A Simple and Rapid Method for the Routine Assay of Total Ascorbic Acid in Serum and Plasma Using Ascorbate Oxidase and o-Phenylenediamine

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Summary A simple and rapid analysis of total ascorbic acid (AsA) in serum and plasma and its automated analysis are described. AsA is oxidized by ascorbate oxidase (AsA oxidase) to dehydroascorbic acid that then reacts with o-phenylenediamine (OPDA) to form a quinoxaline derivative that absorbs at 340nm. The change in absorbance is directly proportional to the total AsA concentration. The assay was validated with a linear concentration range of 0.8-80mg/L, and the within-day and between-day assays precision did not exceed 8.6% and 12.5%, respectively. On 47 sera, the manual enzymatic procedure gave 0.2 mg/L on average lower values than those of an automated enzymatic procedure with a correlation coefficient of 0.847. On another 66 sera, results by automated enzymatic method correlated well with the HPLC method and the regression equation is Y (enzymatic, automated)=0.97 X (HPLC)+0.1, r=0.980, Sy.x=0.6mg/L. An experienced analyst can perform about 24 manual assays per hour whereas the automated procedure gave a rate of 100 assays per hour.

Key Words vitamin C determination, ascorbic acid, ascorbate oxidase, automated assay, enzymatic methods

Ascorbic acid (AsA) is an important component of many biological systems, and various physiological roles have been described for this vitamin. Usually, vitamin C deficiencies can be ascertained by measuring AsA concentrations in plasma or serum (1). The usual practice is to measure plasma and (or) serum AsA concentrations with a dinitrophenylhydrazine (DNPH) method or by high-performance liquid chromatography (HPLC) method (2). The DNPH method requires long incubation times and is too labor intensive for routine laboratory use and the HPLC procedure is difficult to automate. Tulley (3) described an automated enzymatic method using ascorbate oxidase (AsA oxidase: EC 1.10.3.3) and o-phenylenediamine (OPDA); however, falsely high AsA values as a result of suppressed calibration reaction were often observed in his automated method. He also failed to compare the results with a certified method and to demonstrate its accuracy, precision, and sensitivity, particularly at the levels below 4 mg/L. The present study was therefore designed to determine the optimal reaction conditions and analytical performances for the assay of total AsA in serum. Finally, we checked the agreement of the enzymatic method with the established HPLC method.

Materials and Methods

Principle. AsA is oxidized by AsA oxidase to dehydroascorbic acid (DAsA) that is then condensed with o-phenylenediamine to form a fluorescent quinoxaline derivative. The resulting product absorbs at 340 nm, and the change in absorbance is proportional to the amount of AsA present in the specimen.

Reagents. OPDA dihydrochloride, metaphosphoric acid (MPA) and dithiothreitol (DTT) were obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan. AsA (the Japanese Pharmacopoeia Standard) and AsA oxidase (cucumber origin; 146 units per mg solid, 395 units per mg protein of the specific activity) were purchased from the National Institute of Hygienic Sciences, Tokyo, Japan and Oriental Yeast Co., Ltd., Osaka, Japan, respectively. 80mg AsA oxidase was dissolved in 1,000mL of 0.1mol/L, pH 6.5 phosphate buffer; 500mg of OPDA was dissolved in 1,000mL of the same buffer. The protein precipitation reagent containing MPA and DTT was prepared by dissolving 2g of MPA and 42mg of DTT in 5.0mL of distilled water. AsA was dissolved in distilled water to give a 100 mg/L stock solution. Calibrator solution was also prepared fresh daily in distilled water to give an AsA concentration of 25.0 mg/L. We checked the concentration of AsA stock solution after five-fold dilution with 40% MPA by measuring its absorbance at 245 nm, the wavelength of the absorption peak of AsA in acidic solutions. We used 1.22×104 as the molar absorption coefficient of AsA in...
Results

**Manual procedure.** A solution containing 0.05 mL of the MPA/DTT was mixed with 0.5 mL of serum or the AsA calibrator and held at 25°C for 10 min. DTT will reduce any DAsA, if present, to AsA (5); the MPA will deproteinize the serum and stabilize the AsA. DAsA is more susceptible than AsA to hydrolytic ring opening to yield 2,3 diketo-L-gulonic acid. After 10 min of incubation, the mixture was centrifuged at 12,000×g for 5 min. Then 0.25 mL of the supernatant was mixed with 2.0 mL of AsA oxidase solution at 37°C and the specimen was incubated for 1 min at 37°C, after which 0.10 mL of OPDA solution was added. With the latter step as time zero, absorbances were measured at 340 nm and again 60 s later using a Hitachi U-2000 spectrophotometer (Tokyo, Japan) in cuvets thermostated at 37°C. Increases in absorbance were corrected using a reagent blank having the same conditions as the test, but 0.25 mL distilled water was substituted for the serum or calibrator. The serum total AsA concentrations were obtained by comparing the change in absorbance of the unknowns with that of the 25.0 mg/L AsA calibrator.

**Automated procedure.** In the automation version of this assay employing a Synchron CX7 Delta (Beckman Coulter Instruments Inc., Fullerton, CA, USA), 0.2 mL of the AsA oxidase solution was dispensed into the reaction cuvets, and pre-incubated at 37°C for 5 min, primarily to bring the solutions to 37°C. 0.025 mL of the same supernatant was mixed with this solution and incubated for 5 min to oxidize AsA to DAsA. Then 0.01 mL of OPDA solution was added, and an initial rate of reaction was determined by measuring the absorbances at 340 and 700 nm every 16 s for 5 min. The 25.0 mg/L AsA calibrator was also used for the automated procedure.

**Comparison assay.** We used an LC-10A pump, an LC-4C electrochemical detector, and an SCL-10A system controller, all from Shimadzu (Kyoto, Japan). For total AsA (AsA plus DAsA) analysis, we combined 0.1 mL of serum with 0.05 mL of 10 g/L DTT and 0.05 mL of 10% MPA. After standing for 10 min at room temperature, the mixture was centrifuged at 12,000×g for 5 min. After the supernatant was diluted 10- to 20-fold with 0.5% MPA, 0.1 mL aliquots were injected into an Inertsil ODS-2 HPLC column (5 μm, 4.0 mm ID×150 mm L; GL Sciences Inc., Tokyo, Japan). The best separations were achieved by using isocratic elution with a 30 mmol/L, pH 2.3 potassium phosphate solution containing 0.1 mmol/L EDTA and at a flow rate of 0.7 mL/min. To monitor the column effluent, we used an amperometric detector with an applied potential of 600 mV.

**Results**

**Time course of the reaction.** The addition of AsA to AsA oxidase caused a rapid increase in absorbance at 340 nm, and the addition of OPDA produced a second and much greater increase in absorbance (Fig. 1). This is why we chose to measure the absorbance change after the addition of OPDA as time zero, and 60 s later as the measuring interval in the manual method; a 300-s interval was used in the automated procedure. A mixture of AsA with AsA oxidase solution showed an increase of absorbance with time at 340 nm in the absence of the OPDA solution; however, after a 1 min incubation in the manual method, the absorbance stabilized. The change in absorbance at 340 nm was directly proportional to the AsA concentrations between ca. 0.8 mg/L and 80 mg/L. The range of linearity was sufficient to cover a broad range of AsA concentrations in serum ranging from deficient to greater than normal. Although the changes in absorbance at 340 nm of the 10 mg/L AsA concentration had a reaction reading of approximately 0.015–0.022 in the manual procedure, a sufficient reproducibility was obtained as mentioned later since the clinical spectrophotometer was now verified to minimize baseline drift to less than 0.001 units of absorbance per hour, while the lower detection limit was 5 mg/L in the Tulley’s procedure.

**AsA oxidase concentration.** We measured the effect of different concentrations of AsA oxidase on the oxidation of AsA (Fig. 2). The 100 mg/L of AsA in the reaction mixture was completely oxidized in 30 s by the 80 mg/L of AsA oxidase, but only 70% was oxidized by 40 mg/L of the enzyme under the same conditions. To assure complete oxidation of AsA to DAsA, we recommend use of 80 mg/L of AsA oxidase (68 mg/L in final concentration). We also examined another source of AsA oxidase (Sigma Chemical Co., St. Louis, MO, USA: cucurbita species, 197 units per mg solid, 1,000 units per mg protein of the specific activity), because the specific activity of AsA oxidase from Oriental Yeast (cucumbe origin) was not equivalent to that prepared from another source. At 80 mg/L, the AsA oxidase from Sigma or Oriental Yeast gave nearly the same results. Tulley used 1.7 mg/L of AsA oxidase in final concentra-
Next, we determined if the DTT concentration was sufficient to reduce all DAsA to AsA within 10 min at 25°C. We prepared a solution containing 25 mg/L of DAsA and 25 mg/L of AsA by enzymatic oxidation of AsA. When DTT (21–168 mg in 5.0 mL of 40% MPA) was added to the solution, DTT reduced DAsA to AsA. Here, AsA concentrations increased from 25 mg/L to 40 mg/L, thus about 15 mg/L (60%) of DAsA would be reduced. DTT in acidic solution converted DAsA incompletely. Although AsA oxidation is suppressed by excess amount of DTT, DTT at 4.2 mg in our reaction conditions did not interfere with the enzymatic method.

Reproducibility. Two serum pools were prepared: one contained added AsA, and the other did not. Aliquots of these two pools were frozen at −80°C until analysis. Total AsA measurements with the manual procedure were performed on ten consecutive replicates of these two pools. The mean±SD of total AsA in the AsA supplemented pool was 17.0±1.4 mg/L giving a CV of 8.2% and for the unsupplemented plain pool, it was 2.8±0.2 mg/L (CV of 7.1%). For total AsA concentrations determined consecutively for ten days on the same aliquots of pooled serum, we found a mean±SD of 18.8±1.4 mg/L (CV of 7.4%) for the supplemented pool, and 2.4±0.3 mg/L (CV of 12.5%) for the plain pool.

While within-day assays with the Synchron CX7 gave a mean±SD of 16.8±0.3 mg/L (CV of 1.8%) for the supplemented pool and 2.8±0.1 mg/L (CV of 3.6%) for the plain pool, the between-day assays for the same pools and method gave a mean±SD of 19.2±1.0 mg/L (CV of 5.2%) for the supplemented pool and 2.8±0.2 mg/L (CV of 7.1%) for the plain pool.

Analytical recovery. Analytical recovery was obtained by adding 0.5 mL of a 10.0 mg/L AsA solution to 0.5 mL of serum pool having endogenous AsA concentrations of 2.6 mg/L, and determining the total AsA. We found that the calibration reaction was often (but not always) suppressed with the use of more than 200 mg/L of OPDA in final concentration. When 20% MPA was used, the proteins did not precipitate completely, and a clear supernatant was not obtained after centrifugation. Because the change in absorbance at 340 nm tended to decrease with increasing concentrations of MPA, especially above 80%, we used 40% MPA or 4% in the final reaction mixture.

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Analytical recovery. Analytical recovery was obtained by adding 0.5 mL of a 10.0 mg/L AsA solution to 0.5 mL of serum pool having endogenous AsA concentrations of 2.6 mg/L, and determining the total AsA. The serum total AsA concentration after addition of AsA was 6.0 mg/L; thus the analytical recovery was 95%. When 5.0 mg/L AsA solution was added to serum having endogenous AsA concentration of 20.0 mg/L, the analytical value was 12.3 mg/L and recovery was 98%.

Human hemoglobin at 5 g/L, purified bilirubin at 56 mg/L, uric acid at 80 mg/L, and glucose at 3,000 mg/L added to the serum pool had no significant effects on the values of total AsA with the enzymatic method.

Correlation study. We compared total AsA concentrations determined by the manual and automated enzymatic methods on 47 sera: Y (enzymatic, manual)=0.95X (enzymatic, automated)+0.2, r=0.847, S_{xy}=1.6 mg/L, i.e., the manual method gave values that were on average 0.2 mg/L lower. We also measured the total AsA concentrations with the automated enzymatic method and the HPLC method on sera from another 66 patients. Results by automated enzymatic method correlated well with the HPLC method, although the automated enzymatic method gave results that were on average 0.1 mg/L lower. The regression
Fig. 3. Comparison of the total ascorbic acid (AsA) concentrations in 66 sera as measured by the automated enzymatic method using a Synchron CX7 and by the HPLC method. Correlation coefficient, 0.980; regression equation: \( Y = 0.97X + 0.1; \) Sy.x = 0.6 mg/L.

**Discussion**

DNPH reacts with all AsA derivatives, i.e., AsA, DAsA and diketogulonic acid, whereas the enzymatic method described here reacts solely with DAsA and AsA (4). Because AsA was measured after the conversion of AsA to DAsA, DAsA could not be distinguished from AsA in either enzymatic method. However, Dhariwal et al. (6) and Koshiishi et al. (7) reported that DAsA would not be detected in human plasma or serum, and that DAsA could be formed in specimens processed under oxidizing conditions. Thus both enzymatic methods measure AsA and any DAsA that was spontaneously formed after blood sampling. Although diketogulonic acid, a product of AsA breakdown, could not be reduced to AsA by DTT, our enzymatic method converted 60% of DAsA to AsA, that was stable in acid solutions and the 40% remainder reacted directly with OPDA. Because the analytes measured in our enzymatic method were total AsA, i.e., AsA plus DAsA, we think that the addition of DTT is not required prior to the assay of fresh serum specimens for total AsA analysis. If other specimens are employed, particularly those that have been stored, then inactivity of OPDA with diketogulonic acid should be investigated.

In the manual method described here, the AsA calibrator or the sera plus the MPA/DTT to deproteinize the specimen and reduce the DAsA were incubated for 10 min. The AsA oxidase solution was added, the mixture incubated for 1 min to convert all AsA to DAsA, and the OPDA was added immediately thereafter. Five minutes of oxidation was needed in the automated analysis with the Synchron CX7, because increases in absorbance arising in the absence of OPDA interfered with the measurement of AsA (See Fig. 1). The increases in absorbance at 340 nm was probably owing to the formation of semidehydroascorbic acid (8) that generated a higher rate of reaction in the first 16 s in the automated analysis. The same phenomenon most likely also occurred in the manual method but was not observed by us, because the second measurement of absorbance was set at 60 s later.

Our enzymatic method required <10 min for deproteinization. After the clear supernatant was separated, 24 specimens by the manual procedure and at least 100 by the automated procedure could be determined in 60 min. The within-run and between-day imprecision of the manual procedure were higher than that for the automated procedure, but they did not exceed CV's of 8.2% and 12.5% respectively for the manual method. Our enzymatic deproteinization method was not affected by hemolysis when the assay was performed immediately after collection of blood, and good agreement was validated by comparison study with the established HPLC method. Automation should facilitate AsA measurements with equipment that is available in most routine clinical chemistry laboratories. We cautiously estimated the accuracy of the enzymatic method at the “true” value using the purest source of AsA available, and found recoveries of added AsA to serum pool of about 95%.

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