Quantification of Holo-Transcobalamin, a Marker of Vitamin B₁₂ Deficiency, Ebba Nexø, Anna-Lisa Christensen, Anne-Mette Heas, Torben E. Petersen, and Sergey N. Fedosov (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 B₁₂ (cobalamin) precipitate apo-transcobalamin (apoTC) and the holoTC present in the supernatant is measured by ELISA.

Serum holoTC denotes the part of vitamin B₁₂ accessible for the cells of the body and is considered to be a sensitive marker of vitamin B₁₂ deficiency (1–3). Serum holoTC is not easily measured because it accounts for only approximately one-third of the circulating vitamin B₁₂ and because the major part of TC circulates unsaturated with vitamin B₁₂ (apoTC) (1–3). In the few published attempts to measure holoTC, TC is separated from the other vitamin B₁₂-binding protein, haptocorrin, before quantification of vitamin B₁₂. 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 B₁₂ beads and removed apoTC before ELISA quantification (10) of the protein moiety of holoTC. Vitamin B₁₂ (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. At judged from binding studies with apoTC, ~40 nmol of derivatives able to bind TC was coupled to the beads (data not shown).

The B₁₂ 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 B₁₂ 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 B₁₂ 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 cobalamin 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
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 B12 beads (Fig. 1B). Leakage of B12 would increase the amount of holoTC present in samples adsorbed with 20 μL of beads than in samples adsorbed with 10 μL of beads.

The recovery of holoTC was 96.0–1.04 pmol/L (range) and 0.997 pmol/L (mean) as judged from analyzing nine diluted serum samples saturated with vitamin B12 (73–147 pmol/L holoTC) before and after pretreatment with the B12 beads.

When compared with a commercial assay for holoTC based on measurement of B12 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 (S95% = 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 B12 bound to TC was measured after separating TC from the other plasma vitamin B12-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 B12 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 cobalamin 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 \[ y = 0.16x + 33 \text{ pmol/L} \ (r = 0.45) \] and TC saturation \[ y = 0.30x + 0.04 \text{ pmol/L} \ (r = 0.51) \] 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 B12 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 B12 malabsorption and possibly vitamin B12 deficiency (4, 8, 14–16), whereas another study has questioned its usefulness (17).

We studied 10 patients with vitamin B12 deficiency (serum cobalamins, <175 pmol/L; and plasma methylmalonic acid, >0.7 pmol/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 B12 deficiency but also to study the metabolism of TC in other body fluids such as the cerebrospinal fluid.

References