

Ascorbic and dehydroascorbic acid measurement in human serum and plasma¹⁻⁴

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ABSTRACT Plasma supplemented with ascorbic acid was prepared; the stability of these samples was characterized and the accuracy of the supplementation was established. Studies on the accuracy, precision, and sources of methodological bias in the measurement of ascorbic acid were summarized. Measurements of the ratio of ascorbic acid to dehydroascorbic acid in clinical samples was evaluated and was shown to be relatively constant in plasma taken from blood stored at 12 °C for 6 h. These results imply that whole blood has the capacity to maintain a constant ascorbic-dehydroascorbic acid ratio and suggest that this ratio may be of physiological significance. *Am J Clin Nutr* 1991;54:1315S-18S.

KEY WORDS Ascorbic, dehydroascorbic, human serum, stability, reference materials, cancer-free females, juvenile rheumatoid arthritis, laboratory performance, whole blood

Introduction

Throughout the past decade there has been intense interest in the role of antioxidants in the prevention of cancer. Ascorbic acid, one of these antioxidants, has been extensively evaluated clinically as a cancer prevention agent, particularly in gastrointestinal (1-3) and cervical cancer (4). To obtain a clearer assessment of the role of ascorbic acid in cancer prevention, the National Cancer Institute sponsored an interlaboratory study of plasma ascorbic acid in cancer patients. Our studies were initiated to provide a suitable serum-based control material of demonstrated stability and containing an accurately measured amount of ascorbic acid for the purpose of testing the proficiency of the participating laboratories for the measurement of ascorbic acid.

This project consisted of developing a method for preparing stable serum-based materials and an accurate method for quantifying the analyte(s), assessing the precision and accuracy of the measurement, assessing the long-term stability of the ascorbic acid in the candidate serum-based quality control material, conducting round robin analyses of the serum-based control material, and evaluating clinical samples.

Development of a method for preparing stable serum-based reference materials

Various acids with and without chelating agents have been used to preserve ascorbic acid and dehydroascorbic acid (DHAA) in plasma and serum (5). We selected dithiothreitol (DTT) as a

preservative because at a neutral pH, which minimizes protein precipitation, it can maintain ascorbic acid in its reduced form and reduce DHAA to ascorbic acid (6, 7). With this reagent, plasma or serum can be supplemented with ascorbic acid, lyophilized, and conveniently stored at -70 °C without loss of ascorbic acid (6). Thus, a quality control material can be reliably prepared. In the case of colorimetric assays, the DTT interferes with the assay and must first be removed with a reagent such as *N*-ethylmaleimide (7).

To utilize DTT and to improve specificity and sensitivity, we modified a high-performance liquid chromatography (HPLC) method of analysis (8) and used a more stable electrochemical detector (9). For several serum pools this method resolved the ascorbic acid from all other detectable electrochemically active materials [metaphosphoric acid (MPA), uric acid, and DTT] within 8 min (9). Additionally, by first measuring the ascorbic acid in plasma and then treating the plasma with DTT at neutral pH for 30 min, we quantitatively converted DHAA to ascorbic acid and were able to accurately measure DHAA by difference (9).

Assessment of the precision and accuracy of the ascorbic acid measurement

Precision and accuracy were assessed with two candidate reference materials containing different amounts of ascorbic acid. They were prepared by gravimetrically adding ascorbic acid to an ascorbic acid-free serum matrix (9). Representative samples from each candidate material were assayed on 12 different days, over a 21-d period. The results indicated that the method accurately measured the gravimetrically added ascorbic acid. The coefficient of variation of a single measurement was 2%, and no discernable variation was observed in the sample preparation

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² Identification of any commercial product does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the material or equipment identified is necessarily the best available for the purpose.

³ Supported in part by the National Cancer Institute contract no Y01-CP9-0506.

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process, sample extraction step, or HPLC analysis. These material were then used to evaluate quality control in our measurements of clinical samples. Precision and accuracy studies were also performed on ascorbic acid-supplemented plasma preserved with MPA, and similar results were observed.

Assessment of the long-term stability of ascorbic acid in candidate reference materials

Several investigators have studied the short-term stability (1–5 wk) of ascorbic acid and DHAA in serum and plasma under acidic conditions at -20 and -70 °C (10–12). We have extended these stability studies to frozen and lyophilized plasma samples, stored at -70 °C, in which ascorbic acid is preserved with DTT (6). Under these storage conditions the ascorbic acid in the lyophilized plasma was stable for 66 wk and showed only marginal degradation after 80 wk. The ascorbic acid in the frozen plasma was stable for ≥ 57 wk. Recently, these studies were extended to include ascorbic acid and DHAA in plasma preserved with MPA (5 g/100 mL) for 13 wk (9). Although these materials were stable at -70 °C, the sum of ascorbic acid and DHAA in the MPA-preserved plasma showed measurable loss within 24 h at 4 °C. The loss was more pronounced at 25 and 50 °C. These results demonstrate that care must be taken to thaw frozen MPA-preserved plasma samples at temperatures of 20 °C or lower and to assay them promptly (9).

Round-robin analysis of the candidate reference material

Four plasma pools were supplemented with ascorbic acid at different concentrations in our laboratory to form candidate reference materials. The reference material lots from each plasma pool were evaluated in a round-robin study (Table 1). Pools 1–3 (except for lot 46) were lyophilized in the presence of DTT, and pool 4 was preserved with MPA (5 g/100 mL). The results indicate that the participating laboratories generally obtained values comparable to those of the National Institute of Standards and Technology (NIST). The standard deviations of the round-robin ascorbic acid measurements primarily reflect interlaboratory variation. All of the participating laboratories but one used HPLC methods to measure ascorbic acid. One laboratory, not included in Table 1, measured the total ascorbic acid in MPA-stabilized lot 103 by the dinitrophenylhydrazine method (13) and obtained an unexpectedly high mean value of 76.20 $\mu\text{mol/L}$ compared with the NIST value of 66.38 $\mu\text{mol/L}$ (9). This interlaboratory variation in ascorbic acid measurement is similar to the interlaboratory variation NIST has obtained in round-robin measurements of β -carotene, vitamin A, and vitamin E. The source of the interlaboratory variation in ascorbic acid measurements is not clear and may be due to methodological bias and/or to inaccurate standards. Quality-control monitoring with reference materials can improve the accuracy and precision of the measurements by participating laboratories (W May, unpublished observations, 1991) and is a prerequisite for comparing measurements from different laboratories.

Evaluation of clinical samples

The utility of a clinical method lies in its ability to measure an analyte precisely and accurately in multiple samples at low

TABLE 1

Comparison of round-robin and the National Institute of Standards and Technology (NIST) native ascorbic acid values*

Plasma lot	Round-robin results†	NIST results‡
	$\mu\text{mol/L}$	$\mu\text{mol/L}$
Pool 1		
37	35.0 ± 2.3	35.9 ± 1.2
45	53.0 ± 3.5	51.3 ± 2.4
46§	52.5 ± 2.0	49.4 ± 1.6
Pool 2		
58	30.1 ± 3.1	35.8 ± 1.2
60	9.8 ± 1.1	10.3 ± 0.5
Pool 3		
107	24.8 ± 1.2	26.7 ± 0.6
108	62.4 ± 2.8	66.8 ± 1.5
Pool 4		
103§	52.7 ± 2.2	57.3 ± 0.2
104§	22.1 ± 1.2	23.0 ± 0.7

* $\bar{x} \pm \text{SD}$.

† Round-robin measurements on lots 38, 45, and 46 (7 laboratories) were completed 13 wk after the sample preparation, measurements on lots 58 and 60 (5 laboratories) were completed 4 wk after sample preparation, and measurements on lots 103, 104, 107, and 108 (4 laboratories) were completed 56 wk after the sample preparation. Each value represents the average mean of independent duplicate measurements on two different samples (4 measurements).

‡ Data represents an aggregate mean of all data in our stability studies.

§ Lot 46 was frozen only and lots 103 and 104 were preserved in MPA (5 g/100 mL) and frozen. The other lots were freeze-dried.

cost and with short analysis time. We have conducted three studies which examine the utility of our HPLC methods for the measurement of ascorbic acid and DHAA in plasma. In the first study total plasma vitamin C (ascorbic acid plus DHAA) was measured in children with three classes of juvenile rheumatoid arthritis (14). Mean vitamin C concentrations ($\mu\text{mol/L}$) were 19 in the 8 children with systemic disease, 27 in the 14 children with polyarticular disease, 40 in the 12 children with pauciarticular disease, and 37 in 9 children without rheumatoid arthritis. The plasma vitamin C concentrations of the first two groups were significantly different from each other and were significantly lower than the vitamin C levels of the third and fourth groups. Similar results have been reported in adult populations (11, 15); together these studies suggest that low plasma vitamin C may be associated with problems in connective-tissue metabolism.

The observation that for several clinical conditions the ratio of ascorbic acid to DHAA may differ from that of normal subjects (11, 16, 17) and the fact that the oxidation of ascorbic acid to DHAA in serum and plasma (10) is relatively rapid prompted us to evaluate the stability of ascorbic acid and DHAA in blood. Only a limited amount of data has been published on the stability of ascorbic acid and DHAA in whole blood (18, 19). We further examined the stability of ascorbic acid and DHAA in the blood of six normal subjects in the presence and absence of DTT at 4 °C. The blood was drawn into tubes containing anticoagulant and was immediately divided into 2 mL

TABLE 2
The stability of ascorbic acid and DHAA in whole blood, stored at 4 °C

Subject	Total ascorbic acid*	DHAA†		
		Time of measurement		
	$\mu\text{mol/L}$	0 h	3 h	6 h
1	83.3 ± 1.5	8.1	6.2	7.3
2	77.3 ± 1.0	9.4	7.4	6.6
3	80.0 ± 0.6	8.9	7.7	8.0
4	37.9 ± 0.5	4.1	4.5	4.1
5	50.5 ± 0.7	5.6	6.6	4.3
6	67.9 ± 1.2	4.5	4.7	7.7

* Each value represents the mean ± SD of duplicate measurements on plasma samples that were obtained by centrifuging blood samples at 0, 3, 6, and 24 h (ie, 4 samples, 8 measurements).

† Each value was obtained by subtracting the native ascorbic acid from the total ascorbic acid (9) and represents the average of duplicate measurements on a single sample.

aliquots that were allowed to stand at 4 °C for 0, 3, 6, and 24 h. At the end of each time period the samples were centrifuged, and the plasma was added to an equal volume of MPA (10 g/100 mL water) and was frozen at -70 °C. The total ascorbic acid was assayed according to our published method (9). The results in Table 2 summarize the total ascorbic acid for each subject. The values for the total ascorbic acid for the different time periods were combined because the values remained unchanged over the 24-h period. This is reflected in the small SDs of a single measurement. The DHAA values (Table 2) do not appear to vary in any consistent way during the first 6 h. The amount of DHAA measured in these subjects (6.8–12% of the total ascorbic acid) is consistent with the results of some investigators (11, 17, 18), but not for others (7, 16, 20). The stability of the ascorbic acid and DHAA in blood for 6 h and the instability of ascorbic acid in plasma or serum (10) suggest that the blood cells play a significant role in maintaining this ratio and that it may represent the metabolic equilibrium of the system. This is further supported by the inability of DTT to totally reverse this ratio in blood, whereas in plasma it is capable of completely converting the DHAA to ascorbic acid (9). If the presence of DHAA in plasma is an artifact it arises during the blood collection process, because the analytical method is precise and accurate, and the ascorbic acid and DHAA are stable for ≥ 90 d when stored in MPA (5 g/100 mL plasma) as described above (9).

Finally, in a study of 35 cancer-free female subjects, we examined the effect storage time (10–12 mo), patient age, smoking, and vitamin C supplementation on the plasma content of MPA-stabilized ascorbic acid and DHAA. The procedures followed were in accord with the Helsinki Declaration of 1975 as revised in 1983. Preliminary analysis indicated that ascorbic acid increased slightly with age and decreased noticeably with cigarette smoking, which is consistent with the results of other investigators (21–24). Except for cigarette smokers, we observed no other effects. Furthermore, there were no changes in the absolute DHAA content. The lack of any effect of storage over a 10- to 12-mo period further supports our observations on the long-

term stability of ascorbic acid and DHAA in MPA-stabilized plasma.

References

1. Stahelin HB, Gey KF, Brubacher G. Plasma vitamin C and cancer death: the prospective Basel study. *Ann NY Acad Sci* 1987;498:124–31.
2. Sobala GM, Schorah CJ, Sanderson M, et al. Ascorbic acid in the human stomach. *Gastroenterology* 1989;97:357–63.
3. Correa P. The gastric precancerous process. *Cancer Surv* 1983;2:437–50.
4. Romney SL, Basu J, Vermund S, Palan PR, Dutttagupta C. Plasma reduced and total ascorbic acid in human uterine cervix dysplasias and cancer. *Ann NY Acad Sci* 1987;490:132–43.
5. Pelletier O. Vitamin C (L-ascorbic acid and dehydro-L-ascorbic acid). In: Augustin J, Klein BP, eds. *Methods of vitamin assay*. New York: John Wiley and Sons, 1985:303–46.
6. Margolis SA, Davis TP. Stabilization of ascorbic acid in human plasma and its liquid chromatographic measurement. *Clin Chem* 1988;34:2217–23.
7. Okamura M. An improved method for determination of L-Ascorbic acid and L-dehydroascorbic acid in blood plasma. *Clin Chim Acta* 1980;103:259–68.
8. Grun M, Loewus FA. Determination of ascorbic acid in algae by high-performance liquid chromatography on strong cation exchange resin with electrochemical detection. *Anal Biochem* 1983;130:191–8.
9. Margolis SA, Paule RC, Ziegler R. The differential measurement of ascorbic and dehydroascorbic acid in plasma preserved in dithiothreitol or metaphosphoric acid. *Clin Chem* 1990;36:1750–5.
10. Bradley DW, Emery G, Maynard JD. Vitamin C in plasma: a comparative study of the vitamin stabilized with trichloroacetic acid or metaphosphoric acid and the effects of storage at -70°, -20°, 4°, and 25 °C on the stabilized vitamin. *Clin Chim Acta* 1973;44:47–52.
11. Lunce J, Blake DR. The determination of dehydroascorbic acid and ascorbic acid in serum and synovial fluid of patients with rheumatoid arthritis (RA). *Free Radic Res Commun* 1985;1:31–9.
12. Kutnink MA, Skala JH, Sauberlich HE, Omaye ST. Simultaneous determination of ascorbic acid, isoascorbic acid, (erythroic acid) and uric acid in human plasma by high-performance liquid chromatography with amperometric detection. *J Liquid Chromatogr* 1985;8:31–46.
13. Roe JH, Mills MB, Oesterling MJ, Damron CM. The determination of diketo-L-gulonic, dehydro-L-ascorbic acid and L-ascorbic acid in the same tissue extract by the 2,4-dinitrophenylhydrazine method. *J Biol Chem* 1948;174:201–8.
14. Bacon MC, Raiten DJ, Craft N, et al. Nutritional status and growth in juvenile rheumatoid arthritis. *Semin Arthritis Rheum* 1990;20:97–106.
15. Hall MG, Darling RC, Taylor FHL. The vitamin C requirement in rheumatoid arthritis. *Ann Intern Med* 1939;13:415–23.
16. Nagy E, Degrell I. Determination of ascorbic acid and dehydroascorbic acid in plasma and cerebrospinal fluid by liquid chromatography with electrochemical detection. *J Chromatogr* 1989;497:276–81.
17. Schreiber J, Lohmann W, Unversagt D, Otten A. Determination of ascorbic acid, dehydroascorbic acid and isoascorbic acid in blood. *Fres Z Anal Chem* 1986;325:473–5.
18. Lee W, Davis KA, Rettmer RL, Labbe RF. Ascorbic acid status: biochemical and clinical considerations. *Am J Clin Nutr* 1988;48:286–90.
19. Interdepartmental Committee on Nutrition for National Defense. *Manual for nutrition surveys*. Washington, DC: US Government Printing Office, 1957.

20. Nyyssonen K, Pikkarainen S, Parviainen MT, Heinonen K, Mononen I. Quantitative estimation of dehydroascorbic acid and ascorbic acid by high-performance liquid chromatography—application to human milk plasma and leukocytes. *J Liquid Chromatogr* 1988;11:1717-28.
21. Pelletier O. Vitamin C and cigarette smokers. *Ann NY Acad Sci* 1975;258:156-68.
22. Smith JL, Hodges RE. Serum levels of vitamin C in relation to dietary and supplemental intake of vitamin C in smokers and non-smokers. *Ann NY Acad Sci* 1987;498:144-52.
23. Murato A. Smoking and vitamin C. *World Rev Nutr Diet* 1991;64:31-57.
24. Hematological and nutritional biochemistry reference data for persons 6 months-74 years of age: United States 1976-80. Washington, DC: US Government Printing Office, 1982 [DHHS publication (PHS) 83-1682.]