Determination of Dehydroascorbic Acid in Mouse Tissues and Plasma by Using Tris(2-carboxyethyl)phosphine Hydrochloride as Reductant in Metaphosphoric Acid/Ethlenediaminetetraacetic Acid Solution

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Abstract

Ascorbic acid (AA) has a strong anti-oxidant function evident as its ability to scavenge superoxide radicals in vitro. Moreover, AA is an essential ingredient for post-translational proline hydroxylation of collagen molecules. Dehydroascorbic acid (DHA), the oxidized form of AA, is generated from these reactions. In this study, we describe an improved method for assessing DHA in biological samples. The use of 35 mM tris(2-carboxyethyl)-phosphine hydrochloride (TCEP) as a reductant completely reduced DHA to AA after 2 h on ice in a 5% solution of metaphosphoric acid containing 1 mM ethlenediaminetetraacetic acid (EDTA) at pH 1.5. This method enabled us to measure the DHA content in multiple tissues and plasma of 6-weeks-old mice. The percentages of DHA per total AA differed markedly among these tissues, i.e., from 0.8 to 19.5%. The lung, heart, spleen and plasma had the highest levels at more than 10% of DHA per total AA content, whereas the cerebrum, cerebellum, liver, kidney and small intestine had less than 5% of DHA per total AA content. This difference in DHA content may indicate an important disparity of oxidative stress levels among physiologic sites. Therefore, this improved method provides a useful standard for all DHA determinations.

Key words ascorbic acid; dehydroascorbic acid; dithiothreitol; tris(2-carboxyethyl)phosphine hydrochloride; metaphosphoric acid

Ascorbic acid (AA) functions as an electron donor and scavenges free radicals such as superoxide radicals and hydroxyl radicals in vitro. Moreover, AA is essential for post-translational proline hydroxylation of collagen molecules, after which hydroxyproline residues play a critical role in stabilizing the triple helical structure of collagen. Dehydroascorbic acid (DHA), the oxidized form of AA, is generated as a result of these reactions. The biological activity of DHA has been considered equivalent to that of AA, because intracellular DHA is rapidly reduced to AA by dehydroascorbic acid reductase. However, Ogiri et al. reported that DHA had less than 10% of AA's biological efficiency on a molar basis, judging from experiments with the inherently scorbutic ODS rat, which is a convenient animal model for investigating the metabolism of AA in humans. Moreover, May et al. noted that the human mature red blood cells they studied did not have sodium-dependent vitamin C transporters (SVCT1 and SVCT2) yet manifested a very low late uptake of radio-labeled AA. In contrast, human mature red blood cells efficiently took up DHA from blood via a glucose transporter 1 (Glut1). Erythrocyte Glut1 and associated DHA uptake are unique traits of humans and the few other mammals that have lost the ability to synthesize AA in vivo. Furthermore, DHA is known to enter mitochondria via Glut1 and undergo reduction to AA in the mitochondria. AA, which enters mitochondria as DHA, also protects mitochondria from oxidative injury. Thus, because DHA possesses unique physiological features, distinct from those of AA, establishment of a simple and accurate method of assessing the presence of DHA is very important.

AA in biological samples is routinely evaluated by using HPLC systems. AA's maximum absorbance at 265 nm in HPLC coupled with detection by UV light are widely used for such analyses. Other means of AA determination subsequently developed are the ketone derivatization method with 2,4-dinitrophenylhydrazine (DNPH) or o-phenylene-diamine and HPLC coupled with electrochemical detection (ECD) system. HPLC-ECD is widely used at present, because of this assay's great sensitivity and specificity. However, for DHA determination, the UV detector is not suitable, because DHA's maximum absorbance at 220 nm provides only insensitive and nonselective results. DHA is also difficult to detect with HPLC-ECD because of its low electroactivity. Therefore, a DHA concentration is typically calculated by subtracting AA concentration from the total AA (AA+DHA) concentration determined in a different chromatographic run. The total AA concentration is usually calculated by reducing the DHA to AA with sulphydryl compounds including dithiothreitol (DTT) (Fig. 1), mercaptoethanol and homocysteine. However, the reducing ability of these sulphydryl compounds is limited to a narrow neutral pH range. In general, metaphosphoric acid (MPA) and EDTA are used to stabilize the AA and DHA in biological samples. Therefore, raising the samples’ pH to neutral is essential to effectively reduce DHA to AA. Since AA and DHA are unstable at neutral pH, using sulphydryl compounds as a reductant may not be a suitable.

Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), which is easily soluble and stable in aqueous solution, has reducing activity at pH 1.5 to pH 9.0 and has been applied to DHA reduction at pH 4.3 (Fig. 1). Recently, Wächtersbach and Cigic evaluated the reduction of DHA with TCEP at low pH and reported that DHA is fully reduced with 10 mM TCEP in a 2% MPA solution at 25 °C. Therefore,
the reduction of DHA with TCEP at an acidic low pH can now be considered more reliable. However, TCEP has not yet been used as a reductant for testing AA and DHA levels in biological samples such as murine tissues. Therefore, as presented here, we investigated the reduction of DHA with TCEP in MPA solution on ice. Using this improved method to stabilize AA and DHA, we determined the DHA content in mouse tissues and plasma and found significant variability in the percentages of DHA per total AA among the materials examined.

MATERIALS AND METHODS

Materials L-Ascorbic acid (AA), metaphosphoric acid (MPA), bromine, thiourea and dithiothreitol (DTT) were purchased from Wako Pure Chemicals (Osaka, Japan). Ethylenediaminetetraacetic acid (EDTA) was purchased from Dojindo Laboratories (Kumamoto, Japan). Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) was purchased from Tokyo Chemical Industry (Tokyo, Japan).

Animals Male C57BL/6N Cr Slc mice (6 weeks old) were purchased from Japan SLC (Shizuoka, Japan). Throughout the experiments, mice were maintained on a 12-h light/dark cycle in a controlled environment. All experimental procedures using laboratory animals were approved by the Animal Care and Use Committee of the Toho University.

Preparation of Plasma and Tissues Mice were sacrificed, and their blood was collected from the inferior vena cava. Then, 500 µl of blood was gently mixed with 5 µl of 0.5 M EDTA and centrifuged at 880 g for 15 min at 4 °C. The resulting supernatants were used as plasma for further analysis. Afterward, mice were systemically perfused with ice-cold phosphate buffered saline through the left ventricle to wash out remaining blood cells, and tissues of interest were collected and stored at −80 °C until use.

Preparation of DHA DHA was prepared by bromine oxidation of AA. Briefly, 5 µl of bromine (purity >99.9%) was added to 10 ml of 10 mM AA solution for oxidation of AA to DHA. Immediately thereafter, 20 µl of 5% thiourea was added to the solution to remove the small amount of excess bromine and obtain a clear-colored DHA solution. DHA solution was diluted with 5% MPA containing 1 mM EDTA (MPA/EDTA) solution, after which 6.3 mM quantities of DHA were placed in brown-colored vials filled with argon gas for storage at −80 °C until use. DHA solution was used within one week.

Determination of AA AA was analyzed by using a HPLC-ECD method, as described, consisting of a Waters 2695 separations module coupled with a Waters 2465 electrochemical detector (Nihon Waters, Tokyo, Japan). A vacuum degasser, quaternary pump, and thermostated autosampler were included in the Waters 2695 separations module. Separation was achieved on an Atlantis dC18 5 µm column (4.6×150 mm) combined with an Atlantis dC18 5 µm guard column (4.6×20 mm) both from Nihon Waters. The mobile phase consisted of 50 mM phosphate buffer (pH 2.8), 540 µM EDTA and 2% methanol. The flow rate was 1.3 ml/min. Temperatures for the column and Waters 2465 electrochemical detector were set at 30 °C and for the thermostated autosampler at 4 °C. Ten microliters of the sample was injected into the HPLC-ECD system for AA analysis. Electrical signals were recorded by using an electrochemical detector with a glassy carbon electrode at +0.6 V. All electrical signal data from the electrochemical detector were collected by the Waters Empower2 software (Nihon Waters).

Reduction of DHA in MPA/EDTA Solution by TCEP and DTT For reduction of DHA, 100 mM, 175 mM, and 350 mM portions of TCEP and 350 mM of DTT in 5% MPA/EDTA were freshly prepared before use. DHA solution was gently thawed and diluted to 6.3 µM DHA with 5% MPA/EDTA. Eight hundred and ten microliters of 6.3 µM DHA solution (final conc. 5.7 µM DHA) was incubated with 90 µl of 100 mM, 175 mM and 350 mM TCEP (final conc. 10 mM, 17.5 mM and 35 mM TCEP, respectively) or 350 mM DTT (final conc. 35 mM DTT) on ice for 4 h. The reaction mixture was mixed and confirmed as having a pH value of 1.5. After each incubation time, 90 µl of reaction mixture was diluted with 810 µl of 5% MPA/EDTA. Then, the mixture was transferred to an autosampler vial, and AA content was analyzed by HPLC-ECD for the percentage of DHA reduction.

Reduction Efficiency of DHA Added to the Mouse Liver Mouse livers were homogenized with 14 volumes of ice-cold 5.4% MPA/EDTA and centrifuged at 21000 g for 10 min at 4 °C. AA or DHA (0.57 µmol/g tissue) was added...
to the centrifuged supernatant and reduced with 35 mM TCEP on ice for 2 h. After incubation, the reaction mixture was diluted with 5% MPA/EDTA and analyzed for AA by HPLC-ECD.

**Determination of DHA in Tissues and Plasma by Using TCEP and DTT as Reductant** To compare the reduction abilities of TCEP and DTT, we applied both agents separately to tissues and plasma obtained from five mice and measured total AA (AA+DHA) and AA content. Tissues were homogenized with 14 volumes of ice-cold 5.4% MPA/EDTA and centrifuged at 21000 g for 10 min at 4 °C. Plasma was mixed with 1 volume of 10% MPA/EDTA and centrifuged at 21000 g for 10 min at 4 °C. Total AA (AA+DHA) and AA contents in centrifugal supernatants were determined individually, and DHA content was calculated by subtraction of AA from total AA. For determination of total AA by using TCEP as reductant, 90 μl of the centrifugal supernatants were reduced with 10 μl of 350 mM TCEP (final conc. 35 mM TCEP) for 2 h on ice. After reduction, the reaction mixture was diluted with 5% MPA/EDTA and analyzed for total AA by HPLC-ECD. For determination of AA, 100 μl of the centrifugal supernatant was diluted with 5% MPA/EDTA and analyzed by HPLC-ECD. To determine total AA with DTT applied as reductant, we used the method described by Lykkesfeldt et al. with a slight modification. Briefly, 100 μl of centrifugal supernatants were reduced with 100 μl of 5 mM DTT (final conc. 2.5 mM DTT) in 0.5 M Tris–HCl buffer (pH 9.0) for 5 min at 25 °C. The pH value in the reaction mixture was 6.1. After reduction, 50 μl of 0.2 M H₂SO₄ was added to the reaction mixture followed by determination of AA. In the final step, 100 μl of the centrifugal supernatant diluted with 5% MPA/EDTA was subjected to HPLC-ECD for analysis of AA.

**Statistical Analysis** Results are expressed as means±S.E.M. The probability of statistical differences between experimental groups was determined by paired t-test using KaleidaGraph software (Synergy Software, Reading, PA, U.S.A.). Statistical differences were considered significant at p<0.05.

**RESULTS**

**Chromatograms of AA** Since MPA and EDTA are usually used for stabilizing AA in biological samples, our analysis began with those materials. To confirm AA’s chromatographic separation profiles, AA was dissolved in 5% MPA/EDTA then analyzed with our HPLC-ECD system. Figures 2A and B show a typical chromatogram of AA dissolved in 5% MPA/EDTA and 5% MPA/EDTA alone, respectively. Although the retention times changed depending on column batch, flow speed and consistency of the mobile phase, the AA peak was detected at 2.5 min of retention time and was completely separate from MPA/EDTA peaks in this HPLC condition (Figs. 2A, B).

**Characterization of DHA Prepared by Bromine Oxidation of AA** For this study, we prepared DHA solution from AA by bromine oxidation, because commercial DHA powder formed DHA dimers, a structure that is insoluble in aqueous solution. To ensure the AA was completely oxidized to DHA, DHA solution prepared with bromine was tested by HPLC-ECD. No AA peak was present (Fig. 2C). Next, the DHA preparation was reduced again to AA with TCEP to exclude the possibility of DHA and AA breakdown. That is, the soluble DHA preparation was reduced with 35 mM TCEP for 2 h on ice as described in the next section and then analyzed for AA with the HPLC-ECD system. As Fig. 2D depicts, the AA peak was detected at 2.5 min of retention time, and recovery of AA from the theoretical DHA content was 99.9%. These results indicate that AA was completely oxidized to DHA by bromine. In addition, a peak of TCEP was also detected at 3.1 min of retention time, and this peak was completely separate from the AA and MPA/EDTA peaks in this HPLC condition. Therefore, this HPLC-ECD system was used throughout the following study.

**DHA Reduction by TCEP in MPA/EDTA Solution** The pH value of 5% MPA/EDTA solution was 1.5. To determine if DHA was reduced with TCEP and DTT in 5% MPA/EDTA solution, 5.7 μM DHA in 5% MPA/EDTA was incubated with 10 mM, 17.5 mM, 35 mM TCEP and 35 mM DTT for 2 h on ice.

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**Fig. 2.** Representative Chromatograms of AA, DHA, MPA/EDTA, and TCEP

Samples were analyzed by HPLC-ECD as described in Materials and Methods. (A) AA dissolved in 5% MPA/EDTA. (B) 5% MPA/EDTA. (C) DHA dissolved in 5% MPA/EDTA. (D) Reduced DHA dissolved in 5% MPA/EDTA by 35 mM TCEP for 2 h on ice.
DTT for 4 h on ice (Fig. 3). DHA reduction by 10 mM TCEP was approximately 80% at 4 h. Furthermore, DHA reduction by 17.5 mM and 35 mM TCEP approximated 100% at 4 h and 2 h, respectively. However, no DHA reduction was evident when 35 mM DTT was added to the 5% MPA/EDTA solution. From these results, we chose to achieve DHA reduction with 35 mM TCEP incubated for 2 h in 5% MPA/EDTA solution on ice.

**Reduction of DHA Added to the Mouse Liver**

To determine whether TCEP effectively induced this DHA reduction in a biological sample, we combined 0.57 μmol/g tissue of AA or DHA with the centrifugal supernatant of mouse liver then added 35 mM TCEP for a 2 h incubation on ice. The total AA (AA+DHA) contents in a centrifugal supernatant of mouse liver with added AA, added DHA or no additive were 1.88±0.07 μmol/g tissue, 1.89±0.07 μmol/g tissue and 1.26±0.07 μmol/g tissue, respectively (Fig. 4). These results indicate that TCEP completely reduced the added DHA in a biological sample, and its reduction efficiency was almost 100%.

**Content of DHA Reduced by TCEP or DTT in Multi-**

**Fig. 3. Reduction of DHA to AA by Using TCEP and DTT in 5% MPA/EDTA**

5.7 μM DHA was incubated with 10 mM, 17.5 mM, 35 mM TCEP and 35 mM DTT in 5% MPA/EDTA on ice for 4 h. The amount of AA produced by reduction was analyzed by HPLC-ECD system. Values are expressed as means±S.E.M. of three independent experiments.

**Fig. 4. Reduction Efficiency of DHA Added to Mouse Liver Samples**

Livers were homogenized with 14 volumes of ice-cold 5.4% MPA/EDTA and centrifuged at 21000 g for 10 min at 4 °C. 0.57 μmol/g tissue of AA or DHA were added to the centrifugal supernatant of livers and then incubated with 35 mM TCEP for 2 h on ice. Total AA (AA+DHA) contents of AA-added, DHA-added or additive-free centrifugal supernatants of livers were analyzed by HPLC-ECD. Slash area represents the estimated AA and DHA content added to the centrifugal supernatant of liver. Values are expressed as means±S.E.M. of three animals.

**Fig. 5. Comparison of Reductant Ability between TCEP and DTT, and DHA Content in Mouse Tissues and Plasma**

DHA was reduced with either TCEP or DTT as described in Materials and Methods and total AA (AA+DHA) content measured. AA content (closed column) was determined as described in Materials and Methods and DHA content (slashed column) calculated by subtraction. Values are expressed as means±S.E.M. of five animals.
ple Mouse Tissues and Plasma  To compare the reduction abilities of TCEP and DTT, we applied both agents separately to matching tissue preparations and measured total AA (AA+DHA) and AA content. In addition to plasma, the tissues tested were cerebrum, cerebellum, lung, heart, liver, spleen, kidney, adrenal gland, stomach, small intestine and large intestine of 6 weeks old mice. In this experiment, we used conventional DTT reduction methods as describe by Lykkesfeldt et al., and DHA content was calculated by subtraction. Amounts of DHA in the cerebrum, cerebellum, liver, spleen, kidney, adrenal gland, stomach, small intestine and large intestine were not significantly different when either TCEP or DTT was used for reduction (Fig. 5). However, DHA contents in the lung, heart and plasma were significantly higher when TCEP was the reduction agent compared to that by DTT. With TCEP reduction, the percentages of DHA per total AA in the cerebrum, cerebellum, lung, heart, liver, spleen, kidney, adrenal gland, stomach, small intestine, large intestine and plasma were 3.4%, 0.8%, 14.3%, 14.7%, 13.3%, 19.5%, 4.1%, 8.6%, 7.2%, 3.5%, 7.4% and 19.2%, respectively. Moreover, TCEP reduction provided total AA (AA+DHA) yields in the cerebrum, cerebellum, lung, heart, liver, spleen, kidney, adrenal gland, stomach, small intestine, large intestine and plasma of 3.00±0.03 μmol/g tissue, 2.98±0.09 μmol/g tissue, 2.55±0.08 μmol/g tissue, 0.47±0.02 μmol/g tissue, 1.41±0.06 μmol/g tissue, 2.56±0.03 μmol/g tissue, 0.87±0.07 μmol/g tissue, 7.14±0.22 μmol/g tissue, 0.90±0.02 μmol/g tissue, 2.86±0.07 μmol/g tissue, 1.48±0.02 μmol/g tissue and 62.06±1.58 μM, respectively.

DISCUSSION

The present study documents an improved method for calculating DHA content in biological samples by using TCEP as a reduction agent. Here, 35 mM TCEP completely reduced DHA to AA after tissue samples from numerous organs and plasma of mice were incubated for 2 h on ice in 5% MPA/EDTA solution. MPA and EDTA at low pH solution have been used widely for stabilizing AA and DHA in biological samples. The AA was easily separated and have been used widely for stabilizing AA and DHA in biological samples.5,27) Therefore, it is essential to neutralize the pH of biological samples in MPA solution before applying DTT for the reduction of DHA, and re-acidification is required for stabilization of the AA until its injection into HPLC. These procedures are very complicated, and the breakdown of DHA to 2,3-diketogulonic acid, which occurs readily, yields an irreversibly hydrolyzed compound of DHA in the neutralized solution.20) In contrast, TCEP retains its reduction ability in wide pH range from 1.5 to 9.0 and is stable in the solution. In fact, 35 mM TCEP completely reduced 5.7 μM DHA to AA during only 2 h in the MPA/EDTA solution at pH 1.5 on ice (Fig. 3). Thus, DHA in MPA/EDTA solution does not need neutralization for reduction and re-acidification or AA stabilization.

Fig. 5. DHA Determination Protocol in Tissues and Plasma

DHA in the centrifugal supernatant was reduced by using 35 mM TCEP in 5% MPA/EDTA for 2 h on ice and analyzed by HPLC-ECD. DHA content was calculated by the subtraction method.

Lykkesfeldt et al. reported that DHA reduction in MPA/EDTA solution by TCEP was performed at pH 4.3 and 6.2.17) However, their method required the addition of Trizma buffer or McIlvaine buffer containing TCEP to raise the pH value of MPA/EDTA solution. For our improved method, though, the pH value of the MPA/EDTA solution did not need to rise, because TCEP was easily dissolved in MPA/EDTA and sufficiently effective at pH 1.5 on ice. Thus, the advantage of our method is that reduction of DHA can be performed in MPA/EDTA solution without any change in composition or requirement to alter the pH.

One can accelerate the reduction rate of DHA by TCEP in MPA/EDTA solution by increasing the reaction temperature from on ice to 25 °C (data not shown), but we performed all these experimental procedures on ice to avoid possible DHA and AA breakdown at the higher temperature and to develop the simplest method possible for DHA determination.

DHA is known to be transported intracellularly via the glucose transporters, Glut1, Glut3 and Glut4.29) Once DHA enters a cell’s interior, intracellular DHA is readily reduced by dehydroascorbic acid reductase and changed to AA.30) Thereby, levels of DHA in tissues have been kept at low concentrations. Using our improved method, we have determined the DHA content in various tissues and plasma of 6 weeks old mice (Fig. 5). This method indicated that amounts of DHA per total AA differed markedly at separate sites from 0.8 to 19.5%. Especially high DHA levels were found in the lungs, heart, spleen and plasma, i.e., over 10% of DHA per total AA content. On the other hand, cerebrum, cerebellum, liver, kidney and small intestine had less than 5% DHA per total AA content. This variability of DHA content may indicate the different oxidative stress levels among the organs and may represent the related amounts of dehydroascorbic acid reductase activity. Sasaki et al. reported that dehydroascorbic acid reductase activity was highest in the liver and second highest in the brains of chickens.31) Moreover, dehydroascorbic acid reductase activity was lower in the spleen and lungs of these chickens. Paolicchi et al. also reported that the highest levels of dehydroascorbic acid reduc-
tase activity were observed in liver, adrenal gland, intestinal mucosa, submaxillary gland and testis, whereas lower activities were detected in heart, lung, pancreas, skeletal muscle, thyroid and kidney of rats. 32) Although the dehydroascorbic acid reductase activity in mouse tissues is unknown, these results strongly suggest a relationship between DHA levels and dehydroascorbic acid reductase activity in mouse tissues.

In this study, we compared the DHA reduction ability of TCEP to that of DTT. In the lungs, heart and plasma of mice, DHA levels calculated by TCEP reduction significantly exceeded that by DTT reduction (Fig. 5). The far greater sensitivity of TCEP as a reducing agent clearly denotes the superiority of this method for DHA determination in biological samples. The advantages of this method over previous techniques include fast sample preparation by using MPA/EDTA buffer on ice to stabilize DHA and AA during experimental procedures (Fig. 6). Therefore, this method is well qualified to become a standard for DHA determinations in advanced laboratory protocols.

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