

Simultaneous Determination of Urinary Ascorbic Acid and Creatinine by High-performance Liquid Chromatography

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Abstract

A simple high-performance liquid chromatographic method is described for the determination of ascorbic acid and dehydroascorbic acid. Use of tetra-*n*-butylammonium bromide as an ion-pairing reagent in the mobile phase yielded complete separation of ascorbic acid. Total ascorbic acid was determined by reducing the dehydroascorbic acid to ascorbic acid through treatment with DL-homocysteine. The high-performance liquid chromatographic method was then applied to the analysis of human urine. This method can also be available for the simultaneous determination of urinary ascorbic acid and creatinine.

Introduction

Ascorbic acid (AA) and dehydroascorbic acid (DHAA) are equally biologically active forms of vitamin C in human and guinea pig (1).

Therefore, AA and DHAA should be determined to know the total amount of vitamin C in vegetables.

AA has been determined by the dye-titration method using 2,6-dichlorophenolindophenol. Total ascorbic acid (TAA) comprises the sum of AA and DHAA is determined by the colorimetric method using 2,6-dichlorophenolindophenol by oxidizing AA to DHAA and using 2,4-dinitrophenylhydrazine (DNPH) by subsequent formation osazone (2). However, these methods are time-consuming and may overestimate AA and DHAA owing to the presence of oxidizable compounds other than vitamin C in urine samples. In addition, it is difficult to visually determine the titration endpoint.

Recently, several high-performance liquid chromatographic (HPLC) methods have been developed for analysis of AA and DHAA. These methods adopt various column materials, mobile phases and ultraviolet (UV) or electrochemical detectors(3-6). AA was well separated from other compounds in orange juice by use of an ion-pairing reagent in the mobile phase (7,8). Determination of TAA by HPLC requires a rapid method of converting necessary for detection. Hughes (9) described a rapid and complete reduction of DAA to AA by DL-homocysteine.

In this study we report relatively simple HPLC procedure for estimation of DHAA to AA by DL-homocysteine and uses tetra-n-butylammonium bromide as an ion-pairing reagent in the mobile phase. This method was used for simultaneous determination of urinary AA and creatinine (Cr). TAA contents in vegetables were determination in order to compare our HPLC method with the DNPH method.

Materials and methods

Reagents. DL-homocysteine hydrate was obtained from Aldrich Chemical Company, Inc., Milwaukee, Wi, USA. L(+)-ascorbic acid, creatinine, tetra-n-butylammonium bromide and other chemicals were obtained from the Wako Pure Chemical Ind., Ltd., Osaka, Japan. All the reagents used were of reagent grade.

High-performance liquid chromatography. Separation of AA was achieved with a Yanaco L-5000 liquid chromatographic apparatus equipped with a Rheodyne Model 7125 injector. Column effluents were monitored at 265 nm with a Yanaco M-515 variable-wavelength detector. Peak areas were determined using an SIC Chromatocorder 12. A Shodex RSpak DE-613 column (150 mm × 6 mm inside diameter) was used. The mobile phase was composed of 8 mM phosphate buffer, pH 6.8, containing 3 mM tetra-n-butylammonium bromide. The flow rate was 1.0 ml/min.

Sample preparation. Urine; A volunteer (37 year-old, male) took a single oral dose of 225 mg, 150 mg and 75 mg of AA separately. Urine was collected every one hour. Two ml of urine were received 2 ml of 10% metaphosphoric acid. The mixture was centrifuged for 20 min at 3000 rpm. The supernatant was diluted 40-folds with distilled water.

Vegetable; Ten g of Pakuchoi and Tahtuai which are Chinese vegetable were homogenized with 10 ml of 10% metaphosphoric acid in a mortar with sea sand. The slurry obtained was transferred to a centrifuge tube with 20 ml of 5% metaphosphoric acid and centrifuged for 20 min at 3000 rpm. The supernatant was diluted 20-folds with distilled water.

AA assay. One ml of 0.08 M K_2HPO_4 was added to 4 ml of diluted urine sample to give a

final pH of 7.0. For vegetables, a 10-ml of diluted supernatant was diluted with 0.13 ml of 2.5 M K_2HPO_4 to give a final pH of 7.0. A 20- μ l aliquot of this solution was injected into the HPLC system.

TAA assay. TAA was assayed by adding 0.015 g of homocysteine to 5 ml of neutralized sample for AA assay. After 30 min at 25°C, a 20- μ l aliquot of this solution was injected into the HPLC system. The concentration of DHAA was calculated by subtracting the amount of AA from that of TAA.

Results

Separation of AA and Cr. Typical chromatograms are shown in Fig.1 for the elution of standard mixed solutions of AA and Cr (A) and those in urine administered AA orally (B). Retention times were 4.1 min for standard Cr and 4.6 min for standard AA. The peaks of the same retention time as standards were seen in human urine. AA and Cr were completely separated from other unknown peaks. Fig.2 shows chromatograms of AA in vegetables before and after incubation with homocysteine. The homocysteine treatment resulted in the appearance of extra peak at 3.5 min due to homocysteine, but it could be readily separated from AA.

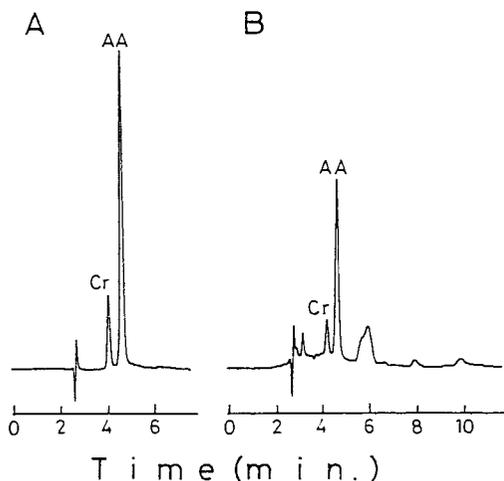


Fig.1 Chromatograms For (A) standard AA (1.6 μ g/ml) and Cr (24 μ g/ml), (B) AA and Cr in human urine.

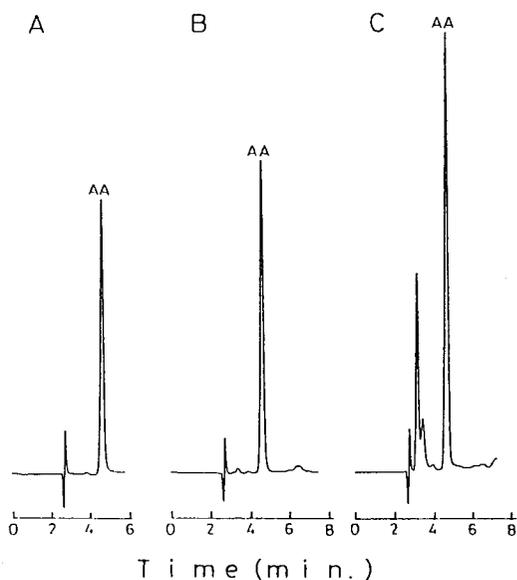


Fig.2 Chromatograms for (A) standard AA(2 μ g/ml), (B)AA in Chinese vegetable, (C) AA in Chinese vegetable incubation with homocysteine.

Table 1 Recoveries of ascorbic acid(AA) and creatinine(Cr) from urine.

	Added (μ g/ml)	Found (μ g/ml)	Recovery (%)
AA	0	18	
	50	66	96.0
	100	117	99.0
	150	168	100.0
	200	222	102.0
Cr	0	1325	
	500	1806	96.2
	1000	2330	100.5

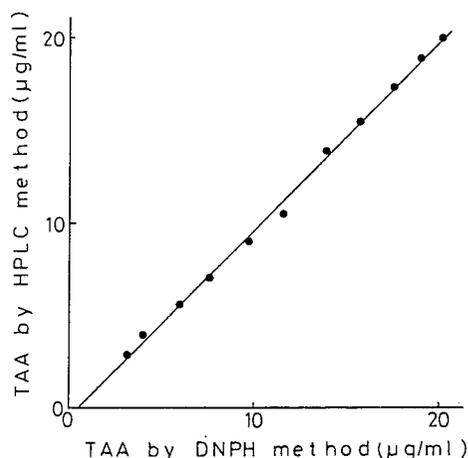


Fig.3 Correlation between quantities of TAA estimated by DNP method and by this HPLC method.

Recovery. Recoveries of AA added to urine ranged from 96.0 to 102.0% and those of Cr from 96.2 to 100.5% (Table 1).

Correlation. A comparison was made of the concentrations of TAA found in various vegetables by DNP method and those by our HPLC method (Fig. 3). The relationship between the values obtained by the two methods was described by a regression line $y=0.9997x - 0.3657$, $r=0.9979$ ($p<0.001$), where x represents the values obtained by DNP method and y represents those obtained by our HPLC method.

Analysis of AA in urine administered AA orally. Urinary TAA, AA and DHAA were analyzed in order to compare the urinary concentrations (Table 2). The urinary excretion of DHAA in TAA ranged from 2.5 to 3.9 %.

Time courses of urinary AA excretion in relation to the Cr excretion after oral administration of 225 mg, 150 mg and 75 mg of AA separately are shown in Fig.4. Urinary excretion of AA related to the Cr excretion reached its maximum values in 3 hours in each dose of AA. A small peak was also observed in 8 hours after administration of all kinds of dose.

Table 2 Comparison of the urinary concentration of DHAA with that of TAA.

Concentration in urine(mg/ml)		
TAA	AA	DHAA
0.435	0.418	0.017(3.9)
0.345	0.333	0.012(3.5)
0.360	0.351	0.009(2.5)
0.212	0.205	0.007(3.5)

Numbers in parentheses indicate percentages(%) of DHAA in TAA.

TAA : total ascorbic acid

AA : ascorbic acid

DHAA : dehydroascorbic acid

Determination of Urinary AA and Cr by HPLC

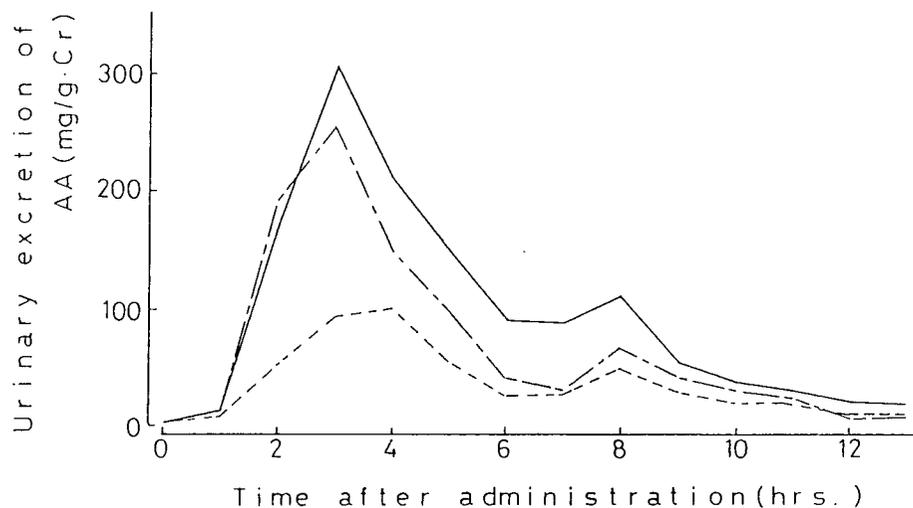


Fig.4 Urinary excretion of AA related to the Cr excretion as a function of time after oral administration of AA(225mg———, 150mg ······, 75mg ······).

Discussion

Determination of TAA by HPLC requires a rapid conversion of DHAA to AA, since DHAA does not absorb UV ray which is necessary for detection. Hughes (9) described a simple and convenient procedure for the reduction of DHAA to AA with DL-homocysteine. This method has been applied to the analysis of TAA by HPLC (10,11). In the present assay, use of this reducing reagent also produced excellent agreement between the colorimetric method using DNPH and our HPLC method as shown by correlation coefficient ($r=0.998$).

The retention time of AA was delayed by the increase in amount of tetra-n-butylammonium bromide as an ion-pairing reagent in the mobile phase. Use of tetra-n-butylammonium bromide in condition of this study gave complete separation of AA, Cr and other unknown peaks in urine. In analysis of AA in vegetables, a clear peak of AA was also obtained, and the separation was not interfered by peak of DL-homocysteine added in sample.

Our HPLC method appears to have a higher specificity than the colorimetric method, because the values obtained by the colorimetric method were higher than the HPLC values in many of the samples.

We determined the amount of urinary excretion of AA after oral administration of AA. AA was the major compound excreted in urine in comparison with DHAA. The purpose of

this investigation was to examine a possibility of monitoring vitamin C intake using the simple assay method of urinary AA. A special merit of this HPLC method is that urinary Cr can be determined simultaneously. Cr adjustment has been thought to be an effective measure in case of the spot urine specimen which is very concentrated or diluted. several studies have been reported for the examination of the urinary excretion of AA after administration of a large dose of AA (12,13). In the present paper, we examined the excretion when a small dosage of AA was taken. Urinary excretion of AA corrected by Cr excretion reached its maximum value in 3 hours after administration of AA. This seems to be related with the fact that the peak plasma vitamin C concentration occurred in 3 hours after oral administration of vitamin C (250 mg) as reported by Murata et al (14). Therefore, we propose that the intake of vitamin C can be monitored by measurement of urinary AA 3 hours after meal.

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