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An Ultramicro Fluorimetric Determination of Total Ascorbic Acid in Human Serum Using 1,2-Diamino-4,5-dimethoxybenzene

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A simple and sensitive fluorimetric method is described for the assay of total ascorbic acid, the sum of ascorbic acid and dehydroascorbic acid, in human serum. This is based on the reaction of dehydroascorbic acid with 1,2-diamino-4,5-dimethoxybenzene to give an intensely fluorescent product; ascorbic acid is oxidized with iodine to dehydroascorbic acid beforehand. The method requires as little as 4 μl of serum and the detection limit of ascorbic acid is 5.1 ng (28.3 pmol).

Keywords——fluorimetry; total ascorbic acid; dehydroascorbic acid; human serum; 1,2-diamino-4,5-dimethoxybenzene

Ascorbic acid and dehydroascorbic acid are equally biologically active. Ascorbic acid is easily oxidized to dehydroascorbic acid during the handling of biological samples (sample collection, transport and storage). Therefore, it is convenient to measure the sum of them (defined as total ascorbic acid).

Many methods have been reported for the determination of total ascorbic acid in human serum or whole blood. Colorimetric methods using 2,4-dinitrophenylhydrazine have been widely utilized as conventional methods in clinical investigations.[1,2] The methods, however, are not very selective and require large amounts of serum (more than 250 μl). More sensitive fluorimetric methods have been developed based on the reaction of dehydroascorbic acid with *o*-phenylenediamine,[3,4] but these methods are not sensitive enough to determine total ascorbic acid in μl amounts of serum or whole blood. High-performance liquid chromatographic methods with photometric,[5] fluorimetric[6,7] and electrochemical[8] detections have also been developed for the determination of ascorbic acid and/or dehydroascorbic acid. These methods are sensitive, but require rather expensive apparatus.

We have previously developed 1,2-diamino-4,5-dimethoxybenzene (DDB) as a fluorogenic reagent for aromatic aldehydes[9,10] and α-keto acids.[11] We recently found that DDB also reacts with dehydroascorbic acid under conditions different from those for aromatic aldehydes and α-keto acids (*i.e.*, in a weakly acidic solution at moderate temperatures) to produce a highly fluorescent compound, and we thus developed a fluorimetric method for the determination of total ascorbic acid in a minute amount of human serum. Ascorbic acid is oxidized with iodine to dehydroascorbic acid before the reaction with DDB.

Experimental

Reagents and Solutions——All chemicals were of reagent grade, unless otherwise noted. Double-distilled water was used. The water was boiled and then rapidly cooled when required for use. DDB·HCl was prepared as described previously[9] and is now available from Dojindo Laboratories (Kumamoto, Japan).

DDB Solution (1.0 mM): DDB·HCl (21.5 mg) was dissolved in 5.0 ml of 0.1 M $Na_2S_2O_3$ (a stabilizer of DDB) and

diluted with 40 mM Britton–Robinson buffer (pH 4.0) to 100 ml. The solution was used within 3 h.

I_2 Solution (1.0 mM): I_2 (127 mg) was dissolved in 100 ml of 1.5 mM KI.

Apparatus——Uncorrected fluorescence spectra and intensities were measured with a Hitachi 650-60 spectrofluorimeter using 10-mm quartz cells; spectral bandwidths of 10 nm were used in both the excitation and emission sides of the monochromator. pH was measured with a Hitachi–Horiba M-7 pH meter at 25 °C.

Procedure——Freshly drawn blood was allowed to stand for 30 min at room temperature (15—25 °C) and centrifuged at 1000 *g* and at 4 °C for 10 min. The serum (4 μl) was mixed with 20 μl of 0.5 M trichloroacetic acid. The mixture was allowed to stand at 0 °C for approximately 5 min and then centrifuged at 1000 *g* for 5 min. To 10 μl of the supernatant (deproteinized serum solution), 1.0 ml of 40 mM Britton–Robinson buffer (pH 4.0) and 0.1 ml of I_2 solution were successively added, and the mixture was allowed to stand at room temperature for 10—20 s. The excess I_2 was decomposed by the addition of 0.1 ml of 0.1 M $Na_2S_2O_3$. Then, 0.5 ml of DDB solution was added, and the resulting solution was warmed at 37 °C for 30 min to develop the fluorescence. The reagent blank was prepared by treating 10 μl of water in the same manner. The fluorescence intensities of the test and blank were measured at 458 nm emission and 371 nm excitation, and the concentration of total ascorbic acid was read from a calibration graph, which was prepared by applying the standard procedure to 10 μl portions of ascorbic acid standard solutions (0.3 pmol—10 nmol (54 ng—1.8 μg)/10 μl).

Results and Discussion

Fluorescence Reaction Conditions

The excitation and emission maxima for the fluorescent product from ascorbic acid were at 371 and 458 nm, respectively (Fig. 1, a_1 and b_1). On irradiation at 371 nm, a weak fluorescence of the reagent blank was observed (Fig. 1, a_3 and b_3); the intensity was 5% of that given by 500 pmol/10 μl ascorbic acid. Raman scattering of the solvent water was not observed under the recommended conditions.

Ascorbic acid was oxidized effectively to dehydroascorbic acid with iodine at concentrations of 0.1 mM or greater in the solution; 1.0 mM was used in the standard procedure. DDB was decomposed with excess iodine to give a red-colored product, which interfered with

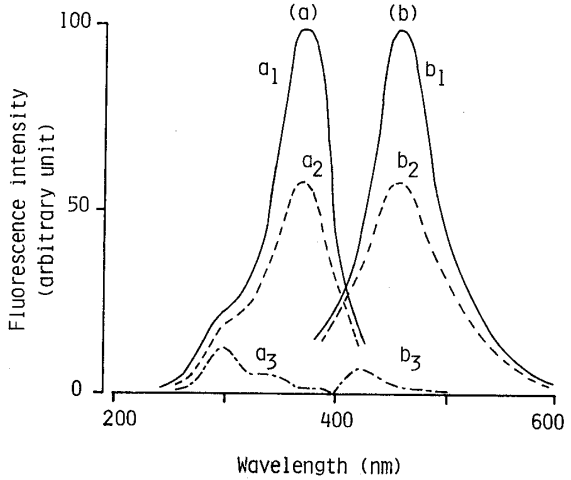

Fig. 1. Fluorescence Excitation and Emission Spectra of the Reaction Mixtures of Ascorbic Acid, Serum and the Reagent Blank

a: excitation spectra; b, emission spectra.
a_1 and b_1: 10 μl of 500 pmol/10 μl ascorbic acid was treated according to the procedure.
a_2 and b_2: 10 μl of the deproteinized serum solution (142.8 ng (793 pmol)/4 μl total ascorbic acid in serum) was treated according to the procedure.
a_3 and b_3: the reagent blank for a_1 and b_1, or a_2 and b_2, respectively.

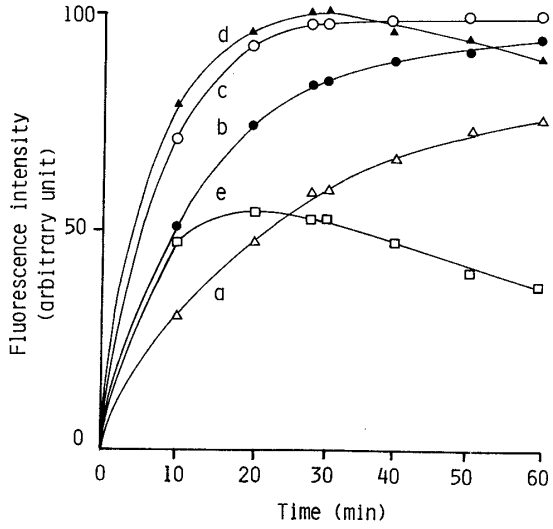

Fig. 2. Effects of pH of 40 mM Britton–Robinson Buffer and Reaction Time on the Fluorescence Development

Portions (10 μl) of 200 pmol/10 μl ascorbic acid were treated according to the recommended procedure but with buffer of various pH. pH: (a) 3.0; (b) 3.5; (c) 4.0; (d) 4.5; (e) 5.0.

the assay of the acid. Therefore, the iodine should be decomposed with sodium thiosulfate. Thiosulfate solution gave a maximum and constant fluorescence intensity in a concentration range of 2.0—200 mM in the solution; 0.1 M was employed in the standard procedure.

The resulting dehydroascorbic acid reacted with DDB in an acidic solution. Britton–Robinson buffer solution in a pH range of 4.0—4.5 gave a maximum and constant fluorescence intensity (Fig. 2). At pH higher than 4.5, the final mixture produced a red color with an absorption maximum at 430 nm, which reduced the fluorescence from dehydroascorbic acid due to an inner filter effect. Below pH 3, a white turbidity due to decomposition of sodium thiosulfate appeared in the DDB solution. The concentration of the buffer did not affect the fluorescence reaction in a range of 10—50 mM. Thus, the deproteinized serum solution was diluted with 40 mM Britton–Robinson buffer (pH 4.0). Phosphate and citrate buffers caused a large background fluorescence for an unknown reason.

DDB solution gave the most intense fluorescence at a concentration greater than about 9 mM in the reagent solution. The background fluorescence intensity increased with increasing concentration of DDB; 10 mM was selected as a sufficient concentration.

The fluorescence intensity from ascorbic acid reached an almost maximum and constant value after warming at 30—37 °C for more than 30 min. Temperatures higher than 50 °C gave lower fluorescence intensities. Therefore, warming at 37 °C for 30 min was selected.

The fluorescence developed under the prescribed conditions did not change on irradiation for 5 min at the excitation maximum of 371 nm, and was stable for at least 45 min in daylight and for at least 2 h in the dark at room temperature.

Fluorescence from Other Substances

Piperonal, pyruvic acid and α-ketoglutaric acid gave weak responses in the recommended procedure with the reagent at a concentration of 10.0 nmol/10 μl; the intensity was 2—3 times that of the reagent blank. All other substances examined did not fluoresce even at a concentration of 10.0 nmol/10 μl: *i.e.*, α-ketovaleric acid, α-ketoisovaleric acid, α-ketocaproic acid, α-ketoisocaproic acid, α-ketoadipic acid, α-ketobutyric acid, α-keto-β-methylvaleric acid, phenylpyruvic acid, *p*-hydroxyphenylpyruvic acid, benzaldehyde, *p*-hydroxybenzaldehyde, 3,4-dihydroxyphenylpyruvic acid, vanillin, 2,3-dimethoxybenzaldehyde, 3,4-dimethoxybenzaldehyde, terephthalaldehyde, 1-naphthaldehyde, *p*-nitrobenzaldehyde, phenylacetaldehyde, isobutylaldehyde, and propionaldehyde. This suggests that the recommended procedure is selective for dehydroascorbic acid.

Determination of Total Ascorbic Acid in Human Serum

The fluorescence excitation and emission spectra of the final mixture obtained through the procedure with serum were identical in shapes and maxima to those of the final mixture obtained with ascorbic acid (Fig. 1, a_2 and b_2). This indicates that a calibration graph can be constructed in the absence of serum. Numerous substances of biological importance, glycine, 17 different L-α-amino acids, glutathione, creatine, creatinine, histamine, tyramine, octopamine, epinephrine, norepinephrine, dopa, dopamine, thiamine, citrulline, allantoin, uric acid, bilirubin, urea, *N*,*N*-dimethylurea, acetone, acetylacetone, acetophenone, lactic acid, acetoacetic acid, homogentisic acid, inositol, D-xylose, D-glucose, D-fructose, D-galactose, D-mannose, D-maltose, D-lactose, epiandrosterone, dehydroepiandrosterone, cortisone and cholesterol, added to normal serum did not interfere with the determination of total ascorbic acid even at unusually high concentrations (15—100 μg/ml serum).

A linear relationship was obtained between the fluorescence intensity and the amount of total ascorbic acid up to at least 0.8 μg (44.4 nmol) in 4 μl of serum. The limit of detection for total ascorbic acid in serum was 5.1 ng (28.3 pmol) per 4 μl, which gave a fluorescence intensity of twice the blank. This sensitivity is at least 70 times higher than that of the methods with *o*-phenylenediamine and is comparable to that of the high-performance liquid chromatographic

methods with fluorescence and electrochemical detections.[6-8] The within-day precision was examined using sera with mean total ascorbic acid values of 19.6 and 105.2 ng (0.11 and 0.58 nmol)/4 μl ($n=15$ in each case). The coefficients of variation were 0.61 and 0.59%, respectively. The recovery of ascorbic acid (100 ng, 0.56 nmol) added to serum with 42.6 ng (0.24 nmol)/4 μl total ascorbic acid was $98.4 \pm 0.6\%$ (mean $\pm$ standard deviation, $n=10$).

Comparison with the 2,4-dinitrophenylhydrazine method[1] (requiring 250 μl of serum) using normal sera containing 5.8—142.8 ng (32—790 pmol) per 4 μl gave a correlation coefficient of 0.998 ($n=10$) and the regression equation for the present method (x) against the 2,4-dinitrophenylhydrazine method (y) was $y=1.03x+0.01$.

The present method requires only 4 μl of serum. Thus, the method is useful in cases where only an extremely small amount of serum is available. The entire procedure takes less than 1 h and more than 30 samples can be determined successively, so this method should be suitable for routine use for the assay of total ascorbic acid in serum or in other biological materials. Studies on the mechanism of the fluorescence reaction are in progress in our laboratories.

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