Active B12: A Rapid, Automated Assay for Holotranscobalamin on the Abbott AxSYM Analyzer

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BACKGROUND: Conventional tests for vitamin B12 deficiency measure total serum vitamin B12, whereas only that portion of vitamin B12 carried by transcobalamin (holotranscobalamin) is metabolically active. Measurement of holotranscobalamin (holoTC) may be more diagnostically accurate for detecting B12 deficiency that requires therapy. We developed an automated assay for holoTC that can be used on the Abbott AxSYM immunoassay analyzer.

METHODS: AxSYM Active B12 is a 2-step sandwich microparticle enzyme immunoassay. In step 1, a holoTC-specific antibody immobilized onto latex micro-particles captures holoTC in samples of serum or plasma. In step 2, the captured holoTC is detected with a conjugate of alkaline phosphatase and antiTC antibody.

RESULTS: Neither apoTC nor haptocorrin exhibited detectable cross-reactivity. The detection limit was ≤0.1 pmol/L. Within-run and total imprecision (CV ranges) were 3.4%–5.1% and 6.3%–8.5%, respectively. Assay CVs were <20% from at least 3 pmol/L to 107 pmol/L. With diluted serum samples, measured concentrations were 104%–114% of the expected values in the working range of the assay. No interference from bilirubin, hemoglobin, triglycerides, erythrocytes, rheumatoid factor, or total protein was detected at expected (abnormal) concentrations. A comparison of the AxSYM Active B12 assay with a commercial RIA for holoTC yielded the regression equation: AxSYM = 0.98 RIA + 4.7 pmol/L (S_y,x 11.4 pmol/L; n = 204). Assay throughput was 45 tests/h. A 95% reference interval of 19–134 pmol/L holoTC was established with samples from 292 healthy individuals.

CONCLUSIONS: The AxSYM Active B12 assay allows rapid, precise, sensitive, specific, and automated measurement of human holoTC in serum and plasma.

Vitamin B12 is essential for 1-carbon metabolism and cell division. Vitamin B12 deficiency is a major public health issue, particularly amongst the elderly. The clinical consequences of vitamin B12 deficiency include megaloblastic anemia and progressive neurologic disease of the central and peripheral nervous systems.

Measurement of the total vitamin B12 concentration in plasma is the current standard clinical test for vitamin B12 deficiency; however, a proportion of individuals with vitamin B12 concentrations that would be considered deficient exhibit no clinical or biochemical evidence of deficiency (1). Conversely, neuropsychiatric (2) and metabolic (1) abnormalities can occur with plasma vitamin B12 concentrations well within the reference interval.

Vitamin B12 is bound to 2 proteins in serum, transcobalamin (TC)3 and haptocorrin (HC). HC binds the majority of circulating vitamin B12 (70%–90%). The exact function of this HC-bound vitamin B12 is unknown, but it is believed to be biologically unavailable to most cells (3). The remaining 10%–30% of vitamin B12 is bound to holoTC. TC carries the vitamin from its site of absorption to the tissues and delivers the vitamin to cells via a specific receptor with a high affinity for holoTC (4).

Genetic absence of HC is rare but not a serious condition and is usually discovered accidentally (5). On the other hand, the genetic absence or an abnormality of TC manifests as the typical hematologic and neurologic pathologies of vitamin B12 deficiency (6). Such deficiency, usually discovered shortly after birth as a failure to thrive, requires aggressive therapy with
vitamin B₁₂ if long-term and irreversible neurologic damage is to be avoided.

The measurement of total serum vitamin B₁₂ suffers from a number of limitations, most particularly that the majority of vitamin B₁₂ measured is that bound to HC. Given that holoTC has a shorter circulating half-life than holoHC, the earliest change that occurs when an individual enters a negative vitamin B₁₂ balance is very likely to be a decrease in the plasma holoTC concentration. Direct measurement of holoTC has been postulated to provide a better indicator of an individual’s vitamin B₁₂ status (7).

Although this hypothesis is now nearly 20 years old, only recently have reliable and sensitive methods for estimating holoTC become available (8, 9). Recent studies have suggested that holoTC is both a better early marker for changes in vitamin B₁₂ status than total vitamin B₁₂ (10–12) and more strongly associated with conditions related to impaired cobalamin function (13). HoloTC concentrations are low in patients with biochemical signs of vitamin B₁₂ deficiency (14). Low values have been reported in vegetarians (15, 16), vegans (17), and the elderly (18).

Current holoTC assays are manual, however, and not suitable for the clinical laboratory workload. Very recently, investigators developed a novel monoclonal antibody (mAb) with a specificity for holoTC that is at least 100-fold greater than for apoTC (19). This antibody will allow the development of simple direct immunoassays for the quantification of holoTC. Such assays will also avoid the need for the various denaturing treatments required to release vitamin B₁₂ from binding proteins used in most vitamin B₁₂ assays and thus will remove a possible cause of preanalytical variability.

We describe the characteristics of an assay we have developed for use on an automated immunoassay analyzer (Abbott AxSYM) that is suitable for testing large numbers of samples. We compare results obtained with this assay with those for the current commercially available RIA, together with the expected concentrations in the healthy population and in untreated pernicious anemia patients.

Materials and Methods

Materials
The production of mouse antihuman holoTC and antihuman TC antibodies has previously been described (19, 20). Human recombinant holoTC and apoTC were from Cobento (21). A sample of purified HC was kindly provided by Ebba Nexø (University of Aarhus, Aarhus, Denmark). We used the holoTC RIA from Axis-Shield Diagnostics and used 0.2-μm OptiLink carboxylate-modified microparticles (Seradyn). We obtained MiniKros hollow-fiber filter modules with a 0.2-μm cutoff from Spectrum Laboratories and alkaline phosphatase (AP) from Biozyme. Sephadex G25 size-exclusion chromatography media was obtained from Sigma-Aldrich and prepared per the manufacturer’s instructions.

Apparatus
The AxSYM is an automated, random-access immunoassay analyzer that uses fluorescence polarization immunoassay, microparticle immunoassay, and ion-capture technologies (22).

Reagents

Microparticle Reagent
For a typical 100-mL coating, 28 mL of anti-holoTC mAb (1 g/L) was mixed with 2.5 mL of 100 g/L microparticle solids and 188 mL of 50 mmol/L MES buffer, pH 5.5. The pH of the mixture was checked and adjusted to 5.5 (within 0.1 pH unit) before conjugation. The reaction was started by the addition of 6.3 mL of 50 g/L of N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride (EDAC), freshly prepared in water; thus, the final concentrations in the reaction mixture were 0.56 g/L mAb, 5 g/L microparticle solids, and 3.3 mmol/L EDAC in 18.8 mmol/L MES, pH 5.5. This coating mixture was stirred for 1 h at room temperature. The particles were then washed by buffer exchange with dialfiltration in 2 coating-mixture volumes of wash buffer (10 mmol/L phosphate buffer containing 1 mL/L Tween 20 and 145 mmol/L NaCl). A MiniKros hollow-fiber filter module (0.2-μm cutoff) was used to exchange buffer with the particles. After washing, the particles were exchanged into 2 coating-mixture volumes of capping buffer (40 mmol/L Tris buffer, 100 mmol/L NaCl, 23 mmol/L EDTA, 10 g/L bovine serum albumin, and 0.9 g/L sodium azide). The particles were then stirred for 1 h at room temperature in the capping buffer. The particles were diluted to 2 coating-mixture volumes in storage buffer (260 g/L sucrose, 40 mmol/L Tris buffer, 100 mmol/L NaCl, 23 mmol/L EDTA, 10 g/L bovine serum albumin, and 0.9 g/L sodium azide) for a final sucrose concentration of 130 g/L. Finally, the particles were stressed by incubation at 37 °C for 16–18 h before storage at 2–8 °C.

Conjugate Reagent
The anti-TC mAb was dialyzed against 100 mmol/L triethanolamine buffer, pH 8.4, containing 0.9 mmol/L EDTA and 100 mmol/L NaCl. AP (about 20 g/L) was dialyzed against 30 mmol/L triethanolamine buffer, pH 7.6, containing 3 mol/L NaCl, 50 μmol/L ZnCl₂, and 1 mmol/L MgCl₂. The mAb was activated (by conversion of primary amine groups to sulfhydryl groups) by reaction for 30 min at room temperature with a
300:1 molar ratio of 2-iminothiolane to mAb. The AP was activated by reaction with a 38:1 molar ratio of sulfosuccinimidyl 4-[(N-maleimidomethyl)-cyclohexane-1-carboxylate to AP and mixing at room temperature for 40–45 min. Both reactions were stopped by adding excess glycine. Size-exclusion chromatography on a Sephadex G25 column was used to remove low molecular weight molecules from the preparations and to exchange the mAb and the AP into their respective conjugation buffers. The mAb preparation was exchanged into 100 mmol/L triethanolamine buffer, pH 7.3, containing 0.9 mmol/L EDTA and 100 mmol/L NaCl; the AP preparation was exchanged into 100 mmol/L triethanolamine buffer, pH 7.0, containing 100 mmol/L NaCl, 50 μmol/L ZnCl₂, and 1 mmol/L MgCl₂. We then mixed activated mAb and AP in a 1:1 molar ratio for 60 min at 37 °C and then quenched the reaction by adding excess maleimide. Finally, we purified the mAb:AP conjugate and exchanged the buffer by size-exclusion chromatography in 100 mmol/L triethanolamine buffer, pH 7.0, containing 100 mmol/L NaCl, 50 μmol/L ZnCl₂, 1 mmol/L MgCl₂, and 1 g/L sodium azide.

CALIBRATORS
HoloTC calibrators were prepared gravimetrically from a 50-nmol/L stock solution of recombinant human holoTC. The purity of the recombinant holoTC was evaluated (a) by denaturing electrophoresis on a polyacrylamide gel containing sodium dodecyl sulfate and (b) spectroscopically by measuring the ratio of the protein component to the vitamin B₁₂ component, the requirement being an A_{280}/A_{362} absorbance ratio of 2.3 (0.1), which indicates a purity of >97% (23). The concentration of the holoTC stock solution was calculated (a) from the absorbance at 362 nm with a molar absorbivity of 30 000 (mol/L)⁻¹ cm⁻¹ (24) and (b) by measurement in a vitamin B₁₂ assay (Elecys B₁₂ immunoassay, Roche Diagnostics). The final formulation of the calibrators contained holoTC diluted in phosphate buffer (10 mmol/L), bovine serum albumin (2.5 g/L), and an excess of vitamin B₁₂ (50 nmol/L). The calibrator concentrations were finally readjusted with a panel of serum samples of known holoTC concentration to correct for differences in ionic strength and pH between the calibrator buffer and serum samples; the interaction between holoTC and the holoTC-specific antibody is sensitive to differences in ionic strength and pH (19).

CONTROLS
The control material was prepared from pooled samples of human serum adjusted with recombinant human holoTC and the phosphate buffer to the desired concentration. The holoTC concentrations of the control material (low and high controls) differed somewhat between preparations.

ASSAY PROTOCOL
Fig. 1 illustrates the assay protocol. The sampling probe pipets the sample or calibrator (0, 8, 16, 32, 64, and 128 pmol/L) and all AxSYM Active B₁₂ reagents required for a test into different wells of a reaction vessel. The diluted sample and microparticles coated with anti-holoTC mAb are combined in the sample well of the reaction vessel. The holoTC present in the sample binds to the coated microparticles. The anti-TC mAb:AP conjugate is pipetted into a second well of the reaction vessel, wash buffer is pipetted into a third well, and Matrix Cell wash solution is pipetted into a fourth well. The reaction vessel is immediately transferred into the Processing Center of the AxSYM instrument. The Processing Probe performs additional pipetting in the Processing Center. A 90-μL aliquot of the reaction mixture containing microparticles and bound holoTC is transferred to the glass-fiber matrix of the Matrix Cell. The Matrix Cell is washed, and 60 μL of the anti-TC mAb:AP conjugate is dispensed onto the Matrix Cell and binds to the captured holoTC. The substrate (4-methylumbelliferyl phosphate) is added after a second wash step, and the rate of substrate dephosphorylation by AP is measured by the optical assembly of the microparticle immunoassay instrument. Calibration used 4-parameter logistic curve fitting.

The sample requirement is 200 μL.

SAMPLES
For studies of the reference interval and method comparison, serum samples were provided by Dr. J. M. Pekelharing, Medical Laboratories, Reiner de Graaf Hospital, Delft, The Netherlands. Samples were collected from healthy volunteer hospital staff; there were no other exclusion or inclusion criteria. Samples were stored frozen at −25 °C to −30 °C until testing. The studies were approved by the regional ethics committee, and the participants gave informed consent. A total of 292 samples were measured once. The participant’s age was available for 278 individuals, and sex data were available for 276 individuals (78 males and 198 females).

For the method comparison, we reassayed 204 samples from the reference population with the HoloTC RIA, a manual RIA, with the protocol supplied by the manufacturer (Axis-Shield).

We also analyzed 5 samples from untreated pernicious anemia patients; these samples were kindly provided by Angel F. Remacha, Hospital de Sant Pau, Barcelona, Spain. The patients had given informed consent.
CHARACTERIZATION OF THE ASSAY

The detection limit (mean + 2 SDs for the zero calibrator) was determined on 2 AxSYM instruments, each of which used 2 lots each of the 0- and 8-pmol/L calibrators. We conducted 3 runs each of 10 replicates for the 0-pmol/L calibrator and 4 replicates for the 8-pmol/L calibrator.

Assay imprecision (CV) was evaluated according to NCCLS (currently CLSI) protocol EP5-A2 (25). We tested 2 samples each in the normal (49 pmol/L) and low-normal (23 pmol/L) regions of the reference interval. Because only the lower part of the reference interval is diagnostically important, we did not include a concentration above the upper limit of the reference interval. We measured the holoTC concentration twice daily in duplicate for 20 days on 2 different AxSYM instruments (n = 160 for each sample).

We estimated the working range (CV <20%) for the assay by measuring the holoTC concentration in 2–5 replicates of 12 undiluted and 4 diluted serum samples in 5 analytical runs conducted over 5 days with 2 different instruments. We took 20–50 measurements of each serum sample.

Analytical selectivity was evaluated in 5 separate experiments by measuring the cross-reactivity with apoTC (500 pmol/L) and HC (5 nmol/L) in AxSYM Line Diluent.

To analyze the effect of dilution on assay linearity, we mixed 3 samples of pooled sera with 72, 87, and 103 pmol/L holoTC with AxSYM Active B12 Low Control (20 pmol/L) at 1:4, 2:3, 3:2, and 4:1 ratios of the serum sample volume to the volume of Low Control. All samples were measured in quadruplicate.

To investigate the use of alternative blood-collection tubes, we analyzed 11 samples with holoTC concentrations of 37–124 pmol/L with serum tubes, gel-separator serum tubes, lithium-heparin plasma tubes, and EDTA plasma tubes.

REFERENCE INTERVAL

To establish a reference interval, we measured holoTC concentrations in serum samples from 292 apparently healthy volunteers (age range, 21–70 years).

STATISTICAL ANALYSES

We calculated total and intraassay imprecision according to CLSI protocol EP5-A2, as described above (25), and assessed assay linearity with regression analysis. To investigate the reference interval, we normalized data by logarithmic transformation before analysis. We used the Student t-test and ANOVA with the post hoc Bonferroni correction. We used Deming regression analysis to compare methods and analyzed residuals with the Bland–Altman plot.
Data were analyzed with Analyse-it (Analyse-it Software) for Microsoft Excel and GraphPad Prism (GraphPad Software).

**Results and Discussion**

A typical calibration curve for the AxSYM Active B12 assay (Fig. 2) shows the relationship between the rate of fluorescent probe production and holoTC concentration. Fitting with the 4-parameter logistic curve produced a good fitting accuracy ($r^2 = 1.00$) and only a small ($<5\%$) fitting error (Fig. 2, inset). The assay features a turnaround time of 20 min and an assay throughput of 45 tests/h.

The mean detection limit was 0.08 pmol/L (observed range, 0.03–0.14 pmol/L). Total imprecision was 6.3%–8.5%, and intraassay imprecision was 3.4%–5.1% (see Table S1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol?/issue?). The working range (CV $<20\%$) was at least 3–107 pmol/L (Fig. 3). The assay was linear over at least the range of 20–103 pmol/L [slope (SD), 1.005 (0.032); y-intercept, 2.8 (1.9) pmol/L; $r^2 = 0.98$] (Fig. 4). Measurements of diluted serum samples yielded concentrations that were 104%–114% of the expected values within the working range of the assay.

Serum contains 2 proteins (apoTC and HC) that have the potential to cross-react with a holoTC-specific antibody. ApoTC is the vitamin B$_{12}$-transporting protein TC uncomplexed with vitamin B$_{12}$. HC is a closely related vitamin B$_{12}$-transporting protein in serum. The cross-reactivities of apoTC and HC were 0.08% and 0.004%, respectively (see Table S2 in the online Data Supplement). We observed no interference with
the following substances added to serum samples: bilirubin, 684 μmol/L (0.4 g/L); hemoglobin, 5 g/L; triglycerides, 17 mmol/L (15 g/L); total protein, 95 g/L; erythrocytes, 4 mL/L; and rheumatoid factor, 500 kIU/L. The measured holoTC values were 92%–110% of the expected values.

HoloTC concentrations measured with the AxSYM Active B12 assay were similar after sample processing with serum, gel-separator serum, and lithium-heparin plasma tubes but were significantly higher after processing with EDTA plasma tubes (139% relative to serum tubes). This effect was not attributable to a direct effect of EDTA on holoTC binding to the antibody, because that interaction is unaffected by EDTA (19). It also cannot be attributed to an effect on AP, because the EDTA-containing plasma is washed off before the AP-conjugated second antibody is added. Whatever the cause, the Active B12 assay cannot be recommended for EDTA-treated plasma.

The comparison of the AxSYM Active B12 assay and the HoloTC RIA yielded a regression equation with a slope (SD) of 0.98 (0.03), a y-intercept of 4.7 (2.0) pmol/L, and a $S_{yx}$ value of 11.4 pmol/L (Fig. 5). The values obtained with the AxSYM Active B12 assay were slightly higher than those obtained with the RIA, as is seen in the Bland–Altman plot (Fig. 5, inset). The principles of the 2 assays are different. The AxSYM Active B12 assay directly measures holoTC in the sample with an immobilized mAb specific for holoTC. In the RIA, an immobilized TC-specific mAb captures the total TC (apoTC plus holoTC) in the sample; the vitamin B₁₂ bound by holoTC is then released and measured with a conventional competitive assay protocol for vitamin B₁₂.

REFERENCE INTERVAL AND EXPECTED VALUES
An ANOVA of the log-transformed data revealed holoTC concentration to be weakly dependent on age but not on sex. Further analysis revealed that the positive correlation with age was confined to women. The 95% reference interval was 19–134 pmol/L (mean, 50 pmol/L), which is similar to the reference interval (24–157 pmol/L) previously obtained with the commercial holoTC assay, HoloTC RIA (8).

Serum samples were obtained from 5 patients who had received a clinical diagnosis of pernicious anemia but had not yet been treated. HoloTC and serum vitamin B₁₂ were measured with the AxSYM Active B12 and Abbott AxSYM B12 assays, respectively. The holoTC and serum vitamin B₁₂ concentrations for the 5 patients were 3, 3, 4, 3, and 4 pmol/L, and 62, 92, 62, 87, and 49 pmol/L, respectively.

In conclusion, AxSYM Active B12 is a sensitive and highly specific high-throughput assay for quantitative measurement of the biologically active fraction of vitamin B₁₂, holoTC, in serum and plasma.

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References

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