free hydrochloric acid (***Table 1***). The peak area of preparation A (with sodium borohydride processing) was 1.3 times as large as that of preparation B (without sodium borohydride processing). The peak area of preparation C (hydrolysis with hydrochloric acid) was 1.1 times as large as that of preparation D (hydrolysis with Fe-free hydrochloric acid). However, the differences were not significant. The coefficient of variation (CV) of pentosidine peak area value was the smallest in preparation D.

Comparing between cases with TCA processing (A and B) and without TCA processing (C and D) in chromatograms, multiple impurity substance peaks were recognized after the peak of pentosidine elution (from 16 to 20 minutes) in C and D chromatograms. From these results, it was assumed that the pentosidine measurement that had less measurement alternation and less impurity substances was performed by the plasma sample pretreatment of preparation A with Fe-free hydrochloric acid (processing with sodium borohydride, protein precipitation with TCA, and hydrolysis with Fe-free hydrochloric acid).

2) Examination of column temperature

A plasma sample of a man in his eighty's (HbA1c: 7.0%) was examined on separations of pentosidine peak, changing column temperature with five conditions (20°C, 25°C, 30°C, 35°C and 40°C) in citric acid HPLC method, after the samples were prepared in processing with sodium borohydride, protein precipitation with TCA, and hydrolysis with Fe-free hydrochloric acid.

Comparing measurement chromatograms by temperature condition indicated that the elution time of pentosidine peak and also the peaks which were at 8 and 12 minutes, became shorter as the column temperature increased. Furthermore, along with the increase in temperature, disorders of the base lines were recognized at 4 and 8 minutes. Meanwhile, at 35°C and 40°C, pentosidine peak and other peaks of impurity substances were unable to be separated. Pentosidine peak area values were at the same level at all of 20°C, 25°C and 30°C. However, miner peaks of I ~ III were found around pentosidine peak (***Fig. 2***). Examination of elution behavior of these minor peaks by temperature indicated that peak I and pentosidine peak were overlapped (***Fig. 3***). Contrarily, the peak of pentosidine was able to be isolated at 20°C and 25°C, as was recognized from data. Therefore, it was assumed that the mix of peak I induced the slight increase in pentosidine peak area value at 30°C. At 25°C, peak I and pentosidine peak were slightly overlapped.

Considering these facts, optimal column temperature was 20°C for plasma pentosidine measurement by citric acid HPLC method.

3) Measurement performance of citric acid HPLC method

Additional recoveries of pentosidine standard material were obtained using human serum albumin (HSA), G-HSA and plasma samples, which were collected from a woman in her twenties and a woman in her seventies (HbA1c: 5.6%) and processed with the optimized pretreatments of sodium borohydride processing, protein precipitation with TCA and hydrolysis with Fe-free hydrochloric acid. Additional pentosidine volume to samples per 20 µL of injection volume of samples with pretreatments to HPLC were 10 pg, 40 pg, 5 pg and 20 pg to HSA, G-HSA, plasma of the woman in her twenties and plasma of the woman in her seventies, respectively, which showed the additional recovery of 109.7%, 91.8%, 96.3% and 101.5%, respectively. All of the additional recovery rates were satisfactory.

4) Comparison in plasma pentosidine measurements between ion-pair and citric acid HPLC method

Fifty-four men and women (23 men, 31 women, between 20 and 99, 66.8 ± 22.6 years old), who underwent anti-aging medical check-up conducted by the Anti-Aging Medical Research Center, Doshisha University, provided plasma of blood collected from their antecubital veins. Pentosidine value (ng/mL) was measured by both the ion-pair HPLC method (contract with LSI Medience) and the optimized citric acid HPLC method. The correlation of plasma pentosidine measured values was recognized between the ion-pair and citric acid HPLC method ($y = 1.3028\ x + 7.7983$, $r = 0.746$, $n = 54$, $p < 0.01$, ***Fig. 4***). However, the plasma pentosidine value measured by ion-pair HPLC method was a larger value than by citric acid HPLC method.

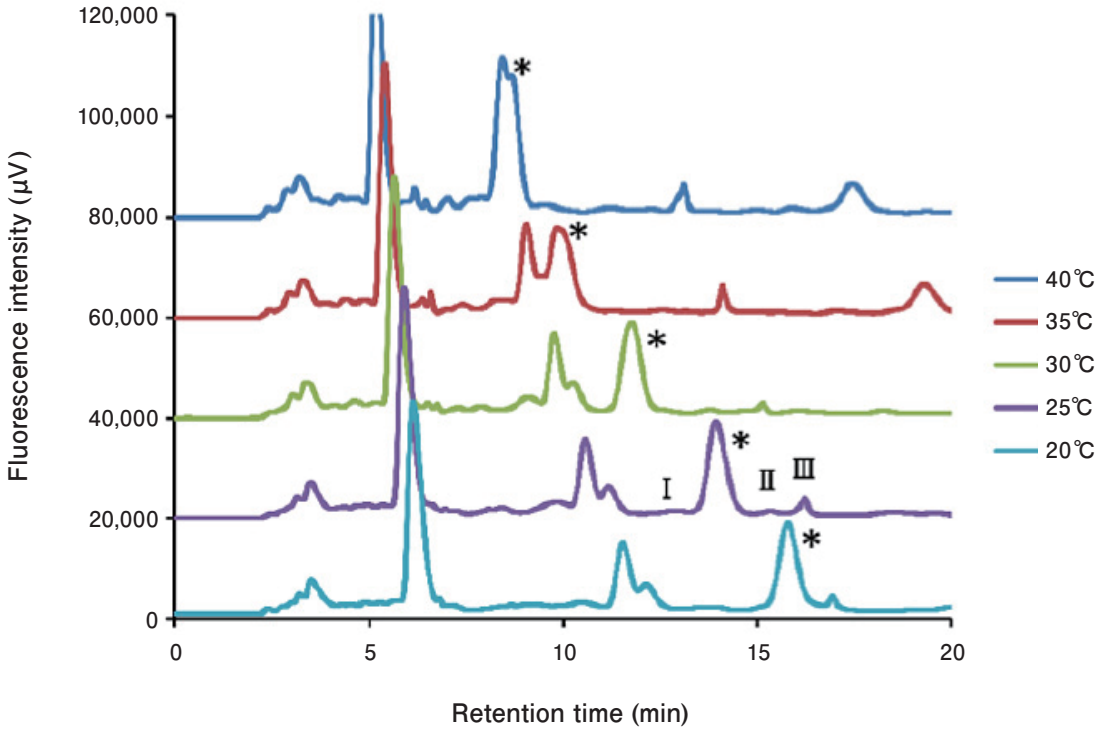

Fig. 2. **Optimization of column temperature.**

Plasma sample; 50 µL (collected from healthy 80 year-old man). Measured by citric acid HPLC method: column; Unison US-C18 (5 µm, 150×4.6 mm I.D), eluent; 100 mmol/L citric acid/ACN (99.5/0.5), flow rate; 1.0 mL/min, detection; ex 335 nm/em 385 nm, injection volume; 20 µL, *; pentosidine peak, miner peak; I ~ III. ACN, acetonitrile; HPLC, high performance liquid chromatography; ex, excitation wavelength; em, emission wavelength.

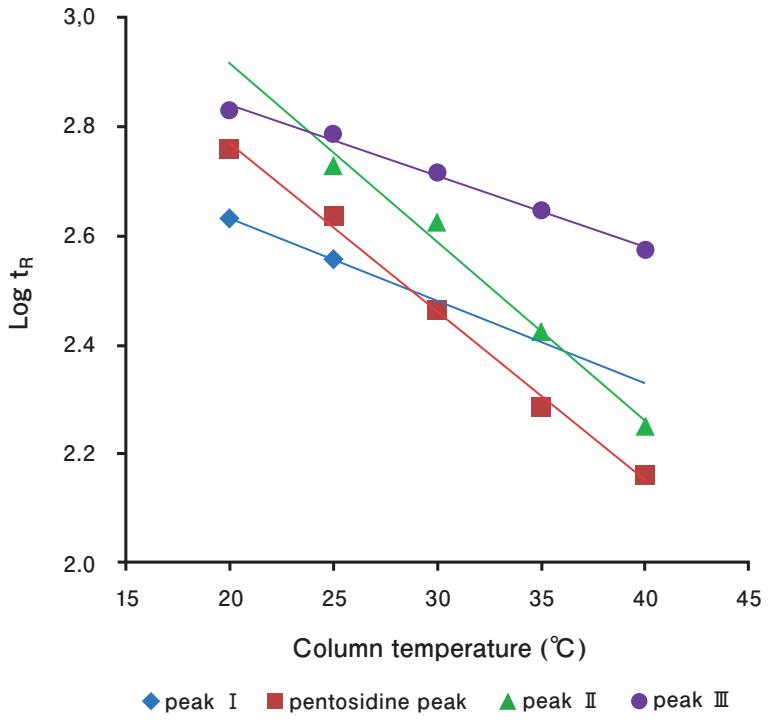

Fig. 3. **The separation position of a minor peak Ⅰ～Ⅲ.**

Plasma sample; 50 μL (collected from healthy 80 year-old man). Measured by citric acid HPLC method: column; Unison US-C18 (5 μm, 150×4.6 mm I.D), eluent; 100 mmol/L citric acid/ACN (99.5/0.5), flow rate; 1.0 mL/min, detection; ex 335 nm/em 385 nm, injection volume; 20 μL, tR; retention time (min); ACN, acetonitrile; HPLC, high performance liquid chromatography; ex, excitation wavelength; em, emission wavelength.

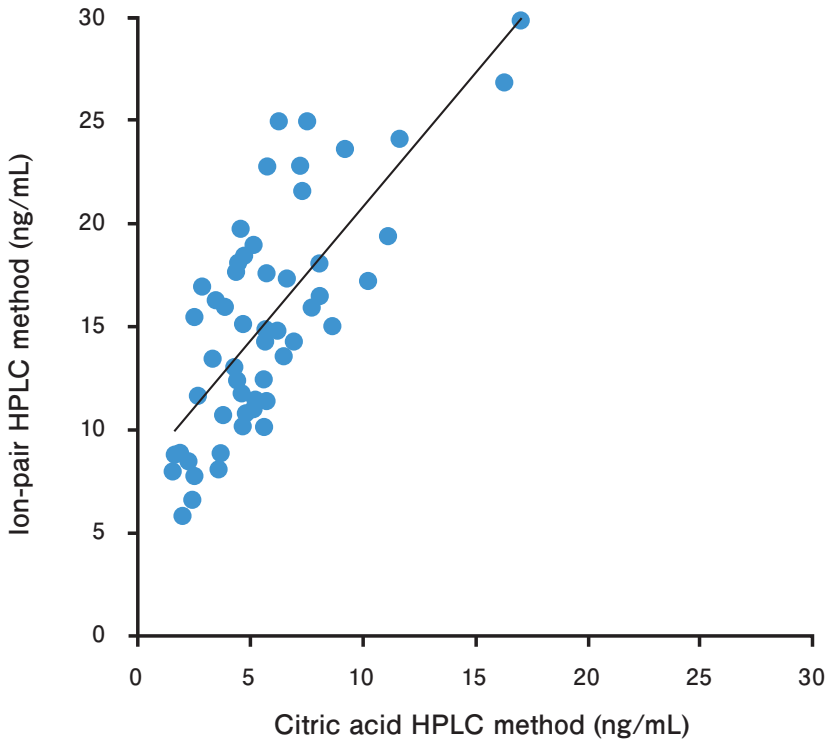

Fig. 4. **Correlation of the two methods in measurement of a pentosidine in plasma.**

Regression line: y = 1.3028x + 7.7983, r = 0.746, n = 54, p < 0.01. Ion-pair HPLC method; measured by LSI Medience. Citric acid HPLC method: column; Unison US-C18 (5 μm, 150×4.6 mm I.D), eluent; 100 mmol/L citric acid/ACN (99.5 / 0.5), flow rate; 1.0 mL/min, detection; ex 335 nm / em 385 nm, column temperature; 20 °C, injection volume; 20 μL. ACN, acetonitrile; HPLC, high performance liquid chromatography; ex, excitation wavelength; em, emission wavelength.

5) Relation of the human plasma pentosidine values measured by citric acid HPLC method with age and aging markers

The plasma pentosidine values (ng/mL) measured by the optimized citric acid HPLC method were examined by relation with age and measured aging marker values of the 83 examinees of anti-aging medical check-up conducted by the Anti-Aging Medical Research Center, Doshisha University (37 men and 46 women, between 20 and 99; 59.9 ± 21.4).

Plasma pentosidine values were 5.4 ± 3.1 ng/mL in men and 4.8 ± 2.7 ng/mL in women ($p = 0.360$). Correlation of plasma pentosidine with age was recognized ($y = 0.06713 x + 1.084$, $r = 0.489$, $n = 83$, $p < 0.01$, ***Fig. 5***). However, there were large differences of measurement values in individuals 60 years old or older.

Measurement values of plasma pentosidine were examined on correlations with aging markers in the anti-aging medical check-up such as calcaneal stiffness value (bone density index), triglyceride, high-density-lipoprotein-cholesterol (HDL-C), low-density-lipoprotein-cholesterol (LDL-C) (index of lipid metabolism), dehydroepiandrosterone-sulfate (DHEA-s) (index of aging-related hormone disorder), blood glucose, HbAlc (index of carbohydrate metabolism), blood cortisol (index of mental and physical stress) and skin AGEs (index of glycative stress) (***Table 2***).

Positive correlations of plasma pentosidine measurement values were recognized with HbA1c ($y = 2.0016x - 6.3391$, $r = 0.433$, $p < 0.01$) and with skin AGEs ($y = 3.1256x - 1.8185$, $r = 0.488$, $p < 0.01$). Contrarily, there were weak negative correlations between plasma pentosidine and DHEA-s ($y = -0.0078x + 6.3181$, $r = -0.341$, $p < 0.01$) and calcaneal stiffness ($y = -0.0411x + 8.8501$, $r = -0.235$, $p = 0.02$). There were no correlations with other measurement markers.

Meanwhile, as for relations between age and evaluation markers, there were positive correlations with HbA1c ($y = 0.015x + 4.8178$, $r = 0.510$, $p < 0.01$) and skin AGEs ($y = 0.0185x + 1.1009$, $r = 0.670$, $p < 0.01$) and negative correlations with DHEA-s ($y = -4.2041x + 406.54$, $r = -0.699$, $p < 0.01$) and calcaneal stiffness ($y = -0.5602x + 120.43$, $r = -0.556$, $p < 0.01$) (***Table 3***). A weak correlation between age and blood glucose was recognized ($y = 0.2541x + 74.676$, $r = 0.347$, $p < 0.01$). Other evaluation markers did not show relations with age.

Discussion

Plasma pentosidine measurement by citric acid HPLC method

Pentosidine measurements of area value were examined using pentosidine standard solution to compare ion-pair and citric acid HPLC method. Both HPLC methods showed extremely high linearity in their calibration curves ($r = 0.999 \sim 1.000$). Furthermore, it was recognized that correlation equations of peak area value in both methods were $y = 1.047x - 585.35$, $r = 1.000$, $p < 0.01$ and both quantitative values of two methods were almost the same. Research data of plasma sample pretreatments revealed that processing with sodium borohydride prevented the elevation of measurement values, and protein precipitation with TCA was effective in the reduction of impurities. Furthermore, it was recognized that pentosidine additional recoveries of *in vitro* HSA plasma sample were 91.8-109.7%. This measuring method was considered to have a satisfactory measurement performance.

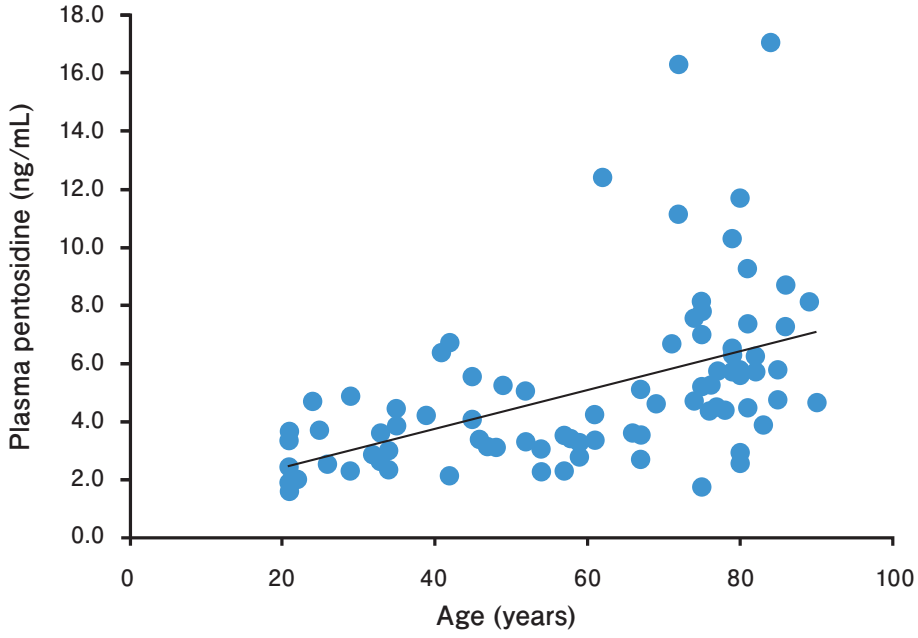

Fig. 5. **Fig. 5. Amount of pentosidine in plasma measured by citric acid HPLC method.**

Regression line: $y = 1.3028x + 7.7983$, $r = 0.746$, $n = 54$, $p < 0.01$. Method: column; Unison US-C18 (5 µm, 150×4.6 mm I.D), eluent; 100 mmol/L citric acid / ACN (99.5 / 0.5), flow rate; 1.0 mL/min, detection; ex 335 nm/em 385 nm, column temperature; 20 °C, injection volume; 20 µL. ACN, acetonitrile; HPLC, high performance liquid chromatography; ex, excitation wavelength; em, emission wavelength.

Table 2. Correlation with the pentosidine concentration in plasma, and each measurement item.

Measurement item	Unit	Regression line	correlation coefficient (r)	p value
Blood glucose	mg/dL	y = 0.0385x + 1.6785	0.209	0.06
HbA1c [NGSP]	%	y = 2.0016x – 6.3391	0.433	< 0.01
Skin AGEs	afu	y = 3.1256x – 1.8185	0.488	< 0.01
Triglyceride	mg/dL	y = -0.0001x + 5.1075	0.001	0.50
HDL-C	mg/dL	y = -0.0021x + 5.2446	-0.014	0.45
LDL-C	mg/dL	y = -0.0114x + 6.5352	-0.113	0.16
Cortisol	μg/dL	y = 0.1318x + 3.764	0.164	0.14
DHEA-s	μg/dL	y = -0.0078x + 6.3181	-0.341	< 0.01
Stiffness value	–	y = -0.0411x + 8.8501	-0.235	0.02

Subject: 20 ~ 80 year-old women and men, n = 83. Plasma pentosidine measured by citric acid HPLC method. NGSP, National Glycohemoglobin Standardization Program; AGEs, advanced glycation end products; HDL-C, high-density-lipoprotein-cholesterol; LDL-C, low-density-lipoprotein-cholesterol; DHEA-s, dehydroepiandrosterone-sulfate; HPLC, high performance liquid chromatography.

Table 3. Correlation with the measurement items and age.

Measurement item	Unit	Regression line	Correlation coefficient (r)	p value
Pentosidine	ng/mL	y = 1.3028x + 7.7983	0.746	< 0.01
Blood glucose	mg/dL	y = 0.2541x + 74.676	0.347	< 0.01
HbA1c [NGSP]	%	y = 0.015x + 4.8178	0.510	< 0.01
Skin AGEs	afu	y = 0.0185x + 1.1009	0.679	< 0.01
Triglyceride	mg/dL	y = 0.7389x + 51.707	0.249	0.98
HDL-C	mg/dL	y = -0.0073x + 65.839	-0.020	0.44
LDL-C	mg/dL	y = 0.0619x + 121.11	0.039	0.64
Cortisol	μg/dL	y = -0.0123x + 10.934	-0.063	0.29
DHEA-s	μg/dL	y = -4.2041x + 406.54	-0.699	< 0.01
Stiffness value	–	y = -0.5602x + 120.43	-0.556	< 0.01

Subject: 20 ~ 80 year-old women and men, n = 83. Plasma pentosidine measured by citric acid HPLC method. NGSP, National Glycohemoglobin Standardization. Program; AGEs, advanced glycation end products; HDL-C, high-density-lipoprotein-cholesterol; LDL-C, low-density-lipoprotein-cholesterol; DHEA-s, dehydroepiandrosterone-sulfate; HPLC, high performance liquid chromatography.

From these research data results, the optimization of plasma pentosidine measuring conditions in citric acid HPLC method was as follows:
Pretreatment, 50 μL of plasma was processed with preparation B and secondarily, hydrolyzed with addition of 100 μL of 6N hydrochloric Acid, Fe free. HPLC sample was obtained.
Conditions of HPLC, column; Unison US-C18 (5 μm, 150×4.6 mm I.D), eluent; 100 mmol/L citric acid /ACN (99.5/0.5), flow rate; 1.0 mL/min, fluorescence detection: excitation wavelength (ex) 335 nm, emission wavelength; (em) 385 nm, column temperature; 20°C, and sample injection volume: 20 μL. The measurement time from the optimized pretreatment of citric acid HPLC method to the completion of the measurement and had a possibility of shortening the time period by approximately 12 hours on the supposition of 24 samples measured in comparison to ion-pair HPLC method [16]. It was recognized the optimization of measurement method could contribute to saving operation time.

However, the plasma sample measurement results showed that the pentosidine measured by ion-pair HPLC method (measured by LSI Medience) showed high values. The correlation equation of plasma sample measurement value between ion-pair and citric acid HPLC method was $y = 1.3028\ x + 7.7983$, $r = 0.746$, $p < 0.01$. There was correlation but both gradient and intercept were high. It was assumed that the reasons of these high levels in ion-pair HPLC methods were that impurities were mixed into the peak of pentosidine and there were differences in purity of the standard material used for the calibration curve.

Correlation between blood pentosidine value and age-related changes

Among anti-aging medical check-up assessment items which the 83 research participants at 20-90 years old, there were weak positive correlations of age with HbAlc, skin AGEs and plasma pentosidine. The blood glucose showed weak correlations with age. Calcaneal stiffness and DHEA-s showed negative correlations with age. However, other measurement items did not show correlation with age.

Plasma pentosidine value had no difference between men and women and was recognized to have correlations with age. However, there were large differences of plasma pentosidine levels in subjects 60 years old or older. Glycative stress is affected by blood glucose level alternations due to lifestyle habits such as diet, excise and sleep [3]. Therefore, there were possibilities that the factors of increase in plasma pentosidine values were affected by aging and differences in life-style habits of subjects.

Pentosidine is one type of products that is formed by glycation binding blood glucose to protein in organisms. Data showed that plasma pentosidine correlated with HbAlc and skin AGEs but did not correlate with blood glucose. It was assumed that measured glucose levels were fasting blood glucose. Furthermore, there was a possibility that time periods were different when pentosidine reflected the influences of blood glucose. HbAlc reflected influences of average blood glucose levels for previous one or two months. HbAlc and skin AGEs values can be changed due to the ingestion of materials with inhibitory effects on glycation [21, 22, 23] for a few months or the improvement of eating habits [24]. Considering these aspects, there were possibilities that plasma pentosidine levels reflected the influences of glycative stress including blood glucose alternations by life-style habits during the previous several months.

Plasma pentosidine showed a weak negative correlation with calcaneal stiffness and increased along with the decrease of calcaneal stiffness. It has been reported that pentosidine formed in bone collagen affects the decline of bone quality due to disordered cross-linking constructions [10]. For this reason, that measurement of plasma pentosidine could be a marker for a bone fracture risk of subjects due to bone quality changes. In addition, plasma pentosidine levels showed a weak correlation with blood DHEA-s level and increased along with the decrease of DHEA-s. DHEA-s is an intermediate metabolite of a hormone and is secreted from the adrenal cortex. It has been reported [25] that DHEA-s decreases with increasing age. For this reason, there was a possibility that plasma pentosidine was a marker to assess the decline of hormone secretion due to glycative stress.

Meanwhile, plasma pentosidine levels did not show correlations with triglyceride, HDL-C and LDL-C levels. It was assumed that there were few relations between the formation of plasma pentosidine and lipid metabolism.

Considering these results, plasma pentosidine levels can be an index for aging and glycative stress.

Conclusion

Optimization of a measurement method of plasma pentosidine in reverse-phase HPLC method using citric acid eluent. The plasma pentosidine measurement level by the optimized method can be an index for aging and glycative stress.

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Statement of conflict of interest

Non contributory.

Reference

1) Yazaki K, Nagai R, Horiuchi S. Creatine plays a direct role as a protein modifier in the formation of a novel advanced glycation end product. *J. Biochem*. 2002; 132: 543-550.
2) Dyer DG, Blackledge JA, Thorpe SR, et al. Formation of pentosidin during noenzymatic browning of proteins by glucose. *J Biol Chem*. 1991; 266: 11654-11660.
3) Ichihashi M, Yagi M, Nomoto K, et al. Glycation stress and photo-aging in skin. *Anti-Aging Medicine*. 2011; 8: 23-29.
4) Spacek P, Adam M. Pentosidine in osteoarthritis: HPLC determination in body fluids and in tissues. *Rheumatol Int*. 2006; 26: 923-927.
5) Sivan SS, Tsitoron E, Wachtel E, et al. Age-related accumulation of pentosidine in aggrecan and collagen from normal and degenerate human intervertebral discs. *J. Biochem*. 2006; 399: 29-35.
6) Takahashi M, Suzuki M, Kushida K, et al. Relation between pentosidine levels in serum and urine and activity in rheumatoid arthritis. *British Jornal of Rheumatology*. 1997; 36: 637-642.
7) Kerkeni M, Saïdi A, Bouzidi H, et al. Pentosidine as a biomarker for microvascular complications in type 2 diabetic patients. *Diabetes & Vascular Disease Research*. 2012; 10: 239-245.
8) Furuya R, Kumagai H, Miyata T, et al. High plasma pentosidine level is accompanied with cardiovascular events in hemodialysis patients. *Clin Exp Nephrol*. 2012; 16: 421-426.
9) Kerkeni M, Santos-Weiss IC, Jaisson S, et al. Increased serum concentrations of pentosidine are related to presence and severity of coronary artery disease. *Thrombosis Research*. 2014; 134: 633-638.
10) Saito M. Bone quality markers: pentosidine, homocysteine, and MTHFR polymorphism. *Rinsho Byori*. 2009; 57: 876-883.
11) Sugiyama S, Miyata T, Ueda Y, et al. Plasma levels of pentosidine in diabetic patients: An advanced glycation end product. *J Am Soc Nephrol*. 1998; 9: 1682-1688.
12) Sanaka T, Funaki T, Tanaka T, et al. Plasma pentosidine levels measured by a newly developed method using ELISA in patients with chronic renal failure. *Nephron*. 2002; 91: 64-73.
13) Nakano M, Kubota M, Owada S, et al. The pentosidine concentraion in human blood specimens is affected by heating. *Amino Acids*. 2013; 44: 1451-1456.
14) Miyata T, Ueda Y, Yamada Y, et al. Accumulation of carbonyls accelerates the formation of pentosidine, an advanced glycation end product: Carbonyl stress in uremia. J Am Soc Nephrol. 1998; 9: 2349-2356.
15) Spacek P, Adam M. HPLC method for pentosidine determination in urine, serum, and tissues as a marker of glycation and oxidation loading of the organism. *J Liq Chromatogr Relat Technol*. 2002; 25: 1807-1820.
16) Shimode A, Yagi M, Naito J, et al. Hair protein glycation reduces hair breaking strength. *Glycative Stress Res*. 2014; 1: 37-45.
17) Ohno R, Nagai R. About measurement of the serum Pentosidine using HPLC. *JMARS News Letter*. 2015; 15: 6-8. (in Japanese)
18) Scheijen JL, Waarenburg MP, Stehouwer CD, et al. Measurement of pentosidine in human plasma protein by a single-column high-performance liquid chromatography method with fluorescence detection. *Journal of Chromatography B*. 2009; 877: 610-614.
19) Hori M, Yagi M, Nomoto K, et al. Inhibition of advanced glycation end product formation by herbal teas and its relation to anti-skin aging. *Anti-Aging Med*. 2012; 9: 125-134.
20) Frantzen F, Grimsrud K, Heggli DE, Sundrehagen E. Protein-boronic acid conjugates and their binding to low-molecular-mass cis-diols and glycated hemoglobin. *J Chromatogr B*. 1995; 670: 37-45.
21) Yonei Y, Miyazaki R, Takahashi Y, et al. Anti-glycation effect of mixed herbal extract in individuals with pre-diabetes mellitus: A double-blind, placebo-controlled, parallel group study. *Anti-Aging Medicine*. 2010; 7: 26-35.
22) Takabe W, Yagi M, Ogura M, et al. Effect of mangosteen pericarp extract-containing black vinegar drink on skin quality through anti-glycative actions. *Glycative Stress Res*. 2017; 4: 158-171.
23) Takeshita S, Ishioka Y, Tomohiro Uemura T, et al. Reducing effect of the long term intake of water chestnut (*Trapa bispinosa* Roxb.) pericap extract on glycative stress in the placebo-controlled double blinded clinical trial and in vitro inhibitory actions on low-density lipoprotein (LDL) glycation. *Glycative Stress Res*. 2017; 4: 299-316.
24) Imai S, Michiaki Fukui M, Kajiyama S. Effect of eating vegetables before carbohydrates on glucose excursions in patients with type 2 diabetes. *J Clin Biochem Nut*r. 2014; 54: 7-11.
25) Nomoto K, Arita S, Yonei Y. Development of a model of functional endocrine age in Japanese people: Serum dehydroepiandrosterone-sulfate (DHEA-s) concentration as an index of Aging. *Anti-Aging Medicine*. 2011; 8: 69-74.