Journal of Chromatography B, 877 (2009) 2168–2172

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Short communication

Fluorometric determination of pantothenic acid in human urine by isocratic reversed-phase ion-pair high-performance liquid chromatography with post-column derivatization

Kei Takahashi, Tsutomu Fukuwatari*, Katsumi Shibata

Department of Food Science and Nutrition, School of Human Cultures, The University of Shiga Prefecture, 2500 Hassaka, Hikone, Shiga 522-8533, Japan

ARTICLE INFO

Article history:
Received 28 January 2009
Accepted 28 May 2009
Available online 6 June 2009

Keywords:
Pantothenic acid
Ion-pair HPLC
Vitamin
Human urine
Fluorometric
Post-column derivatization

ABSTRACT

We describe here a method for the determination of pantothenic acid, vitamin B_5, in human urine by isocratic reversed-phase ion-pair HPLC with post-column derivatization. Pantothenic acid in urine was separated using a Tosoh ODS-80Ts (4.6 i.d. × 250 mm) column with phosphate–sodium hydroxide buffer (pH 7.0) containing dodecyltrimethylammonium chloride. Following the isolation of pantothenic acid it was decomposed to pantoic acid and β-alanine by alkali treatment. The product β-alanine was post-derivatized to the fluorescent 1-alkylthio-2-alkylisoindole with orthophthaldialdehyde in the presence of 3-mercaptopropionic acid. In the proposed method, a urine sample can be directly injected into a HPLC system without any pre-clean up treatment. The limit of detection was 3 pmol (*ca.* 650 pg) per 20 μL of urine at a signal-to-noise ratio of 5:1 and the limit of quantification was 5 pmol (*ca.* 1000 pg) per 20 μL of urine, which was sufficiently sensitive for the determination of pantothenic acid in human urine. The total time required for the analysis was *ca.* 25 min. The proposed method can be used to assess the pantothenic acid content of human urine as an alternative to the standard microbioassay for pantothenic acid.

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1. Introduction

Recent studies have shown that the analysis of urinary compounds can provide a great deal of useful nutritional information. For example, urinary excretion of protein, potassium and sugar [1–3] can be used as biomarkers to estimate the intake of the respective nutrients.

Pantothenic acid (PaA) is a water-soluble vitamin, and there are reports of a correlation between urinary excretion and intake [4]. Urinary excretion of PaA is therefore a suitable surrogate indicator of PaA intake. Several methods for the measurements of PaA have been reported. The most reliable and common method is a microbioassay which uses *Lactobacillus plantarum* ATCC 8014 [5]. The merits of the assay include its sensitivity and the fact that expensive instruments and reagents are not required. The disadvantage of the microbioassay is that it requires specialist knowledge and technique. Alternative chemical assay methods have therefore been developed. For example, the use of radioimmunoassay [6,7] and indirect enzyme immunoassay [8–10] have been reported. Radioisotopes and scintillation counting are required for the former, and non-commercially available antisera are needed for the latter. Methods using gas chromatography–mass spectrometry with multiple ion detection [11]; liquid chromatography–mass spectrometry [12–14]; and liquid chromatography–tandem mass spectrometry [15] have also been reported. Whilst these methods may be associated with high precision, the analytical systems required are expensive and difficult to maintain.

High-performance liquid chromatography (HPLC) assays using ultraviolet (UV) [16–19] or fluorometric detection [20,21] to measure PaA have been reported. The assay using UV detection cannot be applied to urinary PaA because the PaA molecule absorbs very weakly in the UV region. Although the fluorometric determination of PaA as reported by Pakin et al. [21] would be suitable for the rapid determination of the compound in urine, the sample requires pre-treatment and the HPLC separation can be problematic. In this assay, the sample must be purified and the pH adjusted several times which leads to dilution of the sample. We applied this method to the measurement of PaA in human urine; however, we were unable to detect PaA in some samples because of its low concentration in human urine.

In this study, we describe a novel assay for measuring PaA in urine by isocratic reversed-phase ion-pair HPLC with post-column derivatization.

2. Experimental

2.1. Chemicals and reagents

Calcium pantothenate, orthophthalaldehyde (OPA), 3-mercaptopropionic acid (3-MPA), sodium hydroxide, potassium

* Corresponding author. Fax: +81 749 28 8499.
E-mail address: fukkie@shc.usp.ac.jp (T. Fukuwatari).

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doi:10.1016/j.jchromb.2009.05.056

Table 1
Basal data for the new HPLC method for detection of PaA.

Auto-sampler	SIL-10AD$_{VP}$ (SHIMADZU)
Pump-1	L-2130 (HITACHI)
Pump-2	L-2130 (HITACHI)
Pump-3	L-2130 (HITACHI)
Column oven-1	L-2350 (HITACHI)
Column oven-2	655A-52 (HITACHI)
Fluorescence detector	RF-10A$_{XL}$ (SHIMADZU)
Data processor	C-R8A (SHIMADZU)
Column	Tosoh ODS-80Ts (4.6 i.d. × 250 mm)
Length of reactor tube	60 m (PTFE tube, 0.5 mm i.d.)
Temperature (column oven-1)	40 °C
Temperature (column oven-2)	100 °C
Flow-speed 1 (pump-1)	1.0 mL min^{-1}
Flow-speed 2 (pump-2)	0.5 mL min^{-1}
Flow-speed 3 (pump-3)	0.5 mL min^{-1}
Excitation/emission wavelength	345 nm/455 nm
Mobile phase	60 mM KH_2PO_4–NaOH buffer (pH7.0) containing 5 mM DTMA and 5% acetonitrile (v/v)
Solution-1	600 mM NaOH
Solution-2	10 mM OPA and 16 mM 3-MPA

dihydrogenphosphate and acetonitrile were purchased from Wako Pure Chemical Industries, Limited (Osaka, Japan). Tetraethylammonium chloride (TEA), tetrabutylammonium hydrogen sulfate (TBA), hexadecyltrimethylammonium chloride (HDTMA) and tetraheptylammonium bromide (THEPA) were also purchased from Wako. Dodecyltrimethylammonium chloride (DTMA) was supplied by Tokyo Kasei Kogyo Company, Limited (Tokyo, Japan) and tetrahexylammonium hydrogen sulfate (THEXA) and tetraoctylammonium bromide (TOA) were obtained from Fluka (NJ, USA).

2.2. Chromatographic analysis

The instrumentation and the chromatographic condition are shown in Table 1 and Fig. 1.

Separation of PaA in urine was carried out using a Tosoh ODS-80Ts (4.6 i.d. × 250 mm) column (Tokyo, Japan) with pump-1 and column oven-1 (Fig. 1). The mobile phase consisted of 60 mM KH_2PO_4–NaOH buffer (pH 7.0) containing 5 mM ion-pair reagent and 5% acetonitrile; a flow rate of 1.0 mL min^{-1} was used and the column temperature was maintained at 40 °C. Seven ion-pair reagents were compared: TEA, TBA, DTMA, HDTMA, THA, THEPA and TOA.

After the separation was complete, the column effluent was subjected to post-column hot alkaline hydrolysis using NaOH (solution-1), which was delivered via pump-2 at a flow rate of 0.5 mL min^{-1} through a T-connector attached to the polytetrafluoroethylene reactor tube (0.5 mm i.d.) in column oven-2 which was maintained at 100 °C. The effect of various NaOH concentrations (300, 600, 900 and 1500 mM) and reactor tube lengths (40 and 65 m) was compared.

After hot alkaline hydrolysis, an aqueous solution containing 10 mM OPA and 16 mM 3-MPA (solution-2; Fig. 1) was delivered at a flow rate of 0.5 mL min^{-1} via pump-3 through a T-connector to the hydrolyzed effluent to effect post-column derivatization.

2.3. Preparation

Stock solutions of β-alanine and PaA were made up to 1 mM with ultrapure water and stored at −20 °C. Working standard solutions were diluted from the stock solutions to produce a series of concentrations (0.5, 1.0, 2.0, 5.0, 10.0, 20.0, 50.0 and 100 μM); 20 μL of each concentration was then injected into the HPLC system.

Urine collected from three healthy volunteers was used for validation of the method. The samples were compared with the optimal

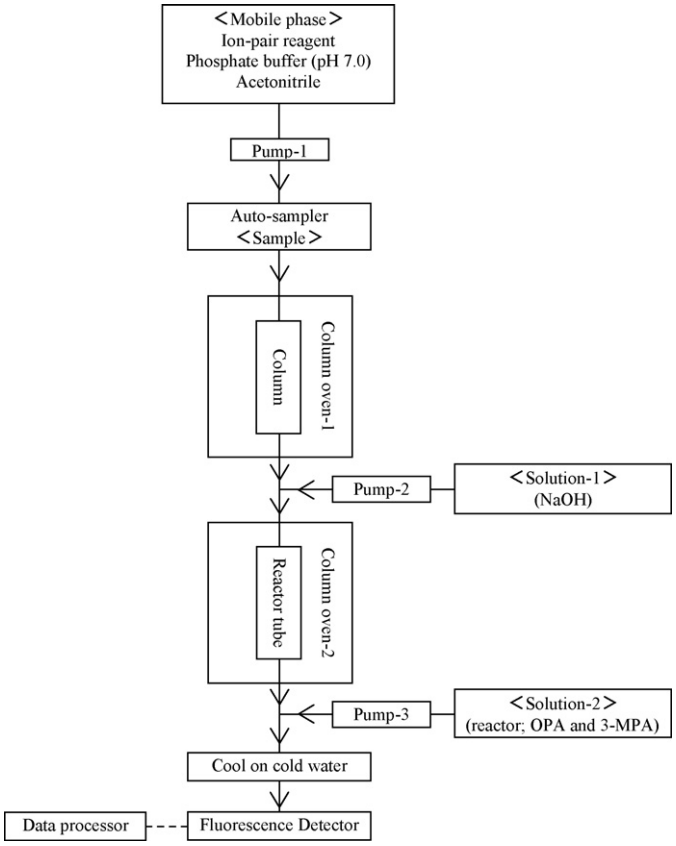

Fig. 1. Determination of PaA in urine (schematic).

basal conditions in the HPLC system which served as the quality control (QC) sample. The results obtained from the HPLC and microbioassay of the 24 h urine samples were compared. The QC and 24 h urine samples were stored at −20 °C and thawed when needed. An aliquot of the urine samples (1.5 mL) was centrifuged at 15,000 × *g* for 10 min at room temperature. The supernatant obtained was filtered through a micro-filter (pore size, 0.45 μm; Millipore, Bedford, USA) and the filtrate (20 μL) injected directly into the HPLC system.

2.4. Assay validation

The within-run precision was calculated by analyzing five replicates of the QC urine on the same day. Between-run precision was determined by triplicate analysis of QC urine on three separate occasions and the value of each was calculated by analyzing five replicates. The coefficient of variation (CV) was used to measure the precision, where:

$$CV(\%) = \left\{ \frac{\text{standard deviation } (SD)}{\text{mean}} \right\} \times 100.$$

Within-run accuracy was measured in different experiments to calculate the precision, and evaluated in the same experiment to ascertain recovery. Accuracy was expressed as relative error (RE) and determined by the equation:

$$RE(\%) = \left\{ \frac{\text{observed concentration} - \text{added concentration}}{\text{added concentration}} \right\} \times 100.$$

The precision and accuracy needed to be within ±10%. Recovery was calculated by the formula:

$$\text{Recovery}(\%) = \left(\frac{\text{observed concentration}}{\text{added concentration}} \right) \times 100.$$

Three additional concentrations (100, 200 and 400 pmol per 20 μL) were analyzed in triplicate. The conversion rate was calculated from the areas under the peaks for β-alanine and PaA using the formula:

$$\text{Conversion rate}(\%) = \left(\frac{\text{peak area for PaA}}{\text{peak area for β-alanine}}\right) \times 100.$$

2.5. Stability

Short-term stability was examined by maintaining the QC urine at 37 °C for 24 h, middle-term stability was evaluated by storing the QC urine at 4 °C for 7 days and long-term stability of PaA was assessed at −20 °C for 30 days. Freeze–thaw stability was determined over three cycles of thawing at 4 °C for 12 h and refreezing for 12 h. For each storage condition, five replicates were analyzed in each batch. The PaA concentration after each storage period was related to the initial concentration determined for the samples, which were freshly prepared and processed immediately. The range of change was calculated by the formula:

$$\text{Range of change}(\%) = \left(\frac{\text{concentration under each condition}}{\text{concentration of freshly preparation}}\right) \times 100.$$

2.6. Comparison with bioassay

To compare the results of the HPLC analysis and the microbioassay (which is the standard method used to determine PaA concentration), PaA in urine samples from 121 volunteers (age range: 9–80 years) was determined using *Lactobacillus plantarum* (ATCC 8014, American Type Culture Collection, USA) as reported by Wright and Skeggs [5].

3. Results and discussion

3.1. HPLC conditions

To determine the PaA concentration in human urine, we modified the fluorometric HPLC method reported by Blanco et al. [20] and Pakin et al. [21] (Table 1 and Fig. 1). The first step is the separation of PaA; the second step is the decomposition of PaA into pantoic acid and β-alanine; and the final step is the reaction between β-alanine, OPA and 3-MPA. The product is 1-alkylthio-2-alkylisoindole, which can be detected at an excitation wavelength of 345 nm and an emission wavelength of 455 nm.

We used 60 mM KH_2PO_4–NaOH buffer (pH 7.0) containing 5% acetonitrile to separate PaA from other compounds in urine using an isocratic-flow system. We used pump-1, column oven-1 and a Tosoh ODS-80Ts column for the separation phase; pump-2, column oven-2 and a PTFE tube (0.5 mm i.d.) as the reactor tube for the alkaline hydrolysis of PaA at an elevated temperature; and pump-3 for the derivatization of β-alanine produced from the decomposition of PaA, with OPA and 3-MPA. The concentrations of the reagents OPA and 3-MPA used in our experiment were those recommended in the literature [20,21].

3.2. Experiment on optimization conditions for PaA measurement in urine without pre-clean up treatment

3.2.1. Derivatization

Although Pakin's system uses a mixture of OPA, 3-MPA and NaOH [21], OPA was found to be unstable to heat and alkali. We investigated whether the mixed solution of NaOH, OPA and 3-MPA was stable after 16 h. The non-mixed solution system was solution-1 (600 mM NaOH) and solution-2 (10 mM OPA and 16 mM 3-MPA).

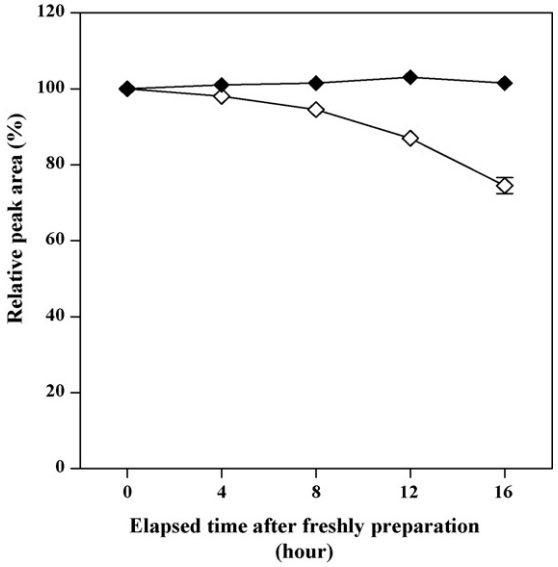

Fig. 2. Effect of using mixed solutions for post-derivatization on the peak area of β-alanine, and comparison of the stability using non-mixed solution and mixed solution. Values are based on the peak area measured at 0 h which is shown as 100%. The results are expressed as mean ± SD ($n = 5$): (◊) mixed solution of 600 mM NaOH, 5 mM OPA and 8 mM 3-MPA; (♦) non-mixed solution, solution-1 was 600 mM NaOH, and solution-2 was 10 mM OPA and 16 mM 3-MPA.

The flow rate of both solutions (solution-1 and solution-2) was 0.5 mL min^{-1}. The mixed solution comprised 600 mM NaOH, 5 mM OPA and 8 mM 3-MPA, and the flow rate was set at 1.0 mL min^{-1} (Fig. 2). Reproducibility was based on each peak area of PaA at 0 h after solution preparation, and was shown to be 100%. In this study, the wide baseline fluctuations did not result from using two pumping devices. The peak area at 0 h for the mixed solution and non-mixed solution did not differ. The peak area produced with the mixed solution gradually decreased to 70% at 16 h after solution preparation. The non-mixed solution was reproducible for 16 h, and was used for subsequent study.

3.2.2. Selection of ion-pair reagents

We compared the representative chromatograms without ion-pair reagents and with seven types of ion-pair reagents. The mobile phase without ion-pair reagent failed to separate PaA from other compounds in urine. The ion-pair reagent (5 mM) was added to the mobile phase. HDTA, THEPA and TOA did not dissolve in mobile phase but the other reagents were found to be soluble, and the QC urine was analyzed using the various mobile phases (Fig. 3). Good separation was obtained with the mobile phases containing TBA, THEXA and DTMA although TBA and THEXA produced tailing. We therefore selected DTMA as the ion-pair reagent. These results did not change even when the concentration was increased to 10 mM.

3.2.3. Effect of NaOH concentration

The alkaline hydrolysis of PaA was markedly affected by the concentration of NaOH and the concentration recommended in the literature [21] did not produce adequate hydrolysis. We therefore examined the optimal concentration of NaOH required to achieve hot alkaline hydrolysis. We compared the peak of β-alanine derived from PaA using 300, 600, 900 and 1500 mM NaOH. The peak area increased when the NaOH concentration increased from 300 to 600 mM. However, the increase in NaOH concentration from 600 to 900 mM, and from 900 to 1500 mM caused the peak area for derived PaA to decrease. We therefore selected 600 mM NaOH as the hydrolyzing solution because the area for derived PaA was the largest.

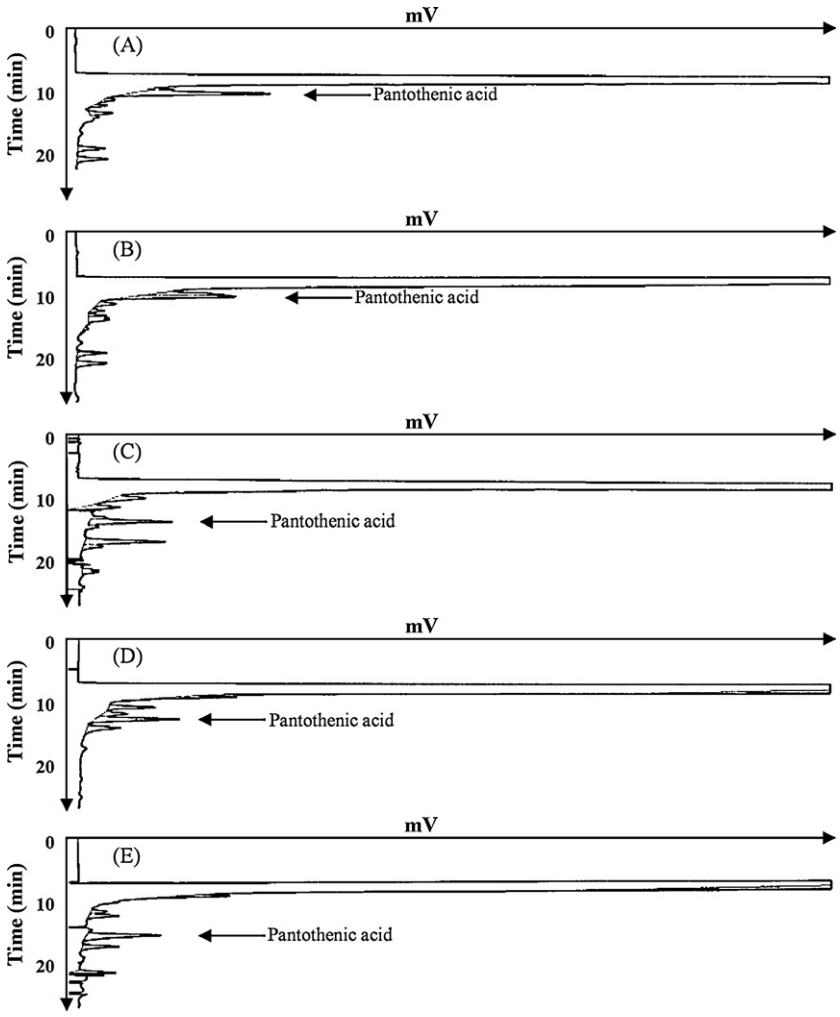

Fig. 3. Effect of the ion-pair reagent on the separation of PaA in urine. Chromatograms showing the influence of various ion-pair reagents on the separation of PaA: chromatogram: (A) no ion-pair reagent; (B) TEA; (C) TBA; (D) THEXA; (E) DTMA. A 20-μL sample of QC urine was injected into the HPLC system. The mobile phase consisted of 60 mM phosphate–NaOH buffer (pH 7.0) containing 5 mM of each ion-pair reagent and 5% acetonitrile. Solution-1 was 600 mM NaOH. Solution-2 consisted of 10 mM OPA and 16 mM 3-MPA.

3.2.4. Effect of the length of the reactor module on hydrolysis with hot alkali

We found that the optimal concentration of NaOH for hydrolysis treatment was 600 mM, but the conversion rate (β-alanine from PaA/β-alanine) was only 58%. We could not obtain a satisfactory conversion rate of PaA to β-alanine. Hot alkaline hydrolysis was markedly affected by not only NaOH concentration, but also by temperature and time. The temperature had been set at 100 °C, and could not be set higher. We therefore tried to use a longer reactor tube to improve the conversion rate of β-alanine.

When the reactor tube was extended from 40 to 65 m, the peak area derived from PaA increased. The increase in the conversion rate was from 58% to 95%. Lengthening the tube by 25 m extended the hydrolysis time by 3.5 min, and the time to hydrolysis was extended from 5 to 8.5 min. The optimal length of reactor tube was selected to be 65 m because the conversion rate obtained was satisfactory. The optimal HPLC conditions are summarized in Table 1.

3.3. Method validation

3.3.1. Linearity of calibration curve and limit of quantification

The linearity of the calibration curve was determined by plotting the peak area (y) of PaA against the standard PaA concentration (x). The correlation coefficient was >0.99, confirming that the calibration curve was linear over the concentration range 5–1000 pmol per 20 μL for the PaA standard. The typical standard curve can be represented by $y = 11370 + 2048x$ ($r = 0.997$). QC urine could be detected at 3 pmol (658 pg) per 20 μL at a signal-to-noise ratio of 5:1, and therefore, the limit of detection was 3 pmol (658 pg) per 20 μL of urine. The limit of quantification was 5 pmol (1096 pg) per 20 μL of urine, which was sufficiently sensitive for the determination of PaA in human urine.

3.3.2. Accuracy and precision

The CV of the within- and between-run precision was 0.63% and 0.79%, respectively. The accuracies as shown by RE were 1.1%, −6.1% and −3.6% at concentrations of 100, 200 and 400 pmol per 20 μL, respectively. These data indicated that the assay was reproducible, accurate and reliable.

3.3.3. Recovery

We added 100, 200 and 400 pmol per injection of standard PaA to urine samples, and the recoveries were 101 ± 3%, 94 ± 1% and 96 ± 3%, respectively.

3.3.4. Stability

We had previously examined the stability of PaA in urine by bioassay, but we evaluated it again in this study. The stability of

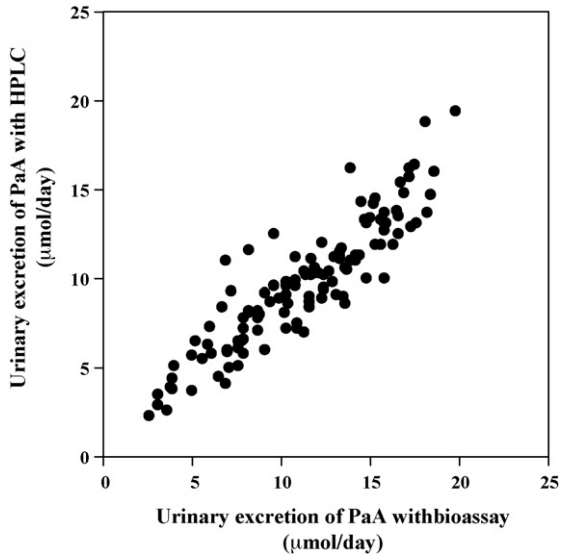

Fig. 4. Comparison of PaA in human urine using the HPLC assay and the microbioassay. The urine samples were collected over 24 h from healthy volunteers (n = 121; age range: 9–80 years). The bioassay method used *Lactobacillus plantarum* ATCC 8014 to determine the level of PaA.

QC urine at 37 °C for 24 h (i.e., short-term stability) was designed to confirm that PaA in urine was stable even though it was collected at a room temperature of 37 °C. Testing the stability at 4 °C for 7 days (i.e., middle-term stability) was designed to confirm that PaA in urine was stable even though it was left in an auto-sampler. The stability at −20 °C for 30 days (i.e., long-term stability) was determined to confirm that PaA was stable even if the HPLC system stopped functioning and the analysis could not be continued for a long period of time. The freeze–thaw stability was investigated in order to confirm that PaA would not decompose during sequential analyses.

The stability of PaA over the short-term, middle-term, long-term, and the freeze–thaw cycles was found to be −4, 0, +2 and −4% change, respectively, compared to the value for fresh urine which was taken as 100%. Under all conditions, PaA in urine was stable.

3.4. Comparison of the PaA content in urine determined using the present HPLC assay and the microbioassay method

To demonstrate that the present HPLC assay could substitute for the microbioassay, we compared urinary excretion of PaA by the two methods.

The values obtained by HPLC were similar to those obtained with the microbioassay (Fig. 4). The linearity of the calibration curve was determined by plotting the values obtained by the HPLC assay (y) against those obtained by the microbioassay (x). The curve can be shown by $y = 1.349 + 0.741x$ ($r = 0.905$). The p value was <0.0001. This result suggested that the HPLC assay is comparable to the microbioassay.

4. Conclusion

An assay was developed to determine the urinary excretion of PaA by isocratic reversed-phase HPLC and an ion-pair reagent. Using an ion-pair reagent and an isocratic-flow system produced a simple, rapid, sensitive, accurate and precise assay. The urinary excretion of PaA analyzed in our assay correlated well with microbioassay, which is currently the "gold standard".

Acknowledgements

This report is part of project: Japanese Dietary Reference Intakes: elucidation of balance of micronutrient and macronutrient intakes (Principal investigator, Katsumi Shibata). The investigation was supported by a grant from the Ministry of Health, Labor and Welfare.

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