Effect of Storage on Serum Vitamin B$_{12}$ and Folate Stability*

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ABSTRACT

To facilitate transport from remote locations, the stability of vitamin B$_{12}$ and folate was investigated in serum specimens. Serum vitamin B$_{12}$ proved to be highly unstable, emphasizing that specimens should be frozen if not analyzed immediately. Light protection is necessary if the sample cannot be analyzed within 4 hours. In contrast, folate is a more robust analyte. In refrigerated serum specimens, folate was stable up to 7 days of storage. In situations where specimen stability is important, vitamin B$_{12}$ status is better assessed with serum or urine methylmalonic acid measurements. Although folate status can be assessed in a similar fashion with homocysteine, specimen stability indicates that direct measurement of folate is a better strategy.

Introduction

Knowledge of the stability of the specimens is critical to determine appropriate conditions for transportation. The laboratory tends to dictate rigorous conditions for transportation since the goal is to ensure the least possible degradation of the analyte. On the other hand, physicians, especially those who work in remote areas, would like to see less stringent transportation conditions. One can appreciate their problem when realizing that freezers and dry ice may not always be readily available. Since there is a need for nutritional evaluation of people living in remote areas, establishment of the least stringent but still adequate transportation conditions is a significant issue.

Little information can be found in the literature or from commercial vendors about the stability of vitamin B$_{12}$ and folate in serum specimens.\textsuperscript{1,2,3,4,5,6} There is a consensus that specimens collected for vitamin B$_{12}$ and folate testing need to be separated from the cells and frozen, however the question of light stability is not generally addressed.\textsuperscript{1,2,3,4,5,6} One study by Mastropaolo et al.\textsuperscript{2} focused on the stability of specimens for retesting, and, consequently, still left doubts about the stability during normal transportation. The purpose of this study was to determine the stability of serum vitamin B$_{12}$ and folate under conditions simulating transport.

Methods

STUDY DESIGN

The stability of vitamin B$_{12}$ and folate was investigated under four different storage con-
ditions: (1) frozen and light protected, (2) frozen and light exposed, (3) refrigerated and light protected, and (4) refrigerated and light exposed. The light protected specimens were wrapped with aluminum foil, while those not light protected were stored without wrapping. All were exposed to light when the refrigerator or freezer was open, but otherwise were not artificially exposed to intense light. This situation was designed to resemble what happens to a specimen under regular processing conditions.

Two volunteers (eight total) were randomly enrolled into each of the four storage condition groups. Blood from each volunteer was collected in serum separator tubes, and centrifuged for 10 min at 3000 g. Serum was divided into six plastic tubes (48 tubes total). One tube was analyzed within 20 min of collection (0 hour specimen), while the subsequent tubes were put into storage and analyzed at 4 and 8 hours, and 1, 3, and 7 days. Three to six parallel measurements were performed on each tube.

Vitamin B\textsubscript{12} and folate were determined by chemiluminescence immunoassay on a Ciba-Corning ACS: 180\textsuperscript{®}. The adult reference interval is 210 to 911 pg/mL for vitamin B\textsubscript{12} and 2.8 to 17.8 ng/mL for folate. Levey-Jennings charts of control materials for vitamin B\textsubscript{12} revealed a day-to-day variation of 18.1, 10.3, and 8.8 percent at concentrations of 303, 630, and 1100 pg/mL; for folate, the day-to-day variation was 17.5, 10.3, and 10.7 percent at concentrations of 1.9, 5.3, and 11.1 ng/mL.

**Calculations**

At each time point, the results were normalized by dividing by the average of the zero hour measurements of each volunteer. The standard error of the mean was calculated from the percentage values and is illustrated as error bars in figures 1 and 2.

To quantitate the effects of light and storage temperature, results at 4 hrs, 8 hrs, 1 day, 3 days, and 7 days were paired so that only the light exposure or storage temperature differed in each comparison. This resulted in five comparisons to assess the effect of light in each storage temperature, and five comparisons to assess the effect of storage temperature with different light exposures. In each comparison, results were compared with the Student t-test. Because of the normalization zero hour, results did not differ. To keep the overall error rate at 5 percent, the following equation was used to calculate the individual error rates allowed in each comparison.\textsuperscript{7,8}

\[ 1 - (1 - \alpha)^c = 0.05 \]

where \( \alpha \) is the error of each individual comparison, and \( c \) is the number of comparisons. For 5 comparisons, the calculated \( \alpha \) value is 0.01. Thus, within each set of conditions, results are considered significantly different only if \( p < 0.01 \).
EFFECT OF STORAGE ON SERUM VITAMIN B₁₂ AND FOLATE STORAGE

140
120
100
80
60

Folate, frozen

light protected
not light protected

-relative conc. / %

time / days

Folate, refrigerated

light protected
not light protected

-relative conc. / %

time / days

Results

VITAMIN B₁₂

The normalized results for vitamin B₁₂ stability are shown in figure 1 (absolute concentrations varied from 193 to 1343 pg/mL). Vitamin B₁₂ concentrations under all four storage conditions followed similar patterns of an initial rise followed by a fall after 1 to 3 days. The frozen specimens showed an initial peak at 1 day of storage, followed by a decrease which dropped below the zero hour concentration at 7 days. The refrigerated specimens also showed a rise in vitamin B₁₂ concentration which was initially fast, and at 3 days reached a level of 18 to 28 percent higher than the initial concentration. After day 3, the relative vitamin B₁₂ concentration decreased by about 25 percent in both those light protected and light exposed. At 7 days the measured results were about equal to the zero hour values.

Compared to light exposed specimens, the light protected specimens were less affected by storage temperatures, and, therefore, had lower vitamin B₁₂ concentrations (closer to the zero hour values). Light exposure had a larger effect on frozen specimens resulting in larger differences in vitamin B₁₂ concentrations, by an average of 10 percent versus 6 percent in the refrigerated ones, between light protected and light exposed specimens. The frozen and light protected specimens were significantly closer to zero hour values at 8 hours and more of storage (p = 0.001).

Storage temperature had a much more pronounced effect on vitamin B₁₂ concentration than light, with refrigerated specimens much more affected than those frozen. The light protection seems to prevent change in vitamin B₁₂ concentration up to 4 hours of storage (p = 0.48 at 4 hours). The light exposed specimens had different vitamin B₁₂ concentrations at all time periods (p < 1 x 10⁻⁵).

Vitamin B₁₂ concentration in the frozen and light protected specimens remained within the coefficient variation (CV) of the assay up to 3 days (p = 0.00005 at 7 days). The frozen light exposed specimens remained stable only for 4 hours (p < 0.001 after 4 hours). The refrigerated specimens were not stable regardless of light exposure (p < 0.0006 at all time periods), see figure 1B.

FOLATE

The normalized results on folate stability are shown in figure 2 (concentrations varied from 6.1 to 40.7 ng/mL). In contrast to vitamin B₁₂, only minor changes were observed in folate concentration under all four storage conditions.

The light protected specimens had higher folate concentrations. Folate concentration in the light protected samples compared to the light exposed statistically differed by 8 percent at 7 days in the frozen specimens (p < 0.002); however, this difference is within the 10 percent CV of the assay.
Unlike in the case of vitamin B₁₂, temperature had minor effect on folate stability. In the light protected specimens, there was a statistically significant difference of 9 percent in folate levels (again within the CV) only at the 3 days of storage (p = 0.0095). However, it should be noted that this p value is very close to our decision level of 0.01. In the light exposed specimens, there was a statistically significant difference in folate levels only after 3 days of storage (p = 0.002 at 7 days).

Statistical calculations confirmed no variation in folate concentration during the observation period in all four storage conditions. The only exception were the refrigerated and light protected specimens at 7 days of storage, which had a statistically significant 6 percent higher folate level than the initial folate concentration (p = 0.0002); however this is within the CV of the assay and is clinically not significant. In the frozen specimens, no significant change was seen during the 7 days of observation. Folate concentration in the frozen specimens remained within ±7 percent from the zero hour measurement, well within the assay CV.

Discussion

Vitamin B₁₂

Vitamin B₁₂ in aqueous solution is quite stable and is typically stored at room temperature in clear glass vials.⁹ In contrast, this study shows that serum vitamin B₁₂ is very unstable. The rise of measured vitamin B₁₂ in serum specimens in short term storage (less than 1 to 3 days) is surprising, but it is seen consistently. It is possible that degradation of vitamin B₁₂ in serum specimens gives rise to metabolites which have a larger response in the chemiluminescence immunoassay. Another possible explanation is that vitamin B₁₂ is slowly released from the binding proteins, and, thus, the free vitamin B₁₂ concentration increases with time. Since the assay uses a sodium hydroxide releasing agent to free all vitamin B₁₂ from binding sites prior to analysis, this second possibility would imply that the releasing step is not complete.

Changes in vitamin B₁₂ concentration can be minimized by freezing. If frozen and analyzed within 4 hours, specimens collected for vitamin B₁₂ analysis do not need to be light protected. The frozen, light protected specimens are stable up to a week. Refrigerated specimens deteriorate rapidly indicating specimens need to be frozen to maintain reasonable stability.

Folate

Serum folate proved to be more stable than was expected from literature sources.¹⁰ While folate is light sensitive in pure form or in aqueous solution,¹⁰ in serum it is not affected by the light conditions employed here. Binding proteins may have a protective effect which prevents light degradation in serum. All stored specimens stayed within ±7 percent from the 0 hour concentration, which is within the CV of the assay. Refrigeration was adequate for up to 7 days of storage.

Testing Strategies

When processing and transporting specimens in remote locations, these stability studies suggest some testing strategies are likely to give more clinically relevant results than others. The measurement of methylmalonic acid is a well-established and sensitive indicator of vitamin B₁₂ status, and methylmalonic acid is also considerably more stable than vitamin B₁₂.¹¹ However, assays for methylmalonic acid are also more expensive.¹² In situations where stability and sensitivity are important, methylmalonic acid is the preferred analyte for the evaluation of vitamin B₁₂ status. Both serum and urine can be used.¹¹,¹³

Unlike vitamin B₁₂, folate is stable and does not require the use of a surrogate marker to give added stability. Homocysteine is a more sensitive indicator of folate status, although homocysteine is elevated in several other conditions, as well.¹⁴ Homocysteine is also relatively unstable and should not replace folate when processing and transport conditions are challenging.
Conclusion

Based on data collected from an ACS:180® analyzer, serum specimens for vitamin B$_{12}$ analysis need to be frozen if not analyzed immediately. Light protection is necessary for vitamin B$_{12}$ if the assay is not done within 4 hours. Refrigerated specimens for serum folate analysis are acceptable if the test is done within 3 days from collection; otherwise, specimens need to be frozen. Light protection is not necessary for folate in the conditions employed here. When collecting specimens in remote locations, serum folate is a robust analyte whereas vitamin B$_{12}$ is not. In the later case, recommendation is made to use methylmalonic acid for the evaluation of vitamin B$_{12}$ status.

References