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Short Communication

Ascorbate and Dehydroascorbic Acid as Reliable Biomarkers of Oxidative Stress: Analytical Reproducibility and Long-term Stability of Plasma Samples Subjected to Acidic Deproteinization

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Abstract

Lack of post-sampling stability of ascorbate and dehydroascorbic acid and failure to block their *in vivo* equilibrium have lowered their value as biomarkers of oxidative stress and limited the ability to further investigate their possible role in disease prevention. In the present article, analytic reproducibility was tested by repeated analysis of plasma aliquots from one individual over 4 years. The plasma was subjected to acidic deproteinization with an equal volume of 10% *meta*-phosphoric acid containing 2 mmol/L of EDTA and analyzed for ascorbate and dehydroascorbic acid by high-performance liquid chromatography with coulometric detection. In a parallel experiment, the stability of human plasma samples treated as above and stored at −80°C for 5 years was tested in a cohort of 131 individuals. No degradation or shift in the equilibrium between ascorbate and dehydroascorbic acid was observed in either of the experiments. In conclusion, ascorbate and dehydroascorbic acid could be adequately preserved in plasma stored at −80°C following acidic deproteinization with *meta*-phosphoric acid containing 2 mmol/L of EDTA. (Cancer Epidemiol Biomarkers Prev 2007;16(11):2513–6)

Introduction

Ascorbate and dehydroascorbic acid (DHA) have been used as biomarkers of oxidative stress for several years (1). Ascorbate acts as a major radical scavenger *in vivo* as well as in regenerating other antioxidants (2). Vitamin C exists predominantly in its reduced state *in vivo* and upon oxidation via the semidehydroascorbyl radical, the resulting DHA is transported into, e.g., erythrocytes and subsequently regenerated intracellularly to ascorbate by one of several possible mechanisms (3). Being placed low in the antioxidant hierarchy (4), ascorbate is considered a biomarker of oxidative stress and offers a general indication of the antioxidant status of an individual. Increased DHA, on the other hand, more specifically suggests a redox imbalance and inadequate recycling capacity (5-7). However, although a considerable body of epidemiologic literature suggests an inverse correlation between plasma ascorbate and the risk of developing cancer and cardiovascular disease, controlled trials have thus far been unsuccessful in showing the health benefits of vitamin C supplementation (1). More recently though, ascorbate is being reexamined for its possible antitumorigenic activity following i.v. infusion (8, 9).

Accurate and reproducible analyses of ascorbate and DHA are necessary prerequisites for their application as biomarkers of oxidative stress as well as understanding their possible roles in disease prevention in general. However, proper quantification of ascorbate and DHA in biological samples poses a significant challenge in analytic clinical chemistry. This is partly due to the labile nature of the compounds as well as the fact that they are in chemical and biological equilibrium with each other. In other words, ascorbate is rapidly oxidized to DHA *ex vivo* if steps are not taken to prevent this reaction (10). In the older literature on ascorbate, these issues were rarely taken into account and this often led to inconsistent and misleading conclusions. In recent years, the stability of the analytes and efficient blocking of their *in vivo* equilibrium has become increasingly recognized as important variables on par with traditional analytic virtues such as specificity, sensitivity, and the absence of interfering compounds. Unfortunately, articles are still published in which sample handling, storage, and analysis has not been properly controlled, suggesting that a continued emphasis on these matters is needed.

One important criterion for a biomarker in general is that the sample from which it is measured should be stable upon storage (11). This is of particular importance in large clinical studies where the collection and analysis of samples takes place over time. For vitamin C analysis, the relevance of this aspect is further emphasized by the lability of the compound. Another significant problem

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with regard to the determination of DHA is that no sensitive, direct, and specific assay for DHA is currently available. Consequently, DHA is usually calculated by subtraction of the ascorbate concentration from that of total ascorbate (ascorbate + DHA) measured after reduction (12). Although this concept remains the best available, it amplifies the requirement for accurate measurement. Consequently, considerable effort has been put into developing and testing various storage conditions and their effect on stability. In particular, Margolis and coworkers have consistently pursued the improvement of reproducibility in vitamin C analyses within and between laboratories (13-16). Most work has focused on the general and less critical measurement of vitamin C or ascorbate, but a few studies have also investigated the combined measurement of ascorbate and DHA and found almost complete preservation of total ascorbate (preserved with reductant) for up to 6 years, but not of ascorbate, suggesting that the *in vivo* equilibrium between ascorbate and DHA was not successfully blocked (14, 15, 17).

The most commonly used method of stabilizing biological samples for vitamin C analysis is by acidic deproteinization with *meta*-phosphoric acid (MPA; refs. 10, 14, 15, 18). We also include EDTA in our sample preparation solution to chelate divalent metal ions which would otherwise promote oxidation of ascorbate to DHA (19). We have previously reported that no significant changes in ascorbate and DHA concentrations could be observed following up to 2 months of storage (10). In the present article, data from two experiments are reported: the reproducibility of ascorbate and total ascorbate measurements in plasma aliquots subjected to repeated analysis over 4 years, and the stability of plasma ascorbate and DHA in a cohort of 131 individuals after 5 years of storage.

Materials and Methods

Experiment I. Venous blood was drawn from one apparently healthy volunteer with a butterfly needle (21-gauge) carefully avoiding hemolysis. Following centrifugation for 5 min at 2,000 × *g*, the plasma was acidified with an equal volume of 10% (w/v) MPA containing 2 mmol/L of disodium-EDTA. The precipitate was removed by centrifugation (5 min, 2,000 × *g*) and the supernatant was stored in 60 aliquots in 0.5 mL microcentrifuge tubes at −80°C until analysis. Ascorbate and total ascorbate were quantified as described previously (20). Briefly, ascorbate, and subsequently, total ascorbate following reduction with tris(2-carboxyethyl)-phosphine hydrochloride were quantified by high-performance liquid chromatography with coulometric detection. DHA was calculated by subtraction. Analysis was carried out in triplicate at 20 different time points over 4 years, i.e., a total of 60 aliquots were analyzed.

Experiment II. Individual blood samples from a cohort of 131 apparently healthy volunteers (21, 22) were obtained and analyzed as described above. After 5 years, the samples were reanalyzed essentially as described in ref. 20, except for the use of an Onyx Monolithic C18 column (100 × 4.6 mm i.d.; Phenomenex) at a flow rate of 1.6 mL/min resulting in a retention time of ascorbate of ~1.3 min. DHA was calculated by subtraction using uric acid as an internal standard (20).

Statistical evaluation (Statistica v. 7.0, StatSoft) of the data was done following Kolmogorov-Smirnov and Lilliefors tests of normality of the distribution pattern. Experiment I was analyzed using linear regression. Experiment II was analyzed by using paired parametric statistics. A $P < 0.05$ was considered significant.

Results

Analytic reproducibility was tested by repeated analysis of aliquots of stabilized plasma obtained from one individual. The results from experiment I are shown in Fig. 1 and Table 1. Within-day coefficients of variation averaged 1.3% and 1.7% for ascorbate and total ascorbate, respectively. Between-day values were 3.3% and 4.1%, respectively, including all data (Table 1). Linear regression analysis showed no significant change in ascorbate or total ascorbate over the course of the 4-year study period.

The stability of plasma samples subjected to acidic deproteinization was further evaluated by reanalyzing a set of samples from a cohort of 131 volunteers. Plasma ascorbate was normally distributed within the cohort (data not shown) and the results of the analyses are presented in Table 2. Overall, very small differences were observed between study start and 5 years later. No statistical differences were observed between ascorbate and DHA measurements at study start and after 5 years, whereas a significant 1.4% increase was observed for total ascorbate (Table 2). Dividing the cohort into quartiles showed no correlation between initial ascorbate

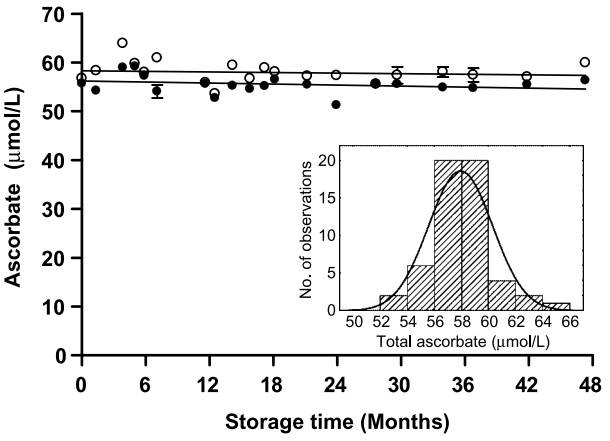

Figure 1. Quantification of ascorbate and total ascorbate (following reduction) in plasma aliquots stabilized with an equal volume of 10% of MPA containing 2% EDTA. The samples were stored at −80°C for up to 48 mo. No significant effect of storage time was observed on ascorbate (●) or total ascorbate (○). *Points,* mean from three individual aliquots of the parent sample; *bars,* SD (absence of error bars indicates that they are smaller than the dot size). *Inset,* the data were normally distributed as tested by the Kolmogorov-Smirnov and Lilliefors tests of normality.

Table 1. Reproducibility of ascorbate and total ascorbate analysis

	Ascorbate	Total ascorbate
Concentration (μmol/L)		
Study start	55.7 ± 0.8	56.7 ± 0.3
After 48 mo	56.3 ± 0.1	59.9 ± 1.1
Average	55.6 ± 1.8	57.7 ± 2.4
%CV (within-day)	1.3	1.7
%CV (between-day)	3.3	4.1
L.R. slope	−0.034	−0.020
L.R. intercept	56.2	58.3
Rate of change (%/y)	−0.7	−0.4

NOTE: Twenty aliquots were analyzed in triplicate over a period of 4 y. The ascorbate and total ascorbate concentrations did not change significantly over the 4-year period.

level and 5-year change (Table 2). A significant inverse correlation between initial DHA concentration and 5-year change was observed ($P < 0.001$).

Discussion

Four years of repeated analyses of ascorbate and total ascorbate in aliquots of stabilized plasma confirmed the robustness of the sample preparation and analytic method. Overall, no significant change in either measure over the 4-year study period was observed (Table 1).

Previously, Comstock et al. examined the stability of ascorbate in MPA-acidified plasma samples for a period of up to 42 months and found no indications of ''important'' loss of ascorbate during storage (23). Margolis and Duewer also studied the stability of ascorbate in MPA-acidified plasma in a particularly well-controlled setup with several participating laboratories (15). In MPA-treated samples stored at −70°C for up to 6 years, ascorbate measurements declined an average of 5.5% per year, whereas total ascorbate declined only 1% per year suggesting that the *in vivo* equilibrium between ascorbate and DHA was not entirely blocked. Inclusion of the reductant dithiotreitol lowered the degradation to <1% per year but also rendered the determination of DHA impossible (15).

Koshiishi and coworkers evaluated the stability of ascorbate following acidic deproteinization of plasma from five individuals without storage (24). They found that acidic deproteinization by means of trichloroacetic acid and perchloric acid promoted the oxidation of ascorbate to DHA, whereas MPA—albeit considerably better than the former—was unable to prevent autooxidation. In contrast to the abovementioned data, the authors concluded that acidic deproteinization is an unsuitable pretreatment of plasma samples intended for ascorbate and DHA analysis (24). However, practical and logistic reasons necessitate that plasma samples are stabilized for short-term, and in particular, long-term storage in large clinical studies. Besides, although it may indeed be practically impossible to avoid marginal post-sampling oxidation of ascorbate, meticulous practice and optimization could substantially lower this phenomenon to 1% to 2% of the total ascorbate (5, 6), which is very far from the 15% to 20% obtained on average in the five samples analyzed by Koshiishi et al. (24). Moreover, our inclusion of 2 mmol/L of EDTA in the sample preparation mixture might also have contributed to the much lower post-sampling oxidation in the present study. Thus, Buettner and Jurkiewicz have shown that catalytic ions are the major reason for the autooxidation of ascorbate in solution (25).

We included EDTA in the sample preparation solution to chelate divalent metal ions which have otherwise been shown to promote oxidation of ascorbate to DHA (19). In the present study, changes in ascorbate and total ascorbate amounted to 0.4% and 0.3% per year, respectively (Table 2). The small increases in ascorbate and total ascorbate between the two measurements are most likely related to small differences between the standard curves as they are well within the calculated between-day variation (Table 1).

On average, DHA did not change over the study period, suggesting that the *in vivo* equilibrium between ascorbate and DHA is well maintained during storage under the conditions applied. Inclusion of an internal standard in the reanalysis of the cohort samples gave rise to a more narrow distribution pattern for DHA compared with the original analysis (Table 2). In the biological sense, negative concentrations have of course no meaning; however, it should be appreciated that DHA is measured as a difference between total ascorbate and ascorbate (6). The concentrations of total ascorbate and

Table 2. Five-year stability of ascorbate and DHA stored at −80°C following acidic deproteinization

	Ascorbate	DHA	Total ascorbate
Plasma concentration (μmol/L)			
Study start (n = 133)	51.9 ± 21.9	4.0 ± 4.7	55.9 ± 22.4
Range	4.7-111.1	−21.4-25.8	5.3-126.2
95% Confidence intervals	48.1-55.7	3.2-4.8	52.0-59.7
Reanalysis after 60 mo	52.9 ± 22.6	3.9 ± 3.0	56.7 ± 23.5*
Range	3.9-123.1	0.1-19.0	5.1-129.8
95% Confidence intervals	49.0-56.8	3.3-4.4	52.7-60.8
Change	0.98 ± 5.9	0.10 ± 5.3	0.88 ± 4.5
Rate of change (%/y)	0.4	−0.5	0.3
Change by quartiles (μmol/L)			
Q_1 (n = 33)	1.4 ± 4.6	4.0 ± 5.1 †	−0.6 ± 1.8
Q_2 (n = 33)	2.0 ± 6.1	1.6 ± 3.6	1.3 ± 5.1
Q_3 (n = 33)	0.1 ± 6.3	−0.5 ± 2.4	0.9 ± 5.3
Q_4 (n = 32)	0.3 ± 6.4	−5.6 ± 4.6	1.9 ± 5.4

*$P < 0.05$ compared with study start by paired t test.
†A significant inverse correlation between DHA concentration and change in DHA concentration over 5 y was found (slope, 0.995; $P < 0.001$).

ascorbate are of the same magnitude and are much larger than their difference, which amounts to a relatively small percentage. Thus, if the DHA concentration is close to zero, the variability of the measurement will result in a normal distribution pattern with a considerable number of negative observations. The original analysis encompassed a relatively larger error and therefore some grossly negative (and positive) observations probably due to pipetting error. Inclusion of uric acid as an internal standard resulted in a narrower range of DHA concentrations and no negative values in spite of the same average concentration (Table 2). The few very large negative and positive DHA concentrations found at study start resulted in changes of the same magnitude but with opposite signs because of the reanalysis being closer to the mean. This gave rise to a significant inverse correlation between the original DHA concentrations and the change over 5 years (slope −0.955; $P < 0.001$; Table 2). Moreover, no correlation between the initial ascorbate concentration and DHA change was observed, supporting the fact that the phenomenon indeed originates from the narrower data distribution of DHA found at reanalysis. The fact that the same average concentration was obtained at reanalysis validates the original results but clearly suggests that the internal standard provides a far more precise estimate of the DHA concentration. Based on these data, the use of internal standards in DHA measurement is highly recommended.

In conclusion, the stabilization of plasma samples intended for vitamin C analysis prior to storage is necessary in order to block the *in vivo* equilibrium between ascorbate and DHA and to prevent the degradation of the analytes. The results of the present study show that ascorbate and DHA could be adequately preserved in plasma stored at −80°C following acidic deproteinization for a period of at least 5 years.

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