Holo-Transcobalamin Concentration and Transcobalamin Saturation Reflect Recent Vitamin B₁₂ Absorption Better than Does Serum Vitamin B₁₂

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Background: We evaluated whether measurement of vitamin B₁₂-saturated transcobalamin (holo-TC) concentrations or TC saturation (holo-TC:total TC) reflects active vitamin B₁₂ absorption in healthy individuals and patients after vitamin B₁₂ intake.

Methods: We obtained blood samples from 31 healthy individuals (age range, 25–57 years) before (days −1 and 0) and after (days 1, 2, and 6) oral administration of three 9-μg doses of vitamin B₁₂. The blood samples from seven patients (age range, 22–39 years) suspected to have decreased vitamin B₁₂ absorption were obtained before and 1 day after the vitamin B₁₂ intake. The blood samples were analyzed for vitamin B₁₂, total TC, and holo-TC. The TC saturation was calculated.

Results: Intraindividual variation was <13% for all measured values, as calculated from samples removed on day −1 and 0. In healthy individuals (n = 31) after intake of vitamin B₁₂, the maximum median (range) increase (as percentages and absolute values) was in TC saturation [52 (−2% to 128)% and 0.04 (0–0.23) as a fraction], closely followed by holo-TC concentrations [39 (0–108)% and 34 (0–149) pmol/L]. All but one healthy individual had an increase of ≥15% in these markers. Serum vitamin B₁₂ showed a smaller increase [14 (−8 to 51)% and 36 (−27 to 290) pmol/L]. After vitamin B₁₂ intake, three patients with Crohn disease had the lowest increases in holo-TC concentration (3, 7, and 14 pmol/L) and in TC saturation (0.004, 0.01, and 0.01) among patients and 30 healthy individuals.

Conclusion: Holo-TC concentrations and TC saturation reflect normal vitamin B₁₂ absorption better than does serum vitamin B₁₂.

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Cobalamin (vitamin B₁₂) is an essential nutrient for one-carbon metabolism and cell division that must be supplied by dietary meat or dairy products; the minimum recommended daily intake is 2.4 μg (1). After ingestion, vitamin B₁₂ is bound to haptocorrin present in saliva. In the small intestine, pancreatic enzymes degrade haptocorrin, and vitamin B₁₂ is transferred to intrinsic factor (IF), a protein synthesized in the parietal cells of the stomach. The IF–vitamin B₁₂ complex is absorbed via the IF–B₁₂ receptor, and vitamin B₁₂ is subsequently bound to transcobalamin (TC) and released into the circulation (2, 3). TC bound to vitamin B₁₂ (holo-TC) facilitates the transport of vitamin B₁₂ from blood to various tissues (4). Accordingly, circulating concentrations of holo-TC may be a marker of vitamin B₁₂ absorption. However, to date, whether changes in holo-TC reflect vitamin B₁₂ absorption has not been evaluated.

In the present study, we evaluated this concept by measuring serum holo-TC and total TC before and after oral intake of three 9-μg doses of vitamin B₁₂ in 31 healthy individuals and 7 patients.

Materials and Methods
The participants in the study included 31 healthy individuals recruited in October 2002. None of them suffered from known disorders related to vitamin B₁₂ deficiency. Persons with chronic systemic disease; persons taking any kind of medications, including vitamins, within the past week; and persons not able to give written informed consent were excluded. The mean age of the healthy individuals was 40 years (age range, 25–57 years). There were 9 men and 22 women. We also included seven patients (age range, 22–39 years; five men and two...
women) who had been referred to the outpatient clinic of the internal medicine department during 2003 for suspected vitamin B$_{12}$ malabsorption. Three of the seven patients had previously been diagnosed as having Crohn disease. The diagnoses for the remaining four patients were not clear.

Written informed consent was obtained from all participants, and the Research Ethics Committee of Aarhus County approved the study protocol (2002.0224).

**STUDY PROTOCOL**

Vitamin B$_{12}$ absorption was evaluated by analysis of serum vitamin B$_{12}$, total TC, and holo-TC in samples obtained before and after oral administration of vitamin B$_{12}$.

In the healthy individuals, samples were taken at 0800 on the day before vitamin B$_{12}$ intake (day −1) and on days 0, 1, 2, and 6. After the blood sample was taken on day 0, the healthy individuals were administered three 9-µg oral doses of vitamin B$_{12}$ (Natur Drogeriet A/S), with 6 h between each dose (0800, 1400, and 2000; time points were allowed to deviate ±45 min). One healthy individual was unavailable for blood sampling on day 6. Vitamin B$_{12}$ absorption in the seven patients was evaluated by the Schilling test I and by the design described above except that the blood samples were obtained only on days 0 and 1. The Schilling test I was performed after our alternative approach.

The vitamin B$_{12}$ tablets were given with either water or orange juice. The participants were allowed to have a light breakfast 30–60 min before blood sampling, not including any diary products, but were otherwise allowed to eat their typical diet. The blood samples were centrifuged within 60 min and were stored at −80°C until further processing.

**SCHILLING TEST I**
The Schilling test I was performed as described previously (4). Briefly, a fasting patient is given a 1-µg oral dose of vitamin B$_{12}$, which is tagged with radioactive cobalt ($^{57}$Co). Two hours after the oral dose, the patient receives by intramuscular injection 1000 µg of nonlabeled vitamin B$_{12}$. A 24-h urine collection is initiated. The percentage of the administered dose excreted in the urine over 24 h is then determined. Urinary excretion of 10–40% of the administered dose is considered normal.

**BIOCHEMICAL ANALYSES**

Serum vitamin B$_{12}$ was determined by a commercial method (Bayer Corporation) on a Centaur analyzer [CV = 6.8% at a mean of 293 pmol/L (n = 272); CV = 5.8% at a mean of 543 pmol/L (n = 280)].

Serum total TC and holo-TC were measured by ELISA as described recently (5, 6), but modified to allow the use of an automated ELISA analyzer (BEP-2000; Dade Behring). The modification was as follows: all incubations were performed at 37°C. The imprecision was 7% for total TC at a mean of 934 pmol/L (n = 91 over 12 months) and was 8% for holo-TC at a mean of 38 pmol/L (n = 41 over 6 months). The reference interval was established by analysis of 161 samples obtained from healthy blood donors (age range, 21–65 years). The reference intervals were 700-1400 pmol/L for total TC, ≥50 pmol/L for holo-TC, and ≥0.05 for TC saturation.

Hematologic indices were assessed with the Coulter Counter STKS1 (Beckman Coulter). Plasma creatinine was measured by the Jaffe method on a Roche Cobas Integra 700 analyzer (CV <3%).

**STATISTICAL ANALYSIS**

The intraindividual variation was calculated from the estimation of variance by ANOVA from the measurements of the analytes from the two samples obtained before the treatment (days −1 and 0).

Changes (increases or decreases) in markers as a function of time were analyzed by comparing the changes obtained for the same individuals relative to baseline (day 0) with the theoretical median "0" assigned for day 0. Because the data did not follow a gaussian distribution, nonparametric testing (Wilcoxon matched-pair test) was used. P values <5% were regarded as statistically significant. Data were analyzed with SPSS10.0 (SPSS Inc.) and GraphPad (Prism 3.03) software.

**Results**

All 31 healthy individuals had normal erythrocyte counts, hemoglobin, mean cell volumes, and creatinine concentrations, as summarized in Table 1. The intraindividual variation was <13% for all analytes (Table 1), as calculated from data obtained for the samples collected before intake of vitamin B$_{12}$ (days −1 and 0).

After oral intake of three 9-µg doses of vitamin B$_{12}$, all markers studied changed as indicated in Fig. 1. The changes relative to baseline (day 0) were highly significant on day 1 (P <0.0002 for all markers) and day 2 (P <0.0005 for holo-TC, TC saturation, and vitamin B$_{12}$; P = 0.02 for total TC). The maximum percentages and absolute increases [median (range)] were 39 (0–108)% and 34 (0–149) pmol/L for holo-TC and 52 (−2 to 128)% and 0.04 (0–0.22) as a fraction for TC saturation, respectively (n = 31). Maximum increases ≥15% for holo-TC and TC saturation were observed at day 1 for 29 individuals and at day 2 for one individual. Only one healthy individual did not have increases in holo-TC concentration and TC saturation.

The increases (as a percentage and absolute values) in serum vitamin B$_{12}$ were less dramatic: 14 (−8 to 51)% and 36 (−27 to 290) pmol/L. In four healthy individuals, vitamin B$_{12}$ did not increase, and in 14 healthy individuals it increased <15%.

Small but significant changes were observed for total TC. The maximum percentage and absolute decreases were 5 (−16 to 9)% and 46 (−180 to 77) pmol/L. Twenty-
three of the 31 healthy individuals had a decrease in total TC concentration at day 1.

After 1 day, the highest values [median (range)] were for holo-TC [118 (56–344) pmol/L], TC saturation [0.13 (0.06–0.43)], and serum vitamin B₁₂ [279 (176–856) pmol/L], whereas total TC reached its lowest concentration [855 (710–1527) pmol/L; Table 2]. After 6 days, the values for holo-TC, total TC, and TC saturation did not differ significantly from baseline, whereas the concentration of serum vitamin B₁₂ remained significantly higher than baseline (P = 0.0086).

On the basis of these results, the calculated TC saturation appears to be a slightly better marker for vitamin B₁₂ absorption because of the observed decrease in total TC together with the increased holo-TC concentration after vitamin B₁₂ intake. Thirty healthy individuals had increases of ≥21% in TC saturation, whereas only 7 had comparable increases in serum vitamin B₁₂ concentration (Fig. 2).

In four of the seven patients suspected of having decreased vitamin B₁₂ absorption, serum holo-TC and vitamin B₁₂ values were below the reference interval (Fig. 3), although their hematologic tests were normal (data not shown). Three of these four patients were previously diagnosed as having Crohn disease. After vitamin B₁₂ intake, the three patients with Crohn disease showed

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### Table 1. Characteristics of the 31 healthy individuals and intraindividual variation in vitamin B₁₂, serum holo-TC, total TC, and TC saturation.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Baseline values, median (range)</th>
<th>Reference intervals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ani</td>
<td>Males</td>
<td>Females</td>
</tr>
<tr>
<td>Age, years</td>
<td>40 (25–57)</td>
<td></td>
</tr>
<tr>
<td>Blood hemoglobin, mmol/L</td>
<td>8.5 (7.8–10.2)</td>
<td>8.4–10.8</td>
</tr>
<tr>
<td>Mean cell volume, fl</td>
<td>89 (79–98)</td>
<td>85–100</td>
</tr>
<tr>
<td>Erythrocyte count, 10¹²/L</td>
<td>4.4 (3.9–5.6)</td>
<td>4.1–6.1</td>
</tr>
<tr>
<td>Plasma creatinine, μmol/L</td>
<td>78 (61–106)</td>
<td>62–133</td>
</tr>
<tr>
<td>Holo-TC, pmol/L</td>
<td>73 (36–281)</td>
<td>≥50</td>
</tr>
<tr>
<td>Total TC, pmol/L</td>
<td>947 (747–1471)</td>
<td>700–1400</td>
</tr>
<tr>
<td>TC saturation, fraction</td>
<td>0.08 (0.02–0.22)</td>
<td>≥0.05</td>
</tr>
<tr>
<td>Vitamin B₁₂, pmol/L</td>
<td>250 (163–661)</td>
<td>200–650</td>
</tr>
</tbody>
</table>

a Laboratory values were determined from the blood samples obtained day −1.
b References intervals for holo-TC, total TC, and TC saturation were based on analyses of 161 samples obtained from healthy blood donors.
c Intraindividual variation was calculated based on values obtained on days −1 and 0 from the 31 healthy individuals before administration of vitamin B₁₂.
d Same reference interval for males and females.
e Calculated as holo-TC:total TC.

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### Table 2. Absolute values for TC saturation, holo-TC, and vitamin B₁₂ before (day 0) and at timed intervals after oral intake of vitamin B₁₂ in 31 healthy individuals.

<table>
<thead>
<tr>
<th>Day</th>
<th>Median (range)</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Marker</th>
<th>Day 0</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Holo-TC, pmol/L</td>
<td>72</td>
<td>118</td>
<td>87</td>
<td>80</td>
</tr>
<tr>
<td>Total TC, pmol/L</td>
<td>905</td>
<td>855</td>
<td>850</td>
<td>885</td>
</tr>
<tr>
<td>TC saturation, fraction</td>
<td>0.08</td>
<td>0.13</td>
<td>0.11</td>
<td>0.09</td>
</tr>
<tr>
<td>Vitamin B₁₂, pmol/L</td>
<td>234</td>
<td>279</td>
<td>253</td>
<td>274</td>
</tr>
</tbody>
</table>

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Fig. 1. Changes in serum vitamin B₁₂ (●), total TC (▲), holo-TC (●), and TC saturation (●) in 31 healthy individuals after ingestion of three 9-μg doses of vitamin B₁₂.

The percentage increase from baseline (day 0) is indicated. Values are the means (SE; error bars). There were highly significant changes from baseline values (day 0) for all markers on day 1 (P < 0.0002 for all markers) and for all markers (P < 0.0005) except TC (P = 0.002) on day 2 after intake of vitamin B₁₂. On day 6 only the change of vitamin B₁₂ was significantly different from the baseline value (P = 0.0024).
negligible increases in holo-TC (3, 7, and 14 pmol/L) and TC saturation (0.004, 0.01, and 0.01; Fig. 3).

In the seven patients who underwent the Schilling test I (Fig. 3), its results (expressed as percentage of vitamin B₁₂ excreted in the urine) were not significantly correlated with the changes in serum holo-TC after vitamin B₁₂ intake ($r = 0.14; P = 0.77$). In six of these patients, however, the two tests were significantly correlated ($r = 0.81; P = 0.05; n = 6$). One of three patients with Crohn disease had an abnormal Schilling test I (2%). The other two had Schilling test I results within the reference interval (10–40%), but their values were in the lower part of the reference interval (10% and 15%, respectively).

**Discussion**

To our knowledge this is the first study to document that serum holo-TC concentration and TC saturation reflect active vitamin B₁₂ absorption. One day after receiving three 9-μg oral doses of vitamin B₁₂, healthy individuals had median increases of ~50% in holo-TC concentration and TC saturation, whereas the increase for serum vitamin B₁₂ was only 14%. These findings strongly suggest that measurement of holo-TC and/or TC saturation after an oral dose of vitamin B₁₂ provides more information regarding active absorption of vitamin B₁₂ than does measurement of serum vitamin B₁₂.

To date, little attention has been paid to the dose of vitamin B₁₂ administered to patients to study the active uptake of vitamin B₁₂ by use of blood tests. Most previous studies have used a relatively larger single oral dose of vitamin B₁₂ (1000 μg) (7, 8). The crucial point here is that with this large dose of vitamin B₁₂, the non-IF-mediated absorption of 1% alone will increase the plasma concentration, thereby giving false results. This increase does not reflect active IF-mediated absorption and thus has limited diagnostic impact regarding the active vitamin B₁₂ absorption.

In our study we designed the intake of vitamin B₁₂ to meet two criteria: (a) we wanted to minimize passive absorption, which accounts for ~1% of the dose supplied (4); and (b) we wanted as much actively absorbed vitamin B₁₂ as possible to accumulate to provide an optimum signal. To meet these two demands, we chose to use a high physiologic dose (9 μg) and to administer this dose three times at 6-h intervals. Absorption of vitamin B₁₂ enters a refractory phase of ~3 h after ingestion of vitamin B₁₂ (4). An additional dose of vitamin B₁₂ is absorbed normally when given ~4–6 h after the initial dose (4). The highest amount of IF-bound vitamin B₁₂ is obtained at a vitamin B₁₂ dose of 10 μg. The dose of 9 μg was chosen because this is commercially available and quite close to 10 μg (4).

We examined seven patients with suspected vitamin B₁₂ malabsorption by both the Schilling test I and our alternative approach. Four of the patients had small increases in holo-TC and TC saturation (Fig. 3), whereas three patients had increases comparable to those for the healthy individuals studied. One of these four patients also had an abnormal Schilling test. The other three had normal Schilling I tests, but the absolute values (urinary excretion of radioactive vitamin B₁₂) for these three patients were in the lower part of the reference interval (Fig. 3).
3. The increase in holo-TC (87 pmol/L) and the result of Schilling test I (12%) were discrepant for one patient. Because this patient had serum vitamin B12 (360 pmol/L) and holo-TC (100 pmol/L) concentrations within the appropriate reference intervals, we believe it most likely that the result obtained by the Schilling test I was falsely low, which is a known problem with this test, most often caused by inappropriate urine collection. As stated in the Results, the correlation was excellent between our approach and the Schilling test I for the remaining six patients. These results thus suggest that our alternative test may be able to identify patients with vitamin B12 malabsorption, but further studies are needed to evaluate the usefulness of the test in the clinical setting. In the context of the present report, the results for the three patients with Crohn disease may be useful, as discussed below. These patients are likely to have no or only limited active absorption of vitamin B12.

One important concern about our study is whether we report, as we anticipate, active absorption of vitamin B12 or whether the results are caused by passive absorption of the vitamin. Comparing our data on patients with Crohn disease with the remaining group makes it very unlikely that our results were caused by passive absorption. In our
design, passive absorption is expected to be ~5% of the total absorption (9). The median increases in the three patients with Crohn disease were 7 pmol/L for holo-TC and 0.01 for TC saturation. These values were ~20% of the median increase observed for healthy controls. We think it more likely that these results reflect a remaining small capacity for active absorption of vitamin B₁₂ in addition to the passive absorption of ~5% (9). Independent of the interpretation, it was possible to distinguish patients with expected low vitamin B₁₂ absorption (patients with Crohn disease) from the healthy individuals.

Evaluation of the intestinal absorption of vitamin B₁₂ is essential to clarify the cause of vitamin B₁₂ deficiency and has also been used to evaluate the absorptive capacity of the small intestine (10). The standard test for vitamin B₁₂ absorption is the Schilling test. The Schilling test is problematic mainly because it uses labeled vitamin B₁₂ and because it requires normal renal function, a complete 24-h urine collection, and large parenteral doses of vitamin B₁₂ that can occasionally obscure the diagnosis (11–13). There thus is a need to find alternative tests for estimating vitamin B₁₂ absorption to both diagnose the cause of vitamin B₁₂ deficiency and estimate the absorptive capacity of the small intestine.

Evaluation of vitamin B₁₂ absorption by the use of blood tests has been explored for a long time. To date such studies have not been unequivocal, most likely because the test used has been serum vitamin B₁₂ (7, 8, 14). Vitamin B₁₂ includes vitamin B₁₂ bound to two binding proteins. The major part is bound to a protein of unknown function, haptocorrin. This portion of the plasma vitamin has a slow turnover with a half-life of 240 h (15, 16), and changes reflecting increased absorption occur relatively late in this fraction of plasma vitamin B₁₂. The minor portion of plasma vitamin B₁₂ is attached to TC. TC transports vitamin B₁₂ to all cells of the body. The turnover is rapid with a half-life of 1–12 h (17, 18). Holo-TC appears to be a superior marker for reflecting sudden changes in vitamin B₁₂ homeostasis (19, 20), but only recently have reliable assays for measurement of holo-TC as well as total TC become available (6, 21).

Our study gave very promising results. All but two healthy individuals showed an increase in holo-TC of 15 pmol/L (15%) and a fractional increase in TC saturation of 0.02 (21%) or more above the baseline values. According to these results, we suggest that vitamin B₁₂ absorption can be considered normal when one of the following two criteria is fulfilled: (a) the increase in serum holo-TC is at least 15 pmol/L and at the same time ≥15% above the baseline value; (b) the increase in TC saturation is 0.02 and at the same time ≥20% above the baseline value.

In healthy individuals, total TC was significantly decreased after the oral dose of vitamin B₁₂. This is in agreement with previous studies (22, 23), but the reason for this decrease is unknown. One possibility is that tissues take up TC to which vitamin B₁₂ is bound more rapidly than unsaturated TC. The consequence of the observed decrease in total TC together with the increased holo-TC concentration after vitamin B₁₂ intake is that calculated TC saturation becomes a slightly better marker for vitamin B₁₂ absorption than measurement of the holo-TC concentration alone. This has been emphasized both in our present study and in a previous study (23) in which a pharmacologic dose of oral vitamin B₁₂ was used. Interestingly, after vitamin B₁₂ intake, total TC was not decreased in the three patients with Crohn disease. It is logical to assume that those patients cannot absorb vitamin B₁₂ and that no increased amount of holo-TC is therefore delivered to the tissues.

The changes in the TC-related markers did not last very long in healthy individuals. The values had returned toward normal by the second day after ingestion of vitamin B₁₂. After 6 days, holo-TC concentrations and TC saturation did not differ significantly from the baseline. It therefore would be possible to repeat the evaluation of vitamin B₁₂ absorption after 1 week.

In conclusion, holo-TC concentrations and TC saturation appear to reflect vitamin B₁₂ absorption better than does serum vitamin B₁₂. Measurement of holo-TC concentrations and/or TC saturation before and after oral ingestion of vitamin B₁₂ could possibly be developed into a test that is suitable as an alternative to the Schilling test I.

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References