

Stability of Ascorbic Acid, Carotenoids, Retinol, and Tocopherols in Plasma Stored at -70°C for 4 Years¹

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Abstract

Aliquots from 40 plasma pools preserved with metaphosphoric acid were assayed for their ascorbic acid values after 12, 24, and 42 months of storage at -70°C . Similarly, aliquots from 16 plasma pools were assayed for values of retinol, several carotenoids, and two tocopherols at 15.5, 27.5, and 51.5 months of storage at -70°C . There were no indications of important losses of these antioxidant micronutrients during storage from the first to the last assay.

Introduction

The value of serum banks for studies of acute infectious diseases has long been recognized (1). Their use for studies of chronic diseases is more recent and is expanding rapidly (2). In view of this widespread use, there is need for elementary knowledge that is currently lacking. An example is the effect of long-term freezer storage on serum or plasma values of many analytes. What little is known about storage stability of antioxidant micronutrients is essentially limited to retinol, β -carotene, and α -tocopherol; it is almost entirely based on observational studies in which the source population is different for each storage time (3). In such studies, changes with duration of storage can be confounded by any differences in serum or plasma concentrations in the various populations.

Much better estimates can be obtained by assays of aliquots of the same serum or plasma pools stored and assayed after different lengths of storage. However, only a few studies have reported on repeated assays of aliquots of the same specimens after storage times of more than 1 year. Ascorbic acid showed only "marginal degradation" after storage at -70°C for 1.5 years when preserved with DTT (4). Serum from 35 women that had been stabilized with metaphosphoric acid showed concentrations of ascorbic acid and dehydroascorbic acid after 4–6 years storage at -70°C that were "generally similar" to fresh drawn samples (5). Retinol was stable for periods up to 8

years both at -20 and -70°C (6–7). No detectable changes were noted in serum values of carotenoids and α -tocopherol after 15 months of storage at -70°C (8), whereas considerable losses of carotenoids were noted after storage at -20°C for as short a time as 6 months (9).

To add to these data, we have periodically assayed aliquots of several large plasma pools. This report records our results for ascorbic acid after 1.0, 2.0, and 3.5 years of storage, and α - and β -carotene, cryptoxanthin, lutein, lycopene, and α - and γ -tocopherol after 1.3, 2.3, and 4.3 years of storage at -70°C .

Materials and Methods

From June to November 1989, a mass campaign was conducted in Washington County, MD, to recruit volunteers to donate 20 ml of blood for a research bank. Blood was drawn into 20-ml heparinized vacutainers (Fisher Scientific, Pittsburgh, PA). The tubes were tilted slowly back and forth to 45° from the horizontal for a total of 8 times to ensure that the heparin dissolved. Blood was refrigerated at 4°C until it was processed, usually within 2–6 h, and always within 24 h of blood drawing. The vacutainers were centrifuged at $1000 \times g$ for 30 min. For the ascorbic acid assays, 0.7 ml of plasma was pipetted into 5-ml blue-capped Vanguard Cryogenic Vials (Sumitomo Bakelite Co., Ltd., Neptune, NJ) containing 0.7 ml of 10% solution of metaphosphoric acid. Equal aliquots of the remaining plasma were pipetted into each of two 5-ml Cryotubes with clear caps to distinguish them from the aliquots for ascorbic acid assays. All aliquots were quickly frozen at -70°C and kept at approximately that temperature until they were withdrawn for various studies.

Plasma for the study of storage effects came from persons who resided in states not contiguous to Washington County. They were selected from specimens donated closest to the end of the blood donation campaign, mostly in October and November 1989. Each pool was composed of plasma from four persons. For ascorbic acid, there were a total of 44 pools, 10 from each of 4 age-sex groups (males born 1910–1939; females born 1910–1939; males born 1940–1969, and females born 1940–1969) plus 4 "comparison" pools derived from a single large pool to estimate intraassay variation at each storage time. For other analytes, there were only 16 pools, 4 from each of the 4 age-sex groups plus 2 additional comparison pools.

The specimens designated for each pool were removed from the freezers and thawed in ice water under dim yellow light. They were then inverted 20 times to remove any possible effects of layering during freezing. After each pool was prepared, 6 aliquots were pipetted into Cryotubes, labeled with pool numbers, and promptly refrozen having been thawed for <30 min. At the designated storage times, one of these aliquots from each pool was shipped in insulated containers containing dry ice to the assay laboratory, where the specimens were stored at -85°C until thawed for assay.

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Assays for ascorbic acid were done at 12, 24, and 42 months after the midpoint of the blood donation dates for the contributors to these pools. For retinol, carotenoids, and tocopherols, assays were done at 15.5, 27.5, and 51.5 months, respectively, after their midpoint collection dates. The laboratory was masked with regard to the source of each aliquot.

For ascorbic acid assays, metaphosphoric acid-stabilized plasma samples were thawed at room temperature while being protected from light (20 min), mixed by vortex (15 s), and then centrifuged (15 min; 4°C; 10,000 × g). The clear supernatant was assayed for ascorbic acid with the use of 2,4-dinitrophenylhydrazine as chromogen (10, 11). The laboratory accuracy for this analytical procedure with the use of both internally and externally prepared quality control specimens is ± 4%, whereas the day-to-day and within-day precision (coefficient of variation) of the laboratory for the assay is <0.05. The analytical instrumentation used to assay ascorbic acid did not vary throughout the 42 months of assay. This equipment consisted of a Gilford Response UV/Vis spectrophotometer (CIBA-Corning Diagnostics Corp., Medfield, MA) plus a Gilford printer/plotter. They were maintained continually under the manufacturer's authorized service agreements. UV/Vis lamps were replaced as necessary.

The other frozen plasma samples were thawed at room temperature while being protected from light (20 min), mixed by vortex (15 s), and then centrifuged (2500 × g; 5 min; 4°C) to obtain clear samples that were assayed for the fat-soluble vitamins and carotenoids with the use of reversed-phase HPLC methodology (8). Laboratory accuracy for this analytical procedure, based on internally and externally prepared quality controls, is 2% for retinol, <5% for tocopherols, and <12% for the individual carotenoids, whereas the day-to-day and within-day precision (coefficient of variation) of the laboratory for these fat-soluble analytes is <0.06. The analytical instrumentation used to assay the fat soluble vitamins and carotenoids did not vary throughout the 51.5 months of assay. This equipment included a ternary-gradient HPLC pump (Model SP8800; Thermo Separation Products, Fremont, CA); a dynamic mixer (Model SP5800; Thermo Separation Products); an 80 position autosampler (Model SP8775; Thermo Separation Products) equipped with a 20-μl sample loop; a guard column (New-Guard C₁₈; Applied Biosystems, Inc., Foster City, CA); an analytical HPLC column (3 μm and 4.6 × 100 mm; Microsorb C₁₈; Rainin Instrument Co., Inc., Woburn, MA); and two analytical, variable wavelength, programmable UV/VIS detectors (Model 783, Kratos Analytical; Applied Biosystems, Inc.) interfaced with two dual-channel, programmable integrators (Model SP4270; Thermo Separation Products). All instrumentation was maintained continually under the manufacturer's authorized service agreement. UV and Vis lamps were replaced as necessary as were the guard and analytical HPLC columns.

To estimate the average rate of change for each of the selected micronutrients over the entire observation period and the baseline value for a storage time of zero, a simple linear regression model was fitted for the concentration of each pool at the three storage times (measured in years) with the use of the least squares method. The intercept represents the estimated "baseline" value. The slope divided by the intercept and multiplied by 100 represents the percentage change in concentration per year, expressed as a percentage of the baseline value.

Results

Intraassay coefficients of variation were based on the comparison specimens that had been assayed along with other speci-

Table 1 Intraassay coefficients of variation^a for concentrations of selected plasma vitamins at each of the three assay times

Vitamins	Assays		
	First	Second	Third
Ascorbic acid	1.7	1.4	3.5
Retinol	1.5	2.6	1.8
Carotenoids			
α-Carotene	10.6	12.2	12.6
β-Carotene	12.9	14.3	16.8
Cryptoxanthin	7.3	9.5	6.1
Lutein	3.3	1.9	2.6
Lycopene	4.0	3.9	1.7
Tocopherols			
α-Tocopherol	8.4	7.1	7.8
γ-Tocopherol	5.8	9.2	10.6

^a Expressed as percentages.

Table 2 Estimated baseline plasma concentrations of selected vitamins by age-sex groups

Vitamins	Units	Estimated baseline values			
		Males		Females	
		20-49 yrs	50-69 yrs	20-49 yrs	50-69 yrs
Ascorbic acid	mg/dl	1.08	1.15	1.23	1.58
Retinol	μg/dl	80.1	81.2	60.5	74.8
Carotenoids					
α-Carotene	μg/dl	3.3	4.9	5.5	6.3
β-Carotene	μg/dl	18.0	22.6	29.1	45.3
Cryptoxanthin	μg/dl	9.14	10.6	10.2	18.3
Lutein	μg/dl	22.6	29.8	23.6	37.4
Lycopene	μg/dl	79.1	50.9	48.9	78.1
Tocopherols					
α-Tocopherol	mg/dl	1.22	1.24	1.02	1.46
γ-Tocopherol	mg/dl	0.22	0.25	0.21	0.29

mens at each storage time. Calculations were based on values for the four comparison aliquots in each ascorbic acid set and the two comparison aliquots in sets assayed for retinol, carotenoids, and tocopherols. Table 1 shows that intraassay variability was the least for retinol and ascorbic acid and the greatest for α- and β-carotenes.

Calculated baseline values of the selected vitamins in plasma are shown in Table 2. Although the donors of plasma for the pools in this study were not selected to be representative of any particular population, it is reassuring that these values are compatible with other published baseline values from American populations (4, 12).

Table 3 shows the mean values at each storage time and the estimated average annual percentage changes in analytes during the period between first and third assays. All changes were slight and well within the limits of chance variability, with overall decreases for ascorbic acid, retinol, and tocopherols and increases for the carotenoids.

Discussion

Although questions can be raised about the fact that specimens in this study were frozen and then thawed some time later for

Table 3 Mean concentrations of selected vitamins in plasma at each of the three assay times, and estimated average percentage changes in concentration per year of storage at -70°C during the study period

Vitamins	Assays			Change per year (%)	
	First	Second	Third	Mean	SE
Ascorbic acid (mg/dl)	1.25	1.23	1.22	-0.66	0.32
Retinol ($\mu\text{g}/\text{dl}$)	73.56	73.68	72.68	-0.49	0.11
Carotenoids (mg/dl)					
α -Carotene	5.01	5.17	5.19	+0.75	0.45
β -Carotene	28.82	29.08	29.12	+0.32	0.30
Cryptoxanthin	12.09	12.42	12.44	+1.03	0.36
Lutein	28.41	28.86	28.68	+0.18	0.36
Lycopene	64.17	64.49	64.31	+0.03	0.19
Tocopherols (mg/dl)					
α -Tocopherol	1.23	1.22	1.21	-0.41	0.13
γ -Tocopherol	0.24	0.24	0.23	-0.92	0.24

the initial assays, this does not appear to be a problem. A number of studies have shown that even repeated freeze-thaw cycles have no demonstrable effect on concentrations of ascorbic acid in stabilized plasma or on concentrations of retinol, total carotenoids, β -carotene, lycopene, total tocopherols, or α -tocopherol.

It is also possible that losses of analytes are greater during the early period of storage than they are later. This study could not detect early losses because the first assays were not done until approximately 1 year after storage was begun. Again, the results of prior studies do not support such a possibility (6, 7, 13–17). Almost all of them involved short periods of storage; at -70°C , losses were inconsequential. If there are only slight losses during the early storage periods, extrapolation from the later assays in the present study, also with slight losses, should give estimates of the true baseline values with relatively little error.

Although the present study encompasses a range of only 3.5–4.3 years, it is the most comprehensive report of the effects of long-term freezer storage of several important antioxidant micronutrients. The effects on ascorbic acid are particularly noteworthy because of the limited information on its changes in concentration when acidified plasma is stored (5). The fact that plasma values of ascorbic acid, retinol, carotenoids, and tocopherols remained almost constant for several years should be

encouraging to all investigators of serological precursors of cancer and other diseases.

References

1. Evans, A. S. Serological surveys: the role of the WHO serum banks. *WHO Chron.*, 21: 185–190, 1967.
2. Winn, D. M., Reichman, M. E., and Gunter, E. Epidemiologic issues in the design and use of biologic specimen banks. *Epidemiol. Rev.*, 12: 56–70, 1990.
3. Comstock, G. W., Alberg, A. J., and Helzlsouer, K. J. Reported effects of long-term freezer storage on concentrations of retinol, β -carotene, and α -tocopherol in serum or plasma summarized. *Clin. Chem.*, 39: 1075–1078, 1993.
4. Margolis, S. A., Ziegler, R. G., and Helzlsouer, K. J. Ascorbic and dehydroascorbic acid measurement in human serum and plasma. *Am. J. Clin. Nutr.*, 54: 1315–1318, 1991.
5. Margolis, S. A., and Ziegler, R. G. Accuracy and precision of ascorbic and dehydroascorbic acid measurements in human blood and plasma. *Nutr. Cancer*, 15: 277–278, 1991.
6. Driskell, W. J., Lackey, A. D., Hewett, J. S., and Bashor, M. M. Stability of vitamin A in frozen sera. *Clin. Chem.*, 31: 871–872, 1985.
7. Gunter, E. W., Driskell, W. J., and Yeager, P. R. Stability of vitamin E in long-term stored serum. *Clin. Chim. Acta*, 175: 329–335, 1988.
8. Craft, N. E., Brown, E. D., and Smith, J. C., Jr. Effects of storage and handling conditions on concentrations of individual carotenoids, retinol, and tocopherol in plasma. *Clin. Chem.*, 34: 44–48, 1988.
9. Mathews-Roth, M. M., and Stampfer, M. J. Some factors affecting determination of carotenoids in serum. *Clin. Chem.*, 30: 459–461, 1984.
10. Roe, J. H. Chemical determination of ascorbic, dehydroascorbic and diketogulonic acids. In: D. Glick (ed.), *Methods of Biochemical Analysis*, pp. 115–139. New York: Interscience Publishers, Inc., 1954.
11. Centers for Disease Control. Laboratory Procedures Used by the Clinical Chemistry Division, Centers for Disease Control, for the Second Health and Nutrition Examination Survey (NHANES II) 1976–1980. IV. Analytical Methods, Vitamin C, pp. 17–19. Atlanta, GA: USPHS, 1981.
12. Stacewicz-Sapuntakis, M., Bowen, P. E., Kikendall, J. W., and Burgess, M. Simultaneous determinations of serum retinol and various carotenoids: their distribution in middle-aged men and women. *J. Micronutr. Anal.*, 3: 27–45, 1987.
13. Bradley, D. W., Emery, G., and Maynard, J. E. 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° on the stabilized vitamin. *Clin. Chim. Acta*, 44: 47–52, 1973.
14. Nierenberg, D. W. Serum and plasma β -carotene levels measured with an improved method of high-performance liquid chromatography. *J. Chromatogr.*, 339: 273–284, 1985.
15. Margolis, S. A., and Davis, T. P. Stabilization of ascorbic acid in human plasma, and its liquid-chromatographic measurement. *Clin. Chem.*, 34: 2217–2223, 1988.
16. Hsing, A. W., Comstock, G. W., and Polk, B. F. Effect of repeated freezing and thawing on vitamins and hormones in serum. *Clin. Chem.*, 35: 2145, 1989.
17. Clevidence, B. A., and Ballard-Barbash, R. Tocopherol contents of lipoproteins from frozen plasma separated by affinity chromatography. *Lipids*, 26: 723–728, 1991.

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