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Note

Determination of total ascorbic acid in human serum by high-performance liquid chromatography with fluorescence detection

TETSUHARU IWATA, MASATOSHI YAMAGUCHI, SHUUJI HARA and MASARU NAKAMURA

Faculty of Pharmaceutical Sciences, Fukuoka University, Nanakuma, Jonan-ku, Fukuoka 814-01 (Japan)

and

YOSUKE OHKURA*

Faculty of Pharmaceutical Sciences, Kyushu University 62, Maidashi, Higashi-Ku, Fukuoka 812 (Japan)

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Ascorbic acid and its oxidized form, 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) and therefore total ascorbic acid (the sum of ascorbic and dehydroascorbic acids) is normally determined.

Several high-performance liquid chromatographic (HPLC) methods with electrochemical [1, 2], UV [3] and fluorescence [4] detection have been reported for the determination of ascorbic and/or dehydroascorbic acid in human serum and whole blood. HPLC methods with electrochemical detection are fairly sensitive but allow the determination only of ascorbic acid. The method with UV detection, based on the reaction of dehydroascorbic acid with *o*-phenylenediamine (OPD), is not sensitive and thus requires a large amount of serum (500 μl). The method with fluorescence detection using OPD as a fluorogenic reagent is more sensitive. However, it does not permit the determination of total ascorbic acid in extremely small amounts of serum.

Recently, we have reported a manual fluorimetric method for the selective

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determination of total ascorbic acid in human serum, based on the reaction of dehydroascorbic acid with 1,2-diamino-4,5-dimethoxybenzene (DDB) to give a highly fluorescent product [5].

In this paper, we describe an ultramicro assay of total ascorbic acid in human serum by HPLC with fluorescence detection using the above reaction. 6,7-Dimethoxy-3-propyl-2(1H)-quinoxalinone (DPQ) was used as an internal standard (I.S.).

EXPERIMENTAL

Reagents and solutions

All chemicals were of analytical-reagent grade unless stated otherwise. Doubly distilled water was used. DDB monohydrochloride was prepared as described previously [6]; it is now available from Dojindo Labs. (Kumamoto, Japan). DPQ was synthesized as described previously [7].

DDB solution (2.0 mM). DDB monohydrochloride (16.4 mg) was dissolved in 5.0 ml of 0.1 *M* sodium thiosulphate (stabilizer of DDB) and diluted to 40 ml with 0.1 *M* acetate buffer (pH 4.5). The solution was stored in the dark and used within a day.

Iodine solution (1.0 mM). Iodine (12.7 mg) was dissolved in 100 ml of 1.5 m*M* potassium iodide.

DPQ (I.S.) solution (10.0 μM). DPQ (2.86 mg) was dissolved in 1000 ml of methanol. The solution was stored at room temperature (15—25°C) in the dark and was usable for at least two months.

Apparatus

Uncorrected fluorescence excitation and emission spectra of the eluate were measured with a Hitachi 650-60 fluorescence spectrophotometer fitted with a 20-μl flow cell, setting the spectral bandwidths at 5 nm in both the excitation and emission monochromators. A Waters 510 high-performance liquid chromatograph equipped with a U6K universal injector and a Shimadzu RF-530 fluorescence spectromonitor equipped with a 12-μl flow cell operating at an excitation wavelength of 371 nm and an emission wavelength of 458 nm were used. A Radial-Pak cartridge phenyl column (100 × 8 mm I.D.; particle size 10 μm; Waters Assoc., Milford, MA, U.S.A.) was used. The column can be used for more than 1000 injections with only a small decrease in theoretical plate number when washed each day with aqueous 50% (v/v) methanol at a flow-rate of 1.2 ml/min for about 30 min after analyses.

Procedure

Freshly drawn blood was allowed to stand for 30 min at room temperature (15—25°C) and centrifuged at 1000 *g* at 4°C for 10 min. The serum (2 μl) was diluted with 5 μl of DPQ solution and 0.1 ml of 0.6 *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 50 μl of the supernatant, 50 μl of 0.3 *M* sodium carbonate in 0.1 *M* acetate buffer (pH 4.5) and 10 μl of iodine solution were successively added, and the mixture was allowed to stand at room temperature for 10—20 sec. The excess of iodine was decomposed by the addition

of 10 μl of 50 m*M* sodium thiosulphate solution. To the mixture were added 50 μl of DDB solution and the resulting solution was warmed at 37°C for 30 min in the dark. The reaction mixture (10 μl) was injected directly into the chromatograph.

A calibration graph was prepared according to the procedure except that 0.1 ml of trichloroacetic acid solution was replaced with a solution containing 1.7—890 pmol (0.3—160 ng) of ascorbic acid. The net peak-height ratio of ascorbic acid to DPQ was plotted against the concentration of the acid added. The concentration of total ascorbic acid was read from the calibration graph.

The mobile phase was aqueous 50% (v/v) methanol at a flow-rate of 2.0 ml/min (ca. 60 kg/cm^2). The column temperature was ambient (ca. 15–25°C).

RESULTS AND DISCUSSION

The conditions used for fluorescence derivatization were as described previously [5].

HPLC conditions

Fig. 1 shows typical chromatograms obtained with ascorbic acid solution and the reagent blank. The peaks for ascorbic acid and DPQ (retention times 2.6 and 8.2 min, respectively) could be completely separated from the components of the reagent blank (Fig. 1; peaks 3—7). The separation of the peaks was first examined using Radial-Pak cartridge C_{18}, C_8 and CN columns with

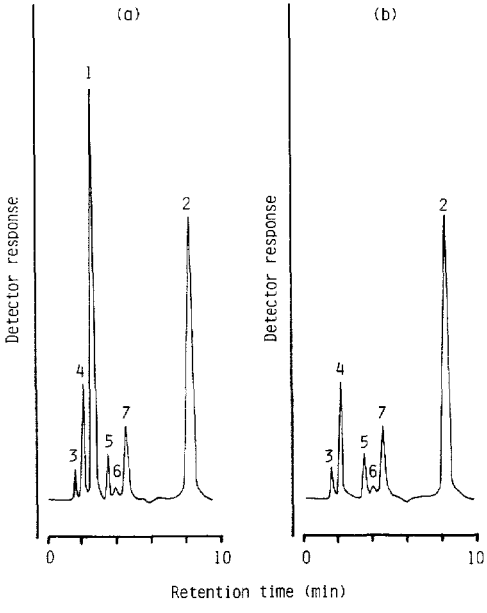

Fig. 1. Chromatograms obtained with (a) ascorbic acid and (b) aqueous solution. Aliquots (2 μl) of ascorbic acid solution [6.4 ng (35.6 pmol) per 2 μl] and of aqueous solution as the reagent blank were treated according to the procedure. Peaks: 1 = ascorbic acid; 2 = DPQ; 3—7 = the blank.

methanol, acetonitrile, water, 30 m*M* phosphate buffer (pH 4–8) and their mixtures as mobile phases. However, none of their combinations gave complete separation of the peaks. Complete separation of the peaks was achieved on a Radial-Pak cartridge phenyl column with aqueous methanol. The methanol concentration in the mobile phase had an effect on the retention times of the peaks. At methanol concentrations higher than 55% (v/v) the peaks slightly overlapped each other, whereas methanol concentrations of 40–45% (v/v) caused a delay in elution with broadening of the peaks. Optimum separation was attained by using aqueous 50% (v/v) methanol as the mobile phase. The peak heights (fluorescence intensity) were not affected by the concentration of methanol.

Determination of total ascorbic acid in serum

A typical chromatogram obtained from normal serum is shown in Fig. 2a. The pattern of the chromatogram was virtually identical with that in Fig. 1a. The component of peak 1, the DDB derivative of dehydroascorbic acid, was identified on the basis of its retention time and the fluorescence excitation (maximum 371 nm) and emission (maximum 458 nm) spectra of the eluate of peak 1 by comparison with those in Fig. 1a, and also by cochromatography of the reaction mixtures of ascorbic acid and serum with aqueous 40, 50 and 53% (v/v) methanol as mobile phase.

The DDB reaction did not give fluorescent derivatives with biological substances having no 1,2-diketo moiety in the molecule, such as amines, amino acids, sugars, lipids, steroids, vitamins and nucleosides, as demonstrated

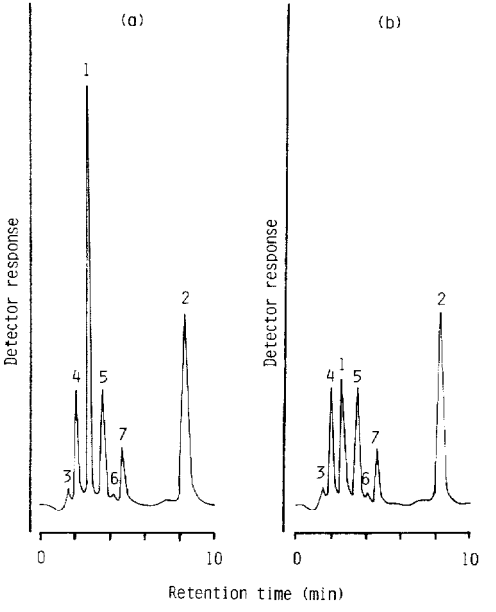

Fig. 2. Chromatograms obtained with (a) normal human serum and (b) a five-fold saline-diluted serum. Aliquots (2 μl) of the samples were treated according to the procedure. Concentration of total ascorbic acid in serum: 10.5 ng (60 pmol) per 2 μl. Peaks as in Fig. 1.

previously [5]. In addition, some 1,2-diketo compounds tested (e.g., α-ketoglutaric acid and pyruvic acid), which react with DDB under the recommended reaction conditions to give weak fluorescence, did not interfere with either the detection or separation of the peak of ascorbic acid, even when they were present at unusually high concentrations in serum (2.0 μmol/ml), because the retention times of α-ketoglutaric acid and pyruvic acid (3.3 and 4.4 min, respectively) are different from those of ascorbic acid and DPQ.

A linear relationship was observed between the ratio of the peak height of ascorbic acid to that of DPQ and the amount of ascorbic acid added in the range 1.7—890 pmol (0.3—160 ng) in 2 μl of serum, and no change in the slope was observed with variation in the serum used. These results indicated that the present internal standard method permits the determination of ascorbic acid in serum over wide ranges of concentration.

The recovery of ascorbic acid added (26.4 ng, 150 pmol) to pooled serum with 4.2 ng (24 pmol) per 2 μl of total ascorbic acid was 98.5 ± 0.1% (mean ± standard deviation; $n = 6$).

The within-day and between-day precisions were examined by performing ten separate analyses using sera with mean total ascorbic acid contents of 34.3 and 6.9 ng (195 and 39 nmol) per 2 μl. The between-day precision was obtained by performing the analyses during ten days with the sera kept frozen at −20°C. The coefficients of variation for the within-day precision were 1.6 and 2.2% and those for the between-day precision were 2.0 and 3.2%, respectively. The limit of detection for total ascorbic acid was 810 pg/ml (4.6 pmol/ml) in serum [8.1 pg (46 fmol) in the injection volume of 10 μl] at a signal-to-noise ratio of 2. The sensitivity is at least 40 times higher than that of the HPLC method with fluorescence detection using OPD. A chromatogram obtained with a five-fold saline-diluted serum is shown in Fig. 2b. This indicates that the method permits the assay of the acid in volumes of normal human serum of 0.5 μl or less.

The method is precise, selective and highly sensitive and requires only 2 μl of human serum, and should be useful in cases where only an extremely small amount of serum is available. The method is also rapid and simple to perform and can therefore be applied for routine use in the assay of total ascorbic acid in serum.

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