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Direct Assay for Cobalamin Bound to Transcobalamin (Holo-Transcobalamin) in Serum

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Background: **Only cobalamin carried by transcobalamin (holo-transcobalamin) is available for cellular uptake and hence is physiologically relevant. However, no reliable or accurate methods for quantifying holo-transcobalamin are available. We report a novel holo-transcobalamin assay based on solid-phase capture of transcobalamin.**

Methods: **A monoclonal antibody specific for human transcobalamin with an affinity constant $>10^{10}$ L/mol was immobilized on magnetic microspheres to capture and concentrate transcobalamin. The cobalamin bound to transcobalamin was then released and assayed by a competitive binding radioassay. The quantification of holo-transcobalamin was accomplished using calibrators composed of recombinant, human holo-transcobalamin.**

Results: **The assay was specific for holo-transcobalamin and had a detection limit of 5 pmol/L. Within-run and total imprecision (CV) was 5% and 8–9%, respectively. The working range (CV <20%) was 5–370 pmol/L. Dilutions of serum were linear in the assay range. The recovery of recombinant, human holo-transcobalamin added to serum was 93–108%. A 95% reference interval of 24–157 pmol/L was established for holo-transcobalamin in 105 healthy volunteers 20–80 years of age. For 72 of these sera, holo-haptocorrin and total cobalamin were also determined. Whereas holo-haptocorrin correlated well ($r^2 = 0.87$) with total cobalamin, holo-transcobalamin correlated poorly ($r^2 = 0.23$) with total cobalamin or holo-haptocorrin.**

Conclusions: **The solid-phase capture assay provides a simple, reliable method for quantitative determination of holo-transcobalamin in serum.**

Vitamin B_{12}[4] (B_{12}; cobalamin; Cbl)[5] is essential for one-carbon metabolism and cell division. Reduced intake of B_{12} from food or impaired intestinal absorption would lead to negative balance and ultimately to severe deficiency when the tissue stores of the vitamin are depleted. The clinical consequences of B_{12} deficiency include megaloblastic anemia and progressive neurologic disease of the central and peripheral nervous systems. An increased plasma homocysteine is another consequence of B_{12} deficiency, and this amino acid is associated with a risk for cardiovascular disease and neural tube defects *(1–4)*. Vitamin B_{12} deficiency is an insidious disorder that takes several years to manifest because of the substantial tissue stores of the vitamin. It is also a public health problem affecting, in particular, the elderly population because of reduced dietary intake and impaired absorption of the vitamin *(5–8)*.

Cbl in serum is bound to two proteins, transcobalamin (TC) and haptocorrin (HC). Less than 25% of total serum Cbl is carried on TC *(9, 10)*. The function of TC is to transport Cbl from its site of absorption in the ileum to tissues and cells that express specific receptors, which internalize the vitamin as the TC-Cbl complex. The function of HC is not well understood, but it is believed that it may serve as a scavenger for potentially harmful Cbl analogs, transporting them to the liver for secretion into

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[4] In this report, vitamin B_{12} refers essentially to the vitamin as a nutritional factor in the diet. Cobalamin refers to the in vivo forms of the vitamin as the methyl, the deoxyadenosyl, or the hydroxo cofactors.

[5] Nonstandard abbreviations: Cbl, cobalamin; TC, transcobalamin (previously transcobalamin II); HC, haptocorrin (previously transcobalamin I and III); holoTC, holo-transcobalamin; mAb, monoclonal antibody; apoTC, apo-transcobalamin; SPR, surface plasmon resonance; holoHC, holo-haptocorrin; and PBS, phosphate-buffered saline.

the bile *(11)*. Thus, it is only the Cbl carried by TC that is available for cellular uptake and hence is physiologically relevant.

With the introduction of assays for total serum homocysteine and methylmalonic acid, it was observed that these metabolites were increased in some patients with serum Cbl concentrations at the low end of the reference interval *(5–7)*. Thus, a need for a reliable method to measure physiologically relevant Cbl was recognized because this marker may provide a more sensitive means to identify subclinical B_{12} deficiency *(12)*. In this report we describe a novel method to measure Cbl bound to TC (holoTC) in human serum or plasma. TC in serum or plasma is "captured" and concentrated by a high-affinity monoclonal antibody (mAb) immobilized on magnetic microspheres; the Cbl bound to TC is then released and measured by a conventional assay protocol for Cbl in serum or plasma.

Materials and Methods

MATERIALS

Encapsulated magnetic microspheres (EM1 100/40; mean diameter, 0.86 μm) coated covalently with goat anti-mouse IgG (H+L) antibody were from Merck-Eurolab SAS. Rabbit anti-mouse Fc-γ used for immobilization of murine mAbs on the BIAcore chip was from Pharmacia Biosensor AB. The production of mouse anti-human TC mAbs and recombinant human TC (apoTC and holoTC) has been described previously *(13, 14)*. Immobilized porcine intrinsic factor and ^{57}Co-labeled cyanocobalamin were from ICN Pharmaceuticals Ltd. A sample of purified human HC was kindly provided by Dr. Ebba Nexø (University of Aarhus, Aarhus, Denmark). Type XS-20 magnetic separators, which hold 20 tubes, were purchased from ABgen House.

SURFACE PLASMON RESONANCE

Surface plasmon resonance (SPR) studies were performed with BIAcore (Pharmacia Biosensor AB). The rabbit anti-mouse Fc-γ antiserum (30 mg/L) was immobilized on the sensor chip surface in 50 mmol/L acetate buffer, pH 5.0. The carboxylated surface of the sensor chip was first activated with *N*-hydroxysuccinimide–*N*-ethyl-*N*′-[3-(dimethylamino)propyl] carbodiimide hydrochloride according to a protocol provided by the manufacturer. The antibody was then immobilized on the chip until a SPR signal of ~2000 resonance units over baseline was obtained. Unreacted *N*-hydroxysuccinimide was blocked by injection of an aliquot (5 μL) of 1 mol/L ethanolamine.

The flow cell was equilibrated with HEPES-buffered saline, pH 7.3, with 3.4 mmol/L EDTA and 50 mg/L BIAcore surfactant at a flow rate of 5 μL/min. The mouse anti-human TC mAb (10 mg/L) was injected in the buffer at a flow rate of 5 μL/min, followed by a wash step with buffer, and finally by the apoTC or the holoTC at 1, 5, 10, 15, 20, and 25 nmol/L. Between each mAb run, the sensor chip surface was regenerated by eluting the mAb and ligand with an injection of 100 mmol/L HCl. The chip with the covalently immobilized rabbit anti-mouse Fc-γ antibody was quite stable because there was no decrease in ligand binding during the course of the experiment. Data points were collected continually during the binding and dissociation processes, and the association rate constant (k_{on}) and the dissociation rate constant (k_{off}) were calculated using the software (BIA Evaluation) supplied by the manufacturer. The dissociation equilibrium constant (K_d) was calculated as the ratio of k_{off}/k_{on}, and the affinity constant (K_a) was calculated as the reciprocal of the K_d.

IMMOBILIZATION OF THE mAb TO HUMAN TC ON MAGNETIC MICROSPHERES

Goat anti-mouse IgG antibody-coated magnetic microspheres were sedimented by a magnet and washed twice in wash buffer (0.1 mol/L sodium phosphate, pH 7.2, 0.15 mol/L sodium chloride, 0.2 mL/L Tween 20). The microspheres were resuspended at 10 g/L in phosphate-buffered saline containing albumin (0.1 mol/L sodium phosphate, pH 7.2, 0.15 mol/L sodium chloride, 5 g/L bovine serum albumin). The anti-TC mAb was added to a final concentration of 10 mg/L, and the microsphere suspension was incubated at ambient temperature for 1 h in a rotary mixer. The microspheres were washed twice with wash buffer, and a 10 g/L suspension was prepared in phosphate-buffered saline containing albumin and 0.8 g/L sodium azide. The antibody-coated microspheres can be kept at 4–8 °C for at least 1 year without loss of activity.

ASSAY PROTOCOL

The holoTC solid-phase capture assay is a two-step competitive assay.[6] Total TC is first sequestered from the serum sample or calibrator and isolated and concentrated by use of magnetic microspheres coated with anti-human TC mAb. The Cbl content of the sequestered holoTC is released under reducing and alkaline conditions, converted to the stable cyano form with potassium cyanide, and quantified in a competitive binding assay with [^{57}Co]Cbl as tracer and immobilized intrinsic factor as the Cbl-binding protein.

Samples and calibrators (400 μL) were diluted 1:1 in phosphate-buffered saline (PBS), and a 40-μL aliquot of a 10 g/L suspension of the mAb-coated microspheres (capture reagent) was added. The microspheres were dispersed by mixing in a whirl-mixer, and the tubes were placed in a rotary mixer for 1 h at ambient temperature. The microspheres were then sedimented with a magnet, washed once with cold wash buffer, and resuspended in 50 μL of PBS containing 10 mmol/L dithiothreitol, 0.05 g/L potassium cyanide, and ~4000 cpm of [^{57}Co]Cbl.

[6] The described holo-transcobalamin assay is available from ICN Diagnostics.

After 15 min, 25 μL of 1 mol/L sodium hydroxide containing 250 mL/L ethanol was added, and the incubation was continued for 10 min. This was followed by the addition of 300 μL of borate buffer, pH 9.6, containing hog intrinsic factor immobilized on a macroporous acrylic copolymer. The suspension was incubated for 1 h in a rotary mixer. The immobilized intrinsic factor was titrated to bind ~50% of the [^{57}Co]Cbl in the absence of any competing unlabeled Cbl. Finally, the suspension was centrifuged for 10 min at ≥1000*g*, the supernatant fraction was discarded, and the pellet was counted in a gamma counter (RIASTAR™ Gamma Counting System; Laborel). A calibration curve was constructed using calibrators with predetermined holoTC concentrations (0, 20, 40, 80, 160, and 320 pmol/L). The points were fitted to a four-parameter logistic function, and the holoTC concentrations of the samples were determined by interpolation from the calibration curve.

measurement of the total cbl concentration in serum

The total serum Cbl concentration (holoTC plus holoHC) was determined with the Abbott automated assay for serum Cbl (IMx® System with IMx Metabolic assay Module, Ver. 4.0). Cbl concentrations in the calibrators were also measured by the IMx method to verify the spectrophotometric assay. Because the method performs poorly below a concentration of 80 pmol/L, the Cbl concentrations of the 20 and 40 pmol/L calibrators could not be determined. However, if the 80, 160, and 320 pmol/L calibrators are correctly assayed from the stock, then 20 and 40 pmol/L calibrators should be stoichiometrically related values.

statistical analyses

Total and intraassay imprecision were calculated using the guidelines (one assay per day) of the NCCLS for performance evaluation of clinical devices. Regression analysis was used to analyze linearity, and a paired *t*-test was used to compare results for EDTA plasma and serum. The GraphPad Prism program (GraphPad Software) was used for these data analyses.

Results

SPR

The k_{on} and k_{off} rate constants for the binding of apoTC and holoTC by four mAbs immobilized on the BIAcore sensor chip are shown in Table 1. Ligands were assayed at 1–25 nmol/L in triplicate and in random order. The four mAbs had subnanomolar K_d values for both apoTC and holoTC. However, mAb 3-9 had the highest computed K_a for both ligands and was therefore used as the TC capture reagent in the assay for holoTC. Two antibodies, mAbs 2-2 and 4-7, showed a small but significantly higher K_a for holoTC than for apoTC, but these values were lower than the K_a for mAb 3-9.

Table 1. Binding kinetics for anti-human TC mAbs as measured by SPR (BIAcore).

Antibody	Antigen	k_{on}, 10^6 L · mol^{-1} · s^{-1}	k_{off}, 10^{-4}/s	K_a, 10^{10} L/mol
2-2	holoTC	3.0	2.4	1.2
2-2	apoTC	1.0	2.3	0.4
3-9	holoTC	3.1	1.3	2.4
3-9	apoTC	2.1	0.56	3.8
3-11	holoTC	3.1	2.4	1.3
3-11	apoTC	1.2	1.6	0.8
4-7	holoTC	1.5	2.4	0.6
4-7	apoTC	0.55	2.5	0.2

testing for removal of tc from serum by immobilized mAb 3-9

Two serum samples (400 μL each) were mixed with 400 μL of PBS and then treated with 40 μL of a 10 g/L suspension of the mAb 3-9-coated magnetic microspheres or 40 μL of a 10 g/L suspension of uncoated magnetic microspheres for 1 h at ambient temperature. The microspheres were sedimented with a magnet, and the supernatant fraction was transferred to another tube. [^{57}Co]Cbl was added to the supernatant fractions, which were then incubated for 15 min at ambient temperature. When analyzed by gel filtration, no apoTC was identified in the sample treated with the mAb-containing microspheres, as shown in Fig. 1. The HC concentration, however, was essentially unchanged by the procedure. The slight increase in the apoHC peak after removal of TC was most likely attributable to the additional [^{57}Co]Cbl available for binding to apoHC in the absence of any apoTC. The same result was obtained when [^{57}Co]Cbl was added to serum before treatment with the mAb-containing magnetic microspheres (data not shown). The mAb did not cross-react with human HC or with TC from other species.

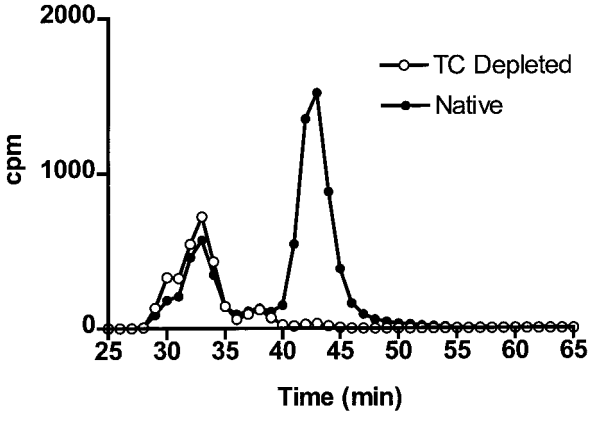

Fig. 1. Gel-filtration chromatogram of native (●) and TC-depleted (○) serum samples.

Aliquots (100 μL) of the supernatants were injected onto a Superdex® 75 column equilibrated with PBS at a flow rate of 1 mL/min. One-minute fractions were collected and counted in a Riastar gamma counter.

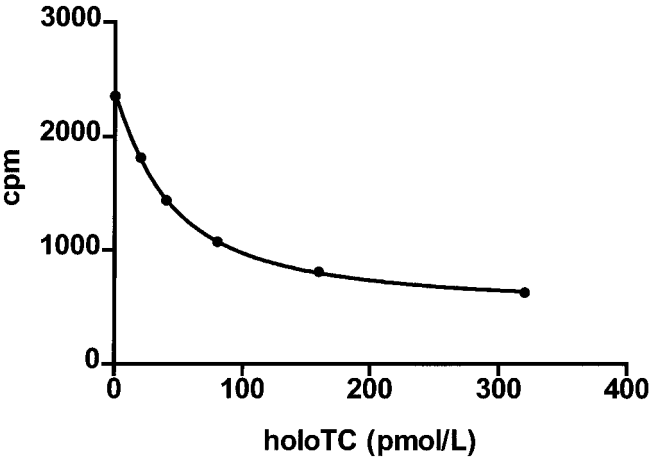

Fig. 2. Calibration curve for holoTC.

Anti-human TC mAb 3-9 immobilized on magnetic microspheres is used to capture and concentrate TC. The CbI bound to TC is then released and quantified by a competitive binding radioassay. Points have been fitted to a four-parameter logistic function. $r^2 = 1.000$.

ASSAY PERFORMANCE

Calibration curve and analytical sensitivity. Fig. 2 shows a calibration curve generated using holoTC calibrators at 0, 20, 40, 80, 160, and 320 pmol/L. Points were fitted to a four-parameter logistic function. HoloTC stock solutions were quantified spectrophotometrically, based on the micromolar absorptivity of bound holoTC *(14)*, and diluted in phosphate-buffered albumin to the holoTC concentrations of the calibrators. The concentrations were confirmed by the Vitamin B_{12} IMx method (Abbott). The detection limit, defined as the mean value for the 0 calibrator minus 3 SD, with three to seven replicates for each of seven assays, was 5.0 ± 3.5 pmol/L.

Assay imprecision. Assay imprecision (CV) was determined by measuring the holoTC concentration of three different sera over 4 weeks. Each serum was assayed in quadruplicate for a total of 20 times over 20 days. Total imprecision was 8–9%, and the intraassay imprecision was 5% (Table 2).

Limit of quantification. The working range (CV <20%) for the assay was estimated by measuring holoTC in 6–17 replicates of 13 undiluted serum samples in one to four analytical runs and computing the total imprecision for each serum. The five lowest serum samples were prepared by mixing the 32 pmol/L sample with serum depleted of TC by treatment with silica *(15)*. For these five samples, a 10 pmol/L calibrator was substituted for the 320 pmol/L calibrator in the construction of the calibration curve. The working range was at least 5–370 pmol/L (Fig. 3).

Table 2. Total and intraassay imprecision.

Mean concentration,[a] pmol/L	Total CV, %	Within-run CV, %
37 ± 3	9	5
56 ± 5	9	5
94 ± 6	8	5

[a] n = 80 per concentration.

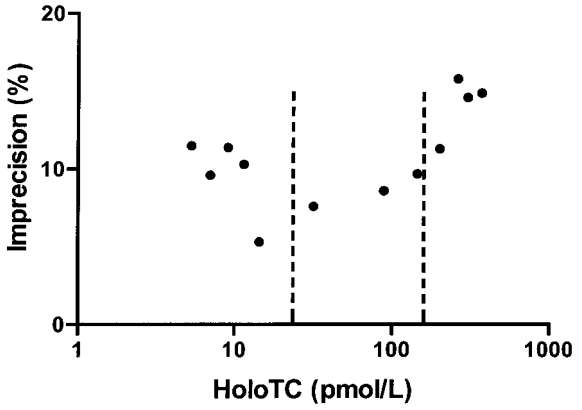

Fig. 3. Limit of quantification.

Imprecision was estimated in 13 serum samples with holoTC concentrations of 5–370 pmol/L. *Dotted lines* show the 95% reference interval (24–157 pmol/L) for holoTC as determined from 105 apparently healthy volunteers (age range, 20–80 years).

Analytical recovery. For recovery analysis, holoTC was added to four serum samples, and the holoTC concentrations were determined before and after supplementation. The recovery of added holoTC was determined by subtracting the value for the unsupplemented sample from the value obtained for the supplemented sample, and dividing by the amount of holoTC added. As shown in Table 3, the mean recovery was 101% (range, 93–108%).

Serial dilution. For dilution analysis, a serum sample was diluted 0-, 1.5-, 2-, 3-, and 6-fold with the zero calibrator to test linearity. The correlation coefficient was 0.99, the slope was 1.06 ± 0.07, and the *y*-intercept was −2.3 ± 4.6 pmol/L (Fig. 4A). The mean (± SD) slope of four experiments was 1.06 ± 0.02. In another experiment, two sera

Table 3. Analytical recovery: holoTC added to human serum.

Initial, pmol/L	Added, pmol/L	Recovery,[a] %
15	38	100
55	12	93
55	25	101
55	38	101
59	20	102
59	41	108
59	82	105
59	164	97
89	29	102
Mean		101

[a] Recovery was determined by subtracting the value for the unsupplemented sample from the value for the supplemented sample and dividing by the amount of holoTC added.

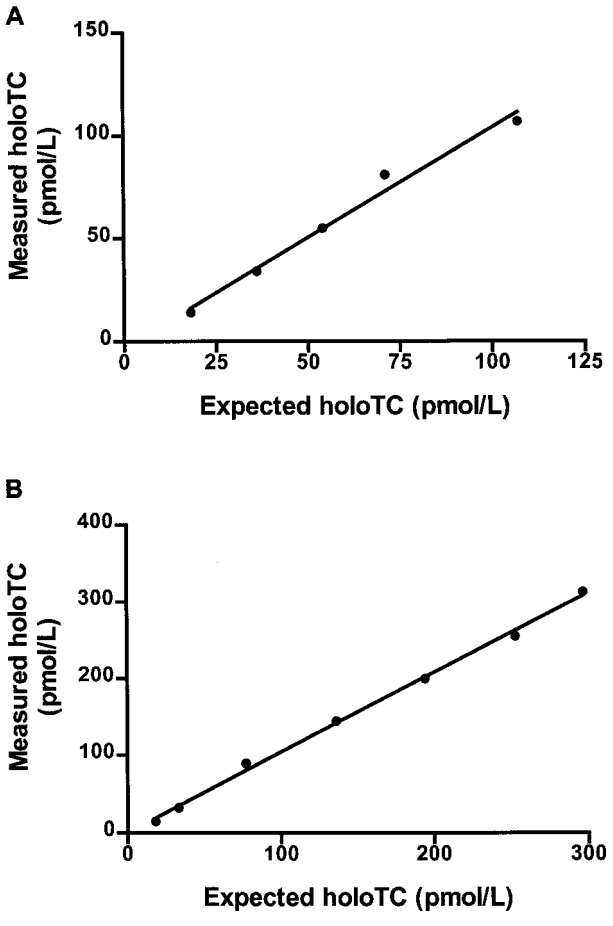

Fig. 4. Serial dilution and linearity.

(*A*), serial dilution of serum sample with zero calibrator. Equation for the line: $y = 1.06x - 2.3$; $r^2 = 0.99$. (*B*), two sera with holoTC concentrations of 18 and 312 pmol/L were mixed in different proportions. Equation for the line: $y = 1.04x + 0.3$ ($r^2 = 1.00$).

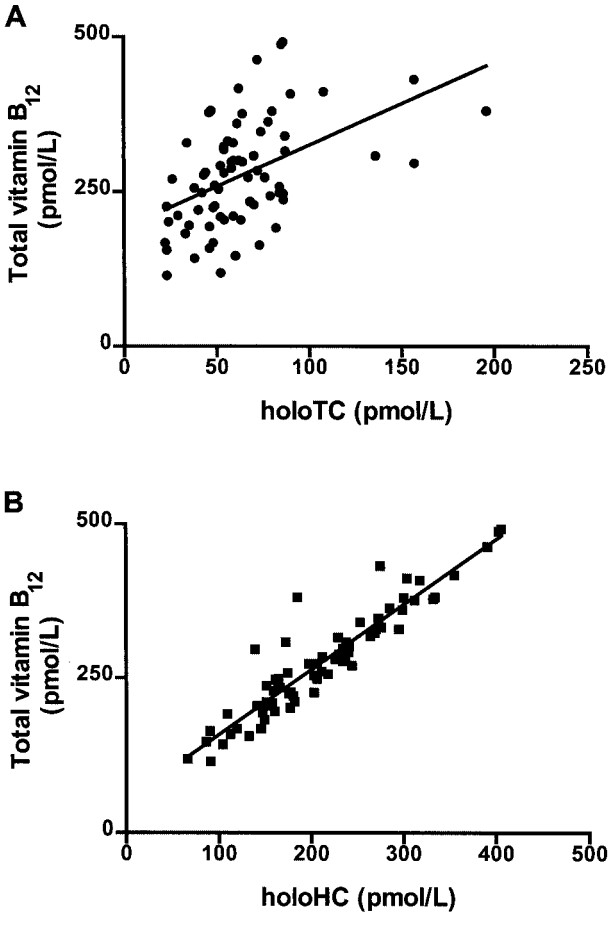

Fig. 5. Correlation between total vitamin B_{12} and holoTC (*A*) and holoHC (*B*) concentrations.

Serum samples were from 72 healthy volunteers. The regression lines obtained were as follows: (*A*), $y = 1.34x + 191$ ($r^2 = 0.230$); and (*B*), $y = 1.06x + 52$ ($r^2 = 0.875$).

with holoTC concentrations of 18 and 312 pmol/L, respectively, were mixed in different proportions, and the holoTC concentration was determined. The correlation coefficient was 1.00, the slope was 1.04 ± 0.02, and the *y*-intercept 0.3 ± 4.1 pmol/L (Fig. 4B)

Interference. No interference was observed with the following substances added to serum: hemoglobin (10 g/L), bilirubin (0.42 g/L), lipid (7.6 g/L), human HC (1 nmol/L), and human apoTC (2.1 nmol/L). Human apoTC added at 5.1 nmol/L caused 15–20% inhibition. However, any possible interference by extreme concentrations of apoTC can be overcome by increasing the amount of capture reagent (mAb-coated microspheres) or by diluting the serum sample.

Comparison of holoTC concentrations in serum and plasma. Serum and EDTA plasma were collected from 27 apparently healthy individuals, and holoTC was measured to determine whether these samples could be used interchangeably. Plasma values obtained were, on average, 8% higher than serum values ($P < 0.001$).

Reference interval and correlation between holoTC and total vitamin B_{12}. HoloTC was measured in serum from 105 apparently healthy volunteers (age range, 20–80 years). The 95% interval for holoTC was 24–157 pmol/L, with a mean of 61 pmol/L. No statistically significant difference for holoTC was observed when we compared men and women or age groups below and above 50 years. For 72 sera, holoTC and total Cbl were measured. Only a weak positive correlation was obtained ($r^2 = 0.23$; Fig. 5A). Omitting the four "outliers" shown in Fig. 5A increased the correlation only slightly ($r^2 = 0.26$). In contrast, the correlation between holoHC and total Cbl was good ($r^2 = 0.87$; Fig. 5B). HoloHC was computed as the difference between total Cbl and holoTC and may thus include other minor holoproteins *(16)*.

Discussion

There are two novel features with this method, which should be emphasized. (*a*) The use of a TC-specific mAb with a $K_a > 10^{10}$ L/mol immobilized on magnetic microspheres assures that virtually all of the TC in serum or

plasma will be sequestered without contamination by HC, which carries the major fraction of Cbl in the serum. (*b*) The small amount of Cbl in the TC fraction is concentrated compared with the serum and thus may be quantified by conventional Cbl assays. Compared with the no-boil Cbl radioassay, which in this respect is most similar to our assay, the Cbl fraction is 8-fold concentrated.

The parameters evaluated for this assay of holoTC, including sensitivity, imprecision, working range, recovery of holoTC added to samples, and linearity, also establish this procedure as a reliable method for measuring TC-bound Cbl at and below the diagnostically crucial low end of the reference interval. The 95% confidence interval of 24–157 pmol/L holoTC and the mean value of 61 pmol/L for 105 healthy adults provide a statistically sound base by which to evaluate patients with clinical disorders that affect vitamin B_{12} homeostasis.

In this study we document the use of the assay only in an apparently healthy population. However, in a recently published study, our assay was used to measure holoTC concentrations in an Asian Indian population, of whom ~75% had metabolic signs of Cbl deficiency (total homocysteine >15 μmol/L and methylmalonic acid >0.26 μmol/L) *(17)*. The authors found a strong correlation ($P < 0.001$) between low holoTC concentrations and increased total homocysteine and methylmalonic acid. In that study, a high correlation ($P < 0.001$) was also reported for holoTC and total B_{12} concentrations, indicating a negative B_{12} balance in existence for many years.

Of interest is the observation that serum and plasma holoTC differed by 8%. This is consistent with a recent report that found total TC to be 6% higher in EDTA plasma than in serum *(18)*. This difference is likely a consequence of the adsorption of TC to the components of the clot, but it is not likely to mask the potential clinical value of a low holoTC concentration.

The physiologic role of TC and the significance of holoTC in maintaining vitamin B_{12} homeostasis are well established *(11, 19)*. The rapid turnover of holoTC *(11, 19–21)* requires a constant source of this protein to maintain normal intracellular Cbl cofactor concentrations.

TC provides an essential function in the assimilation of vitamin B_{12} from the distal ileum into the portal blood *(22, 23)*. In the absence of TC, absorption of vitamin B_{12} is impaired *(19, 24, 25)*. However, when circulating TC is within reference values, a decreased holoTC concentration may be evidence for vitamin B_{12} deficiency, the lack of a sufficient dietary source of B_{12} (i.e., negative vitamin B_{12} balance), or impaired intestinal absorption, which occurs with pathology of the distal ileum. It is for such disorders that measuring holoTC may be helpful *(26)*.

This application for holoTC was first proposed by Herzlich and Herbert *(15)*, but subsequent reports from other laboratories failed to establish this value for holoTC *(27–29)*. A potential cause for these discrepancies could certainly be attributed to the unreliable nature of these methods. In most cases, holoTC was measured indirectly. These procedures usually used QUSO to collectively remove TC in the serum, and the difference in Cbl concentration in serum before and after treatment was considered to be TC-bound Cbl. However, the variable adsorption of TC and the small but significant adsorption of HC by QUSO as well as the inadequate precision of the Cbl assay to distinguish a 10% difference in serum Cbl provided an unreliable estimate of holoTC. Low holoTC concentrations frequently were computed as zero or negative numbers *(28–30)*.

The following facts attest to the superiority of this method over existing methods for quantifying holoTC: (*a*) it provides a direct estimate of holoTC; (*b*) it captures virtually all of the TC by immunoadsorption to microspheres; (*c*) it isolates TC from the major Cbl carrier, HC; (*d*) it concentrates the holoTC so that the Cbl may be accurately assayed, after extraction from the microspheres, by conventional methods; (*e*) it uses recombinant human holoTC, which goes through all the same steps as the samples, to construct the calibration curve, and thus assures accurate quantification; (*f*) it has a detection limit of 5 pmol/L; and (*g*) it has a limit of quantification <10 pmol/L.

In conclusion, we have developed a solid-phase assay that accurately and reliably quantifies the minute amounts of holoTC present in human serum and plasma. The assay is superior to current methods and may serve to identify patients with clinical disorders with altered vitamin B_{12} homeostasis.

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