〈Original Article〉

Determination of dehydroascorbic acid in human urine

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Summary Urinary concentrations of dehydroascorbic acid (DHA) have been proposed as a biomarker for oxidative stress. Contrary to our findings, others have reported that DHA was absent from plasma. Our goal was to measure DHA in urine. Random urine specimens were collected from healthy Japanese adults who did or did not ingest ascorbic acid (AA). DHA and AA concentrations in urine were determined with the 4-hydroxy-2,2,6,6-tetramethylpiperidinyloxy, free radical (TEMPO) and ascorbate oxidase (AO) methods. The TEMPO and AO methods gave recoveries of 110% and 75% of DHA, respectively. DHA values by the AO method correlated well with those by the TEMPO method ($r= 0.889$, $p<0.001$). Among our 12 volunteers, the DHA concentrations by the TEMPO method ranged from 0.9 to 13.8 mg/L. As expected, the amounts of DHA excreted in urine significantly increased following AA ingestion ($p<0.05$); the same was found for serum specimens ($p<0.05$) that were assayed by the TEMPO method. We concluded that we had successfully quantitated urinary DHA from our volunteers.

Key words: Antioxidant, Ascorbate oxidase, Oxidative stress, 4-hydroxy-2,2,6,6-tetramethylpiperidinyloxy, Free radical, Vitamin C

1. Introduction

In addition to its anti-scorbutic activity, ascorbic acid (AA) acts as strong antioxidant in the human body[1)]. AA quenches a variety of reactive oxygen and reactive nitrogen species in an aqueous environment. Dehydroascorbic acid (DHA) and semidehydroascorbic acid (sDHA) are oxidized form of vitamin C arising from AA through oxidative stress *in vivo*. DHA and sDHA are reduced by glutathione[2)] and NADH or NADPH[3)] back to AA, which can then exert antioxidant activity. Because these reduction reactions occur promptly after AA oxidation, DHA and sDHA are difficult to detect in plasma even more so in urine specimens.

Kubin et al.[4)] reported that urinary excretion of DHA was increased in patients undergoing total hip joint endoprosthestic surgery as compared with healthy

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controls. The authors concluded that the stress of surgery increases the rate of AA oxidation and urinary excretion of DHA as a consequence of the enhanced formation of free radicals. Kubin et al. also concluded that the stress of surgery increases the rate of oxidation of AA and urinary excretion of DHA as a consequence of the enhanced formation of free radicals.

The aim of our study was to investigate whether DHA is in fact present in human urine as detected by standard techniques, and whether our analytical technique is reliable.

2. Materials and Methods

1) Subjects

Written informed consent was obtained from all subjects, and our study was in compliance with the rules for human experimentation in our institution. We collected random urine specimens from twelve Japanese adults. Six were men (ages 23-55) and six were women (ages 23-60); all were presumed to be in good health. None of the volunteers smoked or consumed alcohol. None ingested vitamins or any other dietary supplements. Four (W1, 2, 3 and M1) of the volunteers ingested 1 g AA at 9:00 AM in the form of AA powder dissolved in 200 mL of water (BASF Takeda Vitamins Ltd., Tokyo, Japan, or Kanto Chemical Co., Inc., Tokyo, Japan). Subject M1 was subjected to two additional experiments receiving 250 or 500 mg of AA. Their urine specimens were collected before the AA was given and then 2, 4, 6, and 8 hours after intake. In addition, venous blood specimens were collected before AA was given and then 3 and 8 hours later. The tests were performed at 1 to 3 hours after the first- or second-morning urine was collected.

2) Assay methods

DHA and AA concentrations in urine were determined by the 4-hydroxy-2,2,6,6-tetramethylpiperidinyloxy, free radical (TEMPO) method[5] and the ascorbate oxidase (AO) method[6]; both assays were carried out using the Cobas Mira S instrument (Roche Diagnostic System, Montclair, NJ, USA). In addition, DHA and AA concentrations in serum were determined by the TEMPO method on Cobas Mira S. In the TEMPO method, because o-phenyelenediamine (OPDA) does not react with AA, the AA present in urine was first oxidized by TEMPO to DHA; DHA reacts with OPDA to form a color at 340 nm. DHA is measured in the absence of TEMPO when the TEMPO reagent is replaced by phosphate buffer. The difference in the assay values for DHA assessed with or without the TEMPO reagent corresponds to the AA concentration.

Because the AO method reacts exclusively with AA but not with DHA, the latter is measured after the reduction of the DHA to AA by dithiothreithol[7]. The urine pH was neutralized by adding 0.05 mL of $Na_2HPO_4 \cdot 12H_2O$ (150 g/L) to 0.5 mL of urine, followed by 0.1 mL of dithiothreithol (3 g/L) as a reductant. After 10 minutes of incubation at 25℃, the reduction reaction was terminated by an addition of 0.1 mL of *N*-ethylmaleimide (10 g/L). The AA concentration in the mixture was measured by the AO method, and observed values were multiplied by 1.5 in order to correction for a dilution. The difference in the assay values for AA assessed with or without treatment by dithiothreithol or *N*-ethylmaleimide is the DHA concentration.

3. Results

1) Reactivites of DHA

The reagents in the TEMPO and AO methods

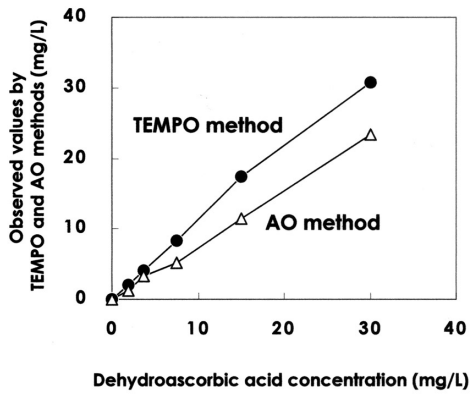

Fig. 1 Reactivites to dehydroascorbic acid by the TEMPO and AO methods.

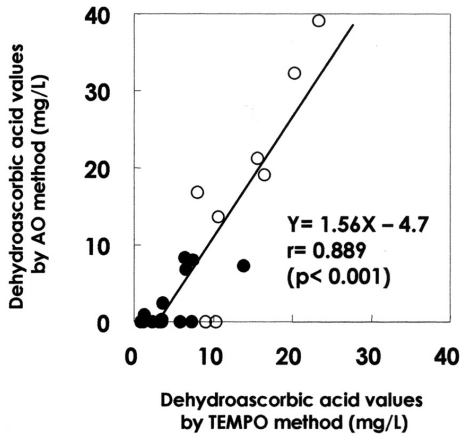

Fig. 2 Comparison of dehydroascorbic acid values found by the TEMPO and AO methods. We found a few negative DHA values and reported these as 0.0 mg/L by the AO method.
(○), urine from volunteers receiving AA;
(●), urine from volunteers not receiving AA.

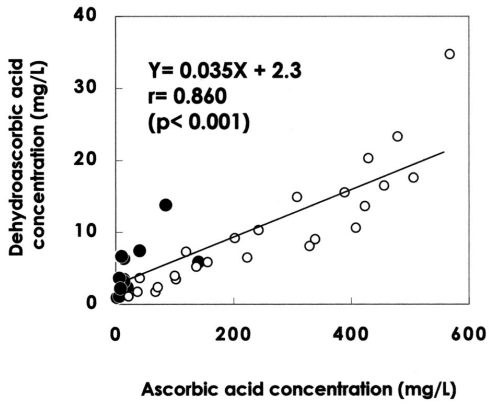

Fig. 3 Correlation of urinary ascorbic and dehydroascorbic acid concentrations in normal subjects. Some healthy volunteers ingested AA orally.
(○), urine from volunteers receiving AA;
(●), urine from volunteers not receiving AA.

react with DHA (Sigma-Aldrich Inc., MO, USA) with an assay range from 0.8 to 30 mg/L (Fig. 1). Recovery of DHA with the TEMPO method was 110% on average. The AO reagent reacted with ca. 75% of the DHA after the reduction treatment. The TEMPO and AO methods yielded a linear Beer's law curves from 0.8 mg/L to 100 mg/L of AA.

2) Within- and between-day reproducibility

Within-day assay of DHA with the TEMPO method for ten consecutive replicates of urine specimens gave a mean ± SD of 11.45 ± 0.14 mg/L, and a between-day assay of DHA for ten days gave a mean ± SD of 10.78 ± 0.83 mg/L. Within-day assay of DHA with the AO method for ten consecutive replicates gave a mean ± SD of 12.27 ± 0.95 mg/L, and a between-day assay of DHA for ten days gave a mean ± SD of 13.60 ± 3.32 mg/L.

3) Method comparison data from our volunteers

Using the TEMPO and AO methods, we measured DHA concentrations in urine from our 12 volunteers (Fig. 2). Although values by the AO method correlated well with those by the TEMPO procedure, nine assays by the AO method gave values of zero or negative values. ($r= 0.889$, $p<0.001$; $Y= 1.56X - 4.7$, $Sy.x= 5.2$ mg/L). We measured urine from subjects who did not receive AA, and some of these also gave values of zero (Fig. 2). Values of AA by the two methods correlated significantly ($r= 0.998$, $p<0.001$; $Y= 1.10X - 3.2$, $Sy.x= 12.4$ mg/L: Figure not shown).

4) DHA and AA concentrations in human urine

We measured DHA and AA concentrations in urine from our 12 volunteers by the TEMPO method, including specimens obtained following 250, 500, and 1,000 mg of AA as the loading dose. As expected, urinary concentration of DHA increased in parallel with the concentration of AA in urine (Fig. 3, $r= 0.860$, $p<0.001$). The ratio of DHA to AA in urine obtained without AA loading was 37.6 ± 24.9% (mean ± SD). Values ranged from 4 to 79% and decreased to 4.2 ± 1.4% (range, 2 to 9%) by AA loading. Absolute amounts of DHA excreted in urine were significantly increased by AA loading (Table 1, $p<0.05$, Wilcoxon matched-pairs signed ranks test). Testing of the volunteers here revealed that serum concentrations of DHA were increased ($p<0.05$) after the AA loading. We also found that the increased

Table 1 Urinary excretion of DHA and AA after AA ingestion

Subject	AA intake (mg)	Analytes	Time of urine collection (hr)[a] 0	2	4	6	8	0-8
W1	1,000	DHA (mg)	0.2	8.7	5.6	3.1	0.7	18.3
		AA (mg)	0.3	141.7	174.2	106.1	28.1	450.3
		DHA/AA (%)	66.7	6.1	3.2	2.9	2.5	4.1
W2	1,000	DHA (mg)	0.1	0.5	2.2	2.1	1.1	6.1
		AA (mg)	0.3	11.3	59.2	54.6	33.8	159.3
		DHA/AA (%)	33.3	4.4	3.7	3.8	3.3	3.8
W3	1,000	DHA (mg)	1.1	0.1	3.4	3.1	2.2	9.9
		AA (mg)	1.4	1.3	70.0	67.6	55.8	196.1
		DHA/AA (%)	78.6	7.7	4.9	4.6	3.9	5.0
M1	1,000	DHA (mg)	0.5	3.1	3.5	2.3	1.1	10.4
		AA (mg)	1.7	72.4	132.5	93.6	23.6	323.8
		DHA/AA (%)	29.4	4.3	2.6	2.5	4.7	3.2
M1	500	DHA (mg)	0.4	1.4	0.5	1.9	0.8	5.0
		AA (mg)	3.1	40.1	19.6	55.7	22.8	141.3
		DHA/AA (%)	12.9	3.5	2.6	3.4	3.5	3.5
M1	250	DHA (mg)	0.3	0.6	0.8	0.7	1.1	3.5
		AA (mg)	0.8	12.1	19.5	14.3	17.8	64.5
		DHA/AA (%)	37.5	5.0	4.1	4.9	6.2	5.4

[a] For example, 2 hours denotes time that expired between dose of AA and collection of urine. Absolute amounts (mg) of DHA and AA excreted in urine were calculated from their concentration (mg/L) and urine volume.

Table 2 Serum concentrations of DHA and AA after AA ingestion

Subject	AA intake (mg)	Analytes	Time of blood collection (hr) 0[a]	3	8
W1	1,000	DHA (mg/L)	0.9	1.3	1.4
		AA (mg/L)	11.1	22.0	23.3
		DHA/AA (%)	8.1	5.9	6.0
W2	1,000	DHA (mg/L)	0.9	1.2	1.3
		AA (mg/L)	12.1	18.3	21.6
		DHA/AA (%)	7.4	6.6	6.0
W3	1,000	DHA (mg/L)	0.9	1.5	1.1
		AA (mg/L)	11.9	25.5	16.7
		DHA/AA (%)	7.6	5.9	6.6
M1	1,000	DHA (mg/L)	1.0	1.4	1.3
		AA (mg/L)	14.4	25.2	22.0
		DHA/AA (%)	6.9	5.6	5.9
M1	500	DHA (mg/L)	0.9	1.3	1.3
		AA (mg/L)	13.3	22.4	22.8
		DHA/AA (%)	6.8	5.8	5.7
M1	250	DHA (mg/L)	0.8	1.2	1.0
		AA (mg/L)	10.8	20.1	15.0
		DHA/AA (%)	7.4	6.0	6.7

[a] For example, 3 hours denotes time that expired between dose of AA and collection of urine.

concentration of DHA was less than 1.4 mg/L, which nevertheless represented a markedly elevated concentration of AA in serum (Table 2).

4. Discussion

We investigated whether DHA in urine could be determined by the TEMPO and AO methods. Both methods reacted linearly with the DHA concentration in a range from 0.8 to 30 mg/L; this covered the wide range of DHA usually observed in human urine. The AO method gave a negative DHA value in those specimens showing DHA concentrations of less than 10 mg/L by the TEMPO method. We decided to determine the low concentration limits of DHA by the TEMPO method (Table 2).

We investigated whether DHA was present in human urine. Results of recent studies on the presence or absence of DHA in biological fluids have been inconsistent. Dharial et al.[8] reported that DHA was not present in the plasma, or serum of normal humans unless assay conditions permitted the oxidation of AA. Koshiishi et al.[9] reported that DHA detected in acidified plasma samples was an artifact resulting from AA oxidation. We considered that if DHA is in fact absent from plasma, it will not detected be in urine. In this study, we found a low concentration of DHA (around 0.8 mg/L) in the sera from healthy volunteers.

Koshiishi et al.[10] reported that DHA was present in circulating blood plasma at a concentration of 0.8 ± 0.3 mg/L. These concentrations agreed with our results. Koshiishi et al.[11] suggested that DHA arose from AA through oxidative stress or disorders where oxidative stress is involved. Lykkesfeldt et al.[12] reported that the DHA concentration in plasma of smokers (0.14 ± 0.40 mg/L) was higher than that in non-smokers (0.02 ± 0.41 mg/L). They determined the total amount of AA, i.e., the sum of AA and DHA, and another assay of AA by HPLC, and calculated the concentration of DHA by the difference between them [(sum AA+DHA)−(AA)]. They noted that their values included negative DHA values, just as we encountered in our AO method. This would account for the lower DHA values they observed.

Based on these observations, we concluded that DHA could have formed in circulating blood plasma. The interconversion of AA to DHA was probably increased owing to the raising level of oxidative stress occurring in the body. In our study with the TEMPO method, we observed 0.9 to 13.8 mg/L (4.8 ± 3.4 mg/L) of DHA in human urine, and 1.1 to 34.7 mg/L (10.3 ± 8.1 mg/L) after AA loading. Koshiishi et al.10) reported 1.4 ± 0.6 mg/L of urinary excretion of DHA from seven healthy adults (ages 22-37). Kubin et al.[4] reported a 150 mg/day of urinary excretion of DHA after intravenous administration of 3,000 mg of sodium ascorbate for two days. Although there are considerable differences between our values and those reported by Koshiishi and Kubin, we can conclude that DHA was truly present in human urine.

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