Spontaneous Decay of Oxidized Ascorbic Acid (Dehydro-L-ascorbic Acid) Evaluated by High-Pressure Liquid Chromatography

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We applied high-pressure liquid chromatography to assess the decomposition of the oxidized form of vitamin C, dehydro-L-ascorbic acid. We selected experimental conditions that might represent a wide variety of clinical and research procedures. Decay of dehydro-L-ascorbic acid proceeded much more rapidly at high pH (7-8) than at low pH (3-5) and was more rapid at 37 or 45 °C than at 0 or 23 °C. When evaluated at pH 6.6, the percent decay was somewhat more rapid from an initial concentration of 1000 μmol/L than at 5-10 μmol/L. The analytical procedure (HPLC) provided useful information about the rate of decay under various conditions. This may facilitate future biological and clinical studies that require a distinction between the oxidized and reduced forms of vitamin C.

Additional Keyphrases: variation, source of radioassay

Although numerous articles are published annually on ascorbic acid, the specific roles of this vitamin in biology are incompletely understood (1). Also, the properties of stability (2) and the relative amounts of the reduced and oxidized forms of the vitamin present in the body have been identified only under limited conditions (3-6). These situations can be attributed, in part, to our incomplete understanding of the characteristics of decay of the oxidized form of vitamin C (dehydro-L-ascorbic acid, DHAA). The decay is a biologically irreversible step that results in formation of the open chain product, diketogulonic acid, which is without known biological function or significance (Figure 1). Evaluating the validity of many previous studies on the biological role of ascorbic acid and DHAA has been difficult, because the studies did not include a consideration of whether the original molecule might have spontaneously decayed to a different form during the procedure. The three compounds shown in Figure 1 have markedly different chemical features, which would affect whether and to what extent they are detected. It has also been difficult to evaluate whether enzymatic degradation of ascorbate occurs in animals as it does in plants (6, 7).

The aim of the present study was to use high-pressure liquid chromatography (HPLC) to evaluate the stability of DHAA under various experimental conditions. The influences of pH, oxygen tension, temperature, time, purity of the incubation buffer, and initial concentration of DHAA have been determined.

Materials and Methods

Reagents. [14C]Ascorbic acid was purchased from Amer sham Corp. (Arlington Heights, IL) and checked routinely for purity (8). [14C]DHAA was prepared as previously described (8) by adding Br2 to an ice-cold solution of [14C]ascorbic acid until the solution turned yellow. The Br2 was then displaced by a stream of N2. The temperature and pH of the incubation buffer were adjusted, then the labeled DHAA was added. The final composition of the buffer was, per liter, 24 mmol of K2HPO4, 4 mmol of KH2PO4, 37 mmol of sucrose, and 0.1 mmol of EDTA. Water used for preparing the incubation buffer was de-ionized and doubly glass-distilled except as otherwise indicated.

Procedure. The incubation buffer was mixed with labeled DHAA at time zero; 0.1-mL samples were taken immediately and after 15, 45, 60, 120, and 240 min. Each sample was pipetted into 0.1 mL of metaphosphoric acid (100 mL/L) and frozen until analysis by HPLC.

Apparatus. The liquid chromatograph used was modified slightly from that used by Bianchi and Rose (3) and consisted of an Altex Model 100A solvent pump (Altex Scientific, Berkeley, CA), a Model 710 injector, Z-module, and a Model 440 absorbance detector (all from Waters Associates, Milford, MA). The fraction collector was from

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LKB (Bromma, Sweden). The column, a Waters Resolve C18 reversed-phase column, was fitted with an RCSS C18 Guard-Pak pre-column (Waters Associates). We injected 20-μL sample aliquots onto the column in a mobile phase of KH2PO4/H3PO4 (0.2 mol/L, pH 3.0) at 0.5 mL/min. The column effluent was collected in 0.4-mL fractions into 3 mL of liquid scintillator. After separation of DHAA and its primary metabolite (diketogulonic acid) by HPLC, we quantified the radiolabeled DHAA and its degradation product by scintillation counting with an LS 5000 TD spectrometer (Beckman Instruments, Brea, CA). The data points in Figures 2–4 are from a single analysis.

Results and Discussion

The rate of spontaneous modification of DHAA was evaluated under conditions typically used for incubations during biological studies, i.e., near-sea-level atmospheric conditions and 23 °C. The concentration of DHAA used was close to physiological values (10 μmol/L). At neutral pH, half of the DHAA was lost in about 100 min (Figure 2). Under these experimental conditions, no detectable amount of the [14C]activity had been converted to the reduced molecule, ascorbic acid. Similarly, in the other evaluation conditions examined, no spontaneous reduction of DHAA was noted, in keeping with the existing literature.

We evaluated the effect of hydrogen ion concentration on delactonization of DHAA over the range of pH 3.0–8.0. The half-life of decay at pH 8.0 was ~20 min. At a pH of 5.0 or below, DHAA was quite stable, decaying by <3% over 4 h. The possible influence of the presence of oxygen was evaluated by equilibrating the reaction mixture before and during the incubation with 100% O2 or with 100% N2. No change in the decay rate of DHAA was obvious with these alterations of atmospheric conditions (data not shown).

The effect of temperature on DHAA decay in a 10 μmol/L solution of DHAA was evaluated at pH 6.6. At 45 °C, half of the DHAA had decayed within 15 min (Figure 3, top), whereas at 0 °C, decay was minimal. When the incubation buffer was maintained at pH 7.2, decay of DHAA was more rapid at all temperatures (Figure 3, bottom).

We evaluated the decay rate of DHAA at initial concentrations of DHAA ranging from 5 to 1000 μmol/L; we kept the pH at 6.6. The half-life of DHAA decay at a concentration of 10 μmol/L was threefold greater than at 1000 μmol/L (Figure 4). In contrast, when the pH was maintained at 3.0, the decay of DHAA was much slower at initial concentrations ranging from 10 to 1000 μmol/L (data not presented). Also, at that pH, there was no difference in decay rate between the various initial concentrations; the values at the last interval measured (i.e., after 240 min of incubation) were all between 92.4% and 94.9% of the original DHAA content. This information might help investigators decide which pH is most appropriate for storing samples intended for future assay of DHAA, e.g., in metabolic or transport studies.

The effect of water purity in the incubation buffer was evaluated by using single-glass-distilled or tap water to prepare the solutions. There was no noticeable difference in the decay of DHAA when this change was made (data not presented), in contrast to the known effect that water purity has on degradation of ascorbic acid. Knowledge of DHAA decay in tap water might be of practical value in, e.g., nutritional studies that involve dietary supplementation with vitamin C by dissolving it in drinking water.

We have evaluated the properties of DHAA decay under various conditions selected to be of interest to investigators who evaluate ascorbate metabolism under physiological conditions. The results should also be useful to those who must store biological samples for later quantification of ascorbate and its byproducts. Furthermore, the existing literature on DHAA incubations at warm temperatures or at high pH values may now be evaluated in terms of the likely stability of the molecule in those studies.

The decay of many nutrients is affected by the ambient gaseous atmosphere. For instance, the degradation of ascorbic acid to DHAA (an oxidative process) is promoted under an oxidizing atmosphere. The lack of response of DHAA decay to the amount of oxygen present reflects the fact that the underlying chemical event is hydration, which is not likely to be influenced by the presence or absence of oxygen.

Because it is most convenient to perform studies on micronutrients without having to consider whether the
The concentration of the substrate is an important factor in the rate of decay of the substrate, we evaluated the effect of DHAA concentration on DHAA decay. Ascorbate was previously found to be more stable when present at a high concentration than at a low concentration; however, the opposite is true for DHAA stability (Figure 4).

In conclusion, an HPLC assay previously designed to determine tracer concentrations of 14C-labeled ascorbate is suitable for quantifying DHAA and its degradation products. This approach is useful and allows for a prediction of the stability of DHAA under a variety of conditions that might be encountered during biological or medical studies.

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References

Evaluation of the Clinical Usefulness of a Chemiluminometric Method for Measuring Creatine Kinase MB
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The use of creatine kinase isoenzymes (CK-MB) in the diagnosis of acute myocardial infarction (AMI) is well established. We evaluated the use of a new chemiluminometric method (CK-Ciba) for measuring CK-MB by calculating its sensitivity, specificity, positive and negative predictive values, and diagnostic efficiency for diagnosing AMI. We tested 333 samples from 229 patients within 4 h of receipt. The patients were divided into four groups: (1) patients who had an AMI, (2) patients who had AMI ruled out, (3) patients who had CK-MB measured for reasons other than to rule out AMI, and (4) patients who had only one sample drawn. Only patients in Groups 1 and 2 were used in the study. AMI was diagnosed by a cardiologist. The prevalence of AMI in our population was 0.18. A receiver–operator characteristic curve was used to establish optimal values for identifying MI with the CK-Ciba results: CK-MB ≥10 µg/L and a CK-MB index of ≥3.0 (µg of CK-MB per U of CK × 100). Using these values, we calculated a sensitivity of 1.00, specificity of 0.97, positive predictive value of 0.87, and a diagnostic efficiency of 0.97. We conclude that the CK-Ciba method has high sensitivity, high specificity, and good predictive values for CK-MB and is appropriate to use to rule out AMI.

Additional Keyphrases: myocardial infarction · electrophoresis compared · receiver–operator characteristic curve

The efficacy of creatine kinase (CK; EC 2.7.3.2) isoenzyme MB (CK-MB) measurement in the diagnosis of acute myocardial infarction (AMI) is well documented (1, 2). Recently, some laboratories have changed from the widely used semiquantitative electrophoresis method to quantitative immunochromatographic methods. Although the immunochromatographic methods decrease labor costs and improve result turnaround, most require long incubation periods or are subject to interference by macro CK-1 and high concentrations of CK-MM and CK-BB (3, 4). Recently, a new two-site chemiluminimetric (sandwich) immunoassay (Magic Lite CK-MB (CK-Ciba); Ciba Corning Diagnostics Corp., Medford, MA 02052) was reported to have a short incubation period and to be free of these interferences (5). However, other investigators reported that the CK-Ciba method had poor specificity (0.57) and poor diagnostic efficiency (0.69, 3).