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## SERUM ASCORBIC ACID STABILITY OVER AN EXTENDED PERIOD: RELEVANCE TO EPIDEMIOLOGICAL STUDIES

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### ABSTRACT

Epidemiological evidence suggests that intake of vitamin C and of fruit and vegetables rich in vitamin C may reduce the risk of certain cancers. Most epidemiological studies have relied on estimates of vitamin C based on questionnaires because serum ascorbic acid (AA) can be very unstable. The objective of this study was to investigate the effect of extended storage at  $-70^{\circ}\text{C}$  and of multiple free-thaws on AA concentration in stabilized human serum from a large multi-center cervical cancer case-control study; the serum samples had been stabilized by acidification with meta-phosphoric acid and stored at  $-70^{\circ}\text{C}$ . The results showed that under these conditions human serum samples are stable for periods of at least 2 years. There were indications that sample preparation in the field and freezing and thawing the stabilized serum samples could affect reproducibility and validity. Overall, our results indicate that with this stabilization method, biological material collected in large-scale, multicenter studies remains useful for AA analysis for at least 2 years after collection.

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**KEY WORDS:** Ascorbic acid, Vitamin C, Stabilization, Epidemiological studies.

### INTRODUCTION

Epidemiological evidence suggests that intake of vitamin C and of fruits and vegetables rich in vitamin C may reduce the risk of certain types of cancers (1-8). Most of these epidemiological studies have evaluated the role of vitamin C with dietary intake information obtained from questionnaires. However, estimates of vitamin C intake from dietary instruments can be imprecise because of recall bias, inaccuracy of food composition values, and loss of vitamin C during storage and cooking (9,10).

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Direct assessment of serum AA (11,12) may provide a more valid measure of nutrient status for studies of cancer etiology. However, vitamin C in human serum is rapidly and irreversibly oxidized so that very few studies have been able to measure vitamin C directly. In these studies, serum AA had to be measured shortly after the blood was collected (6,13,14). Therefore, guaranteeing the stability of AA in blood samples stored over extended periods is crucial for large prospective or case-control studies as it can take years to accrue sufficient cancer cases for analyses (15-17).

In this report, we examine several methodological issues pertinent to collection and storage of meta-phosphoric acid stabilized serum held for later AA analysis, these include the effect of prolonged storage at low-temperature, the influence of multiple freeze-thaw cycles, and the effect of sample collection and sample preparation by personnel at various times and at different study sites.

### MATERIALS AND METHODS

Serum samples were obtained from a case-control study of cervical cancer carried out in five metropolitan areas (Birmingham, Chicago, Denver, Miami, and Philadelphia) from 1983 through 1985. For all study participants, a pair of aliquots of serum (i.e., duplicates) were stored. Details of the study design have been described elsewhere (18-20). For this study, 66 control subjects were randomly selected from non-smoking white study participants aged 35-59. Subjects were selected so that they were representative of the 5 study centers, the different phases of entry into the study (early -- March 1983 to February 1984; middle -- March 1984 to December 1984; and late -- January 1985 to October 1985), and of AA concentrations as determined in 1989: (low --  $\leq 0.879$  mg/dl; medium -- 0.880 - 1.263 mg/dl; and high -- 1.267 - 2.316 mg/dl). In addition, two sets of serum samples (24 and 21 samples, respectively) were selected from the 66 to investigate the effect of thawing and freezing on AA concentrations; these latter samples were not representative of the five sites or of AA concentrations.

Whole blood was collected by venipuncture, allowed to clot at room temperature for 40 to 60 minutes, and centrifuged at 3,500 rpm in a non-refrigerated table-top centrifuge for 10 minutes. Then 1 ml. of serum and 1 ml. of 10% freshly-made meta-phosphoric acid were mixed and immediately stored at 4°C. Within 24 hours these samples were frozen at -70°C. Serum from each subject was aliquoted in duplicate.

Periodically, batches of study samples and interspersed quality control samples were shipped on dry ice to the analysis laboratory where they were stored at -85°C until thawed for spectrophotometric determination of serum AA using 2,4-dinitrophenylhydrazine as chromogen (A,B) (21). The quality control samples consisted of two pools of human sera, prepared by the Centers for Disease Control, containing either "low" or "normal" concentrations of AA.

One objective of this study was to investigate the effect of extended storage at -70°C on AA concentration in stabilized human serum. In 1989, 55 "low" and 55 "normal"

quality control samples were analyzed. Twelve "low" and 12 "normal" quality control samples were analyzed in 1991 and compared to the 1989 results (experiment 1). Differences in AA concentrations for the quality control samples assayed in 1989 (n=55) and 1991 (n=12) were tested for significance using a 2-sample t-test.

Stability was also investigated via analyses at two different times, of paired aliquots stored for each of the 66 controls (experiment 2). In 1989 (between June and August), a single aliquot was randomly selected from each pair of aliquots, thawed, and assayed for AA. The remaining aliquots were assayed in November 1991. The difference in values for each pair was tested for significance using a paired t-test. If a significant overall difference was found for these 66 paired samples, the influence of study site, collection time (e.g., personnel experience as the study progressed), and AA concentration was assessed using a general linear model. For descriptive purposes, least square mean differences were calculated using the difference in values for each pair of aliquots. Least square means represent the means that would be expected if the study design had been balanced on the three factors: study site, collection time, and AA concentration.

The effects of freezing and thawing were also investigated in two different ways. First, we used each sample as its own control, successively thawing and refreezing the same samples (experiment 3). Twenty-four serum samples were initially assayed for AA in 1989, refrozen, stored at  $-70^{\circ}\text{C}$ , and again thawed and assayed in 1991. The effects on AA concentrations of this regimen were tested via a paired t-test. Finally, we investigated the effect on stabilized serum AA of multiple freezes and thaws (experiment 4). Twenty-one samples underwent three thaw-freeze cycles; AA concentration was assayed at each thaw. Serum was frozen at  $-70^{\circ}\text{C}$  for 2 week intervals between thaws. A one-way analysis of variance was used to analyze these data. Significant differences were examined using contrasts.

## RESULTS

Results of experiment 1 showed AA concentration in stabilized quality control serum pools to be stable at  $-70^{\circ}\text{C}$  for 2 years. The mean AA value for the "low" quality control samples was  $0.85 \pm 0.03$  mg/dL (mean  $\pm$  standard deviation) both in 1989 and 1991; for "normal" quality control samples the values were  $1.25 \pm 0.04$  mg/dL in 1989 and  $1.28 \pm 0.03$  mg/dL in 1991, a difference that was neither statistically nor biologically significant. These pooled samples provide a precise test of the effect on AA concentration of 2 years of storage; as these samples were pooled samples prepared at the same time, there was no inter-individual variability.

Similarly, the AA in the stabilized serum of the 66 subjects (experiment 2) showed no biologically meaningful degradation during 2 years of storage. The mean serum AA in the set of aliquots measured in 1989 was 1.22 mg/dL, while that in the second set of aliquots, measured in 1991, was 1.25 mg/dL. While this small increase in concentration is likely to be biologically insignificant, it was found to be statistically significant ( $p < 0.0001$ )

by a paired t-test. To investigate this increase further we used a general linear regression model.

Results from linear regression (Table I) indicated that different study sites did not have a significant effect on the increase in serum AA concentration, but that both collection time and level of initial (1989) AA concentration did. Contrasts showed that there was a significant increase in AA concentration during the early and middle phases of the study as compared to the late phase. A comparison of the least square means for the differences (1991 minus 1989) for the early, middle, and late phases of the study indicated that they were small (0.044, 0.037, and -0.004 mg/dL, respectively) and decreased over time.

TABLE I: The Effect of Study Site, Collection Time, and AA Concentration on the Difference in AA Concentration (1991 - 1989) in Duplicate Serum Samples from the Same Subject

	P-value
Study Site <sup>2</sup>	0.10
Collection Time <sup>3</sup>	0.04
Ascorbic Acid Concentration 1989 <sup>4</sup>	0.002

<sup>1</sup> Factors in a linear regression model and associated p-values.

<sup>2</sup> Five study sites: Birmingham, Chicago, Denver, Miami, and Philadelphia.

<sup>3</sup> Three collection times during the study: early - 3/83-2/84; middle - 3/84-12/84; late - 1/85-10/85.

<sup>4</sup> Three concentrations: low - 0 to 0.879 mg/dl; medium - 0.880 to 1.263 mg/dl; and high - 1.267 to 2.316 mg/dl.

The least square mean differences in serum AA (1991 minus 1989) on duplicate samples at low, medium, and high concentrations in 1989 were -0.002, 0.015, and 0.064 mg/dL, respectively. There was a significant increase in AA concentration when duplicates with high levels of AA (1.267-2.316 mg/dL) were compared.

Our investigation of the effects of successive thaws showed a small but statistically significant ( $p < 0.0001$ ) increases in the serum AA concentration between the first and the second thaw in both experiments 3 and 4, but no additional increase between the second and the third thaws (Table II). The increase after the first thaw-freeze cycle and storage for 2 weeks was similar in magnitude to that seen in samples that were thawed and frozen and stored for 2 years. In both situations serum AA increased by 0.07 to 0.09 mg/dL.

TABLE II: Effect of Repeated Freeze-Thaw Cycles on AA in Meta-Phosphoric Acid Stabilized Serum Samples

Study Design	N	Freeze/Thaw cycle	AA conc. (mg/dL) (mean + SD <sup>1</sup> )
<u>Two week cycle</u>			
1991	21	1	1.14 ± 0.65
1991	21	2	1.23 ± 0.67 <sup>2</sup>
1991	21	3	1.22 ± 0.68 <sup>2</sup>
<u>Two year cycle</u>			
1989	24	1	1.13 ± 0.59
1991	24	2	1.20 ± 0.60 <sup>2</sup>

<sup>1</sup> Interindividual standard deviation.

<sup>2</sup> Significantly different from cycle 1 values.

### DISCUSSION

The results of this study indicated that the AA concentration in stabilized serum is stable at -70°C for 2 years. Furthermore, collection of blood samples at different sites did not affect the AA concentrations. Both these issues are important in epidemiological designs as many such studies are carried out in different locations, so that the samples are collected at different places. Furthermore, in large studies, samples have to be stored for many years before analyses can take place, thus, the long-term stability of AA is very important.

We found that during the late stage of the study the difference in AA concentration between the duplicate samples were small. These results suggest that the technicians may have improved in sample preparation over time. It is important that adequate training be given to all technical staff before initiating a study collecting biological samples for AA measurements.

The observed increase in AA concentrations in various types of samples seen in experiments 3 and 4 needs to be investigated further. The apparent increase in the AA concentration in samples with high AA may be an artifact of the assay, as these values are at the top end of the standard curve. Alternatively, there may be some unknown factor operating to produce a concentrating effect at these high levels or due to evaporation of the acidified aqueous phase during repeated sample handling. To avoid such problems multiple aliquots from each subject should be stored so that unfrozen aliquots can be used if repeat measures need to be taken.

In conclusion, this study shows that AA in human serum samples that have been stabilized with meta-phosphoric acid is stable during storage at -70°C for periods of 2

years or more. This encouraging finding indicates that blood collected in large-scale, multicenter studies are useful for AA analysis several years after collection using the method described here.

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