Short Communication

Long-term Cryoconservation and Stability of Vitamin C in Serum Samples of the European Prospective Investigation into Cancer and Nutrition

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Abstract

Plasma vitamin C level may be associated with risk of some chronic diseases. The rapid degradability of vitamin C in biological samples necessitates its stabilization with metaphosphoric acid or similar agents. However, in most cohort studies, prospectively collected biological samples are not treated with stabilizing agents before long-term frozen storage and it is not known whether vitamin C can be properly measured in such samples. The objective of this study was to determine the degree of vitamin C degradation in plasma samples stored without stabilization for 7 to 11 years at ~196°C. Spearman’s correlation coefficients indicate a moderate correlation between baseline and final plasma vitamin C levels in both men (r = 0.57, P < 0.0001) and women (r = 0.52, P < 0.0001). Samples were also categorized based on low or high baseline levels of plasma vitamin C, with the latter category showing the highest rate of loss per year of frozen storage in men (1.96 μmol/L, P value for difference <0.0001; percent loss 24.6%) and women (2.35 μmol/L, P value for difference <0.0001; percent loss 24.2%), as determined by multiple regression analysis adjusted for smoking status, age, and body mass index. In men, both baseline and final plasma vitamin C values were lower in smokers than never smokers, but for both men and women the rate of vitamin C loss during storage was not significantly different between smokers and never smokers. The results of this study show that vitamin C can be measured with reasonable reliability in plasma samples frozen for long periods of time without addition of any stabilizing agents. (Cancer Epidemiol Biomarkers Prev 2005;14(7):1837–40)

Introduction

Vitamin C from the diet has been suggested to be inversely associated with risk of some cancers (1, 2) and cardiovascular disease (2), likely due to its antioxidant properties (3). With the advent of appropriate analysis techniques, measures of vitamin C in biological samples have been used as a biomarker of health status [e.g., in the study of gastric cancer (4, 5) and total mortality (6)]. Because vitamin C in biological samples can easily degrade or be readily oxidized, it is often stabilized before sample storage by the addition of stabilizing agents such as EDTA (a metal chelator), perchloric acid, DTT, or metaphosphoric acid, all of which act as protein-precipitating agents (7). However, in the case of large, multipurpose prospective studies, biological samples are collected and often stored for many years before analysis for a multitude of biomarkers. Thus, blood samples are not always treated specifically for the preservation of vitamin C before storage. Present literature on vitamin C stability deals with losses during the handling, processing, and freezing procedures of blood samples (7-12), after short periods of frozen storage without stabilization (11, 12) or for longer time periods of up to 6 years with stabilization (13, 14). However, there is currently very little information on the feasibility, stability, and validity of vitamin C analysis in blood samples stored by freezing at very low temperatures (~196°C) for very long periods of time without the addition of stabilizing compounds such as metaphosphoric acid. Thus, the objective of this study was to determine the stability of vitamin C in plasma samples stored for 7 to 11 years in liquid nitrogen and not treated with any stabilizing agents during storage compared with samples analyzed at baseline with stabilization. The samples were collected from a subset of subjects enrolled in the European Prospective Investigation into Cancer and Nutrition (EPIC) study.

Materials and Methods

The EPIC Study and the EPIC-Norfolk Subcohort. The EPIC study (15-17) is an ongoing prospective multicenter cohort study with 521,468 subjects from 23 centers in 10 European countries. The EPIC biorepositories are the world’s largest, hosting over 9 million aliquots maintained at ~196°C. For the present study, subjects were selected from the Norfolk (Cambridge, United Kingdom) center of the EPIC study (EPIC-Norfolk). The enrollment, recruitment, and sample collection
methods of the EPIC-Norfolk cohort are detailed elsewhere (18). It was chosen for the present study because in addition to the standardized EPIC protocol, it also collected an extra series of plasma samples with metaphosphoric acid preservation specifically for vitamin C analysis. These samples, referred to as "baseline samples" in the present study, were stored at −70°C and analyzed within 1 week of collection.

**Study Design.** For the present study, 144 subjects were randomly selected from all EPIC-Norfolk subjects for whom baseline plasma vitamin C measures exist. Equal numbers of men and women were chosen based on baseline smoking status (current or never smokers), year of enrollment into the EPIC study (1993, 1995, or 1997), and the quintile of baseline vitamin C values (those in the lowest or highest gender-specific quintiles based on the entire EPIC-Norfolk subcohort baseline vitamin C values; men, lowest: 20.8, highest: 72.6; women, lowest: 30.3, highest: 85.1 μmol/L). In 2004, the plasma vitamin C levels of these subjects were remeasured (referred to as "final samples") in frozen plasma samples not previously treated with any stabilizing agents.

Both baseline and final measurements were done at Addenbrookes Hospital (Cambridge, United Kingdom) using the same fluorometric analysis method (19). Baseline analyses were done on 0.25 mL plasma collected in citrate tubes, stabilized with 0.5 mL of 10% metaphosphoric acid (prepared fresh weekly), and stored at −70°C (maximum 1 week) until analysis. The relation between baseline vitamin C levels and mortality in the EPIC Norfolk subcohort has been reported elsewhere (6). For the final vitamin C measures, plasma from blood initially drawn into citrate tubes and frozen (−196°C) for up to 11 years was removed from storage in the central EPIC biorepository, thawed, and standardized with a standardized volume of freshly prepared metaphosphoric acid. Samples were quickly refrozen to −70°C and shipped on dry ice to Addenbrookes Hospital for analysis. For 12 of the 144 subjects, the amount of plasma in the straws was insufficient for vitamin C analysis, leaving a total of 132 subjects. For 24 of the subjects, existing plasma vitamin C data from a pilot phase of this study, conducted in 2002 using the exact procedures as described above, are also included in this report (referred to as "intermediate samples").

**Statistical Analyses.** To evaluate the effect of time on vitamin C loss, as well as to perform paired tests, a multivariate linear growth (mixed) regression model was used, in which baseline and final vitamin C levels were modeled as the response variable, and the effects of study subjects were included as random effects. To evaluate differences in baseline and final plasma vitamin C, an indicator variable (0 = baseline; 1 = final) was included in the model. To assess the difference in vitamin C measurements at baseline and final, by smoking status and quintile of vitamin C, as well as their interaction with the indicator term for baseline versus final, were included in the model. Analyses were adjusted by year of enrollment. Separate analyses were done to quantify the effect of time on vitamin C loss by using the number of years in frozen storage as a predictor. Interaction terms for the number of years in frozen storage with smoking status and the quintile of vitamin C were considered, but only the latter was retained because smoking status was not determined to be an effect modifier. Sex-specific analyses were consistently conducted. All models were run using nontransformed data and with the intermediate measurements for 24 subjects included. A t test was used to assess the percent loss between the baseline and final time points. For all analyses, P < 0.05 was considered statistically significant. All statistical models were run with SAS Statistical Software (SAS, Cary, NC).

To assess the correlation between baseline and final vitamin C, Spearman’s rank coefficients were calculated, adjusting for smoking status, year of enrollment, and the quintile of baseline vitamin C where necessary. The coefficients of variation (SD as a percentage of the mean) were also calculated for baseline and final vitamin C measures.

**Results**

Figure 1 plots the baseline versus the final vitamin C values. A moderate Spearman’s rank correlation was observed between the two measures for men and women combined (r = 0.62, P < 0.0001) and separate (men: r = 0.57, P < 0.0001; women: r = 0.52, P < 0.0001). When stratified by quintile of baseline vitamin C, the correlations were stronger in the lower quintile (men: r = 0.62, P = 0.0004; women: r = 0.72, P < 0.0001) than in the highest quintile (men: r = 0.54, P = 0.0013; women: r = 0.36, P = 0.0423). Table 1 shows the baseline and final vitamin C values by gender, stratified by smoking status and quintile of baseline vitamin C. In both the lowest and highest quintiles of baseline vitamin C, the final vitamin C values were significantly lower than the baseline (Table 1). Men, but not women, smokers had significantly lower baseline (P = 0.0007) and final (P = 0.0398) vitamin C values than never smokers (Table 1).

The multivariate regression model shows that for each year in frozen storage, vitamin C levels decreased by 0.26 μmol/L in men and by 0.69 μmol/L in women in the lowest quintile of baseline vitamin C, and by 1.96 μmol/L in men and by 2.35 μmol/L in women in the highest quintile of baseline vitamin C (Table 2). Initial models showed an interaction effect between the period of time in frozen storage and the quintile of baseline vitamin C for men and women. The average percent losses of vitamin C between baseline and final time points are also shown in Table 2. They show that in both men and women, the loss of vitamin C with frozen storage is greater in the highest quintile of baseline vitamin C than in the lowest quintile.

The rate of vitamin C loss in both men and women was not significantly different between smokers and never smokers. The coefficients of variation for the analyses were comparable for the baseline (53.9%) versus the final (54.9%) time points.
This study has shown, in repeated measures of a subsample of women (n = 65), that average loss of plasma vitamin C from baseline to final time points, % (SE, \( \frac{P}{C_0} \)) women, lowest: 30.3 mol/L, highest: 85.1 mol/L. This is important because it has been suggested that although metaphosphoric acid is an effective stabilizer at low temperatures, it may not be appropriate for thawed samples (20). Here, metaphosphoric acid was added to the repeat serum samples in preparation for analysis at both the baseline and final time points, between (a) men versus women; (b) never and current smokers, in men and women; and (c) the lowest and highest quintiles of baseline vitamin C, in men and women. \( P \) values were derived from the same multivariate regression model described above.

\[ * \text{As determined by the gender-specific distribution of the baseline plasma vitamin C values of the entire EPIC-Norfolk subcohort.} \]

**Table 1. Baseline and final plasma vitamin C values by gender, year of collection, smoking status and quintile of vitamin C at baseline**

<table>
<thead>
<tr>
<th>Smoking status</th>
<th>Men</th>
<th>Women</th>
<th>( \frac{P}{C_0} )</th>
<th>( \frac{P}{C_0} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Current smokers</td>
<td>55.29 ± 5.56</td>
<td>42.52 ± 4.20</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Never smokers</td>
<td>59.59 ± 4.51</td>
<td>35.13 ± 3.52</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Level of plasma vitamin C at baseline</td>
<td>21.58 ± 1.36</td>
<td>18.84 ± 1.38</td>
<td>0.0015</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Lowest quintile</td>
<td>21.58 ± 1.36</td>
<td>18.84 ± 1.38</td>
<td>0.0015</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Highest quintile</td>
<td>75.32 ± 2.07</td>
<td>56.72 ± 2.35</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Level of plasma vitamin C at baseline</td>
<td>32.36 ± 1.68</td>
<td>25.61 ± 1.50</td>
<td>0.0015</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Lowest quintile</td>
<td>86.15 ± 2.28</td>
<td>64.51 ± 2.65</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

**Discussion**

This study has shown, in repeated measures of a subsample of EPIC, that vitamin C can be measured with reasonable reliability in plasma frozen for up to 11 years without addition of any stabilizing agents. Previously, it has been observed that plasma vitamin C, stabilized before freezing and stored at \(-70^\circ\text{C}\) for 4 (13) to 6 (14) years, shows no significant losses from baseline values. However, no previous data exist on vitamin C levels in nonstabilized samples. Conceivably, vitamin C loss may be related to sample handling before freezing. However, it has been shown that refrigeration of fresh blood samples for various time periods up to 24 hours before processing and freezing does not appreciably affect the levels of vitamin C in metaphosphoric acid–stabilized serum or plasma (8). This is important because it has been suggested that although metaphosphoric acid is an effective stabilizer at low temperatures, it may not be appropriate for thawed samples (20). Here, metaphosphoric acid was added to the repeat serum samples in preparation for analysis at both the baseline and final time points, between (a) men versus women; (b) never and current smokers, in men and women; and (c) the lowest and highest quintiles of baseline vitamin C, in men and women. \( P \) values were derived from the same multivariate regression model described above.

**Table 2. The effect of various variables on the extent of vitamin C loss in men and women, as determined from a multivariate regression model**

<table>
<thead>
<tr>
<th>Level of plasma vitamin C at baseline</th>
<th>Men</th>
<th>Women</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extent of change in plasma vitamin C levels per year of frozen storage, ( \frac{P}{C_0} )</td>
<td>0.26 (0.13, ( P = 0.0553 ))</td>
<td>-0.69 (0.14, ( P &lt; 0.0001 ))</td>
</tr>
<tr>
<td>Highest quintile</td>
<td>-1.97 (0.20, ( P &lt; 0.0001 ))</td>
<td>-2.36 (0.35, ( P &lt; 0.0001 ))</td>
</tr>
<tr>
<td>Average loss of plasma vitamin C from baseline to final time points, % (SE, ( P ))</td>
<td>8.2 (6.8, ( P = 0.2406 ))</td>
<td>17.6 (4.4, ( P = 0.0003 ))</td>
</tr>
<tr>
<td>Lowest quintile</td>
<td>24.6 (2.5, ( P &lt; 0.0001 ))</td>
<td>24.2 (3.0, ( P &lt; 0.0001 ))</td>
</tr>
</tbody>
</table>

\*P values derived from a multivariate regression model comparing the plasma vitamin C values at baseline and final time points (determined by an indicator term of 0 = baseline and 1 = final), adjusted by smoking status, quintile of plasma vitamin C at baseline, and year of enrollment in the EPIC study and with interaction terms for the baseline/final indicator term with smoking status and with the quintile of baseline vitamin C, stratified by sex (except the test for men and women combined). \( P \) values for comparison of plasma vitamin C values, at the baseline and final time points, between (a) men versus women; (b) never and current smokers, in men and women; and (c) the lowest and highest quintiles of baseline vitamin C, in men and women. \( P \) values were derived from the same multivariate regression model described above.

\*As determined by the gender-specific distribution of the baseline plasma vitamin C values of the entire EPIC-Norfolk subcohort.

\*Values not in parentheses indicate change from baseline in the level of plasma vitamin C (\( \frac{P}{C_0} \)) with each year of frozen storage, adjusted for smoking status. Values in parentheses indicate the SE and \( P \) value.

\*From \( t \) tests assessing the percent loss from baseline to final. Values not in parentheses indicate average loss of plasma vitamin C (%) with each year of frozen storage. Values in parentheses indicate the SE and \( P \) value.
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between the baseline and final vitamin C values. To assess changes in vitamin C levels with storage time, this study considered a detailed mixed regression model, taking into account important variables with a potential to affect the stability or level of vitamin C. These results (Table 2) show a clear difference between the level of baseline vitamin C and the extent of loss due to frozen storage without stabilization, indicating that the extent of loss is more rapid at higher levels of baseline plasma vitamin C. This is also clearly observed in the percent average loss of vitamin C in the same table. Hypothetically, this may be important, for example, in populations such as vegetarians, who may have higher plasma vitamin C values than non-vegetarians (21).

The results of this study also suggest a greater amount of vitamin C loss for women than men at both the lower and higher quintiles of baseline vitamin C. This may be due, in part, to the higher baseline vitamin C levels in women, possibly leading to more potential for degradation, or it may be the result of other gender-specific dietary or physiologic differences.

In addition to storage time and initial level of vitamin C, smoking status may also be of importance in affecting vitamin C stability, particularly for men whose vitamin C values were lower at both baseline and final time points in smokers versus never smokers (Table 1). Smokers have previously been shown to have lower blood vitamin C levels versus nonsmokers in the entire EPIC-Norfolk cohort (6) and elsewhere (22). Because smokers have been shown to be exposed to increased levels of oxidative stress (23, 24), it can be posited that smoking may lead to a faster degradation of antioxidants such as vitamin C (25). Alternatively, there may be dietary and therefore plasma level differences in the level of intake of vitamin C, as well as of other antioxidants (e.g., vitamin E), between smokers and never smokers (26). However, in the present study, interaction tests between smoking status and the rate of vitamin C loss were not statistically significant, although adjustments were made for smoking status in all analysis models.

To maintain consistency, the same method of vitamin C analysis was used for both the baseline and final measures. The coefficients of variation for series are comparable, suggesting a similar degree of variation. However, other factors (e.g., different reagents, personnel, etc.) may have affected the variation of the results to some degree. Nonetheless, because all samples were handled with the same protocols, it is probable that the changes observed here between the baseline and final measurements are time related and mostly due to actual degradation during storage. Overall, these results indicate that there is some loss of information, likely due to either systematic decline with time or lack of metaphosphoric acid stabilization during storage, or both. Further research using a larger sample size and a broader range of vitamin C levels will provide useful information for the interpretation of studies on the effect of vitamin C on disease risk.

In conclusion, this study has shown that although freezing of non-metaphosphoric acid-stabilized plasma samples at ultralow temperatures does cause some degree of vitamin C loss, measurement of vitamin C in these samples is possible and can provide valuable information as a biomarker. This is the first study to report on the stability of vitamin C after up to 11 years in frozen storage without any form of stabilization during the period of storage. The results have important implications because many biorepositories originating from prospective cohort studies may have collected their biological specimens for a large number of potential analyses and so may not have used stabilizing procedures specifically for vitamin C before freezing.

Acknowledgments

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References

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