Ascorbate in Plasma as Measured by Liquid Chromatography and by Dichlorophenolindophenol Colorimetry

Dorothy J. Vander Jagt, Philip J. Garry, and William C. Hunt

Ascorbic acid was measured in 125 plasma samples by an automated colorimetric method involving dichlorophenolindophenol and by a "high-performance" liquid-chromatographic procedure with electrochemical detection. The two methods gave comparable results for samples with ascorbate concentrations of 1 to 20 mg/L (r = 0.97). We also measured the amount of total ascorbate (ascorbic acid + dehydroascorbic acid) in the same samples by a liquid-chromatographic procedure with precolumn derivitization of ascorbic acid. We confirmed that plasma contains little dehydroascorbic acid.

Additional Keyphrases: electrochemical detection · chromatography, reversed-phase

Ascorbic acid in plasma or serum has been measured most often by colorimetric methods based on the oxidation-reduction properties of ascorbic acid or the formation of chromogenic derivatives, such as hydrazones (1). A standard redox reagent used in ascorbate analysis is the blue dye, 2,6-dichlorophenolindophenol (DCIP): ascorbic acid is oxidized to dehydroascorbic acid, and oxidized DCIP is reduced to the colorless form at low pH. The reaction can be followed spectrophotometrically. DCIP reacts only with ascorbic acid; however, the amount of dehydroascorbic acid in plasma has been reported to be much less (<5% of the total) than ascorbic acid (2, 3). The assay of ascorbate in plasma with use of DCIP has been automated (4, 5), and an automated method has been used in several nutritional surveys (6, 7).

More recently, "high-performance" liquid chromatography (HPLC) methods have been developed for determining ascorbic acid in physiological fluids and tissues (8). The combination of chromatographic separation and highly sensitive detection methods, such as electrochemical detection, provides greater specificity and sensitivity for ascorbic acid than in the colorimetric methods.

We have measured ascorbic acid in a large number of plasma samples by both an automated DCIP procedure (4) and an HPLC method in a comparison of the two methods. In addition, we measured total ascorbate (ascorbic acid plus dehydroascorbic acid) in the same samples by using a different HPLC procedure to confirm that plasma contains low amounts of dehydroascorbic acid.

Materials and Methods

Samples

Samples were obtained from individuals after an overnight fast. Blood was collected in evacuated heparinized tubes, and the plasma was separated from cells by centrifugation within 30 min after collection. The ascorbic acid was stabilized by mixing 300 µL of plasma with an equal volume of metaphosphoric acid (100 g/L). We then assayed the samples immediately or stored them at -70 °C. Before analysis, samples were centrifuged (3500 × g, 4 min) to remove precipitated protein.

Automated Colorimetric Procedure

For the DCIP analyses we used an AutoAnalyzer II continuous-flow system (Technicon Instruments Corp., Tarrytown, NY 10591). The reagents, preparation of standards, and flow scheme have been previously described in detail (4).

References

HPLC Determination of Reduced Ascorbic Acid

**Apparatus**: The chromatographic system consisted of a high-pressure pump (Model A; Eldex Laboratories, San Carlos, CA 94070), a Rheodyne injector (Model 7125) with a 20-μL sample loop, a 5-μm reversed-phase (C18) column, 4.5 mm (i.d.) × 25 cm (IBM, Danbury, CT 06810), an amperometric LC detector (EC-230, IBM) with potential set at +0.7 V, and a potentiometric recorder (Curtken, Hawleyville, CT 06440). We also used a 4.6 mm (i.d.) × 5 cm precolumn, packed with 40-μm C18 pellicular media, to prevent contamination of the analytical column.

**Reagents**: The mobile phase was sodium acetate buffer (50 mM mol/L), adjusted to pH 4.75 with glacial acetic acid. Before use, the buffer was filtered through a 0.2-μm pore-size filter (Millipore, Bedford, MA 01730) to remove particulate matter, then 0.17 mL of n-octylamine (Aldrich Chemical Co., Milwaukee, WI 53233) was added per liter of buffer. The mobile phase was continuously purged with helium gas during use, to eliminate air from the system.

An ascorbic acid stock solution (1 g/L) was prepared by dissolving 100 mg of ascorbic acid (Fisher Scientific Co., Dallas, TX 75074) in 100 mL of metaphosphoric acid (12 g/L). Working standards in the range of 0.25 to 5 mg/L were prepared by diluting the stock solution with 50 g/L metaphosphoric acid solution.

**Procedure**: Dilute samples (100 μL) with an equal volume of distilled water, then inject into the HPLC system; inject standard solutions without dilution. Repeat assays of standards frequently, approximately every 10 samples, to check for the stability of the electrode response. Elute the samples with the acetate buffer at a flow rate of 0.8 mL/min; under these conditions, ascorbic acid is eluted in about 5 min. Calculate sample concentrations from peak heights.

**HPLC Determination of Total Ascorbic Acid**

We used a modification of a method for the determination of total ascorbate in whole blood (9). Ascorbic acid was enzymatically oxidized to dehydroascorbate by ascorbate oxidase (EC 1.10.3.3), and the total dehydroascorbic acid was reacted with o-phenylendiamine to produce the fluorescent derivative 3-(1,2-dihydroxyethyl)fluoro(3,4-b)quinoxaline-1-one.

**Apparatus**: We made the following changes in the system described above: a 100-μL sample loop was used in the injector, and a fluorometer (FS 950; Kratos, Ramsey, NJ), equipped with a 365-nm interference-type excitation filter and a 418-nm cutoff emission filter, was the detector.

**Reagents**: Sodium acetate solution (1.5 mol/L) was prepared by dissolving 12.5 g of sodium acetate in 100 mL of distilled water. Ascorbate oxidase solution was prepared by dissolving lyophilized ascorbate oxidase (Sigma Chemical Co., St. Louis, MO 63178) in an equimolecular mixture of 0.1 mol/L potassium phosphate buffer and glycerol; final enzyme activity was 50 kU/L o-Phenylendiamine (5 mmol/L), prepared by dissolving 10 mg of o-phenylendiamine dihydrochloride (Sigma Chemical Co.) in 10 mL of distilled water, was stable for 30 min. Because pure dehydroascorbic acid is not available, we used ascorbic acid standards (1 to 10 mg/L) in metaphosphoric acid (50 g/L) for calibration, after the enzymatic oxidation of ascorbic acid to dehydroascorbic acid.

**Procedure**: Add 100 μL of the sodium acetate solution to 100 μL of sample or standard to adjust the pH to 5.5. Add 10 μL (0.5 U) of enzyme solution and let the reaction proceed for 5 min at room temperature. (We had previously determined that this was sufficient time for conversion of all the ascorbate to dehydroascorbate.) Add 100 μL of o-phenylendiamine solution to the oxidized sample and let react for 10 min at room temperature, keeping the samples protected from light during the reaction because the derivative is light sensitive. Inject 100 μL of this reaction mixture into the chromatographic system and elute with potassium phosphate buffer (80 mmol/L/methanol (80/20 by vol) at a flow rate of 0.75 mL/min. Under these conditions, the dehydroascorbic acid derivative is eluted after about 10 min. Calculate sample concentrations from their peak heights.

**Precision Studies**

Before assaying the samples, we determined the precision of each method by repeated assays of a pooled plasma sample. We had divided the plasma pool into 300-μL aliquots and had stabilized and stored them as described above. We assayed 10 aliquots each day for four consecutive days by each method.

**Results**

Precision data for the methods are summarized in Table 1. The within-day variance was determined as the average of the variances obtained for each day. The between-day variance was estimated by determining the variance of the means obtained for each day, which was then adjusted for the within-day variance component.

Although the variance components for the HPLC assay for ascorbic acid were slightly smaller than those of the other two procedures, the three methods had comparable precision. The DCIP method had a significantly larger within-day component of variance (p = 0.044) than did the HPLC procedure for ascorbic acid. The HPLC procedure for total ascorbate had a significantly larger between-day component (p = 0.045).

Plasma samples from 125 different individuals and containing a wide range of ascorbate concentrations were analyzed for ascorbic acid by the automated DCIP method and by HPLC. The results are summarized in Figure 1. The relationship between the methods is linear for ascorbate concentrations of 1 to 20 mg/L, with a correlation coefficient of 0.97. However, the slope departs substantially from 1.0, and the DCIP colorimetric procedure gave slightly higher values than the HPLC procedure at ascorbate concentrations less than 13 mg/L. The average difference over the entire range of concentrations was 0.69 mg/L.

We also compared the concentrations of total ascorbate (ascorbid acid + dehydroascorbic acid) and reduced ascorbate in the same samples (Figure 2). The relationship between the two concentrations was linear, with a correlation coefficient of 0.96. Again, the slope differed from 1.0, and the total ascorbate concentrations were higher than the

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**Table 1. Precision of Methods for Determination of Ascorbic Acid in Plasma**

<table>
<thead>
<tr>
<th>Method</th>
<th>Ascorbic Acid, mg/L</th>
<th>SD, %</th>
<th>SD&lt;sub&gt;total&lt;/sub&gt;, %</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPLC (reduced)</td>
<td>12.1</td>
<td>0.39</td>
<td>0.12</td>
<td>3.3</td>
</tr>
<tr>
<td>DCIP</td>
<td>12.5</td>
<td>0.52</td>
<td>0.22</td>
<td>4.5</td>
</tr>
<tr>
<td>HPLC (total)</td>
<td>12.5</td>
<td>0.42</td>
<td>0.38</td>
<td>4.5</td>
</tr>
</tbody>
</table>

SD<sub>total</sub> = between-day standard deviation. SD<sub>within</sub> = between-day standard deviation. SD<sub>total</sub> = \sqrt{SD<sub>within</sub>^2 + SD<sub>within</sub>^2}. CV = (SD<sub>within</sub>/A)100.
ascorbic acid concentrations at values below 10 mg/L. The average difference was 0.32 mg/L.

Discussion

HPLC procedures are being used more frequently for the determination of ascorbic acid in biological samples because of the greater sensitivity and specificity of these methods as compared with colorimetric procedures. The sensitivity of the DCIP method is adequate for measuring ascorbic acid at the concentrations usually found in plasma or serum, but the specificity of the reaction has been questioned, because DCIP reacts with other reducing substances in plasma, e.g., glutathione. This interference is minimized in the automat-
ed DCIP method by precisely timing the reaction between DCIP and the sample, and by adjusting the pH of the reaction mixture to 3.5. The reaction of DCIP and ascorbate proceeds at a faster rate than the reaction of DCIP with other potentially interfering substances (10). Ascorbate is essentially the only substance that will reduce DCIP at pH 1 to 4 (11).

By comparing the DCIP method with a highly specific chromatographic procedure with electrochemical detection, we were able to assess the specificity of the DCIP reaction. Both procedures gave comparable results for a wide range of plasma ascorbate concentrations, but, as Figure 1 indicates, values by the automated DCIP method are slightly higher than those by the HPLC method. This difference may be important at low plasma ascorbate concentrations, e.g., when a cutoff value of 4 mg/L is used to determine "risk levels" in large population surveys (7).

Because the sampling rate of the automated DCIP method, approximately 40 per hour, is greater than would be possible with an HPLC procedure, the DCIP method would be preferable for use in nutritional surveys or other studies requiring the analysis of a large number of samples. The agreement between results by the methods makes it possible to compare results of different studies if either of the methods was used.

The agreement between the ascorbic acid and total ascorbate concentrations, determined by two separate HPLC procedures, confirms that most of the ascorbate in plasma is present as ascorbic acid.

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References