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Oxidative Decomposition of Vitamin C in Drinking Water

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We have previously shown that vitamin C (ascorbic acid) can initiate hydroxyl radical formation in copper contaminated household drinking water. In the present study, we have examined the stability of vitamin C in copper and bicarbonate containing household drinking water. In drinking water samples, contaminated with copper from the pipes and buffered with bicarbonate, 35% of the added vitamin C was oxidized to dehydroascorbic acid within 15 min. After 3 h incubation at room temperature, 93% of the added (2 mM) ascorbic acid had been oxidized. The dehydroascorbic acid formed was further decomposed to oxalic acid and threonic acid by the hydrogen peroxide generated from the copper (I) autooxidation in the presence of oxygen. A very modest oxidation of vitamin C occurred in Milli-Q water and in household water samples not contaminated by copper ions. Moreover, addition of vitamin C to commercially sold domestic bottled water samples did not result in vitamin C oxidation. Our results demonstrate that ascorbic acid is rapidly oxidized to dehydroascorbic acid and further decomposed to oxalic- and threonic acid in copper contaminated household tap water that is buffered with bicarbonate. The impact of consuming ascorbic acid together with copper and bicarbonate containing drinking water on human health is discussed.

Keywords: Vitamin C; Dehydroascorbic acid; Oxalic acid; Threonic acid; Water; Copper

INTRODUCTION

Ascorbic acid (vitamin C) is a natural antioxidant that is nowadays added as a vitamin and preservative to a variety of food sources, e.g. fruit juices in high quantities. The intake of supplemental vitamin C has also increased considerably and supplements containing up to 1–2 g of vitamin C/tablet can be found in any grocery store. This amount of ascorbic acid is well above the recommended daily intake (RDI) that is 60 mg/day for adult women or men. Thus, the mean daily intake of vitamin C has significantly increased in many countries during the past 20 years. High intake is generally not believed to be harmful since vitamin C is a water-soluble compound that is not stored in the body and the excess ingested vitamin is excreted in the urine.

Numerous studies have shown that the antioxidant, ascorbic acid, has beneficial effect on many age-related diseases such as: atherosclerosis, cancer, some neurodegenerative and ocular diseases.[1–7] However, it has also been shown that, in the presence of transition metals such as copper and iron, ascorbic acid can in fact function as a strong pro-oxidant.[8–10] In line with this, we have recently demonstrated by using coumarin-3-carboxylic acid that ascorbic acid can induce hydroxyl radical formation in copper contaminated, bicarbonate buffered household drinking water.[11] During this process, ascorbic acid is oxidized to dehydroascorbic acid by the copper ions present in the water sample.

Since the oxidized form of ascorbic acid, dehydroascorbic acid, has been shown to be toxic and to generate oxidative stress in various cell systems[12–14] we have here studied the formation and the stability of this compound in tap water samples. We demonstrate here, that ascorbic acid is very rapidly oxidized to dehydroascorbic acid and further decomposed to oxalic and threonic acid in copper contaminated bicarbonate-buffered drinking water.

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MATERIALS AND METHODS

Chemicals

Ascorbic acid, threonic acid and cupric chloride dihydrate were purchased from Fluka, Riedel-deHaen, Germany. Tris[hydroxymethyl]-aminomethane (Tris base) and *o*-phenylenediamine dihydrochloride (OPD), oxalic acid, diethyldithiocarbamic acid, dithiothreitol (DTT), *p*-hydroxybenzoic acid, sodium bicarbonate and dehydroascorbic acid were from Sigma, St Louis, USA. Stock solutions of the chemicals used were prepared in Milli-Q water (18 MΩ cm) and protected from light. Samples of tap water were collected in sterile 15 ml polypropylene test tubes (Greiner) and stored at 4°C in the dark until used. All stock solutions of the reagents used in the assay were prepared fresh daily.

Measurement of Copper and Bicarbonate Concentration in the Water Samples

The copper and bicarbonate concentration in the water samples were measured as previously described.[11,15]

Measurement of Dehydroascorbic Acid with *o*-phenylenediamine

In contrast to ascorbic acid, dehydroascorbic acid absorb UV light very poorly. To measure dehydroascorbic acid formation we therefore used the reagent *o*-phenylenediamine, a reagent that form a fluorescent complex with dehydroascorbic acid.[16,17] For the assay, 2 mM ascorbic acid was added to the water samples and incubated at room temperature for various time periods. After this, 2 mM of *o*-phenylenediamine was added to the tubes followed by 8 mM Tris buffer. The fluorescent complex was excited at 370 nm, and the emission at 440 nm was measured by a Hitachi F-2000 fluorescence spectrophotometer. The fluorescence values were converted into dehydroascorbic acid concentration from a standard curve, where known amounts of dehydroascorbic acid was used. The actual concentration of dehydroascorbic acid in the prepared standards was controlled by addition of 10 mM DTT followed by HPLC analysis on the ascorbic acid formed. All measurements were done at room temperature.

Measurement of Ascorbic Acid Metabolites by Using HPLC

An isocratic HPLC system (Waters model 1515) equipped with a manual Rheodyne injection valve (25 μl loop) and a 2-channel UV/VIS detector (Waters model 2487) was used. The column used for quantitation of ascorbic acid and oxalic acid was a Nucleosil C18 150 × 4.1 mm I.D, 10 μm particle size column (Supelco Inc.). Chromatography was performed using isocratic elution using 10 mM phosphate buffer (KH_2PO_4) containing 5% methanol, pH 3.3 (H_3PO_4). The flow rate was 0.5 ml/min. The peaks were detected at 210 nm and analyzed by using the Waters Breeze software. For the identification and calibration we used standards of threonic-, oxalic-, dehydroascorbic- and ascorbic-acid in Milli-Q water. All separations were performed at room temperature.

When the Nucleosil C18 column was used, the peaks for the threonic acid and hydrogen peroxide standards overlapped in the chromatogram. Therefore, to quantitate threonic acid, an anion column PRP-X100 (Hamilton), 150 × 4.1 mm I.D, 10 mm particle size equipped with a PRP-X100 guard column (25.0 × 2.3 mm I.D) was also used. Chromatography was performed using isocratic elution using 4.0 mM *p*-hydroxybenzoic acid containing 2.5% methanol, pH 8.5 (NaOH). The flow rate was 2.0 ml/min. The peaks were detected by indirect UV at 310 nm and analyzed by using the Waters Breeze software.

RESULTS

Ascorbic Acid Oxidation in Drinking Water

Ascorbic acid oxidation in two tap water samples (sample 1 and 2), a Milli-Q water sample supplemented with 100 mg/l HCO_3^- and 0.5 mg/l Cu^{2+} and a control Milli-Q water sample were followed for 3 h by HPLC analysis (Fig. 1A). The two water samples had been sampled in the same way, directly drawn from the tap, but they originated from two different municipal water suppliers. When 2 mM ascorbic acid was added to sample 1, that contained 25.9 mg/l bicarbonate and 0.19 mg/l copper, a very modest degradation of the vitamin occurred with time. In this water sample 38.6% of the added ascorbic acid had been oxidized during the 3 h incubation. On the contrary, when 2 mM ascorbic acid was added to the water sample 2, that contained 150.4 mg/l bicarbonate and 0.53 mg/l copper, the vitamin was almost completely (92.6%) oxidized during the 3 h incubation. A similar oxidation process could be seen when 2 mM vitamin C was added to Milli-Q water supplemented with 100 mg/l bicarbonate and 0.5 mg/l copper. Addition of 2 mM ascorbic acid to Milli-Q water resulted in a very modest oxidation of the vitamin with time.

Dehydroascorbic Acid Formation in Drinking Water

The formation of dehydroascorbic acid, was measured by using *o*-phenylenediamine. As can

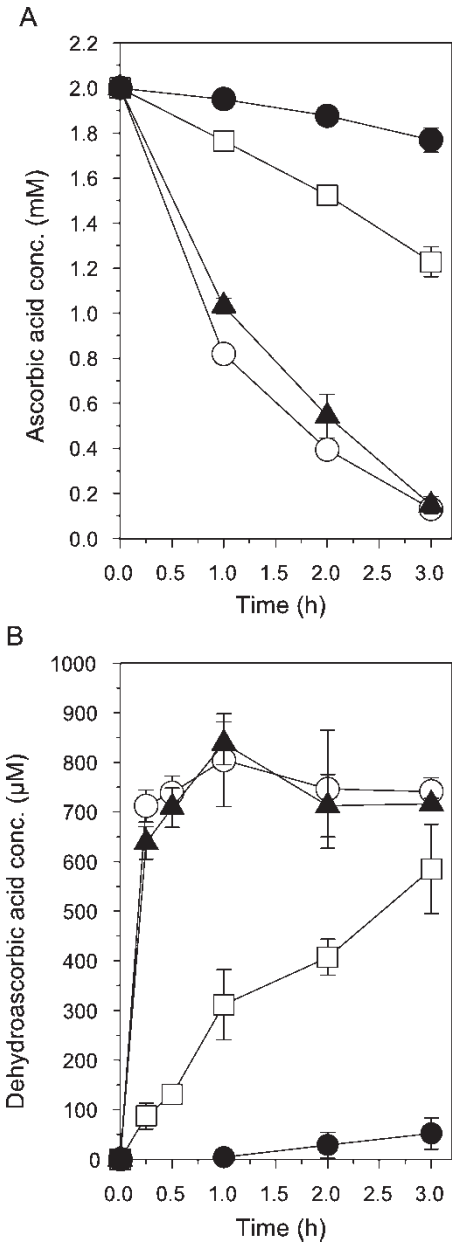

FIGURE 1 Ascorbic acid degradation (A), and dehydroascorbic acid formation (B), in household drinking water 2 mM ascorbic acid was added to two household tap water samples; sample 1 (□) and sample 2 (▲), a Milli-Q water sample (●) and a Milli-Q water sample supplemented with 100 mg/l bicarbonate and 0.5 mg/l copper (○). The concentration of ascorbic acid was measured at indicated time points by using HPLC analysis. Dehydroascorbic acid was measured by using the reagent *o*-phenylenediamine. Data shown are mean ± SD of triplicates from one representative experiment out of three conducted.

be seen in Fig. 1B, dehydroascorbic acid was rapidly formed in the tap water sample 2. After 15 min incubation, 638 ± 33 μM of dehydroascorbic acid had been formed. However, at 15 min, only 87 ± 26 μM of dehydroascorbic acid had been formed in sample 1. Addition of 2 mM ascorbic acid to Milli-Q water supplemented with 100 mg/l bicarbonate and 0.5 mg/l copper resulted in rapid formation of dehydroascorbic acid. In the control sample where ascorbic acid had been added to Milli-Q water, very low concentrations of dehydroascorbic acid could be detected. The amount of dehydroascorbic acid formed in four commercially sold domestic bottled water samples, after ascorbic acid addition (15 min incubation), was in the range of 0–3 μM.

Dehydroascorbic Acid Reacts with Hydrogen Peroxide and Generate Oxalic Acid and Threonic Acid

A typical chromatogram of a catalytic drinking water sample incubated with 2 mM ascorbic acid for 3 h is shown in Fig. 2A. In this chromatogram, based on retention time, the peak that appeared at 5.6 min was identified as oxalic acid (peak 1). At 5.9 min, a peak appeared that had a retention time similar to the hydrogen peroxide- and threonic acid standards (peak 2). During the 3 h incubation 2 mM ascorbic acid (rentention time 7.9 min, peak 4) had been oxidized, in this bicarbonate rich and copper contaminated drinking water sample to 713 ± 63 μM dehydroascorbic acid (*o*-phenylenediamine assay), 488 ± 28 μM oxalic acid and 77 ± 14 μM threonic acid (quantitated by using an anion column). The peak for dehydroascorbic acid (peak 3) appeared in the chromatogram at 6.8 min together with an unknown metabolite that had a retention time of 7.1 min. In a similar way, addition of 4 mM hydrogen peroxide to 2 mM ascorbic acid in Milli-Q water supplemented with 100 mg/l bicarbonate resulted in 839 ± 72 μM oxalic acid and 192 ± 24 μM threonic acid within 3 h (Fig. 2B). Moreover, very rapid formation of oxalic acid and threonic acid could be obtained when 4 mM hydrogen peroxide was added to 2 mM dehydroascorbic acid in Milli-Q water supplemented with 100 mg/l bicarbonate. A 10-min incubation at room temperature resulted in 640 ± 24 μM oxalic acid and 181 ± 24 μM threonic acid (Fig. 2C). In control experiments, 2 mM dehydroascorbic acid was added to Milli-Q water supplemented with 100 mg/l bicarbonate. In the absence of hydrogen peroxide, 79% less oxalic acid was formed (Fig. 2D).

To verify that dehydroascorbic was formed in our drinking water samples, we used DTT, a reducing agent that can turn dehydroascorbic acid back into ascorbic acid. When DTT was added to copper and bicarbonate supplemented Milli-Q water that had been incubated with 2 mM ascorbic acid for 3 h, the ascorbic acid peak reappeared in the chromatogram (approximately 680 μM ascorbic acid was formed). Dehydroascorbic acid has previously been shown to be spontaneously decomposed to L-diketogulonate

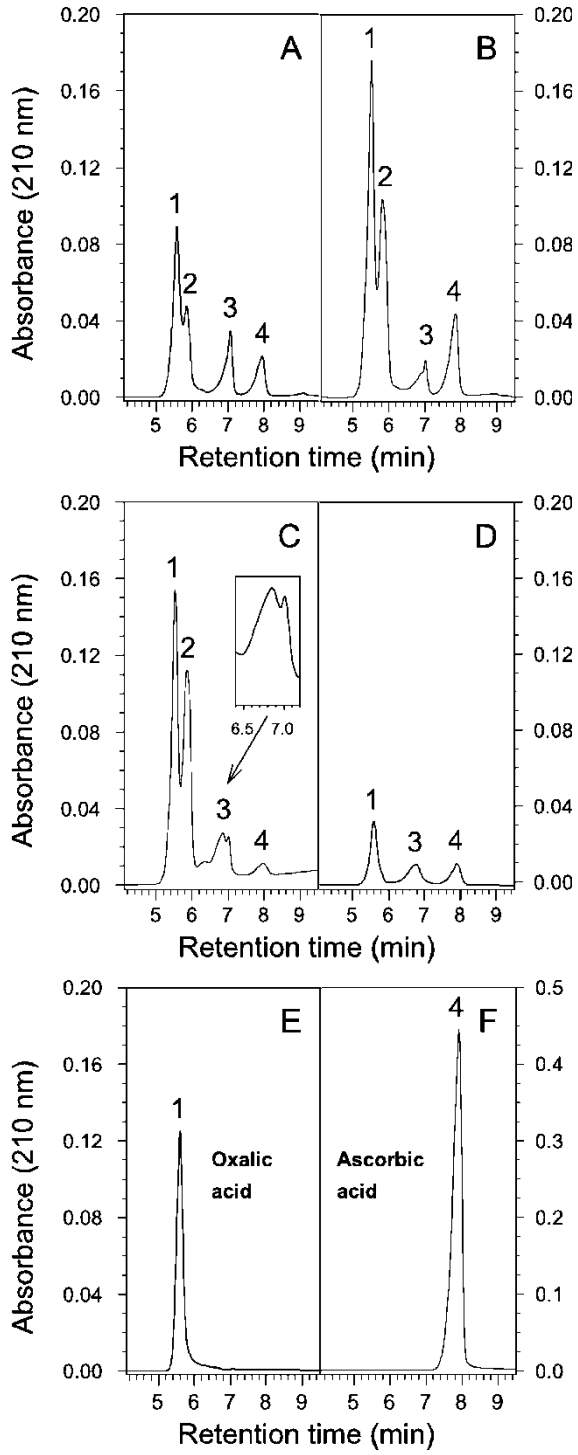

FIGURE 2 HPLC analysis of ascorbic acid metabolites in drinking water. (A) 2 mM ascorbic acid was added to a drinking water sample and incubated at room temperature for 3 h. (B) 2 mM ascorbic acid and 4 mM hydrogen peroxide was added to a Milli-Q water sample supplemented with 100 mg/l bicarbonate and incubated for 3 h at room temperature. (C) 2 mM dehydroascorbic acid and 4 mM hydrogen peroxide was added to a Milli-Q water sample supplemented with 100 mg/l bicarbonate and incubated for 10 min at room temperature. The inset show the peaks for dehydroascorbic acid (at 6.8 min) and an unknown compound (at 7.1 min). (D) 2 mM dehydroascorbic acid was added to a Milli-Q water sample supplemented with 100 mg/l bicarbonate and incubated for 10 min at room temperature. (E) Oxalic acid standard (0.6 mM) in Milli-Q water. (F) Ascorbic acid standard (2 mM) in Milli-Q water. Please note the different scaling in chromatogram F.

(2,3-DKG) or erythroascorbate.[18–20] Our results indicated that dehydroascorbic acid and not DKG was present in the sample.

DISCUSSION

The drinking water reaching the consumer at their homes can easily be contaminated by copper ions due to corrosion in the copper pipes in the house (building). Especially, the first-draw water used in the morning can readily be contaminated by copper ions.[21,22] In light of these facts, and our previous studies showing that ascorbic acid can drive a hydroxyl radical generating process in copper and bicarbonate containing drinking water[11,15] we decided to study how fast and to what extent ascorbic acid is oxidized in a copper contaminated drinking water sample. Our results show that ascorbic acid is oxidized relatively fast in bicarbonate rich water samples that are contaminated by copper ions. Approximately 32% of the vitamin C had been oxidized after 15 min and the vitamin was almost completely oxidized within 3 h (Fig. 1A and B). The oxidation process could easily be mimicked by adding vitamin C to Milli-Q water supplemented with 100 mg/l HCO_3^- and 0.5 mg/l Cu^{2+} because, the oxidation process require copper ions and a pH around 4–5.[15]

When ascorbic acid is oxidized in the presence of copper ions, dehydroascorbic acid is formed (Fig. 1B). The dehydroascorbic acid formation, in the bicarbonate rich water sample contaminated with copper, was very rapid and up to 650 μM dehydroascorbic acid could be formed within 15 min. The amount of dehydroascorbic acid formed within 15 min in the various tap water samples tested were in the range of 100–650 μM. On the contrary, when commercially sold domestic bottled water was used in the assay very modest degradation of ascorbic acid took place. Some of the bottled water samples tested (mineral waters) were buffered with bicarbonate. However, in the absence of copper ions, oxidation of vitamin C cannot take place. This reflects the importance of copper ions in the oxidation process.

HPLC analysis, performed on the water samples that had been incubated with 2 mM ascorbic acid for 3 h at room temperature, clearly indicated that the vitamin had been oxidized into dehydroascorbic acid and further decomposed into two major metabolites, oxalic acid and threonic acid. In the water sample 2, that was contaminated with copper ions and had high concentration of bicarbonate, only 131 ± 13 μM of the added 2 mM ascorbic acid was left after a 3 h incubation. During the 3 h incubation period, the added ascorbic acid had been oxidized to 488 ± 28 μM oxalic acid. High concentrations of oxalic acid (calcium oxalate) are toxic and

can promote the formation of kidney stones.[23,24] Interestingly, calcium oxalate microcalcification has also been observed in benign and malignant breast biopsy specimens.[25,26] Whether the oxalic acid present in the breast tissue originate from the ascorbic acid- or amino acid metabolism is currently not known.

When ascorbic acid is oxidized in the presence of divalent copper, monovalent copper is formed. When the reduced copper is re-oxidized in the presence of oxygen, superoxide is generated. In the presence of a proton donor, the superoxide is further reduced to hydrogen peroxide. Most likely, the hydrogen peroxide formed in the reaction, when ascorbic acid is oxidized by copper, promote the cleavage of dehydroascorbic acid to oxalic acid and threonic acid. In line with this assumption we found that addition of hydrogen peroxide directly to ascorbic acid, in the presence of 100 mg/l bicarbonate, resulted in the formation of oxalic acid and threonic acid during the 3 h incubation (Fig. 2B). However, when hydrogen peroxide was added directly to dehydroascorbic acid in the presence of 100 mg/l bicarbonate, the same amount of oxalic acid and threonic acid could be obtained within 10 min incubation (Fig. 2C). Our results indicate that the hydrogen peroxide formed, and not the hydroxyl radicals generated during the reaction, is responsible for the dehydroascorbic acid decomposition in the water samples.

In conclusion, our results show that significant amounts of either dietary or supplementary ascorbic acid can be rapidly oxidized to dehydroascorbic acid when added to bicarbonate rich (buffered) copper contaminated drinking water. Thus people consuming this type of water will most likely ingest, or generate more dehydroascorbic acid in their stomach, than people using bottled water. Dehydroascorbic acid can enter cells via the GLUT glucose transporter.[27] Intracellular reduction of large amounts of dehydroascorbic acid to ascorbic acid by NADPH- and glutathione-dependent reactions may markedly decrease the cellular concentrations of NADPH and glutathione in some celltypes.[28–30] In line with these findings, dehydroascorbic acid has been shown to cause oxidative stress and apoptosis in pancreatic and neural cells by depleting their intracellular store of reduced glutathione.[14,31,32] The impact of long-term intake of dehydroascorbic acid, the oxidized form of ascorbic acid, on human health, remains to be studied.

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