

Assessment of Vitamin B₁₂ Absorption Based on the Accumulation of Orally Administered Cyanocobalamin on Transcobalamin

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BACKGROUND: Vitamin B₁₂, or cobalamin (Cbl), is absorbed in the intestine and transported to the cells bound to transcobalamin (TC). We hypothesize that cyanocobalamin (CNCbl) is absorbed unchanged, thereby allowing measurement of the complex of CNCbl bound to TC (TC–CNCbl) to be used for studying the absorption of the vitamin.

METHODS: TC was immunoprecipitated from serum samples obtained from healthy donors at baseline and at 24 h after oral administration of three 9- μ g CNCbl doses over 1 day. Cbl was released by treatment with subtilisin Carlsberg. The different forms of Cbl were isolated by HPLC and subsequently quantified with an ELISA-based Cbl assay.

RESULTS: At baseline, the median TC–CNCbl concentration was 1 pmol/L (range, 0–10 pmol/L); the intra-individual variation (SD) was 1.6 pmol/L ($n = 31$). After CNCbl administration, the TC–CNCbl concentration increased significantly ($P = 0.0003$, paired t -test), whereas no major changes were observed in any of the other Cbl forms bound to TC ($n = 10$). Only a moderate additional increase in TC–CNCbl was observed with prolonged (5 days) CNCbl administration ($n = 10$). We designed an absorption test based on measuring TC–CNCbl at baseline and 24 h after CNCbl intake and established a reference interval for the increase in TC–CNCbl ($n = 78$). The median absolute increase was 23 pmol/L (range, 6–64 pmol/L), and the relative increase was >3 -fold.

CONCLUSIONS: Our data demonstrate that CNCbl is absorbed unchanged and accumulates on circulating TC. We suggest that measuring TC–CNCbl will improve the assessment of vitamin B₁₂ absorption.

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Cobalamin (Cbl),⁴ or vitamin B₁₂, is a nutrient necessary for normal DNA synthesis, red blood cell production, and maintenance of the nervous system. Cbl deficiency is caused either by malabsorption or low dietary intake of Cbl. Cbl uptake from food involves the binding of intrinsic factor to dietary Cbl in the intestine (1). After binding of intrinsic factor–Cbl to the receptor complex cubam, which is localized in the apical pole of the ileal epithelium, the cubam–intrinsic factor–Cbl complex is internalized via endocytosis (2). In the circulation, Cbl is bound to 2 transport proteins, transcobalamin (TC) and haptocorrin. TC transports Cbl to the cells of the body, whereas the function of haptocorrin remains to be clarified. Only about 20% of the circulating Cbl is bound to TC (holoTC) and thus available for cellular uptake. Therefore, plasma holoTC has been suggested as a more sensitive marker for Cbl status than total plasma Cbl (3–6).

For decades, the Schilling test has been used to measure Cbl absorption in humans for identifying patients with an acquired inability to absorb Cbl (7). This procedure has become increasingly unacceptable, however, because it uses radioactively labeled Cbl (8). Moreover, radiolabeled Cbl has become less available. Recently, a new approach, the CoblSorb test, has been introduced for the study of Cbl uptake. The CoblSorb assay is based on measuring holoTC before and after intake of a test dose of Cbl (9–11). Although the test seems to demonstrate excellent sensitivity, showing no increase in holoTC in patients unable to absorb Cbl (12), the test's specificity depends on the baseline holoTC concentration. Obviously, the specificity of the test would be improved if it could selectively measure the newly absorbed vitamin. We speculated that such measurement was possible, provided that cyanocobalamin (CNCbl) is

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⁴ Nonstandard abbreviations: Cbl, cobalamin; TC, transcobalamin; CNCbl, cyanocobalamin; OHcbl, hydroxocobalamin; AdoCbl, 5'-deoxyadenosylcobalamin; MeCbl, methylcobalamin; TC–CNCbl, TC-bound CNCbl.

absorbed unmodified. In individuals on a typical diet, CNCbl accounts for only a minor part of the Cbl in the circulation. The major forms are hydroxocobalamin or aquocobalamin (OHCbl) and the 2 coenzyme forms of Cbl, 5'-deoxyadenosylcobalamin (AdoCbl) and methylcobalamin (MeCbl) (1, 13).

The aim of the present study was to document whether CNCbl is absorbed unmodified and if this is the case, to explore the use of measuring TC-bound CNCbl (TC-CNCbl) for assessing vitamin B₁₂ absorption.

Experimental Design and Methods

STUDY PARTICIPANTS AND SAMPLES

Serum samples were obtained from 3 previously characterized populations, all consisting of healthy donors. Except for the samples in cohort A, all samples were collected without any protection from light. Cohort A samples were collected into foil-wrapped test tubes, and all subsequent handling steps were performed in dim red light until the sample was applied to the HPLC instrument.

Cohort A consisted of 4 females and 10 males between 18 and 49 years of age. These individuals were randomly chosen from a population that received oral supplements of 9 µg CNCbl at 0, 5.5, and 11 h after collection of the baseline sample (9). Ten light-protected pairs of serum samples obtained at baseline and at 24 h after intake of the first CNCbl dose were analyzed for all forms of Cbl on TC.

Cohort B consisted of 9 males and 22 females between 25 and 57 years of age (11). We analyzed samples obtained on 2 consecutive days before vitamin B₁₂ supplementation.

Cohort C consisted of 78 individuals, 40 males and 38 females between 21 and 81 years of age (10). These individuals received oral supplements of three 9-µg CNCbl doses at 0, 6, and 12 h after collection of the baseline sample; they continued to take 3 vitamin B₁₂ doses per day for 5 days. Blood samples were collected at baseline (day 0) and on days 1, 2, 3, 4, and 7. We analyzed the samples collected at baseline and at 24 h for all individuals in this cohort. In addition, we evaluated all samples obtained from 10 randomly chosen individuals. HoloTC was previously measured in all of the samples from cohort C (10).

ENZYMATIC EXTRACTION OF Cbl FROM TC

All handling of serum samples and material obtained from serum was done in 2-mL polypropylene tubes (Sarstedt) or 200-µL conical vials (MicroLab). TC in the samples was immunoprecipitated by incubating 200 µL serum with 25 µL magnetic microspheres cov-

ered with monoclonal antibodies to TC (kindly donated by Axis-Shield, Dundee, Scotland, UK). We treated the immunoprecipitated TC for 2 h at 37 °C with 100 µL of subtilisin Carlsberg from *Bacillus licheniformis* (EC 3.4.21.14; Sigma-Aldrich) in 0.1 mol/L Tris-HCl, pH 7.4 (Sigma-Aldrich), as previously described (14).

HPLC OF RELEASED Cbl

The Cbl forms were separated by HPLC, as described by Jacobsen et al. (15). The HPLC apparatus (HP 1100; Agilent Technologies) was fitted with a precolumn (SecurityGuard cartridge C18, 4 × 3 mm; Phenomenex) followed by a reversed-phase column (Luna 3 µm C18, 150 mm × 4.6 mm; Phenomenex). Both columns were thermostated at 20 °C. A gradient of acetonitrile (J.T. Baker) in 0.05 mol/L phosphoric acid, pH 3, was applied 4 min after injection of 90 µL of sample or calibrator. Eluted fractions were collected, and the eluent in the fractions was lyophilized (HetoVac).

Standard solutions, calibrators, and samples. Standard solutions containing 100 pmol/L OHCbl, CNCbl, AdoCbl, and MeCbl (all from Sigma-Aldrich) were separated with HPLC to determine the retention times of the different Cbls. Seven calibrators in the concentration interval of 0–436 pmol/L were prepared by diluting CNCbl with the subtilisin solution. One hundred microliters of calibrator or sample were transferred to light-protected vials (MicroLab), and 10 µL of an aqueous solution of 10-g/L acetaminophen (GlaxoSmithKline) was added as an internal standard before HPLC. For the standard solutions of the different Cbls, postcolumn fractions were collected every 30 s between 10 min and 22 min after sample injection. For the calibrators, only 1 fraction, corresponding to the retention time of CNCbl (15–16 min), was collected. For cohort A, fractions were collected every 30 s between 10 min and 22 min after sample injection. For cohorts B and C, only fractions corresponding to the retention time of CNCbl were collected.

QUANTIFICATION OF Cbl IN POSTCOLUMN FRACTIONS

Recombinant unsaturated TC (350 pmol/L; kindly donated by Cobento Biotech, Aarhus, Denmark) in 0.1 mol/L phosphate buffer, pH 8, containing 1 g/L BSA (Sigma-Aldrich) was added to the lyophilized column fractions and incubated at room temperature for 1 h. The amount of the resulting holoTC was measured with an ELISA (16). Quantification of Cbl in the postcolumn fractions was based on the ratio of the absorbance signal from the ELISA to the acetaminophen peak areas, as determined with the chromatography

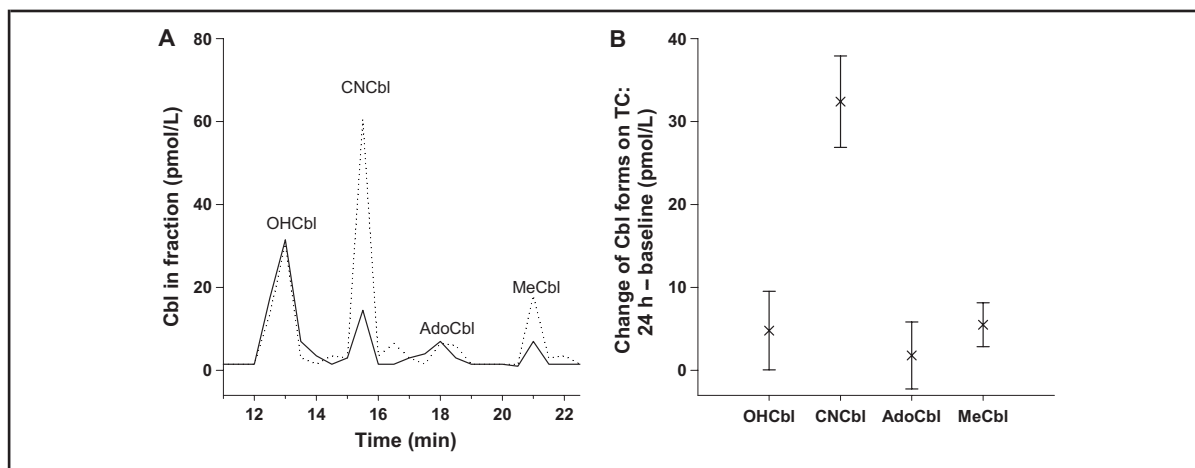


Fig. 1. Forms of Cbl on TC before and 24 h after CNCbl intake.

Ten healthy donors were administered 3 oral doses of 9 μg CNCbl on a single day. Light-protected blood samples were withdrawn at baseline and 24 h after the first dose. Cbl on serum TC was isolated and separated by HPLC. (A), Chromatograms from 1 representative donor showing the baseline Cbl forms on TC (solid line) and the forms on TC 24 h after supplementation (dotted line). (B), The changes in OHCbl, CNCbl, AdoCbl, and MeCbl found on TC for all 10 donors at 24 h after supplementation. Data are presented as the mean and SD.

software. The 7 calibrators containing CNCbl were used to construct calibration curves.

METHOD VALIDATION AND STATISTICAL ANALYSIS

Method imprecision was evaluated with duplicate estimates of a high (180 pmol/L) and a low (15 pmol/L) concentration of TC–CNCbl in 10 analytical runs performed over a period of 2 months. Calculations of intraassay imprecision and total imprecision were based on the recommendations of Krouwer and Rabinowitz (17). Statistical tests were performed with Prism software (version 4.0; GraphPad Software).

Results

The method we have developed combines TC immunoprecipitation, enzymatic release of Cbl, HPLC separation, and measurement of Cbl in the post-column fractions. The method had an intraassay (total) imprecision of 7%–9% (11–14), as judged from 10 analytical runs of duplicate serum samples with high and low TC–CNCbl concentrations (i.e., 180 pmol/L and 15 pmol/L) measured on 10 days over a 2-month period.

We investigated the change in the Cbl pattern on TC in light-protected serum samples obtained from healthy individuals at baseline and 24 h after oral CNCbl administration (cohort A; Fig. 1A). The mean (SD) increase in OHCbl, CNCbl, AdoCbl, and MeCbl on TC was 4 (4) pmol/L, 32 (6) pmol/L, 2 (4) pmol/L, and 5 (3) pmol/L, respectively (Fig. 1B). Paired *t*-tests

for each of the Cbl forms at baseline and 24 h after oral CNCbl administration showed a highly significant increase in CNCbl concentration ($P = 0.0003$) and a marginal, although significant, increase in MeCbl ($P = 0.03$), whereas no changes were observed for OHCbl ($P = 0.25$) and AdoCbl ($P = 0.37$). These results suggest that a major fraction of CNCbl is transferred unmodified over the ileal epithelium and that measurement of TC–CNCbl could be a sensitive marker of Cbl absorption.

Furthermore, we examined the intraindividual variation of TC–CNCbl by measuring paired samples drawn on 2 separate days from individuals who were not given vitamin B₁₂ supplements ($n = 31$, cohort B). The mean (SD) TC–CNCbl concentration was 1 (1.6) pmol/L (range, 0–10 pmol/L).

To explore the increase in TC–CNCbl amount with prolonged CNCbl administration, we analyzed samples taken before, during, and after 5 days of CNCbl administration ($n = 10$, randomly chosen from cohort C). The baseline samples contained 0–4 pmol/L TC–CNCbl (median, 1 pmol/L). After 1 day, the TC–CNCbl concentration had increased to 7–48 pmol/L (median, 26 pmol/L). Only small additional increases were observed on days 2, 3, and 4 (median, 9, 5, and –2.5 pmol/L, respectively). Two days after cessation of oral intake of CNCbl, the TC–CNCbl had returned almost to baseline concentrations (median, 5 pmol/L; range, 0–14 pmol/L) (Fig. 2).

Our initial studies suggested that measuring TC–CNCbl before and 24 h after an oral CNCbl dose

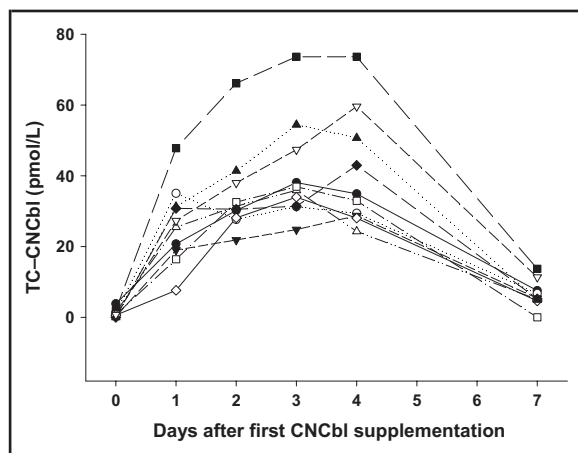


Fig. 2. Accumulation of CNCbl on TC after intake of CNCbl.

Healthy donors ($n = 10$) were supplemented daily with 3 oral doses of $9 \mu\text{g}$ CNCbl for 5 days. TC-CNCbl was measured in serum from blood samples withdrawn at baseline (day 0) and on days 1, 2, 3, 4, and 7 after the first dose.

is more useful for assessing the ability to absorb CNCbl than the previously published method involving measuring holoTC before and after 2 days of CNCbl intake (10). To explore this possibility, we examined a third cohort (cohort C, $n = 78$). The median TC-CNCbl concentration at baseline and 24 h was 2 pmol/L (range, 0–14 pmol/L) and 24 pmol/L (range, 7–65 pmol/L), respectively. The median absolute increase in TC-CNCbl concentration was 23 pmol/L (range, 6–64 pmol/L), and the fractional increase was >3 -fold (the increase is indefinite in samples with baseline CNCbl concentrations of 0 pmol/L) in all but 1 patient. This patient had a high baseline TC-CNCbl concentration (14 pmol/L), whereas all of the others had baseline concentrations between 0 and 7 pmol/L.

Discussion

We report that a major part of a test dose of CNCbl is absorbed from the intestinal cells without modification, and we propose that the capacity to absorb vitamin B_{12} can be evaluated by measuring TC-CNCbl before and after intake of a test dose of CNCbl. Our proposed test is an extension of the CobaSorb Cbl-absorption test, which is based on measuring holoTC before and after CNCbl intake (10). The sensitivity of the CobaSorb test has been judged excellent (12), a finding that supports the conclusion that no passive absorption occurs after administration of a high phys-

iological dose (three $9\text{-}\mu\text{g}$ doses) of vitamin B_{12} . In contrast, the specificity of the CobaSorb test depends on the baseline concentration of holoTC, and use of this test is not recommended if the baseline holoTC concentration is >75 pmol/L (10). Our new test, which we have named C-CobaSorb, has a markedly improved specificity, a conclusion reached by comparing the results obtained for 78 healthy individuals with the C-CobaSorb test (present study) and the CobaSorb test (10).

With the C-CobaSorb test, all but 1 of the studied individuals (baseline CNCbl-TC, 14 pmol/L) were classified as capable of absorbing vitamin B_{12} according to the following criteria: (a) an absolute TC-CNCbl increase of >6 pmol/L (>3 times the intraindividual variation of 1.6 pmol/L) and (b) a relative TC-CNCbl increase of >3 -fold from the baseline sample to the 24-h sample. Until further experience has been accumulated, we suggest the test be used only if the baseline TC-CNCbl concentration is <10 pmol/L. With the

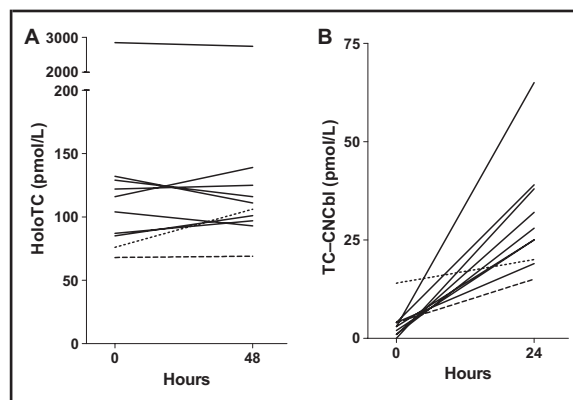


Fig. 3. Absorption of vitamin B_{12} (CNCbl), as judged by holoTC measurement (CobaSorb) or TC-CNCbl measurement (C-CobaSorb).

Donors ($n = 78$) were administered 3 oral doses of $9 \mu\text{g}$ CNCbl per day for 5 days. Blood samples were taken at baseline and at timed intervals after the start of supplementation and analyzed. Results for individuals judged able to absorb vitamin B_{12} ($n = 68$) with both methods are not indicated. Results for individuals that could not be classified with CobaSorb ($n = 8$) are indicated with a solid line. A dashed line indicates a donor judged by the CobaSorb assay to be unable to absorb vitamin B_{12} ; the dotted line indicates a donor that could not be classified with C-CobaSorb. (A), The CobaSorb test. Measurement of holoTC at baseline and 48 h after first test dose of vitamin B_{12} . Data on the measurement of holoTC is obtained from Hvas et al. (10). (B), The C-CobaSorb test. Measurement of TC-CNCbl at baseline and 24 h after first test dose of vitamin B_{12} .

CobaSorb test, only 69 of the individuals were classified as capable of absorbing vitamin B₁₂, as judged by an absolute holoTC increase of >10 pmol/L and a relative increase of >22% (11). Of the 9 individuals that the CobaSorb test either could not classify as absorbing vitamin B₁₂ (baseline holoTC >75 pmol/L; n = 8) or had classified as unable to absorb vitamin B₁₂ (baseline holoTC, 68 pmol/L; n = 1), the C-CobaSorb assay classified all of these individuals as absorbers of vitamin B₁₂ (Fig. 3). The results suggest that C-CobaSorb may prove a valuable complement to the previously introduced CobaSorb test.

Previous studies suggested that TC carries primarily OHCbl and to some degree AdoCbl (18). The results with our improved methods for extracting and quantifying circulating TC-Cbl support these findings and show that OHCbl accounts for a mean of 50% of the Cbl on TC at baseline. CNCbl intake produced a substantial increase in TC-CNCbl, and the concentration of TC-MeCbl showed a small but significant increase. This finding suggests that a minor fraction of the test dose of CNCbl is converted into the coenzyme MeCbl form after decyanation in the cytosol (19), whereas the major fraction remains unchanged on release from the intestinal cell and into the circulation.

In conclusion, we suggest that most of the CNCbl from oral supplements remains unchanged during intestinal absorption and that the resulting increase in

circulating TC-CNCbl can be used as a sensitive marker for the absorption of vitamin B₁₂.

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