Spectrophotometric Determination of Ascorbic Acid and Dehydroascorbic Acid

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We present a method for measuring ascorbic acid in methanol/trichloroacetic acid extracts prepared from human plasma after enzymatic oxidation of ascorbic acid to dehydroascorbic acid by ascorbate oxidase. Samples were assayed by spectrophotometrically monitoring the kinetics of the concentration-dependent absorbance changes of dehydroascorbic acid with phosphate-citrate-methanol buffers. Ascorbic acid was determined as the difference between dehydroascorbic acid and total ascorbic acid content. The detection limit was <0.5 μmol/L. The calibration curve was linear (r > 0.995) over the range 0–1000 μmol/L. Analytical recovery of ascorbic acid added to plasma was 93–105%. The between-day variance was <7%. Comparison of the spectrophotometric determination (y) with a chromatographic procedure (x) gave y = 1.02x – 0.653 (S_yx = 3.61) over the range of physiologically relevant concentrations. Total analysis time is <10 min per sample and allows the simultaneous analysis of multiple samples.

Indexing Terms: enzymatic methods/assay kinetics/chromatography, liquid/reducing agents/vitamin C/intermethod comparison

Ascorbic acid is of great importance in biochemical reactions as a reducing agent. For example, recycling of antioxidants such as vitamin E by ascorbic acid has been shown to be protective against oxidative stress (1). Numerous ascorbic acid assays have been applied for determining the physiological roles of the vitamin. Spectrophotometric procedures include the oxidation of ascorbic acid with 2,6-dichloroindophenol, the reduction of dehydroascorbic acid with 2,4-dinitrophenylhydrazine, or the reduction of metal ions to form light-absorbing derivatives. These assays lack sensitivity and specificity because of interfering compounds such as sugars, amino acids, and glucuronic acid. Such disadvantages make them unattractive for use with cells, subcellular organelles, or plasma (2).

Because of these problems, HPLC techniques have been developed to determine ascorbic acid. Unfortunately, ultraviolet detection is also subject to interference by other absorbing substances present in the sample, e.g., uric acid, hypoxanthine, or xanthine. Electrochemical detection provides better specificity, but both methods have to deal with the instability of ascorbic acid during the long time needed for sample analysis, especially when automated sampling devices are used.

Elsewhere we have described a procedure that facilitates the direct measurement of dehydroascorbic acid in human plasma by spectrophotometrically monitoring the concentration-dependent kinetics of the absorbance changes of dehydroascorbic acid in phosphate/methanol-containing solutions (3). The method allows multiple sample analysis and avoids time-consuming sample processing. The reaction produces a mixture of ketals by adding methanol to the carboxyl groups in position 2 and 3 of dehydroascorbic acid (4). Here we describe the application of this spectrophotometric procedure to determine ascorbic acid after its enzymatic oxidation to dehydroascorbic acid by ascorbate oxidase, calculating the ascorbic acid concentration as the difference between initial dehydroascorbic acid and total ascorbic acid concentrations.

Materials and Methods

Reagents. Ascorbic acid, ascorbic acid oxidase (EC 1.10.3.3), citric acid 1-hydrate, monosodium phosphate, dithiothreitol, and desferrioxamine were obtained from Sigma Chemical Co., St. Louis, MO. Methanol (HPLC-grade) was supplied by Mallinckrodt Chemicals, Paris, KY. Chelex 100 (200–400 mesh) was purchased from Bio-Rad Labs., Richmond, CA. Chelexed buffers were prepared by stirring 100 mL of buffer with 10 g of Chelex resin for 60 min at room temperature to remove trace metal residues, then filtering the buffer from the resin.

Spectrophotometry. Spectrophotometric measurements were taken with a Perkin-Elmer Lambda 2 Spectrophotometer (Perkin-Elmer, Norwalk, CT) equipped with a thermostating system. We used 1-cm-pathlength cuvettes and a sample volume of 1 mL.

HPLC analysis. HPLC was performed with an Isco Model 2350 pump (Isco, Lincoln, NE) with a gradient programmer, an UV-VIS detector, and a Hitachi F-1300 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). The column was a 5-μm (particle size) Sephasil C18 column, 250 × 4.6 mm from Pharmacia LKB, Uppsala, Sweden.

Dehydroascorbic acid was determined by precolumn derivatization of dehydroascorbic acid with o-phenylenediamine to its quinoxaline derivative (5). In brief, equal volumes of calibrators and samples were mixed with a 10 mmol/L solution of o-phenylenediamine and incubated at 37 °C for 30 min in the dark. Of this reaction mixture 200 μL was filtered and injected into the HPLC apparatus. The mobile phase consisted of potassium phosphate buffer (80 mmol/L) and 200 mL/L methanol adjusted to pH 6 and filtered through a 0.2-μm pore-size filter (Millipore, Bedford, MA). The flow rate was 0.75 mL/min. The fluorescence detector
was set to 365 nm excitation and 418 nm emission with 15-nm slit bandwidth. Under these conditions the fluorescent derivative was eluted after 7 min.

HPLC determination of ascorbic acid with ultraviolet detection was done with a modified ion-pair reversed-phase technique, as described previously (6), also with a 5-μm (particle size) Sephasil C18 column, 250 × 4.6 mm. The mobile phase consisted of two buffers: 4 mmol/L tetrabutylammonium hydroxide, 10 mmol/L KH2PO4, and 10 mL/L methanol, pH 6.5 (buffer A); and 1.4 mmol/L tetrabutylammonium hydroxide, 10 mmol/L KH2PO4, and 300 mL/L methanol, pH 5.5 (buffer B). Buffers were filtered (0.2-μm pore-size filters) and analysis was performed by gradient separation: 5 min at 1000 mL/L buffer A, 2 min increasing to 400 mL/L buffer B, 9 min increasing to 440 mL/L buffer B, 10 min increasing to 1000 mL/L buffer B, and held there for 10 min. The flow rate was set to 1.2 mL/min, and absorbance was detected at 266 nm.

**Derivatization procedure.** Ascorbate oxidase (250 U) was dissolved in 2.5 mL of glycerol plus 2.5 mL of a 100 mmol/L monopotassium phosphate solution adjusted to pH 6; divided into portions; and stored at −80 °C. Ascorbic acid calibrators were dissolved in water and used immediately, because ascorbic acid degrades spontaneously in aqueous solutions. To 800 μL of ascorbic acid calibrators or samples we added 20 μL of ascorbic acid oxidase solution and let this react for 5 min at room temperature. During this period ascorbic acid was completely oxidized as we determined previously. Methanol (400 μL) and 700 g/L trichloroacetic acid solution (40 μL) were added separately for protein precipitation. The samples were cooled on ice and centrifuged at 10,000g for 10 min at 4 °C; 800 μL of the supernate was aspirated. Before analysis, 20 μL of a freshly prepared 200 mmol/L desferrioxamine solution was added; the samples were then neutralized by adding 20 μL of a 4 mol/L potassium hydroxide solution. A double-Chelexed solution (200 μL) containing 2 mol/L monosodium phosphate and 0.333 mol/L citric acid 1-hydrate adjusted to pH 7.5 was added. Absorbance changes were monitored at 346 nm and 37 °C. Samples and calibrators for dehydroascorbic acid determination were treated as described above except that ascorbic acid oxidase was replaced by distilled water.

**Human plasma samples.** Human blood was freshly drawn from healthy donors into citrate–phosphate–dextrose medium. The samples were centrifuged at 1500g for 10 min at 4 °C. The plasma was treated and deproteinized as described above. Plasma samples were deproteinized immediately after centrifugation [to determine initial (normal) dehydroascorbic acid concentrations] and after the 5 min incubation with ascorbate oxidase (to measure the total ascorbic acid content). The experimental protocol was conducted according to the Helsinki Declaration of 1975, as revised in 1983.

**Data analysis.** Each experimental result as shown in the figures is the mean ± SD for at least three measurements.

**Results**

**Optimum assay conditions.** The dependence of the absorbance changes on the concentration of methanol, phosphate, and pH has been described previously (3). The kinetics increase linearly with the methanol concentration but show a saturable maximum for increasing phosphate concentrations. The rate of absorbance changes increases up to pH 8; no absorbance change was detectable at higher pH values. The temperature dependence of the reaction kinetics is shown in Fig. 1. Thermostating devices and standardized temperature conditions during sample and calibrator preparation are required for this assay. Arrhenius plots of lnA/dt against 1/T gave linear regression curves. The energy of activation, calculated from the slope of the Arrhenius plot, was 95.5 kJ · mol⁻¹ with a temperature coefficient (Qₐ₁₀) of 1.28.

**Calibration curves.** Ascorbic acid calibrators were prepared and analyzed as described in Materials and Methods. Typical calibration curves of formation rate against ascorbic acid concentrations in the ranges 0–100 μmol/L and 0–1000 μmol/L are shown in Fig. 2. The response to various concentrations of ascorbic acid was linear up to 1000 μmol/L ascorbic acid (r >0.995). The reaction was followed for 300 s; the absorbance change per time unit was calculated from the linear period of the reaction, between 150 and 300 s after starting the measurement. The lower detection limit of the assay, estimated as the concentration corresponding to a signal 3 SD above the mean for a calibrator free of analyte, was determined by measuring distilled

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**Fig. 1.** Dependence of the absorbance changes at 346 nm of a 100 μmol/L calibration solution of dehydroascorbic acid on temperature.

Inset: Arrhenius plot. The regression curve was linear (r =0.99). Energy of activation was found to be 95.5 kJ · mol⁻¹ with a temperature coefficient (Q₁₀) of 1.28. Figs. 1, 2, and 4: results are shown as mean ± SD, except when SD is smaller than the data symbol.
water 12 times in exactly the same way as plasma samples. The detection limit was thus calculated to be 490 nmol/L. For a sample volume of 800 µL the detection limit for ascorbic acid is therefore 392 pmol/sample.

Ascorbic acid in biological samples. The ascorbic acid concentration in human plasma was between 15 and 85 µmol/L (n = 18); this decreased to nearly 0 after several freezing and thawing cycles, which have been described to convert ascorbic acid to dehydroascorbic acid (7). Five freezing and thawing cycles reduced the ascorbic acid content to <10% of the initial amount.

Analytical recovery from biological samples. Human plasma samples were supplemented with different amounts of ascorbic acid to determine the extent of ascorbic acid recovery using this assay. All extracts showed virtually complete ascorbic acid recovery (Table 1), and the correlation of ascorbic acid added with ascorbic acid recovered was linear (r > 0.999).

Assay specificity. Human plasma samples were analyzed for ascorbic acid by oxidation of ascorbic acid by ascorbate oxidase, either spectrophotometrically as described in Materials and Methods or by HPLC (5). The samples for the spectrophotometric measurements were assayed in triplicate. The correlation between the methods is linear (r > 0.98), as shown in Fig. 3. The intercept and the slope of the regression line were −0.635 and 1.02, respectively. The mean difference between the methods was 2.4 µmol/L ascorbic acid over a range of 0 to 85 µmol/L (Sxy = 3.61). The correlation between HPLC and spectrophotometric determinations was confirmed by reassaying three samples with an HPLC method involving direct ultraviolet detection.

Stability. The instability of ascorbic acid is the main problem of ascorbic acid or dehydroascorbic acid assays. Sufficient stability of ascorbic acid, both inter- and intraassay, is a prerequisite to avoid overestimating dehydroascorbic acid and underestimating ascorbic acid. To assess the interassay stability of ascorbic acid, we stored samples and calibrators at room temperature (25 °C) and at 4 °C, −20 °C, and −80 °C after the addition of methanol and trichloroacetic acid, as described in Materials and Methods. No loss of ascorbic acid was detectable in human plasma samples for 24 h at 4 °C or during 4 weeks when stored at −80 °C (Fig. 4). Samples were stable at room temperature for 60 min (100.8% ± 2.5% of the ascorbic acid concentration) but decreased to 71.7% ± 12.4% of the initial amount within 24 h. Stored at −20 °C, samples contained 110% ± 7.3% of the initial ascorbic acid value after 2 weeks and 64.5% ± 2.6% after 4 weeks.

The intraassay stability of ascorbic acid was investigated by incubating 100 µmol/L ascorbic acid calibrators under assay conditions. The absorbance changes at 346 nm, indicating the conversion of ascorbic acid to dehydroascorbic acid during the assay, were monitored. Trace metal complexing reagents have been

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Table 1. Recovery of ascorbic acid from human plasma.

<table>
<thead>
<tr>
<th>Ascorbic acid added, µmol/L</th>
<th>Kinetics, A/s (×10^6)</th>
<th>Ascorbic acid detected, µmol/L</th>
<th>Recovery, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>224 ± 7</td>
<td>63.1</td>
<td>—</td>
</tr>
<tr>
<td>10</td>
<td>257 ± 11</td>
<td>72.4</td>
<td>93</td>
</tr>
<tr>
<td>50</td>
<td>410 ± 8.6</td>
<td>115.6</td>
<td>105</td>
</tr>
<tr>
<td>100</td>
<td>579 ± 13.8</td>
<td>163.3</td>
<td>100</td>
</tr>
</tbody>
</table>

A human plasma sample containing 63.1 µmol/L ascorbic acid was supplemented with various concentrations of ascorbic acid and assayed. The kinetics of the absorbance changes was calculated from 150 to 300 s after starting the measurement and is given as dA/dt (A/s × 10^6). Ascorbic acid concentrations were determined after calibration with appropriate amounts of ascorbic acid.

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Fig. 3. Correlation of the spectrophotometric method with the HPLC method for measuring ascorbic acid.

Human plasma samples were assayed for ascorbic acid (AA) by monitoring the absorbance changes at 346 nm spectrophotometrically as described in the text and by HPLC with precolumn derivatization of dehydroascorbic acid with o-phenylenediamine to give a fluorescent derivative (8). The samples for the spectrophotometric measurements were run in triplicate; the correlation was linear (r > 0.98). The spectrophotometric determination was also compared with HPLC with ultraviolet detection for three samples (C).
described to stabilize ascorbic acid (8). Without the addition of a trace metal complexing reagent, there was a constant loss of ascorbic acid under assay conditions, which was only slightly influenced by albumin, as shown in Table 2. The addition of desferrioxamine prevented ascorbic acid from undergoing conversion to dehydroascorbic acid in a dose-dependent manner, and EDTA accelerated the ascorbic acid degradation over that in a solution containing no chelator, as reported previously (3). We were able to stabilize the ascorbic acid by removing trace metals with double-Chelexed phosphate–citrate solutions and adding 4 mmol/L desferrioxamine. The residual absorbance changes reflect impurities of the ascorbic acid calibrator, as revealed by HPLC (data not shown). Intraassay stability was also determined for three plasma samples and gave similar results when compared with the calibrators, as shown in Table 2.

**Table 2. Intraassay stability of ascorbic acid.**

<table>
<thead>
<tr>
<th>Incubation</th>
<th>Ascorbic acid loss, % (mean ± SD)</th>
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<tbody>
<tr>
<td></td>
<td>Calibrators</td>
</tr>
<tr>
<td>Control*</td>
<td>6.03 ± 0.27</td>
</tr>
<tr>
<td>+1 g/L bovine serum albumin</td>
<td>4.96 ± 0.35</td>
</tr>
<tr>
<td>+1 mmol/L EDTA</td>
<td>12.29 ± 0.34</td>
</tr>
<tr>
<td>+10 mmol/L EDTA</td>
<td>17.52 ± 1.22</td>
</tr>
<tr>
<td>Double-Chelexed buffer</td>
<td>1.30 ± 0.34</td>
</tr>
<tr>
<td>+2 mmol/L desferrioxamine</td>
<td>0.96 ± 0.16</td>
</tr>
<tr>
<td>+4 mmol/L desferrioxamine</td>
<td>0.80 ± 0.13</td>
</tr>
</tbody>
</table>

* Control incubations for calibrators contained 100 μmol/L ascorbic acid and were prepared as described in the text except that desferrioxamine was omitted and the phosphate–citrate buffer was not chelexed. Absorbance changes at 346 nm, indicating the conversion of ascorbic acid to dehydroascorbic acid, were monitored. We estimated the intraassay stability of ascorbic acid in three plasma samples with original ascorbic acid concentrations of 34.7, 42.5, and 74.1 μmol/L. After adding 100 μmol/L ascorbic acid to the plasma samples, we measured the appearance of its oxidation product, dehydroascorbic acid, under assay conditions. Values were corrected for the initial ascorbic acid and dehydroascorbic acid content. All concentrations are given as final concentrations.

**Precision data.** To determine the precision of this assay, we estimated the ascorbic acid content of a pooled sample (nominally 24 μmol/L) six times in succession for six consecutive days. Human plasma extracts were prepared as described in Materials and Methods and the methanol–trichloroacetic acid extracts were stored at −80 °C. The within-day variance was calculated as the mean of the variances obtained for each day. The between-day variance was estimated by determining the variance of the daily means. The within-assay CV was 4.6% (23.9 ± 1.10 μmol/L), the between-assay CV 6.8% (23.9 ± 1.63 μmol/L), and the total CV was 8.2% (23.9 ± 1.96 μmol/L).

**Discussion**

The method we describe determines the ascorbic acid content of human plasma samples as the difference between two measurements with and without oxidation of ascorbic acid, by spectrophotometrically monitoring the concentration-dependent absorbance changes of dehydroascorbic acid with phosphate–methanol solutions. The spectrophotometric equipment is easy to use, and sample preparation time is short. Multicell cuvette holders allow simultaneous spectrophotometric determinations of numerous samples. By obviating the time-consuming sample preparation required for most HPLC assays and using a high sampling rate, costs per sample are reduced. The intraassay stability of ascorbic acid and the short time needed for sample preparation and analysis improve the reliability of this ascorbic acid assay.

The derivatization procedure is also easy to perform and avoids another problem of HPLC assays by stabilizing the ascorbic acid during the time needed to assay multiple samples. The intraassay stability provided by this spectrophotometric assay allows large-scale analyses of numerous samples.

Trace metal residues in the reaction mixture can be reduced by using double-Chelexed buffers and by adding desferrioxamine, a potent iron chelator that prevents the reduction of iron by ascorbic acid with the concomitant formation of hydroxyl radicals, which perpetuate the loss of ascorbic acid. The enhancement of trace metal-induced peroxidative processes by EDTA has been described (9), and the EDTA-induced ascorbic acid degradation in phosphate–citrate buffers is in concordance with our previous results (3).

The reaction product was identified as a mixture of ketals by use of fast atom bombardment mass spectrometry in a sodium chloride–glycerol matrix, with a methyl diketone being the main reaction product. Further mass spectrometric analyses could not confirm this structure of the reaction product (molecular mass of 266 Da) because mass spectrometric peaks with an m/z ratio of 267 (MH⁺) can be found even without the addition of the dehydroascorbic acid derivative and might result from the interaction of glycerol with sodium ions. Moreover, the dehydroascorbic acid derivative to the matrix does not result in an increase of the parent molecular ion (unpublished observations). Fur-
ther analyses must be done to validate the proposed structure.

We compared the spectrophotometric determination of ascorbic acid with a chromatographic procedure with fluorescence detection to assess the specificity of our assay. Both procedures gave comparable results over a broad range of physiologically relevant concentrations. The spectrophotometric determination of ascorbic acid shortens the time needed to assay numerous samples and gives results comparable with those by HPLC procedures. This assay has the potential to become a suitable alternative to ascorbic acid determinations by HPLC, especially if large-scale analyses are required.

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References