Quantification of Holo-Transcobalamin, a Marker of Vitamin B_{12} Deficiency, *Ebba Nexo,[1*] Anna-Lisa Christensen,[1] Anne-Mette Hvas,[1] Torben E. Petersen,[2] and Sergey N. Fedosov[2]* ([1] Department of Clinical Biochemistry, Aarhus University Hospital, DK-8000 Aarhus C, Denmark; [2] The Laboratory for Protein Chemistry, University of Aarhus, DK-8000 Aarhus C, Denmark; * address correspondence to this author at: Department of Clinical Biochemistry, AKH, Aarhus University Hospital, Norrebrogade 44, DK-8000 Aarhus C, Denmark; fax 45-8949-3060, e-mail E.Nexo@dadlnet.dk)

We report a new method for measurement of holotranscobalamin (holoTC), in which magnetic beads coated with vitamin B_{12} (cobalamins) precipitate apo-transcobalamin (apoTC) and the holoTC present in the supernatant is measured by ELISA.

Serum holoTC denotes the part of vitamin B_{12} accessible for the cells of the body and is considered to be a sensitive marker of vitamin B_{12} deficiency *(1–3)*. Serum holoTC is not easily measured because it accounts for only approximately one-third of the circulating vitamin B_{12} and because the major part of TC circulates unsaturated with vitamin B_{12} (apoTC) *(1–3)*. In the few published attempts to measure holoTC, TC is separated from the other vitamin B_{12}-binding protein, haptocorrin, before quantification of vitamin B_{12}. This allows a direct measurement of the cobalamins attached to TC *(4–6)* or an indirect calculation of holoTC from measurement of total plasma cobalamins and the plasma cobalamins not attached to TC *(7–9)*. We have chosen another path.

We produced B_{12} beads and removed apoTC before ELISA quantification *(10)* of the protein moiety of holoTC. Vitamin B_{12} (400 mg; Fluka) was modified by acid treatment *(11)* to produce derivatives with a free COOH group. Magnetic beads (DB M-270 amine; DYNAL) from 10 mL of the original suspension were incubated overnight at room temperature with 10 mL of 2 mmol/L derivatives in 0.1 mol/L 2-morpholinoethane sulfonic acid, pH 5.5, in the presence of 10 mmol/L 1-ethyl-3-(dimethylaminopropyl)carbodiimide (Sigma). The beads were then washed twice with 50 mL of 1 mol/L Tris, pH 7.5, twice with 50 mL of 1 mol/L ammonium acetate, pH 4.6, and 10 times with 50 mL of 0.1 mol/L Tris–0.5 mol/L NaCl, pH 7.5. As judged from binding studies with apoTC, ~40 nmol of derivatives able to bind TC was coupled to the beads (data not shown).

The B_{12} beads dissolved in 0.2 mol/L sodium phosphate, pH 7.5, were kept at 4 °C, and, before use, the beads (typically 150 μL containing 0.8 mg of beads) were washed four times with 4 mL of Tris buffer (0.05 mol/L Tris–1 mol/L sodium chloride, pH 8.0), twice with 4 mL of assay buffer [0.1 mol/L sodium phosphate–1 g/L human albumin (Behringwerke), pH 8.0], and finally dissolved in 450 μL of assay buffer (assay solution). No detectable leakage of cobalamins from the beads was observed as judged from measurement of cobalamins in the supernatant of 250 μL of stock solution of the washed beads redisolved in 250 μL of buffer (<33 pmol/L cobalamin).

The amount of B_{12} beads needed for removal of apoTC was determined from experiments where increasing amounts of beads were added to 100 μL of serum (Fig. 1A). For the routine assay, 100 μL serum was incubated for 1 h with 10 μL B_{12} beads (assay solution). Before the magnetic separation of the beads from the sample, 290 μL of assay buffer was added. The supernatant containing the holoTC in a final dilution of 1:3 was measured by ELISA *(10)*. Likewise, total TC was measured in serum prediluted 1:19 with assay buffer. Serum cobalamins and cobalamins in the supernatants from the beads were analyzed by a commercial protein-binding assay (Bayer a/s) on Centaur equipment (reference interval, 200–600 pmol/L). Plasma methylmalonic acid was measured by gas chromatography–mass spectrometry *(12)* (reference interval, 0.08–0.28 μmol/L). Linear regression, unpaired *t*-test, and ANOVA were performed with the GraphPad Prism program (GraphPad Software).

The total imprecision (CV) of the holoTC assay was 8% at 33 pmol/L and 7% at 88 pmol/L as judged from measurement of a low and a high serum control sample in

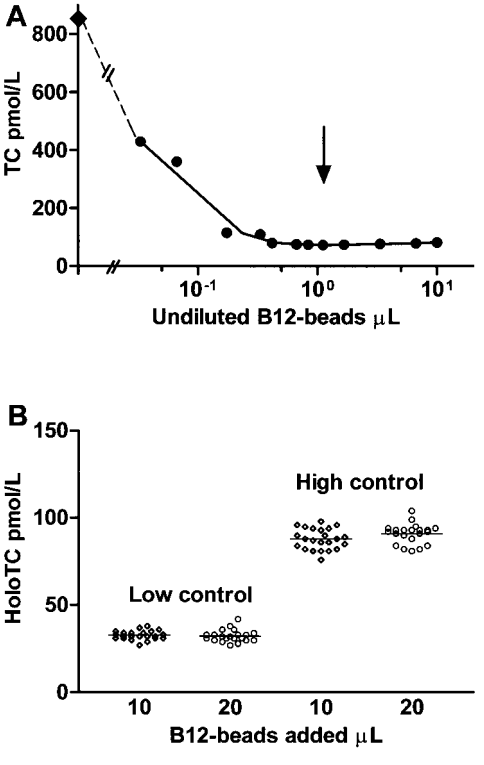

Fig. 1. Evaluation of the holoTC assay.

(*A*), titration of B_{12} beads. Serum (100 μL) was adsorbed with increasing amounts of magnetic beads (*x axis*; logarithmic scale) coated with vitamin B_{12} derivatives, and TC was measured in the supernatant (*y axis*). The *diamond* on the *y* axis indicates the total amount of TC present in the sample. The *arrow* indicates the amount of beads used in the final design of the assay. (B), quality control evaluation. holoTC in control samples absorbed with 10 μL (*10*) or 20 μL (*20*) of B_{12} beads before measurement of holoTC by ELISA. Results from low (mean, 33 pmol/L; CV, 8%; n = 22) and high (mean, 88 pmol/L; CV, 7%; n = 22). No statistical significant difference was observed for samples pretreated with 10 or 20 μL of B_{12} beads.

22 analytical runs over a period of 3 months (Fig. 1B). The controls gave identical mean results whether adsorbed with 10 or 20 μL of B_{12} beads (Fig. 1B). Leakage of B_{12} would increase the amount of holoTC present in the sample, and if leakage had been a problem, we would have expected to see higher values of holoTC in samples adsorbed with 20 μL of beads than in samples adsorbed with 10 μL of beads.

The recovery of holoTC was 0.96–1.04 pmol/L (range) and 0.997 pmol/L (mean) as judged from analyzing nine diluted serum samples saturated with vitamin B_{12} (73–147 pmol/L holoTC) before and after pretreatment with the B_{12} beads.

When compared with a commercial assay for holoTC based on measurement of B_{12} attached to TC (Axis-Shield), on 18 serum samples ranging from 40 to 170 pmol/L holoTC by both methods, we found $y = (0.75x - 13$ pmol/L ($S_{y|x}$ = 9.6 pmol/L; r = 0.94), where y represents values obtained for holoTC by the commercial assay, and x represents values obtained for holoTC by our method. The 95% confidence intervals for the slope and intercept were 0.60 to 0.90 and −20 to 6.6 pmol/L, respectively.

The reference intervals for holoTC and TC saturation were determined from analysis of serum samples obtained from 137 healthy blood donors (21–65 years of age) with serum total TC within the reference interval (560–1550 pmol/L) *(10)*. No difference was observed between males (n = 68) and females (n = 69) nor between those ≥50 years (n = 68) as compared with those <50 years (n = 69). The central 95% reference interval for holoTC was 40–150 pmol/L (mean, 75 pmol/L) and was comparable to previously described values obtained by methods where the amount of B_{12} bound to TC was measured after separating TC from the other plasma vitamin B_{12}-binding protein, haptocorrin *(4, 6, 9)*.

Because our assay allowed us to measure both total TC and holoTC, we were able to also calculate the TC saturation (holoTC/total TC). Approximately 10% of the circulating TC was saturated with vitamin B_{12} with a central 95% reference interval of 0.05–0.20% (mean, 0.09%). The results are in agreement with previous data from analysis of holo- and apoTC *(6, 8, 9)*.

The fraction of the total serum cobalamins attached to TC was 0.15–0.50% (central 95% confidence interval; mean, 0.30%), values that are well in accordance with those reported previously *(7, 9, 13)*. Both holoTC [$y = 0.16x + 33$ pmol/L (r = 0.45)] and TC saturation [$y = 0.30x + 0.04$ pmol/L (r = 0.51)] showed a positive correlation with serum cobalamins.

For >15 years, it has been speculated that holoTC and, possibly, TC saturation would be better indicators of early vitamin B_{12} deficiency than total serum cobalamins *(9)*. These speculations are justified both because TC is needed to transport cobalamins into the cells and because the major part of circulating cobalamin is unavailable for most of the cells of the body *(1)*. To date, only a few experimental studies have suggested that holoTC is an early and sensitive marker of vitamin B_{12} malabsorption and possibly vitamin B_{12} deficiency *(4, 8, 14–16)*, whereas another study has questioned its usefulness *(17)*.

We studied 10 patients with vitamin B_{12} deficiency (serum cobalamins, <175 pmol/L; and plasma methylmalonic acid, >0.7 μmol/L). The values obtained for both holoTC (range, 2–34 pmol/L) and TC saturation (range, 0.004–0.03) were well below the reference interval. Our results therefore confirm the potential usefulness of measuring holoTC and TC saturation.

The major problem in clarifying the usefulness of holoTC measurements has been the lack of a suitable method. We believe that our approach has solved the methodologic problems. We combined a sensitive ELISA for TC *(10)* with a simple procedure for removal of apoTC and established an assay that could determine both holoTC and total TC in a volume of <200 μL of serum. We believe this method will be useful not only to clarify the role for holoTC and TC saturation as diagnostic tests for vitamin B_{12} deficiency but also to study the metabolism of TC in other body fluids such as the cerebrospinal fluid.

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