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Stability of Total Ascorbic Acid in DTT-Preserved Plasma Stored at 4˚C

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ABSTRACT

In order to evaluate the stability of total ascorbic acid (TAA) after storage in refrigeration at 4˚C, EDTA-blood was collected from 12 volunteers and preserved with dithiothreitol (DTT) immediately. After sampling, the blood was portioned into 19 micro-centrifuge tubes. One tube of sample was examined immediately by the capillary electrophoresis (CE) method, while the others were stored in refrigeration at 4˚C until analysis. TAA analysis were performed once every 4 hr for a total of 72 hr after the sample collection. Our results showed that TAA concentrations in these specimens did not alter significantly within the first 16 hr of storage; however, TAA levels started to decline significantly ($p < 0.05$) thereafter. These data demonstrated that DTT could be an effective preservative in maintaining the stability of TAA in a refrigerated temperature for up to 16 hr. Thus, it is advised that if the determination of TAA cannot be performed immediately, specimens must be preserved with DTT at the refrigerated temperature and assayed within 16 hr in order to prevent the occurrence of inaccurate results.

Key words: total ascorbic acid, dithiothreitol, capillary electrophoresis

INTRODUCTION

There has been increasing attention to the importance of oxidative damage of lipids, proteins and other constituents of biological structures in diseases and aging[(1)]. Strong evidence can be found in the literature supporting vitamin C as the most active water-soluble antioxidant[(2)]. Vitamin C provides antioxidant protection against degenerative diseases (such as cancer and cardiovascular disorders) through its free-radical scavenging properties[(3)]. Therefore, the vitamin C assay in body fluids has gained its popularity for the management of certain clinical conditions. Vitamin C exists in a reduced form, namely ascorbic acid (AA), and an oxidized form, dehydroascorbic acid (DHAA), *in vivo*. Oxidative stress is believed to be responsible for the loss of AA and the resulting increase in the DHAA concentration in plasma. Since AA is unstable and easily oxidized to DHAA, it is imperative that specimens are properly prepared. Okamura[(4)] reported that once DHAA was reduced to AA by incubating the sample with dithiothreitol (DTT), the total ascorbic acid (TAA, the sum of AA and DHAA) could be determined. Margolis and Duewer[(5)] demonstrated that the TAA in lyophilized plasma samples containing DTT was stable for at least 6 years period when it was stored at -70˚C. In addition, the TAA in frozen serum preserved with metaphosphoric acid (MPA) degraded no more than 1% of the total mass per year over a 2-year period when it was stored at -70˚C. The measurement of DHAA and AA concentrations is sensitive to variuos factors, including the sample collection method, storage condition, assay condition, and assay procedure[(6)]. Some investigators had evaluated the stability of AA in plasma or serum by assaying the concentrations of the AA after different storage periods[(7-9)]. However, the specimens for those studies were either plasma or serum pools that were stored at -70˚C or -25˚C prior to analysis. It was troublesome to separate the serum and freeze the specimen immediately for clinical tests. For this reason, it is convenient if the specimens in transit are preserved under refrigerated temperature. However, no related study for the TAA determination had been reported using repeated assays of the same specimens under refrigerated temperature. Our report was to evaluate the suitability of utilizing DTT as a protector for the maintenance of specimen integrity prior to the determination of TAA by capillary electrophoresis (CE) at a refrigerated temperature. Our data indicated that if the DTT-preserved specimen was preserved under a refrigerated temperature, the TAA could remain stable for 16 hr without deterioration.

MATERIALS AND METHODS

I. *Sample Procurement and Processing*

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Venous blood from 12 volunteers, including 4 males and 8 females, was collected in ethylenediamine tetraacetic acid (EDTA) vacuum tubes (Becton Dickinson). Volunteers were asked to fast overnight prior to sample collection. All blood samples (10 mL) were preserved with 15 μL of 1 M DTT immediately and mixed well. Each volunteer's blood was portioned into 19 micro-centrifuge tubes (500 μL), one of which was assayed by the capillary electrophoresis (CE) method immediately, while the remaining were then assayed in 4-hr intervals for a total of 72 hr. The processed blood was stored at 4˚C till analysis. Using a modified method of Koh *et al*[10], each aliquot of the blood was centrifuged, and the plasma (200 μL) was separated and mixed with isoascorbic acid (50 μg/mL) as an internal standard. The mixtures of plasma were then mixed with 0.1 mL of 12% trichloroacetic acid (TCA) to deproteinate the sample. The solution was vortexed for 30 sec. After centrifugation of 12,000 rpm for 10 min at 4˚C, the supernatants were extracted with an equal volume of diethyl ether to remove the remaining protein. The aqueous phase was filtered through a 0.20-μm membrane syringe filter prior to use.

II. *Standard Solution Preparation*

Stock standard solution of ascorbic acid (1 g/L) and internal standard solution of isoascorbic acid (1 g/L) were prepared in the solution containing 1 mM EDTA/3% MPA freshly. Then, the ascorbic acid and isoascorbic acid were diluted to make various concentrations of solution to construct the calibration curve.

III. *Total Ascorbic Acid Determination*

Capillary Zone Electrophoresis (CZE) separation was carried out on a capillary electrophoretic instrument (P/ACE MDQ system, Beckman, Palo Alto, CA, USA). The uncoated fused-silica capillary was purchased from Polymicro Technologies (Scottsdale, AZ, USA). Each sample was pressure-injected into the capillary for 10 sec by an autosampler. Between successive electrophoretic runs, each capillary was rinsed sequentially with 0.1N NaOH, distilled water and running buffer for 2 min.

The size of the capillary was 60 cm × 75 μm i.d. and the length to the detector was 50 cm. After the capillary column was installed into a capillary cartridge, the capillary was conditioned by pressuring with ethanol and 1N HCl for 10 min, water for 5 min, 1N NaOH for 10 min and water for 5 min, followed by equilibration with a 100 mM running buffer (Tricine buffer pH 8.8) for 10 min. The separation step was conducted at a constant temperature of 25.0 ± 0.1˚C by means of continuous circulation of fluorocarbon liquid through the cartridge. The applied voltage was set at 17 kV across the capillary, and on-column UV absorption at 254 nm was used for the quantitation of the TAA.

IV. *Statistics*

Data were expressed as mean ± standard derivation. The analyzed values in this study were compared by the repeated-measures analysis of the variance method. Moreover, the analyzed values of the 12 volunteers at each time interval were compared using the least significant difference test. When the probability was less than 0.05, the difference was considered statistically significant.

RESULTS AND DISCUSSION

Some investigations demonstrate that CE is a precise analytical method for the quantitative analysis of AA[10,11]. In this study, the quantitative analysis of the TAA by CE was also useful as evident by excellent coefficient of variation (CV) obtained (Table 1). The mean analytical recovery for the TAA was 102.4% and the standard deviation was 5.7% in the recovery test. A representative CE chromatogram of the TAA analysis is shown in Figure 1.

AA, an essential vitamin found in fruits and vegetables, has been studied extensively in its role as an antioxidant. Furthermore, the most important role of AA is formation and stabilization of collagen, a major component of all connective tissues, by hydroxylation of proline and lysine for cross-linking. AA also functions in the conversion of tyrosine to catecholamines and is present in high concentrations in the human brain. It not only enhances the absorption of iron, but also possibly involves heme function. Therefore, the determination of AA is very important in clinical tests. DHAA should be undetectable in plasma samples, which were treated with an anticogulant and then centrifuged at 1,000×g for 15 min at room temper-

Table 1. Precision data of total ascorbic acid determination by the capillary electrophoresis method

Specimen	N	Mean ± SD (mg/L)	CV (%)
I	9	4.98 ± 0.12	2.5
II	9	9.75 ± 0.35	3.6
III	9	19.16 ± 0.28	1.5

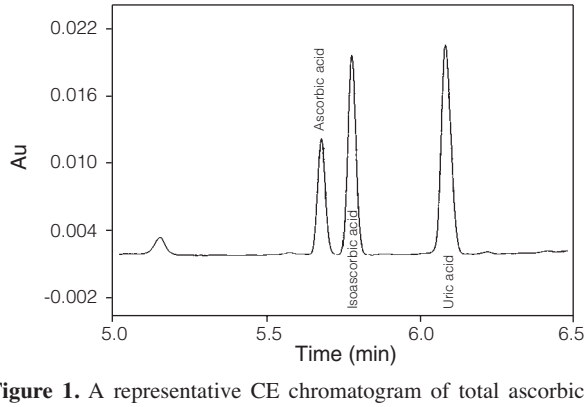

Figure 1. A representative CE chromatogram of total ascorbic acid determined in a sample.

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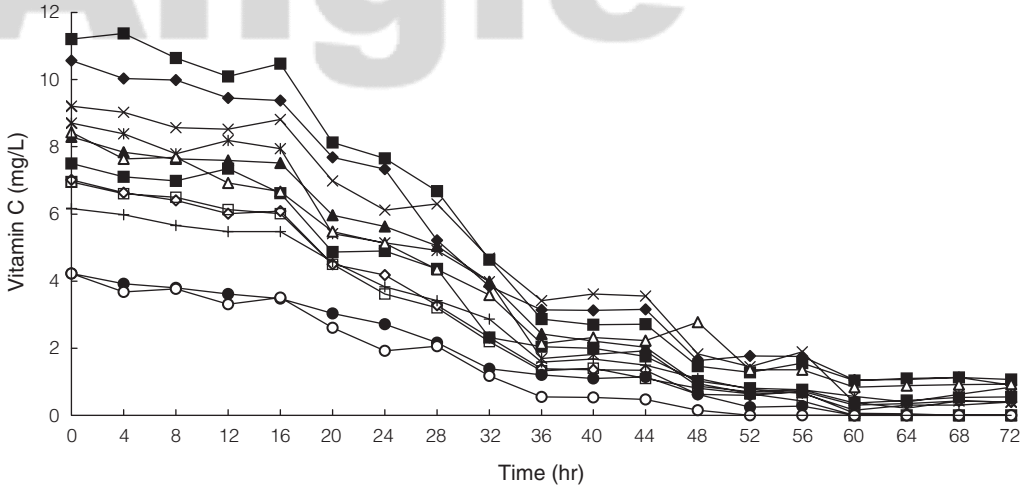

Figure 2. Stabilities of total ascorbic acid determined by capillary electrophoresis on 12 plasma specimens preserved with DTT and stored at 4˚C. The assay was performed sequentially in 4-hr intervals for a total of 72 hr after the sample collection.

ature[12]. Because AA is unstable and easily oxidized to form DHAA which is in term quickly degraded to 2,3-diketogulonate ($t_{1/2}$ = 1-2 min.)[12]. On the contrary, in many reports acidic deproteinization was adopted as a pretreatment of plasma samples for the quantitation of AA and DHAA[13,14]. MPA and TCA are two commonly used acids[6]. Although acidic deproteinization has been the accepted sample pretreatment for TAA quantitation, Stempak *et al*.[15] found out that numerous MPA concentrations (5%~15%) tested were inefficient at removing protein from the samples. In general, TCA has better efficiency of deproteinization with (5%~15%) than MPA. Therefore, the plasma samples for the measurement of AA and DHAA had been deproteinized with 12% TCA in this study. Because DTT reduces DHAA to AA, DTT acts as an ideal antioxidant for TAA analysis. As a result, the TAA concentrations of the samples preserved in the presence of DTT were stable for more than one year under storage at -80˚C. However, the samples required rapid pretreatment with DTT, as well as further acidification and deproteinization with TCA. Such procedure is troublesome in clinical studies, where immediate treatment and freeze may be technically difficult. Frozen specimens, therefore, were suitable for epidemiological studies. It is convenient to store the specimens in transit under the refrigerated temperature in clinical performances.

In our study, all blood specimens were preserved with DTT and stored at 4˚C until analysis. The concentrations of TAA determined by capillary electrophoresis are shown in Figure 2. The TAA concentrations did not show significant variation during the first 16 hr. Nevertheless, the concentrations dropped sharply thereafter. The TAA concentrations of plasma at the 16th hour after sample collection show significant differences ($p < 0.05$) from those at the 20th hour. Fresh plasma for TAA determination is the best choice in clinical performances. However, if the specimens for TAA determination cannot be performed immediately, attention must be paid to the period of storage. Our data indicated that if the DTT-preserved specimen was stored under a refrigerated temperature, TAA could remain stable for 16 hr without deterioration.

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