TECHNICAL NOTES

Specific Determination of Ascorbic Acid with Chemical Derivatization and High-Performance Liquid Chromatography

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INTRODUCTION

Radical reactions have received wide and growing attention in biological sciences. For systematic investigations on lipid peroxidation, it is essential to establish specific and sensitive method to analyze key molecules involved in radical reactions and defence mechanisms. We recently reported the specific method to determine malondialdehyde which is commonly used to estimate the extent of lipid peroxidation.[1] As the next extension, we gave special attention to ascorbic acid, which is one of the important antioxidants in biological system.[2]

Many methods are reported on the determination of ascorbic acid in animal tissue extracts and fluids.[3-6] The colorimetric assay originally developed by Roe et al.[7] has still been widely used as the conventional method. The (dinitrophenyl)hydrazine (DNPH) method involves the formation of bis((2,4-dinitrophenyl)hydrazone) (osazone) with dehydroascorbic acid and diketogulonic acid, which is rapidly formed from the former under the physiological condition accompanying the irreversible loss of vitamin C activity.[8] It has been claimed that the colorimetric assay lacks sensitivity and specificity for the measurement of ascorbic acid in biological samples and foods.[4] Moreover, it is a serious problem that the structure of the osazone has not been well characterized by modern analytical methods.[9]

Recently the traditional method is being replaced by high-performance liquid chromatography (HPLC).[10-13] The use of HPLC has increased the specificity and sensitivity of ascorbate assays and reduced analysis time compared with chemical methods. However, some disadvantages remain in current HPLC assays. Since ascorbic acid is highly hydrophilic, the direct application of HPLC using a commercially available C_{18} column may not be appropriate for its specific separation in biological materials containing a great variety of water-soluble compounds. The difficulty in the separation occurs even when the detection is made with a highly sensitive electrochemical detector,[6,10,12] and the method requires an expensive apparatus.

In this paper, we report a precolumn method involving chemical derivatization of ascorbic acid into bis((dinitrophenyl)hydrazone) of dehydroascorbic acid (osazone), which is sufficiently hydrophobic to allow us specific determination of the vitamin using a reversed-phase C_{18} column and widely prevailed absorption detector. This paper is also concerned with the structure of the osazone.

EXPERIMENTAL SECTION

Materials. Anhydrous dehydroascorbic acid and barium diketoglutonate were prepared according to the literature.[14]

Mass, NMR, and Infrared (IR) Spectroscopy. The mass, NMR, and IR spectra were recorded with a JEOL, JMS-HX110, a JEOL JNM-GSX400, and a Shimadzu infrared spectrophotometer IR-420, respectively.

Preparation of Dehydro-L-ascorbic Acid Bis((dinitrophenyl)hydrazone) (Osazone). Two percent (dinitrophenyl)hydrazine in 4.5 mol/L sulfuric acid (100 mL) and stannous chloride (0.3 g) was added to a solution of anhydrous dehydroascorbic acid (1 g) dissolved in 5% metaphosphoric acid (10 mL). The mixture was stirred at 37 °C for 3 h to give the dark red precipitates. The dehydroascorbic acid bis((dinitrophenyl)hydrazone) was extracted with ethyl acetate from the reaction mixture. The organic layer was washed with water and evaporated to dryness. The residue was washed with 50% phosphoric acid, water, ethanol, and ethyl ether. The product was purified by recrystallization from a mixture of acetone and hexane, giving 0.46 g of the product whose mp was 299–301 °C. Major peaks in the IR spectrum (KBr): 3421, 3099, 1745, 1616, 1602, 1587, 1500, 1432, 1342, 1315, 1141, 1119, 1055, 1036, 924, 833, 787, 741, 636, and 453 cm^{-1}. Mass spectrum, *m/e* (for more than 100): 534 (M^+, 100), 535 (M + 1, 28), 536 (M + 2, 3), 516 (13), 474 (20), 183 (8).

The barium salt of 2,3-diketogulonate was converted to the osazone by a similar procedure as described above.

HPLC Analysis. A Shimadzu LC-6A pump was used. The osazone was applied to a μ-Bondasphere 5-μm C_{18}-100A column (3.9 × 150 mm, Waters), eluted with 55% acetonitrile (1 mL/min), and the absorption at 505 nm was recorded with a Shimadzu SPD-6AV spectrophotometer.

Determination of Ascorbic Acid. One hundred microliters of ascorbic acid solution in the concentration range of 1–50 μmol/L was oxidized with 0.2% 2,6-dichloroindophenol (5 μL). The solution was mixed with 1% stannous chloride (50 μL) in 5% metaphosphoric acid solution and 2% (dinitrophenyl)hydrazine (120 μL) in 4.5 mol/L sulfuric acid. The mixture was incubated

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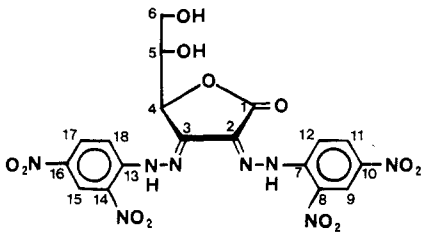

Figure 1. Structure of the osazone (bis((2,4-dinitrophenyl)hydrazone) of dehydroascorbic acid).

for 3 h at 37 °C. Ethyl acetate (1 mL) and water (1 mL) were added to the reaction mixture. After shaking and centrifugation (2500 rpm, 10 min), 400 μL of the ethyl acetate layer was taken and evaporated to dryness. The residue was dissolved in 100 μL of acetonitrile, and 10 μL of the sample was applied to HPLC.

Determination of Ascorbic Acid, Dehydroascorbic Acid, and 2,3-Diketogulonate in Rat Plasma. The freshly prepared rat plasma (100 μL) was added to 0.9 mL of ice-cold 20% metaphosphoric acid containing 1% stannous chloride, mixed thoroughly, and centrifuged at 5000 rpm for 10 min at 4 °C to get the deproteinized plasma sample. For the determination of ascorbic acid, dehydroascorbic acid, and diketogulonic acid (total ascorbic acid), 100 μL of the supernatant was subjected to the assay described in above section.

For the determination of diketogulonate in the plasma, 0.1% dithiothreitol (20 μL) was added to 100 μL of plasma. The mixture was kept at room temperature for 10 min[15] and then treated with 20% metaphosphoric acid (880 μL) containing 1% stannous chloride and centrifuged. To 100 μL of the supernatant were added 1% $SnCl_2$ (50 μL) in 5% metaphosphoric acid and 2% DNPH solution (120 μL) in 4.5 mol/L sulfuric acid. The following procedure was the same as described above.

For the determination of dehydroascorbate and diketogulonate, 1% $SnCl_2$ (50 μL) dissolved in 5% metaphosphoric acid and 2% DNPH (120 μL) in 4.5 mol/L H_2SO_4 were added to 100 μL of the original deproteinized plasma sample. The following procedure was performed in a similar manner as described above. Subtracting the value obtained here from the diketogulonate value determined above gives the content of dehydroascorbic acid.

Data Analysis. Experimental points represent the mean ± SD of at least three samples.

RESULTS AND DISCUSSION

Preparation of the Standard Compounds. In order to determine ascorbic acid, authentic samples for HPLC analysis, such as dehydroascorbic acid, diketogulonic acid, and their osazones, were prepared. Dehydroascorbic acid and diketogulonic acid were prepared according to the method of Kenyon and Munro.[14] Dehydroascorbic acid was obtained as colorless crystals and had mp 223–225 °C dec (lit.[13] mp 225 °C). The IR absorption spectrum of dehydroascorbic acid showed a strong and broad carbonyl band at 1780 cm^{-1}.

Dehydroascorbic acid was coupled with DNPH. Based on mass and IR spectra described in experimental section, the structure of the reaction product was proposed as shown in Figure 1. This structure was further supported by NMR measurements. Based on ^1H and ^{13}C NMR spectra, DEPT (135°), ^1H–^1H COSY, ^{13}C–^1H COSY, and HMBC spectra, the assignments of all hydrogens and carbons were made. All carbon atoms of the osazone were numbered as shown in Figure 1. ^{13}C NMR (100 MHz, in DMSO and its peak was designated as 39.5 ppm): δ (assigned carbon number) 61.5 (6), 72.3 (5), 80.4 (4), 129.7 (3), 142.6 (2), 163.8 (1), 141.3 (13), 117.5 (18), 129.5 (17), 141.2 (16), 122.3 (15), 133.4 (14), 143.9 (7), 117.0 (12), 130.2 (11), 139.4 (10), 122.5 (9), and 131.3 (8). In the assignment, two phenyl groups were not discriminated strictly. The result of ^1H NMR (400 MHz, in DMSO + D_2O) was as follows: δ (assignment) 3.50 (2 H attached to C-6, d, J = 7.2 Hz), 3.95 (1 H attached to C-5, td, J = 7.2 and 1.2 Hz), 5.45 (1 H attached to C-4, d, J = 1.2 Hz). The coupling constant (1.2 Hz) between the protons on C-4 and C-5 was very small. This may be due to the preferred conformation of the C-C bond similar to the case of dehydroascorbic acid.[15] In the absence of deuterium oxide, protons bound to C-5 and C-6 appeared as quartet-like and a broad featureless peak, respectively. This result indicates that both C-5 and C-6 have hydroxyl protons to cause a small coupling. On the other hand, the peak designated as the proton linked to C-4 was not affected by deuterium oxide as expected from the structure as shown in Figure 1. Other aromatic protons were observed as 8.55, 8.45, 8.95, 8.90, 8.20, and 8.55 ppm and were assigned to the proton bound to C-18, 17, 15, 9, 12, and 11, respectively.

Diketogulonic acid was converted to the osazone, whose structure was found to be identical with the osazone prepared from dehydroascorbic acid and DNPH based on absorption, IR, NMR, and mass spectra as well as comelting.

Chromatography of Standards. Ascorbic acid was measured by oxidizing to hydroascorbic acid for subsequent analysis. Among several oxidizing agents, 2,6-dichloroindophenol allowed quantitative oxidation of the vitamin and did not interfere with the chromatography. Figure 2a shows an HPLC elution profile of the authentic osazone of dehydroascorbic acid, detected by the absorbance of 505 nm. The retention time of the compound was 5.6 min under the used condition. The detection limit was 1 pmol in the injected sample of 10 μL. A typical chromatogram of the osazone of dehydroascorbic acid, prepared from ascorbic acid involving the indophenol oxidation, is shown in Figure 2b. Complete and optimum separation of the peaks was achieved on a Waters C_{18} column with 55% acetonitrile as the mobile phase. A linear relationship between peak area and ascorbic acid concentration was obtained for a 100 μL aliquot, the concentration of which was 0.1–100 μmol/L and the recovery of ascorbic acid was quantitative in the concentration range. The correlation coefficient was 1.00.

Determination of Ascorbic Acid in Rat Plasma. The method was applied to determine total vitamin C in rat plasma. The plasma was treated with 20% metaphosphoric acid containing 1% stannous chloride to prepare the deproteinized plasma sample. The values of total ascorbic acid obtained by our method for plasma samples were compared with the values by the conventional colorimetric DNPH method.[7] Total ascorbic acid measured by our method was 58.3 ± 0.57 μmol/L. On the other hand, the same plasma was subjected to the conventional DNPH method, and the total ascorbic acid content was 160.0 ± 4.40 μmol/L. For the determination of recovery of ascorbic acid, the vitamin was added to the plasma in the concentration range of 20–200 μmol/L. After deproteinization, the sample was subjected to our assay method. Ascorbate added to plasma was recovered in 85–90%. The detection limit was 1 pmol in the injected sample of 10 μL, similar to the standard described above.

In addition to the determination of total ascorbic acid, dehydroascorbic acid and diketogulonic acid contents of the plasma were also determined. When the oxidation with dichloroindophenol was omitted, the level of dehydroascorbic acid and diketogulonic acid was obtained. For the measurement of diketogulonate content, dehydroascorbic acid of the plasma was, at first, reduced to ascorbic acid with dithiothreitol by a slightly modified method of Okamura.[16] The subsequent procedures were described in the Experimental Section. Dehydroascorbic acid and diketogulonic acid determined by our HPLC method were 1.9 ± 0.20 and 0.7 ± 0.04

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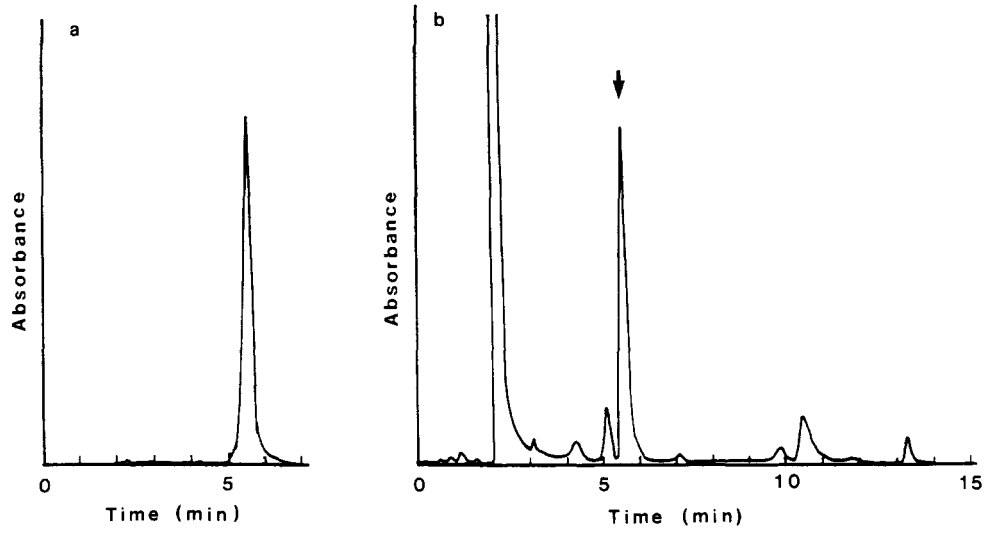

Figure 2. HPLC elution profiles of the osazone (a) and the osazone prepared from ascorbic acid involving indophenol oxidation (b). (a) Ten microliters of 10 μmol/L of the osazone was injected. (b) Ascorbic acid solution (50 μmol/L) was treated according to the procedure described in the Experimental Section. The osazone peak was designated by the arrow.

μmol/L, respectively. Therefore, the amount of oxidized forms (the sum of dehydroascorbic acid and diketogulonic acid) measured by the present method was calculated to be only 4.5% of total ascorbic acid. In addition to determination of ascorbic acid in plasma within one SD strain rat, the total ascorbate level of three normal SD rats were determined. The ascorbic acid level in plasma determined by our method and that by the conventional DNPH method were 56.4 ± 2.69 and 154.5 ± 4.45 μmol/L, respectively.

Ascorbic acid level in rat plasma measured by our HPLC method was only 36.5% of the value obtained by the colorimetric DNPH method. These results indicate that substances present in biological samples, such as carbohydrates and ketones, may interfere in the traditional DNPH method to yield high values. Although the conventional DNPH method has still been widely used, our experiments show that the re-evaluation is urgently necessary concerning the specificity in the measurement of the vitamin, especially in biological samples. It is worthwhile to note that our present method needs only 5 μL of plasma, whereas the conventional DNPH method requires a large amount of plasma (more than 200 μL). The assay is suitable for the determination of vitamin C in biological materials where only a small amount of sample is available or low amount of ascorbic acid is found.

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