Research Article

Stability of Folate and Vitamin B\textsubscript{12} in Human Serum after Long-Term Storage: A Follow-Up after 13 Years

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Received 30 October 2017; Revised 29 December 2017; Accepted 22 January 2018; Published 13 March 2018

In epidemiological and nutrition research, it is very important to evaluate the stability of biomarkers as function of both storagetime and temperature. In this study, the stability offolate and vitamin B\textsubscript{12} in human serum samples has been tested after long-term storage at −80°C up to 13 years. Serum samples of 16 individualswere used in this study. The concentration offolate and vitamin B\textsubscript{12} has been determined at $t = 0$ and at 1, 8, and 13 years after storage at −80°C. The folate concentrations in serum samples remained stable at −80°C. The concentration of vitamin B\textsubscript{12} was decreasing during the time of the study to about 50%. The correlation of the folate and also of the vitamin B\textsubscript{12} concentrations in the stored samples compared with the starting values was still good. Therefore, although the concentration of vitamin B\textsubscript{12} decreased upon storage, reliable comparative analyses can still be performed.

1. Introduction

Folate and vitamin B\textsubscript{12} are both vitamins from the one-carbon metabolism and serve as compounds that repair DNA and remethylate homocysteine [1–3]. The folate status is associated with several chronic diseases with a large impact on public health [4]. Folate deficiency is also associated with birth defects [5]. Both vitamins are related to both cardiovascular diseases [3, 6, 7, 9] and cognition [7–10]. Therefore, they are very important in nutrition studies to assess the dietary patterns. In addition, in epidemiological and clinical research, these vitamins are often used to make risk assessments in prospective studies. In these studies, often serum or plasma samples were used from biobanks which have been stored for several years and sometimes even for decades. Therefore, the assessment of the stability of these biomarkers during long-term storage is of great importance [11].

The measurements of folate and to a lower extend vitamin B\textsubscript{12} are important biomarkers in clinical medicine and in epidemiologic studies. Both vitamins can be measured by a variety of methods, but the use of autoanalyzers have become common practice [12].

In the present study, folate and vitamin B\textsubscript{12} have been measured with a commercially dedicated autoimmunoanalyzer. The stability of folate and vitamin B\textsubscript{12} in serum samples was determined after a long-term storage of up to 13 years at −80°C.

2. Materials and Methods

2.1. Blood Withdrawal and Storage. For the stability study, serum samples of 16 human volunteers (blood donors) were used. Samples were obtained from the Central Blood Laboratory of the Red Cross (Amsterdam, the Netherlands) with written permission of the volunteers. After blood withdrawal, serum samples were prepared within two hours, divided in aliquots, and stored at −20°C and −70°C. In this study, only samples that have been stored at −70°C were used.

The initial concentrations of folate and vitamin B\textsubscript{12} at $t = 0$ have been determined within 4 hours after centrifugation. For long-term stability, samples have been stored for 1, 8, and 13 years, at −70°C for one year and at −80°C for the remaining period. Samples stored at −70°C were kept in a freezer equipped with temperature recorder and sound alarm. From 1 year onwards, the samples were stored at
2.2. Measurements of Biomarkers. The serum samples were kept on ice for less than 1 hr before the first measurements at \( t = 0 \). The measurements were performed as a single measurement, except on day 0 (duplicate measurements). At time points 0 and 1 year, folate and vitamin \( B_{12} \) have been determined with an autoanalyzer (Hitachi 912, Roche Diagnostics, Almere, the Netherlands). A reference serum (AHSL-1) was included in each assay. During the first year of storage, the intra-assay variation for folate was 1.9% (\( N = 8 \)). The inter-assay variation for vitamin \( B_{12} \) was 3.4% (\( N = 8 \)).

At time points 8 and 13 years, folate and vitamin \( B_{12} \) have been determined in serum with an immunoanalyzer (Access-2, Beckman-Coulter, Woerden, the Netherlands). A pretreatment converts the folate polyglutamic acid forms to the monoglutamic acid form predominant in serum. The measurements were performed as a single measurement, with dedicated kits from Beckman-Coulter. At time point 8 years, the in-assay variation was determined with a reference serum (Biorad number: 40192). For folate, the in-assay variation was 1.1% (\( N = 2 \)). The in-assay variation for vitamin \( B_{12} \) was 4.3% (\( N = 2 \)). At time point 13 years, the in-assay variation was determined with three reference sera (Biorad-1, -2, and AHLS-2). For folate, the mean in-assay variation was 1.6% (\( N = 4 \)). The mean in-assay variation for vitamin \( B_{12} \) was 2.7% (\( N = 4 \)).

The statistical differences (95% confidence interval) of the values from the initial value at \( t = 0 \) were determined with a \( t \)-test for two-samples assuming equal variances.

3. Results

At the start of the study (\( t = 0 \)), the initial concentrations of folate and vitamin \( B_{12} \) of the 16 volunteers were determined. The mean concentration for folate at \( t = 0 \) was \( 6.1 \pm 1.7 \) ng/mL, and the median value was 5.8 ng/mL, with a range of 3.9–10.5 ng/mL. The mean concentration for vitamin \( B_{12} \) at \( t = 0 \) was 471 ± 75 pg/mL, and the median value was 464 pg/mL, with a range of 381–709 pg/mL.

3.1. Stability of Folate and Vitamin \( B_{12} \). The stability of both folate and vitamin \( B_{12} \) in human serum was followed during 13 years. In Figure 2, the mean values of the 16 volunteers are shown at each time point, expressed as percentage of the mean value of their concentrations at \( t = 0 \) (before freezing of the samples).

The levels for folate are rather constant during 13 years of storage at \(-80^\circ C\). Somewhat lower values, but statistically significant, were observed at time points 1 and 13 years of 94.4% and 92.0%, respectively.

For vitamin \( B_{12} \), an almost linear decrease was observed during the time period of storage. The mean level of vitamin \( B_{12} \) decreased from 100% to 85.6%, 62.2%, and 50.5% at time points 1, 8, and 13 years, respectively.

3.2. Correlations for Folate. In Figure 3, the correlation lines of the folate concentrations of the 16 volunteers before the storage of the sera and after storage at 1, 8, and 13 years are shown. The correlations of the time points with the starting values are very good. The correlation lines have the following equations: \( y = 0.870x + 1.02 \), \( y = 0.703x + 3.53 \), and \( y = 0.552x + 5.11 \) for 1, 8, and 13 years, respectively. The correlation coefficients (\( R^2 \)) were 0.961, 0.867, and 0.889 for 1, 8, and 13 years, respectively. The statistics between 2 sets of data are \( P = 0.0019 \) for 0 versus 1 year, \( P = 0.155 \) for 0 versus 8 years, and \( P = 0.031 \) for 0 versus 13 years as determined with a \( t \)-test for equal variances.

3.3. Correlations for Vitamin \( B_{12} \). In Figure 4, the correlation lines of the vitamin \( B_{12} \) concentrations of the 16 volunteers before the storage of the sera and after storage at 1, 8, and 13 years are shown. Although the concentration of vitamin \( B_{12} \)
Several short-term stability studies on folate and vitamin B$_{12}$ were performed in the past [13–17]. But only one long-term stability study on folate was found in EDTA plasma for 48 months at −20°C [18]. After 6 months of storage the mean value of folate decreased to 25% of its original value, and this level remained constant until 48 months. For vitamin B$_{12}$, only one long-term stability study was found [14] and showed a good stability during 48 months of storage in EDTA plasma at −20°C, which is a different finding with our present results.

The present study shows in which folate and vitamin B$_{12}$ were measured after 1, 8, and 13 years of storage at −80°C that the measurements of folate still can be done without any major change in the concentration. For vitamin B$_{12}$, there is a consistent decrease to about 50% after 13 years of storage at −80°C. It must be noted that from 8 years on, the autoanalyzer and reagents were changed so that a possible level change could be expected, although a comparative study was performed that showed the same results for both methods. In a previous report after 1 year of storage [19], the decrease of vitamin B$_{12}$ was already visible, but then it was not feasible to make a strong conclusion based on only one time point. Now after 13 years, it is clear that this decrease was consistent.

For epidemiological studies, however, this decrease in concentration of vitamin B$_{12}$ has only little effect on the rank order and correlations with the starting concentrations. Rather good correlation coefficients ($R^2$) were found between 0.87 and 0.89 after storage. For folate, the concentrations of the stored samples remain close to the starting concentrations with good correlations with the starting concentrations; for example, the correlation coefficient ($R^2$) at 13 years was 0.89.

There has been one study on the long-term stability of B vitamins [20] in which storage at −25°C during 4, 6, 17, and 29 years was reported. Folate was measured as p-aminobenzoylglutamate declined at a slow rate of 0.98%/year and about 80% of the folate was recovered after 29 years of storage as determined with LC-MS/MS. For folate, p-aminobenzoylglutamate appeared to be the method of choice for the determination of folate status in stored serum samples. In our method, a pretreatment also converts the folate polyglutamic acid forms to the monoglutamic acid form which is predominant in serum.

In the same study, vitamin B$_{12}$ status did not show any decline, as measured with a microbiological assay [20]. This is in contrast with our results. Maybe the method of choice has an influence of the outcome of the study.

In conclusion, this study shows that long-term storage of serum samples at −80°C, intended for future measurements of folate and vitamin B$_{12}$, can still be used for data analyses, although the concentration of vitamin B$_{12}$ decreases steadily with longer storage time.
Ethical Approval

Samples were obtained from blood donors of the Central Blood Laboratory of the Red Cross (Amsterdam, the Netherlands) with written permission of each of the volunteers. In this study, principles of the Declaration of Helsinki were followed.

Disclosure

All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

Conflicts of Interest

No potential conflicts of interest were reported by the authors.

Acknowledgments

Part of the study was conducted with the financial support from the Board of Directors of the National Institute of Public Health and the Environment (Bilthoven, the Netherlands), within the strategic project on antioxidant supplements for aging.

References
