Clin Chem Lab Med 2010;48(2):249–253 © 2010 by Walter de Gruyter • Berlin • New York. DOI 10.1515/CCLM.2010.032

Determination of serum holotranscobalamin concentrations with the AxSYM active B_{12} assay: cut-off point evaluation in the clinical laboratory

Fabrizia Bamonti[1,*], Giovanna Antonella Moscato[2], Cristina Novembrino[3], Dario Gregori[4], Claudia Novi[2], Rachele De Giuseppe[1], Claudio Galli[5], Valentina Uva[1], Silvia Lonati[1] and Rita Maiavacca[6]

[1] Dipartimento Scienze Mediche, Università degli Studi di Milano, Ospedale Maggiore Policlinico, Mangiagalli e Regina Elena, Fondazione IRCCS, Milan, Italy
[2] U.O. Laboratorio di Analisi Chimico-Cliniche Ospedale di Cisanello, Azienda Ospedaliero-Universitaria Pisana, Pisa, Italy
[3] Dipartimento Scienze Neurologiche, Centro ''Dino Ferrari'', Università degli Studi di Milano, Ospedale Maggiore Policlinico, Mangiagalli e Regina Elena, Fondazione IRCCS, Milan, Italy
[4] Department of Environmental Medicine and Public Health, Labs of Biostatistics and Epidemiological Methods, University of Padova, Padua, Italy
[5] Abbott Diagnostics, Rome, Italy
[6] Dipartimento Area Servizi Diagnostici, Laboratorio di Patologia Clinica, Ospedale Maggiore Policlinico, Mangiagalli e Regina Elena, Fondazione IRCCS, Milan, Italy

Abstract

Background: A reliable early marker is required for diagnosis of cobalamin deficiency. We calculated an appropriate holotranscobalamin (HoloTC) cut-off point for identifying cobalamin deficiency using an immunoenzymatic assay.
Methods: Determination of the cut-off threshold and correlation between HoloTC and the other diagnostic parameters routinely used for vitamin B_{12} deficiency [total vitamin B_{12} (tB_{12}), folate, homocysteine] were measured in 250 routine blood specimens from 107 men (mean age 59.0 ± 18.8 years) and 143 women (mean age 54.2 ± 23.1 years). The inclusion criterion was serum tB_{12} concentration ≤221 pmol/L.
Results: Analytical performance results agreed with those reported by others. A weak correlation (R = 0.42) was found between HoloTC and tB_{12}. A 40 pmol/L cut-off threshold was chosen for HoloTC and the associated sensitivity and specificity was 0.86 and 0.66, respectively. Out of 250 tested samples, 126 showed tB_{12} concentrations 139–221 pmol/L (gray zone, GZ) and 124 had tB_{12} concentrations <139 pmol/L (low, L). Values less than the cut-off for HoloTC were present in 68.2% and 37.9% of cases in the GZ and L group, respectively ($p < 0.01$), and in 53.2% of subjects.
Conclusions: Our results confirmed the analytical reliability of the AxSYM HoloTC assay. The method is adequate for routine use and a cut-off threshold of 40 pmol/L is appropriate for assessing cobalamin deficiency in populations with reduced tB_{12} values.
Clin Chem Lab Med 2010;48:249–53.

Keywords: analytical performance; holotranscobalamin; immunoenzymatic assay; vitamin B_{12}.

Introduction

Vitamin B_{12} or cobalamin, a micronutrient supplied by meat and dairy products, is essential for mammalian intracellular metabolism, particularly metabolism of one-carbon groups and cell proliferation and differentiation (1, 2).

Low nutritional intake or impaired intestinal absorption of vitamin B_{12} may lead to a negative balance and eventually to functional deficiency when tissue storages are depleted.

Cobalamin deficiency has clinical consequences such as megaloblastic anemia in severely deficient individuals, and a variety of progressive neurological diseases that occur in the absence of hematological complications, and hyperhomocysteinemia, associated with several pathological conditions including cardiovascular diseases, birth defects, neuropsychiatric disorders and dementia (3–5).

Determination of vitamin B_{12} concentrations is useful in the prevention, diagnosis and/or prognosis of a variety of disorders directly or indirectly associated with defects in the metabolic pathways of this vitamin. However, serum total vitamin B_{12} (tB_{12}) concentrations are a dubious marker of actual functional B_{12} status because in some cases it correlates poorly with hematologic indices (6). Cobalamin deficiency develops insidiously over the years, caused either by an autoimmune disease, such as pernicious anemia, or due to nutritional deficiency. Therefore, early and reliable diagnosis of vitamin B_{12} deficiency is essential because of the latent nature of this disorder and the possible risk of irreversible neurological damage (7) which may be prevented by vitamin supplementation.

*Corresponding author: Fabrizia Bamonti, Dipartimento Scienze Mediche, Università degli Studi di Milano, Ospedale Maggiore Policlinico, Mangiagalli e Regina Elena, Fondazione IRCCS, Via F. Sforza, 35, 20122 Milan, Italy
Phone: +390255033473, Fax: +390250320403,
E-mail: fabrizia.bamonti@unimi.it
Received July 22, 2009; accepted September 24, 2009; previously published online December 7, 2009

The determination of cobalamin status by measuring holotranscobalamin (HoloTC, or active B_{12}) concentrations represents a new approach for diagnosing subtle cobalamin deficiency (7, 8). HoloTC, the transcobalamin (TC)-cobalamin complex representing the biologically active form of the vitamin and consisting of ~10%–30% of total serum B_{12}, is recognized by ubiquitous specific membrane receptors (5, 7) and could have high diagnostic value as a marker of storage (5).

Several studies indicate that HoloTC is a more sensitive marker of vitamin B_{12} status compared with total serum cobalamin (5), and that it could be the earliest and most sensitive marker for vitamin B_{12} deficiency (1). In fact, HoloTC levels are low in most, but not all, patients with biochemical signs of vitamin B_{12} deficiency. Notably, low values have been reported in both vegetarians and vegans, and in populations with a low intake of vitamin B_{12} (9–11).

According to some studies, there is enough evidence to suggest that HoloTC is an early marker of changes in cobalamin homeostasis (12), and determination of a suitable HoloTC cut-off point is essential due to the fact that the cut-off threshold reported by different studies ranges from 35 to 45 pmol/L (13, 14).

The goals of this study were to evaluate the analytical performance of this new immunoenzymatic method for HoloTC (Active B_{12}, Abbott Diagnostics, Wiesbaden, Germany) using an automated analyzer (AxSYM, Abbott), and to calculate an appropriate cut-off threshold for HoloTC that would identify cobalamin deficiency in an Italian population.

Materials and methods

The active B_{12} immunoassay, based on a microparticle enzyme immunoassay (MEIA) technique with calibration range of 0–128 pmol/L, was performed using the AxSYM analyzer (15).

Analytical performance was assessed using standard procedures. Inter- and intra-assay imprecision were determined according to the Clinical and Laboratory Standards Institute (CLSI) protocol EP5-A2 (16) using a control sample that we prepared from pooled serum (HoloTC concentration 76 pmol/L) and two levels of control material supplied by the manufacturer (HoloTC concentration 23 pmol/L and 49 pmol/L).

Linearity was assessed by measuring samples prepared from dilutions of two home-made serum pools containing high (143.3 pmol/L) and low (8.8 pmol/L) concentrations of HoloTC. Recovery was assessed using 10 plasma samples containing known concentrations of HoloTC (range: 4.0–111.6 pmol/L) spiked with plasma from a sample with a known HoloTC concentration (59.0 pmol/L). Each aliquot was measured in duplicate and recovery was calculated as the percentage of observed vs. the expected value.

The limit of detection was established by testing the assay calibrator A (0 pmol/L) 25 times and calculating the mean + 2 SDs of the measured results.

Possible correlation between HoloTC and the other diagnostic parameters routinely used for vitamin B_{12} deficiency (tB_{12}, folate, homocysteine) was assessed using 250 routine blood specimens, obtained from 107 men (mean age 59.0 ± 18.8 years) and 143 women (mean age 54.2 ± 23.1 years). The only inclusion criterion was that the serum tB_{12} concentration be ≤221 pmol/L.

Each blood sample, collected in light protected tubes either without additive for determining serum tB_{12}, active B_{12}, folate and creatinine concentrations, or containing EDTA for plasma homocysteine analysis, was tested fresh or stored at −80°C until analysis. To minimize analytical variation, a single technician assayed all samples with the same instrument: serum tB_{12} and active B_{12} together with serum folate concentrations using a MEIA (Abbott Diagnostics); plasma total homocysteine (tHcy) by a fluorescence polarization immunoassay (FPIA, Abbott Diagnostics) with the AxSYM analyzer. Measurement of serum creatinine was performed using a colorimetric assay with the Modular analyzer (Roche Diagnostics, Basel, Switzerland).

Total B_{12} cut-off points were identified as those maximizing the ϕ correlation coefficient $(TP \cdot TN - FP \cdot FN)/\sqrt{(TP+FN) \cdot (TN+FP) \cdot (TP+FP) \cdot (TN+FN)}$, where TP = true positive, FP = false positive, TN = true negative and FN = false negative. The product yields a number between −1 and 1, with 1 indicating a perfect prediction and 0 random prediction. Values below 0 indicate worse than random prediction. Next, HoloTC cut-off thresholds were derived and the area under ROC curve (AUC), sensitivity and specificity were calculated, along with their 95% confidence intervals (CI), estimated via bootstrap (1000 runs). All analyses were performed using the R System (17).

Results

Mean analytical imprecision (intra-assay and inter-assay) ranged between 2.9% and 4.1% CV for assay controls, and between 6.0% and 7.7% CV for our home-made serum controls. HoloTC linearity was confirmed for the range from 8.8 to 143.3 pmol/L ($r = 0.99$). The assay mean recovery for spiked specimens was 95% (interval: 90%–100%), and mean linearity determined by dilution was 100% (interval: 93%–111%). The detection limit was 0.07 pmol/L.

Total B_{12} values maximizing the ϕ correlation coefficient were in the range of 138–186, with two local maxima, one at 146 and another at 174. At these concentrations, the maximum ϕ correlation coefficient was at 40 pmol/L HoloTC (95% CI for ϕ 0.225–0.283). Table 1 shows the AUC, HoloTC sensitivity and specificity for the selected tB_{12} cut-off thresholds.

HoloTC values were not affected by gender and age, and estimated cut-off thresholds did not change according to age and gender (p-value of the gender and age group difference 0.54 and 0.298, respectively).

The 250 serum specimens used for this study showed an even distribution of tB_{12} values between the gray zone (GZ: 139–221 pmol/L; 126 subjects) and low values (L: <139 pmol/L; 124 subjects). HoloTC mean concentrations were 46.5 ± 16.2 pmol/L in GZ and 34.2 ± 14.0 pmol/L in L samples ($p < 0.005$). Moreover, the frequency of low HoloTC value (<40 pmol/L) in subjects with low tB_{12} concentrations was 68.2% compared to 37.9% in subjects with tB_{12} in GZ ($p < 0.01$).

Poor correlation was found between HoloTC and tB_{12}, not only in all 250 specimens ($R = 0.420$), but also in samples with low values of tB_{12} only ($R = 0.337$).

No correlation was found between HoloTC and other parameters that are metabolically correlated to either tB_{12} (folate

Table 1 AUC, HoloTC sensitivity and specificity for the selected tB_{12} cut-off thresholds.

		tB_{12}					
		<140	≥140				
HoloTC	<40	104	96				
	≥40	13	37				
		95% CI			AUC	95% CI	
Sens	0.74	0.62	0.86	Folate	0.61	0.47	0.75
Spec	0.52	0.38	0.66	Homocysteine	0.32	0.19	0.45
AUC	0.75	0.63	0.87	Creatinine	0.42	0.28	0.56

Left side: predictive capability of HoloTC at selected cut-off thresholds for total B_{12}. Right side: areas under the ROC curve and 95% CIs for other putative predictors. tB_{12}, total B_{12}; HoloTC, holotranscobalamin; Sens, sensitivity; Spec, specificity; AUC, area under ROC curve.

and homocysteine) or creatinine. Linear regression analysis showed correlation coefficients of 0.14, 0.14 and 0.10 for folate, homocysteine and creatinine, respectively.

Qualitative agreement between HoloTC and tB_{12} was 65.2% ($p<0.05$). Interestingly, HoloTC and tB_{12} measured in the 250 subjects identified 84 subjects with normal values for both parameters and 79 with abnormal values for both parameters. Additionally, tB_{12} values were low and HoloTC concentrations were normal in 33 subjects, whereas 54 subjects with normal values of total cobalamin (i.e., GZ) showed low concentrations of HoloTC (Table 2).

Agreement of HoloTC with folate was 55.2% ($p<0.0001$), with Hcy 51.6% ($p<0.0001$) and with creatinine 45.6% ($p<0.0001$). These were lower than the agreement seen between tB_{12} and these same parameters.

Discussion

There is concern about the feasibility of an early diagnosis of cobalamin deficiency in asymptomatic subjects since the prevalence of sub-clinical functional cobalamin deficiency is higher than expected. Current assays measure serum tB_{12} concentration, a small percentage (10%–30%) of which, HoloTC, is metabolically active (18). Recently, studies focused on HoloTC to evaluate the potential reliability and diagnostic usefulness of an active B_{12} assay for predicting vitamin B_{12} status in different clinical settings (1, 2, 8, 10). It is essential to establish a proper threshold point for active B_{12} deficiency, as previous studies do not agree on cut-off thresholds that range from 35 to 45 pmol/L.

We first checked the analytical performance of the assay, which was good, and our data were comparable to those obtained by Brady et al. (16). On the basis of our experimental findings, the new AxSYM active B_{12} assay showed good analytical reliability and ease of performance due to the simple pre-analytical phase and complete automation of the AxSYM analyzer, confirming the adequacy of the assay for routine use.

The 40 pmol/L cut-off value we selected was also reported by other authors (18–20). We also verified the predictive ability of HoloTC at this threshold for selected tB_{12} cut-off points. The AUC data were more predictive than other putative predictors of cobalamin deficiency such as folate and Hcy.

The poor correlation between active B_{12} and tB_{12} values, and the lack of correlation between HoloTC and the other parameters that might be related to vitamin B_{12} status represents another relevant finding of this study. Our results confirm the suitability of the 40 pmol/L cut-off threshold for assessing cobalamin deficiency in populations with reduced tB_{12} values, showing a considerable percentage (53.2%) of subjects with low levels of the most metabolically important fraction of cobalamin.

Measurement of serum HoloTC concentration may prove helpful in evaluating the absorption of vitamin B_{12} as HoloTC concentrations and TC saturation reflect recent vitamin B_{12} absorption better than serum tB_{12} (21). However, according to Chen et al., HoloTC concentrations most probably reflect vitamin B_{12} status independently of its recent absorption (22). Questions are still raised concerning the specificity of the HoloTC assay. There is concern about subtle cobalamin deficiency, especially in populations at risk. As reported by Miller et al. (23) in a study of an elderly cohort, and confirmed by Gonzalez-Gross et al. (24), measurement of both HoloTC and tB_{12} concentrations provide better screening for cobalamin deficiency than either assay alone.

Table 2 Qualitative agreement between total B_{12} and HoloTC in 250 serum samples with total B_{12} <221 pmol/L.

Agreement $p<0.05$	65.2%	HoloTC		Total
		Normal	Pathological	
tB_{12}	Normal	84	54	138
	Pathological	33	79	112
	Total	117	133	250

Pathological values were <139 pmol/L for total B_{12} and <40 pmol/L for HoloTC. tB_{12}, total B_{12}; HoloTC, holotranscobalamin.

The graded predictive classification of vitamin B_{12} deficiency proposed by Miller et al. (23) showed that the measurement of both parameters would help identify more at-risk subjects. This classification may be used by physicians to plan further diagnostic testing and/or treatment and in some cases avoid over-treatment. In fact, as reported by Carmel et al. (3), metabolic studies showed that not all the subjects presenting with sub-clinical cobalamin insufficiency were actually vitamin-deficient.

However, a recent study by Clarke et al. (18) showed that using cut-off thresholds of equal sensitivity and specificity (45 and 200 pmol/L for HoloTC and tB_{12}, respectively), HoloTC had slightly better diagnostic accuracy compared with vitamin B_{12} in detecting actual vitamin B_{12} deficiency in subjects with normal renal function, although neither test can be recommended to screen asymptomatic individuals.

This is in contrast somewhat to an earlier study by our group demonstrating the importance of HoloTC assay in monitoring cobalamin status in asymptomatic subjects at risk of developing sub-clinical vitamin B_{12} deficiency due to some physio-pathological condition (e.g., elderly, obese subjects) and/or life-style risk factor (e.g., smokers, vegans) (25). Thus, accurate identification and reliable diagnosis of vitamin B_{12} deficiency is important.

Finally, in agreement with Gonzalez-Gross's observation (24), and on the basis of our own experience, given the high prevalence of hyperhomocysteinemia (frequently due to vitamin B deficiency) careful monitoring of the metabolic markers of cobalamin status is suggested (26, 27).

In conclusion, determination of HoloTC concentrations may be used as a complementary diagnostic strategy to avoid the development of pathological conditions (macrocytic anemia or neurological disease) before symptoms emerge, and should also be used for large scale screening of subjects at latent risk of cobalamin deficiency.

Acknowledgements

The authors are very grateful to Mrs. Mary Coduri for linguistic consultation and to Miss Gloria Defilippi and Miss Maria Pina Manca for their assistance in preparing and assaying samples.

Conflict of interest statement

Authors' conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article. Study support by Abbott Diagnostics played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.
Research funding: This study was supported partly by the Abbott Diagnostics and partly by grants to Prof. Fabrizia Bamonti from the Ministero dell'Università e della Ricerca Scientifica e Tecnologica, Rome, Italy.
Employment or leadership: One of the authors (C.G.) is an employee of Abbott Diagnostics.
Honorarium: None declared.

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