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High-performance liquid chromatographic determination of plasma ascorbic acid in relationship to health care

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ABSTRACT

We have developed a simple reversed-phase high-performance liquid chromatographic method for determining plasma ascorbic acid level and studied the relationship between its plasma concentration and fruit and vegetable intake and plasma dopamine-β-hydroxylase activity. The samples were pretreated by precipitating the proteins and injected onto the column. Elution with a methanol gradient in sodium phosphate buffer was carried out by monitoring the absorbance at 265 nm, and the peak corresponding to ascorbic acid was well separated from other peaks of reagents used for pretreatment and from plasma endogenous components. The proposed method correlated well with the conventional dichlorophenol–indophenol method. Mean levels of ascorbic acid in normal human plasma were 0.86 ± 0.36 mg/dl for males (twenty subjects, 19–28 years old) and 1.01 ± 0.30 mg/dl for females (twenty subjects, 19–21 years old). There was good correlation between plasma ascorbic acid levels and dopamine-β-hydroxylase levels, reflecting activities of daily living, but no correlation was found between these levels and dietary consumption of vegetables or fruits.

INTRODUCTION

Ascorbic acid is a normal constituent of body fluids. Its relationship to health and the nutritional status of an individual and the decrease in plasma ascorbic acid that occurs in some diseases, such as diabetes mellitus, and in patients undergoing surgery have been reported [1–3]. On the other hand, there have been several reports on the relationship between ascorbic acid status and free radicals, ageing, immunity, cancers and regulation of enzymatic and cellular function [4–9]. Serum ascorbic acid therefore seems to be a index of wellness, because it might protect against cancer, ageing and lipid peroxidation as an antioxidant, and against diabetes mellitus.

Current assays of ascorbic acid in blood, plasma and urine involve chemical and colorimetric methods, and the values obtained vary [1,3]. HPLC systems have been reported to be useful for simple and specific determination of ascorbic acid [10,11].

We have devised a simple and convenient HPLC system to measure ascorbic acid levels in plasma, and its relationship to dietary intake and to dopamine-β-hydroxylase (DBH) activity in plasma as an index of activities of daily living (ADL).

EXPERIMENTAL

Samples and reagents

Heparinized blood samples for determining plasma ascorbic acid levels in males and females,

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and the correlation between them and dietary consumption, were obtained from healthy student volunteers (twenty males, 19–28 years old; twenty females, 19–21 years old), after informed consent was given. In the study of correlation between ascorbic acid and DBH, blood samples from six males and seven females (19–21 years old) were used; in this study ascorbic acid levels and DBH activities could be measured simultaneously.

All chemicals and reagents were of analytical grade or equivalent. All solvents and solutions used in HPLC were filtered through Millipore filters (Millex-GV 0.22 μm, SLGV025LS, Japan Millipore, Tokyo, Japan).

Dichlorophenolindophenol method

To determine the level of ascorbic acid in plasma, a modified colorimetric assay as described by Koba *et al.* [12] was used.

Assay of dopamine-β-hydroxylase (DBH) activity

A modification of the batch method of Watanabe *et al.* [13] was used to determine serum DBH activity.

HPLC instrumentation and conditions

The HPLC system consisted of a Tosoh gradient HPLC system with a Model CCPM pump, a Model UV-8000 detector and a Model FC microprocessor controller (Tosoh, Tokyo, Japan). A Hitachi (Tokyo, Japan) Model D-2000 chromato-integrator was also used with this system. A Tosoh ODS-80TM octadecyl silica gel column (150 mm × 4.6 mm I.D., 5 μm particle size) was obtained from Tosoh. The following mobile phases were used: (I) 2% methanol in 0.1 mol/l sodium phosphate monobasic, pH 4.4, and (II) 10% methanol in the same solution. Mobile phase I was pumped through the column at a flow-rate of 0.5 ml/min at room temperature. Samples of 5 μl were injected. Analyses were carried out at a wavelength of 265 nm and 1 cm/min chart speed. After chromatography, the column was purged with mobile phase II for 7 min before re-equilibration with mobile phase I, to remove more quickly other plasma components such as uric acid.

Pretreatment of plasma sample

Immediately or within a day after sampling by venepuncture, to 0.2 ml of each plasma separated, 0.8 ml of developer I and 0.2 ml of deproteinizing solution (0.5% thioglycolic acid in 5% metaphosphoric acid, 10% trichloroacetic acid or 5% acetic acid) were added. After subsequent centrifugation for 5 min at 1500 *g*, 5 μl of the supernatant were used as the sample injected into the column. All samples were assayed within a day to avoid oxidation of ascorbic acid to dehydroascorbic acid.

Estimation of dietary food consumption

We used the method of Morimoto *et al.* [14] to estimate food consumption. In this method individuals completed a questionnaire regarding their daily food intake for each of six groups: protein sources such as fish and meat; carbohydrate sources such as potatoes; fats and oils; milk and its products; fruits; and vegetables (red, green and yellow vegetables including strawberries and tomatoes). Scores of 1–4 (high, medium, low and none) were used to assess fruit and vegetable intake. The mean intake in a week was converted into a daily intake. Dietary information from forty volunteer students was obtained by personal interview in the last quarter of 1990.

RESULTS

Preparation of plasma samples

In preliminary experiments for deproteinization, we compared metaphosphoric acid, trichloroacetic acid and acetic acid. Recoveries of 1 mg of ascorbic acid added to plasma were 90.6–98.5% (mean = 93.9%, $n = 3$), 78.7–87.6% (mean = 82.6%, $n = 3$) and 53.8–76.6% (mean = 62.3%, $n = 3$), respectively. It was found that 5% metaphosphoric acid including 0.5% thioglycolic acid was most effective in recovering essential amounts of ascorbic acid in plasma supernatant after deproteinization.

Chromatographic separation

A binary solvent system (water–acetonitrile) failed to separate ascorbic acid by this HPLC method, because it showed a marked absorption at the maximal absorbance wavelength (265 nm) of ascorbic acid. We therefore used methanol–sodium phosphate monobasic solution as a mobile phase, and could separate ascorbic acid, metaphosphoric acid and thioglycolic acid in about 5 min at room temperature. After investigating chromatographic conditions such as volume of sample injected, flow-rate and concentrations of methanol and sodium phosphoric acid, baseline resolution of ascorbic acid was achieved under chromatographic conditions described in the Experimental section. Under these conditions, the retention times of metaphosphoric acid, ascorbic acid, thioglycolic acid and maleic acid as internal standard were 2.96, 4.47, 5.23 and 8.67 min, respectively (Fig. 1). The ascorbic acid peak was well separated from other peaks.

We confirmed that this peak is derived from ascorbic acid by preincubating the plasma with ascorbate oxidase (EC 1.10.3.3) (Fig. 2).

Determination of ascorbic acid in human blood

We compared the levels of ascorbic acid in plasma and serum by this HPLC method. No difference between the samples was observed. Heparin (sodium salt) and EDTA (sodium salt) were compared as anticoagulants: the former was chosen, because the retention time of the latter (3.91 min) was too close to that of ascorbic acid. After storage of plasma at 4°C for two days, an approximately 10% decrease in ascorbic acid level was observed (mean of two plasma samples measured, 0.99 → 0.88, 0.83 → 0.75 mg/dl); subsequently there was a further 2.9% decrease in one week.

Endogenous substances such as uric acid, fumaric acid and pyridine nucleotides did not interfere with ascorbic acid or the internal standard, maleic acid, when small amounts of each were added to plasma. Furthermore, the levels of these substances in plasma were low enough to be detected on the chromatogram.

Calibration curves used for quantitation of ascorbic acid in plasma, obtained by plotting peak heights against sample concentrations, were linear in the concentration range evaluated (0.5–3.0 mg/dl).

The accuracy and precision of the method were measured in the concentration range 0.5–3.0 mg/dl. The accuracy was high (mean recovery 101.2%), and within-day precision varied be-

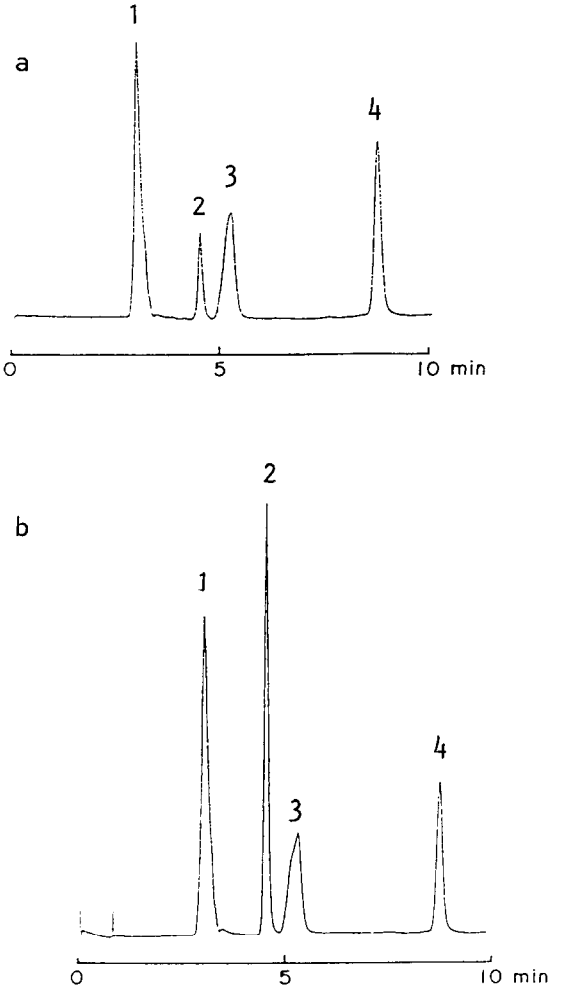

Fig. 1. HPLC of ascorbic acid in plasma and standard solution. Column: TSK gel ODS-80TM, 150 mm × 4.6 mm I.D. Mobile phates: (I) 2% methanol + 0.1 mol/l sodium dihydrogenphosphate, pH 4.4; (II) 10% methanol + 0.1 mol/l sodium dihydrogenphosphate, pH 4.4. Flow-rate: 0.5 ml/min. Detector: UV 265 nm. Peaks: 1 = metaphosphoric acid used for pretreatment; 2 = ascorbic acid in plasma (a) and 3 mg/dl standard solution (b); 3 = thioglycolic acid used as an antioxidant; 4 = maleic acid as an internal standard.

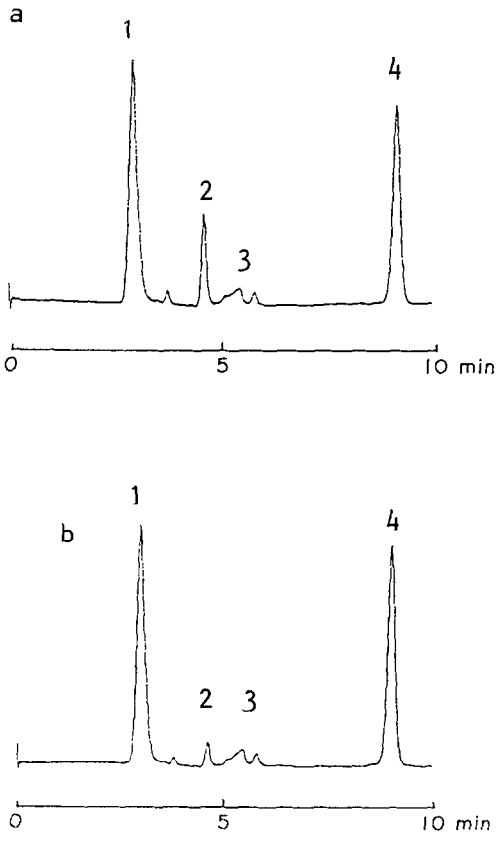

Fig. 2. HPLC of ascorbic acid in plasma before and after treatment with ascorbate oxidase. Plasma was incubated with 0.2 U/ml ascorbate oxidase at 30°C and at pH 5.6 for 5 min. (a) Before treatment, (b) after treatment. Peaks: 1 = metaphosphoric acid; 2 = ascorbic acid; 3 = thioglycolic acid; 4 = maleic acid.

tween 3.4 and 5.7%. The precision determined by repeated assays on four consecutive days of pooled plasma samples, which were stored in aliquots at −80°C after deproteinization with metaphosphoric acid, gave day-to-day values of 4.8–6.2% for three kinds of plasma and 4.2% for plasma spiked with 1.0 mg/dl ascorbic acid.

The detection limit of this method was 0.01 mg/dl ascorbic acid. The correlation coefficient was 0.714 between this HPLC method and the conventional dichlorophenolindophenol (DCIP) method (Fig. 3), but plasma ascorbic acid levels obtained by the present method were slightly lower than those obtained by the conventional method.

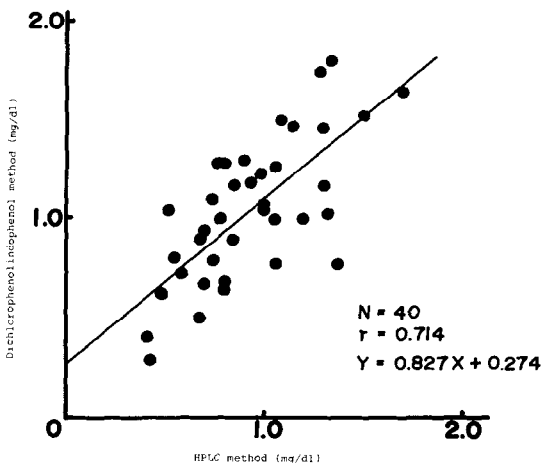

Fig. 3. Correlation between the present HPLC method and the dichlorophenolindophenol method for determination of plasma ascorbic acid levels.

Application to health and nutritional profiles

The plasma concentrations of ascorbic acid measured in twenty healthy subjects, and dietary food intake, are shown in Table I. No significant difference between mean values of males and females was observed as determined by Student's *t*-test. The differences in dietary vegetables and fruits between males and females were also non-significant. Serum levels of DBH and ascorbic acid measured in thirteen subjects are shown in Table II. The significance of coefficients was tested by paired *t*-test for simple correlation coefficients. A correlation between them was observed, with a correlation coefficient of 0.725. However, no correlation was noted between the levels of ascorbic acid and dietary consumption of vegetables and fruits.

DISCUSSION

We consider that the present HPLC method for assaying plasma ascorbic acid levels is sufficiently simple and easy to use for routine application in any laboratory, in spite of the analysis time (20 min) and the use of a two mobile phase system. However, ascorbic acid is readily oxidized to dehydroascorbic acid owing to its acidity [15]. After allowing plasma to stand at 4°C for

TABLE I

ASCORBIC ACID LEVEL IN PLASMA AND DIETARY CONSUMPTION OF VEGETABLES AND FRUITS

	Male	Female	Difference
Ascorbic acid (mean ± S.D.) (mg/dl)	0.86 ± 0.36	1.01 ± 0.30	$p > 0.100$ $n = 20$
Dietary consumptions of vegetables and fruits (score[a], mean ± S.D.)	1.25 ± 0.51	1.29 ± 0.31	$p > 0.100$ $n = 20$

[a] The total number of servings of fruit and red, green and yellow vegetables per day as follows. Vegetables: 0, none; 0.5, low intake (cut vegetables on one hand grip); 1.0, medium intake; and >1.0, high intake. Fruits: 0, none; 0.5, low intake; 1.0, medium intake (one medium size apple, for example); and >1.0, high intake.

two days, a 10% decrease in ascorbic acid level was observed, followed by a further 2.9% decrease after one week. We assayed all samples within a day to avoid this decrease. As a result, we found no effect of dehydroascorbic acid on ascorbic acid determination, although Lopez-Anaya and Mayersohn [10] have found a non-negligible concentration of dehydroascorbic acid in some plasma. VanderJagt *et al.* [16] and Dharival *et al.* [17] reported that dehydroascorbic acid was not detectable in plasma and urine using their HPLC methods.

Regarding the slightly lower values of plasma ascorbic acid level found in the correlation experiment compared with those of the conventional DCIP method, it is considered that UV radiation probably caused some reduction in ascorbic acid during HPLC analysis.

The DCIP method correlates well with the HPLC method [16]. It was confirmed that cysteine, glutathione, glucose, sucrose and glutamic acid do not interfere with the determination under DCIP titration method. However, this method has the disadvantage that stopped-flow apparatus is necessary for more accurate and rapid kinetic assay, which is not routinely used [18,19].

Several reports have determined normal human plasma levels of ascorbic acid using HPLC [11,17,20,21]. These levels, expressed in mg/dl (normal human beings aged 18–63 years), are

TABLE II

CORRELATION BETWEEN PLASMA ASCORBIC ACID LEVEL, DBH LEVEL AND DIETARY CONSUMPTION OF VEGETABLES AND FRUITS

DBH = Dopamine-β-hydroxylase activity. Values are mean ± S.D.

Ascorbic acid (mg/dl)	DBH (μmol/min/l)	Dietary consumption (score)	Significance of coefficients
0.77 ± 0.23	17.9 ± 8.8		$r = 0.725$ df = 11[a] $p < 0.010$
0.93 ± 0.31		1.26 ± 0.41	$r = 0.286$ df = 38[b] $0.025 < p < 0.050$

[a] Sample size = 13 (six males and seven females, 19–21 years old); $y = 0.019x + 0.422$.

[b] Sample size = 40 (twenty males and twenty females, 19–28 years old); $y = 0.380x + 0.913$.

0.75–1.92 (male and female), 0.36–1.75 (male) and 0.71–1.57 (female) and 0.83–1.10 and 0.86 ± 0.54 (male and female). Mean values in our study are in agreement with these reports. Fujino *et al.* [2] noted a difference in mean values between males and females, but no such difference was observed in our study.

It has been speculated that the size of the ascorbic acid pool in human is only 1.5 g, that it can be maintained by daily physiological intakes of this vitamin, and that excretion into the urine also affects its intake [22,23]. It is possible that ascorbic acid levels in plasma are dependent upon both the pool size and excretion rate of ascorbic acid, *i.e.* amounts of intakes.

We compared the dietary consumption of vegetables and fruits and plasma levels of ascorbic acid in males and females, but no correlation between them was observed. Regarding this lack of correlation, it is necessary for us to study further details of the dietary questionnair on daily food intake according to the ordinary survey method, because the method of Morimoto *et al.* [14] is a "convenient method" for the estimation of dietary consumption.

DBH is a copper-metalloenzyme, and its inactive form is converted into an active form by ascorbic acid [15]. It is considered that a high plasma ascorbic acid level and the greater body pool might enhance DBH activity. This suggests that plasma ascorbic acid level can be used as an index of ADL. DBH activity in plasma is considered to reflect ADL [24–28]. Since DBH and noradrenaline are both released from the sympathetic nervous system during stress and exercise [28], their release seems to be proportional to ascorbic acid release. Further study of plasma ascorbic acid levels is required for physiological or pathological status, and health care. Our HPLC method for ascorbic acid determination in plasma seems to be convenient for this purpose.

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