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An International Standard for holotranscobalamin (holoTC): international collaborative study to assign a holoTC value to the International Standard for vitamin B12 and serum folate

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Abstract

Background: Investigation of possible B12 and folate deficiencies requires measurement of these vitamins in serum. There is evidence that holotranscobalamin (holoTC), the active portion of B12 available to cells, is a more specific marker of early B12 deficiency than total B12. The availability of immunoassays for holoTC prompted an international collaborative study to assign a holoTC value to the World Health Organization (WHO) 1st International Standard (IS) for vitamin B12 and serum folate, 03/178.

Methods: The IS, 03/178, and three serum samples with different holoTC levels were assayed by 12 laboratories in eight countries using manual and automated immunoassays for holoTC; one laboratory additionally performed an in-house assay. Fourteen sets of data were analysed.

Results: Overall, the IS, 03/178, and the three serum samples demonstrated assay linearity and parallelism. An overall geometric mean (GM) holoTC value of 106.8 pmol/L was obtained for 03/178, with an inter-laboratory geometric coefficient of variation (GCV) of 10.5%. There was a reduction in inter-laboratory variability when the holoTC

levels in the serum samples were determined relative to the IS with an assigned holoTC value rather than to the assays' calibration. Accelerated degradation studies showed that 03/178 was sufficiently stable to serve as an IS for holoTC.

Conclusions: The WHO Expert Committee on Biological Standardization endorsed the proposal to assign a holoTC value of 107 pmol/L to 03/178, corresponding to 0.107 pmol per ampoule, for use as the 1st IS for vitamin B12, serum folate, and holoTC.

Keywords: active B12; anaemia; cobalamin; standardisation.

Introduction

Vitamin B12 is a porphyrin-like molecule containing cobalt, termed cobalamin. As a cofactor for two enzymes catalysing methyl group transfer reactions, namely methionine synthase and methylmalonyl CoA mutase, B12 is necessary for the transformation of methyltetrahydrofolate to tetrahydrofolate for DNA synthesis, and for fatty acid metabolism. B12 deficiency can therefore lead to functional folate deficiency and impaired DNA synthesis, resulting in pernicious (megaloblastic) anaemia, and demyelinating neuropathy [reviewed in 1, 2].

Approximately 20% of circulating B12 is bound to the protein carrier transcobalamin, which transports B12 into the cells. This complex, holotranscobalamin (holoTC), is therefore the 'active' portion of B12 available to cells; the remaining approximately 80% of B12 that is bound to the carrier haptocorrin has no known biological function, although both forms are measured in conventional assays for B12. There is evidence that holoTC is a better marker of early B12 deficiency than total B12 [3, 4]. Furthermore, holoTC measurement has additional advantages over B12 measurement: it may give fewer indeterminate results, particularly for patients over 65; holoTC assays

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give a better indication of B12 status in pregnancy and for patients taking oral contraceptives [4–6].

The World Health Organization (WHO) International Standard (IS) for vitamin B12 and serum folate (03/178) was established in 2005 with an assigned B12 value of 480 pg/mL and an assigned folate value of 12.1 nmol/L (5.33 ng/mL) [7]. Immunoassays for the measurement of holoTC are now commercially available [8, 9] and the aim of this study was to assign a holoTC value to 03/178 following an international collaborative study. The availability of an international reference material for holoTC should facilitate further development of assay methodologies, and ensure continuity of unitage and comparability of results across methods.

Materials and methods

International Standard for vitamin B12 and serum folate (03/178) and serum samples

The IS for vitamin B12 and serum folate, 03/178, consists of a pool of human serum from seven donors lyophilised in glass ampoules (approx. 1 mL/ampoule; CV 0.08%) [7]. Three patient serum samples, known to vary in their holoTC content, were similarly lyophilised for use in the collaborative study.

Collaborative study participants

A total of 12 laboratories in eight countries participated in the study (Appendix 1), returning 14 sets of data. Each laboratory was assigned a code number, which does not reflect the order of listing. The participants included manufacturers, clinical laboratories and research laboratories.

Methods

Laboratories performed commercial immunoassays (Axis-Shield Diagnostics) utilising holoTC-specific antibodies, in manual and/or automated (Abbott Architect) formats, and standardised by the manufacturer with recombinant holoTC. One laboratory additionally performed an in-house method [10]. The methods used by participating laboratories are shown in Appendix 2.

Study design

Each participant was provided with three ampoules of each of the IS, 03/178, and serum samples 1, 2 and 3. Participants were requested to reconstitute ampoule contents with 1.0 mL distilled or deionised water on the day of assay. They were asked to assay two independent

doubling dilution series of each preparation on each of 3 days, using fresh ampoules each day, to give a total of three holoTC estimates for each of 03/178 and samples 1, 2 and 3. Based on the known approximate levels of holoTC in the study samples, participants were requested to assay 03/178 and serum sample 3 from neat to 1/16 dilution, serum sample 2 from neat to 1/8 dilution, and serum sample 1 neat and diluted 1/2 only. Participants were requested to return their results (as raw data or holoTC concentrations in pmol/L for each dilution) on results sheets provided.

Statistical analysis

Results in pmol/L reported by the participants for 03/178 and serum samples 1, 2 and 3 were corrected for dilution factor and used for further analysis. On day 3, laboratory 12 analysed each replicate dilution series using a different reagent kit lot; these were treated as individual assays. All mean results shown in this report are unweighted geometric means (GMs). Variability between assays within and between laboratories was expressed using geometric coefficients of variation [$GCV = (10^s - 1) \times 100\%$ where s is the standard deviation of the \log_{10} transformed estimates]. Comparison of \log_{10} pmol/L results was carried out in Minitab 17 (Minitab Inc., State College, PA, USA) by fitting a general linear model with laboratory and dilution as factors, with post hoc Tukey's test being used to compare results obtained using different dilutions. Grubbs' test on log potencies was used to identify any outlier laboratory mean results.

Accelerated degradation studies

Ampoules of 03/178 that had been stored at a range of temperatures (-70°C , -20°C , $+4^\circ\text{C}$, $+20^\circ\text{C}$, $+37^\circ\text{C}$) for 9 years and 8 months were assayed using a manual ELISA kit. Replicate dilution series were assayed in two independent assay runs using a fresh set of ampoules per run. The holoTC content of the ampoules stored at temperatures ranging from -20°C to $+37^\circ\text{C}$ was expressed as a percentage of that of ampoules stored at -70°C . The mean results from the two assays were analysed using the Arrhenius model for accelerated degradation studies [11].

Results

Estimated holoTC content of 03/178

An overall analysis to compare results obtained using different dilutions, as described above, showed no significant differences between the 1/2, 1/4 and 1/8 dilutions. Final results were calculated using these dilutions only. The holoTC values (in pmol/L) for individual assays along with the laboratory GMs and intra-laboratory variability, expressed as %GCV, are shown in Table 1 and Figure 1. Intra-laboratory repeatability was good with all

Table 1: Laboratory estimates of holoTC (pmol/L) and assay variability (%GCV) for 03/178 and the study samples.

Laboratory	holoTC pmol/L (%GCV)			
	03/178	Sample 1	Sample 2	Sample 3
1	95.7 (5.0)	10.7 (19.8)	25.9 (8.8)	77.7 (9.0)
2	110.3 (3.2)	15.1 (1.6)	33.8 (1.4)	91.2 (5.9)
3a	122.5 (1.4)	15.3 (2.6)	34.2 (3.6)	103.9 (2.7)
3b	112.1 (2.3)	13.2 (6.4)	29.4 (3.9)	94.1 (2.7)
4a	101.3 (1.7)	17.5 (9.5)	19.9 (7.2)	67.8 (6.0)
4b	82.8 (7.5)	10.7 (28.1)	24.3 (18.0)	75.2 (10.8)
5	101.8 (2.7)	14.6 (1.6)	32.1 (2.9)	88.7 (0.8)
6	104.5 (2.2)	15.0 (0.4)	34.3 (0.5)	89.9 (1.5)
7	110.4 (0.3)	15.8 (2.4)	36.1 (2.0)	96.3 (5.0)
8	109.8 (1.5)	13.3 (0.8)	33.3 (1.4)	99.7 (0.9)
9	122.5 (8.6)	18.2 (26.6)	37.0 (17.9)	105.6 (17.3)
10	111.0 (1.3)	16.9 (1.2)	36.9 (4.0)	94.2 (2.5)
11	113.2 (29.4)	14.7 (12.7)	29.7 (30.9)	118.8 (25.5)
12	104.7 (3.7)	13.5 (14.2)	33.9 (8.1)	92.6 (2.2)
Geometric mean	106.8	14.4	31.1	91.7
Inter-laboratory GCV	10.5%	17.3%	19.6%	15.6%
95% CL	100.8–113.2	13.2–15.8	28.0–34.4	84.3–99.7

GCV, geometric coefficient of variation; CL, confidence limits.

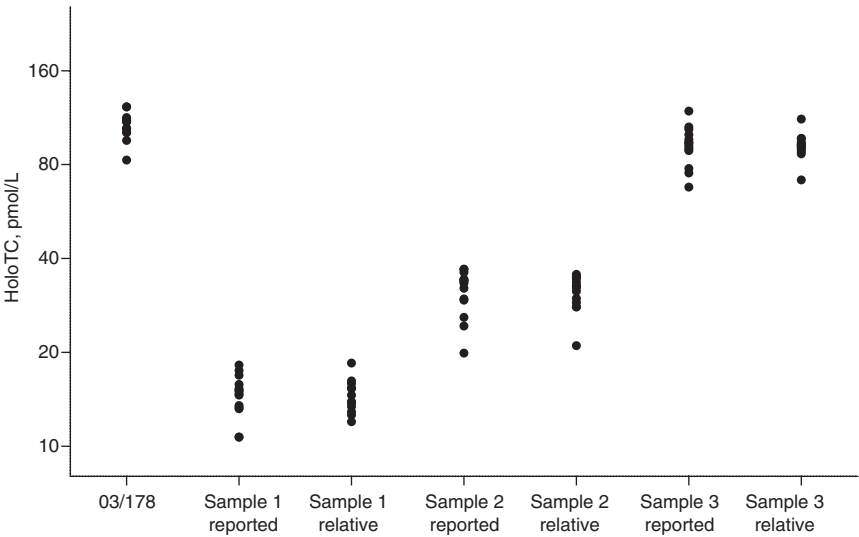


Figure 1: High-low plots showing mean laboratory holoTC estimates of IS 03/178 and samples 1, 2 and 3 relative to assay calibrants (reported potencies), and holoTC estimates of samples 1, 2 and 3 calculated relative to the IS 03/178 with an assigned potency of 106.8 pmol/L (relative potencies).

laboratories having GCVs below 10%, with the exception of laboratory 11. An overall GM value of 106.8 pmol/L was obtained, with an inter-laboratory GCV of 10.5% (Table 1).

Parallelism of serum samples and 03/178

Parallelism of the dose-response lines for 03/178 with serum samples 2 and 3 was assessed using the slope

ratios shown in Supplemental Table 1. Serum sample 1 was not considered, as only two dilutions of this sample were tested. Taking 0.80–1.25 as a typical range of slope ratios that can be used to conclude parallelism, the data demonstrated an acceptable level of parallelism. Slope ratios <0.80 and >1.25 were noted in only six (7%) and four cases (5%), respectively, i.e. similar numbers above and below the range illustrating no overall ‘trend’ in the slope ratios.

Estimated holoTC content of serum samples 1, 2 and 3

As for 03/178, final results were calculated after exclusion of some dilutions for serum sample 2 (1/8 excluded) and serum sample 3 ('neat' and 1/16 excluded) due to significant differences in results between these dilutions and the other dilutions tested. The holoTC values (in pmol/L) for individual assays, relative to the assay calibrants, along with the laboratory GMs and intra-laboratory variability, expressed as %GCV, are shown in Table 1 and Figure 1. The estimated holoTC content of the serum samples relative to 03/178 is shown in Table 2 and Figure 1, where the pmol/L values were calculated by taking the content of 03/178 to be 106.8 pmol/L.

For all serum samples, inter-laboratory %GCV values were lower for the relative potencies (12.3%, 14.9% and 9.9% for serum samples 1, 2 and 3, respectively) when compared to those for directly estimated pmol/L contents (17.3%, 19.6% and 15.6%, respectively; Tables 1 and 2). For serum samples 2 and 3, laboratory 4a was noted as an outlier ($p < 0.05$) and the exclusion of this laboratory further reduced inter-laboratory %GCV values for the relative potencies of these samples to 8.9% and 6.6%, respectively.

Thus, despite the already good agreement between laboratories on the estimated holoTC values, there was a

further reduction in inter-laboratory variability when the holoTC content of the samples was determined relative to the IS.

Stability

The holoTC content of ampoules stored at -20°C , $+4^{\circ}\text{C}$, $+20^{\circ}\text{C}$ and $+37^{\circ}\text{C}$, expressed as a percentage of the holoTC content of ampoules stored at -70°C , was 99.1%, 98.4%, 87.4% and 62.5%, respectively. These data give a predicted loss of 0.035% potency per year when stored at -20°C , which represents good stability.

Discussion

There was overall good agreement between laboratories on the holoTC content of 03/178 (%GCV, 10.5%). Although the number of different methods was limited, with an unavoidable bias towards a single analytical platform, i.e. Abbott Architect, this assay is both CE-marked and US FDA-approved and is the predicate device for future holoTC assays. It is also important that an IS is available before the development of further assays for holoTC to ensure consistency between assay methods as introducing a new IS after the development of a number of assays for diagnostic analytes can be too late to ensure effective standardisation. This was clearly demonstrated by the difficulty in persuading manufacturers to recalibrate against the WHO Reference Reagent for Serum Transferrin Receptor (sTfR), 07/202, once it had been established, and where assay results were not even being reported in the same units [12].

The candidate IS for holoTC, 03/178, demonstrated overall parallelism to the patient samples included in the study, demonstrating 'like against like' properties. The inclusion of these samples, with different holoTC levels, demonstrated a reduction in inter-laboratory variability when the holoTC levels in the samples were determined relative to the IS (holoTC value of 106.8 pmol/L assigned) rather than to the assays' calibration. Thus 03/178 showed commutability, albeit with a limited number of patient samples, and the data indicated that its use as a standard would minimise inter-method variability. These results along with the stability data thus show that 03/178 is fit for use as a WHO IS to standardise assays for serum holoTC.

Although the holoTC content of 03/178 is relatively high compared to the usual cut-off threshold of around

Table 2: Estimates of holoTC (pmol/L) and assay variability (%GCV) for the study samples using 03/178 as standard with an assigned value of 106.8 pmol/L.

Laboratory	holoTC pmol/L (%GCV) relative to 03/178		
	Sample 1	Sample 2	Sample 3
1	12.0 (14.1)	28.9 (5.7)	86.7 (4.2)
2	14.6 (3.7)	32.8 (4.6)	88.3 (8.4)
3a	13.4 (1.7)	29.8 (2.5)	90.7 (1.3)
3b	12.6 (8.0)	28.0 (5.1)	89.6 (5.0)
4a	18.5 (7.7)	21.0 (5.5)	71.4 (4.4)
4b	13.8 (28.5)	31.4 (15.4)	97.0 (13.5)
5	15.4 (2.1)	33.6 (1.4)	93.1 (2.0)
6	15.4 (2.7)	35.0 (2.1)	91.8 (1.3)
7	15.3 (2.5)	34.9 (1.9)	93.1 (4.8)
8	12.9 (1.7)	32.5 (0.7)	97.0 (0.7)
9	15.9 (17.1)	32.3 (9.0)	92.1 (8.1)
10	16.2 (0.9)	35.6 (2.7)	90.7 (2.3)
11	13.9 (14.9)	28.0 (6.9)	112.1 (35.6)
12	13.8 (10.4)	34.5 (5.0)	94.4 (4.3)
Geometric mean	14.4	31.1	91.7
Inter-laboratory GCV	12.3%	14.9%	9.9%
95% CL	13.5–15.5	28.6–33.6	86.8–96.8

GCV, geometric coefficient of variation; CL, confidence limits.

32 pmol/L for identifying B12 deficiency [13], it allows for the testing of serial dilution series to assess linearity and parallelism, and spiked recovery experiments over the relevant holoTC measuring range. The holoTC value of 03/178 is also consistent with the assigned total B12 value of 480 pg/mL (equivalent to 354 pmol/L) in that holoTC typically comprises 10%–30% of the total B12 in a normal serum [2, 4, 8].

On the basis of the data summarised in this report, the WHO Expert Committee on Biological Standardization endorsed the proposal to assign a holoTC value of 107 pmol/L to 03/178 (when reconstituted with 1.0 mL), corresponding to 0.107 pmol (4.6 ng assuming a molecular weight of 43 kDa) [14] per ampoule, for use as the 1st IS for vitamin B12, serum folate, and holoTC.

The availability of an IS for holoTC should facilitate the development of new assay methodologies and further assessment of the clinical utility of holoTC.

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Appendix

Appendix 1: Participants of the collaborative study (in alphabetical order of country)

- Benjamin Teis, Sullivan Nicolaidis Pathology, Australia
- Ebba Nexø and Cindy Søndersø Knudsen, Aarhus University Hospital, Denmark
- Eija Stenholm and Annukka Paju, HUSLAB, Meilahti Hospital, Finland
- Liisa Ahola, United Medix Laboratoriot Oy, Finland

Frank Holger Perschel and Ellen Richter, Labor Berlin – Charite Vivantes GmbH, Laboratoriumsmedizin, Germany

Arjan de Mare and A Knuif, Medlon and Medisch Spectrum Twente, The Netherlands

Lanja Saleh, Institute for Clinical Chemistry, University Hospital of Zurich, Switzerland

Lorraine Simpson and Pauline Wicks, Axis-Shield Diagnostics, UK

Graham Roberts, National Institute for Biological Standards and Control, UK

Agata Sobczynska-Malefora, Nutristasis Unit, Viapath, St. Thomas’ Hospital, UK

Kate Guberg, Nutritional Biochemistry Laboratory, MRC, UK

Joshua W Miller and Melissa Murphy, Miller Research Laboratory at Rutgers University, USA

Appendix 2: Methods used by participating laboratories

Laboratory number	Method (as reported by the laboratory)
1	Manual ELISA
2	Automated ELISA
3a	Automated ELISA
3b	Manual ELISA
4a	In-house method
4b	Manual ELISA
5	Automated ELISA
6	Automated ELISA
7	Automated ELISA
8	Automated ELISA
9	Manual ELISA
10	Automated ELISA
11	Manual ELISA
12	Automated ELISA

References

1. Hunt A, Harrington D, Robinson S. Vitamin B12 deficiency. *Br Med J* 2014;349:g5226.

2. Green R. Indicators for assessing folate and vitamin B-12 status and for monitoring the efficacy of intervention strategies. *Am J Clin Nutr* 2011;94(suppl):666S–72S.

3. Herzlich B, Herbert V. Depletion of serum holotranscobalamin II: an early sign of negative vitamin B12 balance. *Lab Invest* 1988;58:332–7.

4. Nexo E, Hoffmann-Lücke E. Holotranscobalamin, a marker of vitamin B-12 status: analytical aspects and clinical utility. *Am J Clin Nutr* 2011;94:359S–65S.

5. Valente E, Scott JM, Ueland P-M, Cunningham C, Casey M, Molloy AM. Diagnostic accuracy of holotranscobalamin, methylmalonic acid, serum cobalamin, and other indicators of tissue vitamin B12 status in the elderly. *Clin Chem* 2011;57: 856–63.
 6. Devalia V, Hamilton MS, Molloy AM, on behalf of the British Committee for Standards in Haematology. Guidelines for the diagnosis and treatment of cobalamin and folate disorders. *Br J Haematol* 2014;166:496–513.
 7. Thorpe SJ, Heath A, Blackmore S, Lee A, Hamilton M, O'Broin S, et al. International Standard for serum vitamin B12 and serum folate: international collaborative study to evaluate a batch of lyophilised serum for B12 and folate content. *Clin Chem Lab Med* 2007;45:380–6.
 8. Brady J, Wilson L, McGregor L, Valente E, Orning L. Active B12: A Rapid, Automated Assay for Holotranscobalamin on the Abbott AxSYM Analyzer. *Clin Chem* 2008;54:567–73.
 9. Bamonti F, Moscato GA, Novembrino C, Gregori D, Novi C, De Giuseppe R, et al. Determination of serum holotranscobalamin concentrations with the AxSYM active B(12) assay: cut-off point evaluation in the clinical laboratory. *Clin Chem Lab Med* 2010;48:249–53.
 10. Nexø E, Christensen AL, Hvas AM, Petersen TE, Fedosov SN. Quantitation of holo-transcobalamin, a marker of vitamin B12 deficiency. *Clin Chem* 2002;48:561–2.
 11. Kirkwood TB. Predicting the stability of biological standards and products. *Biometrics* 1977;33:736–42.
 12. Thorpe SJ, Heath A, Sharp G, Cook J, Ellis R, Worwood M. A WHO Reference Reagent for the Serum Transferrin Receptor (sTfR): international collaborative study to evaluate a recombinant soluble transferrin receptor preparation. *Clin Chem Lab Med* 2010;48:815–20.
 13. Heil SG, de Jonge R, de Rotte MC, van Wijnen M, Heiner-Fokkema MR, Muller Kobold AC, et al. Screening for metabolic vitamin B12 deficiency by holotranscobalamin in patients suspected of vitamin B12 deficiency: a multicentre study. *Ann Clin Biochem* 2012;49:184–9.
 14. Quadros EV, Rothenberg SP, Pan Y-C, Stein S. Purification and molecular characterization of human transcobalamin II. *J Biol Chem* 1986;261:15455–60.
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- Supplemental Material:** The online version of this article (DOI: 10.1515/cclm-2015-1167) offers supplementary material, available to authorized users.