Experimental vitamin B\textsubscript{12} deficiency in a human subject: a longitudinal investigation of the performance of the holotranscobalamin (HoloTC, Active-B12) immunoassay

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Abstract

Based on Victor Herbert’s model for sequential stages in the development of vitamin B\textsubscript{12} deficiency, the holotranscobalamin (HoloTC) immunoassay has controversially been promoted as a more specific and sensitive replacement for the total vitamin B\textsubscript{12} test, for the diagnosis of deficiency. There have been no longitudinal studies, by means of experimental cobalamin deficiency, because ethical considerations prevent such risky studies on patients or healthy human volunteers. The objective was to provide a detailed record of the response of HoloTC, compared to total vitamin B\textsubscript{12} and metabolites, to the development of experimental vitamin B\textsubscript{12} deficiency in an initially replete human subject. This 54 year old male, with a vitamin B\textsubscript{12} deficiency possibly caused by a defect in the intracellular cobalamin metabolism, ensured an initially replete condition by means of oral doses of cyanocobalamin supplements at 1000 μg/day for 12 weeks. The subject then depleted himself of vitamin B\textsubscript{12}, by withholding treatment and using a low-cobalamin diet, until significant metabolic disturbances were observed. The responses of serum total vitamin B\textsubscript{12} and HoloTC and the two metabolites, plasma methylmalonic acid and homocysteine, were monitored by weekly blood tests. HoloTC was not significantly more sensitive than either total serum vitamin B\textsubscript{12} or total homocysteine, and was much less sensitive than methylmalonic acid. HoloTC decreased from an initial concentration of >128 pmol/L to a minimum of 33 pmol/L on day 742, the only day on which it fell below the lower limit of the reference interval. Total vitamin B\textsubscript{12} decreased from an initial concentration of 606 pmol/L to a minimum of 171 pmol/L on day 728. Total homocysteine increased from an initial concentration of 8.4 μmol/L to a maximum of 14.2 μmol/L on day 609. Methylmalonic acid unexpectedly contained four distinct peaks; initially at 0.17 μmol/L, it first exceeded the upper limit of the reference interval on day 386, finally reaching a maximum peak of 0.90 μmol/L on day 658. The results of this experiment are inconsistent with Herbert’s hypothesis that HoloTC is the earliest marker of vitamin B\textsubscript{12} deficiency, and therefore do not support his model for the staged development of vitamin B\textsubscript{12} deficiency.

Keywords: Holotranscobalamin, HoloTC, Active-B12, Vitamin B\textsubscript{12}, Methylmalonic acid, Self-experimentation

Background

Based on Victor Herbert’s model for sequential stages in the development of vitamin B\textsubscript{12} deficiency (Herbert 1987a, 1994), the holotranscobalamin (HoloTC) immunoassay has controversially been promoted as a more specific and sensitive replacement for the total vitamin B\textsubscript{12} test, for the diagnosis of deficiency (Axis-Shield and Abbott Laboratories 2006a, b, 2007a, b; Axis-Shield 2007a, b, 2012, 2014a, b).

Other researchers have reviewed the HoloTC immunoassay (Morkbak et al. 2005; Aparicio-Ugarriza et al. 2014). This author’s detailed review of Herbert’s model and the HoloTC immunoassay, and suggested alternative hypothesis for the development of vitamin B\textsubscript{12} deficiency, has been submitted to this journal as a separate article. Although there have been many published reports supporting the use of the HoloTC...
immunoassay, several experimenters reported results that do not support the claim that HoloTC is the earliest and most sensitive indicator of vitamin B12 deficiency (Miller et al. 2005; Clarke et al. 2007; Schrempf et al. 2011; Palacios et al. 2013; Remacha et al. 2014).

There have been no longitudinal studies, by means of experimental cobalamin deficiency, because ethical considerations prevent such risky studies on patients or healthy human volunteers.

Commencing in March 2007, this author used himself as the subject of an experiment to investigate the sensitivity of total vitamin B12, HoloTC and the two metabolites, methylmalonic acid (MMA) and total homocysteine (tHcy), to the onset of vitamin B12 deficiency.

**Objective**
The objective of this study was to investigate the performance of the HoloTC (Active-B12) immunoassay during the development of experimental vitamin B12 deficiency in an initially replete human subject, by means of self-experimentation.

**Methods**

**Ethics statement**
As a member of Committee on Publication Ethics (COPE), this journal requires that experiments on human subjects adhere to the ethical standards of the Declaration of Helsinki. In particular, there must be informed consent of subjects, and the experiment must be approved and overseen by a research ethics committee or institutional review board. Because this author did not obtain informed consent, and the study did not receive ethics committee approval, it is necessary for the author to explain the reasons why publication of this report is ethical.

Firstly, the author was both the experimenter and the single subject, so the requirement for informed consent does not apply. There was no institutional involvement, so there was no possibility of coercion. The subject was assessed by a psychiatrist, a Fellow of the Royal Australian and New Zealand College of Psychiatrists, before the experiment commenced, and found to be competent to evaluate the risks and benefits, and to accept full responsibility for the conduct of the experiment.

Secondly, the Declaration of Helsinki is silent on self-experimentation, because it is concerned with the conduct of research on patients or healthy volunteers by others. The requirement for ethics committee approval therefore does not apply where the single subject is also the sole experimenter. Also, because there was no institution involved in the study, with the experiment conducted by an independent researcher, no ethics committee existed.

Thirdly, the experiment was not performed recklessly or carelessly; the subject’s condition was monitored weekly by a general practitioner and the psychiatrist; neither had any conflict of interests. Being qualified medical practitioners receiving all weekly pathology reports, both doctors were able to continually assess the condition of the subject. The subject instigated, designed and performed the experiment, and the doctors’ only role was monitoring for safety.

Lastly, the motivation for performing the experiment was ethical, and involved no conflict of interests. The author wanted to investigate the performance of the new HoloTC (Active B12) immunoassay, for the diagnosis of vitamin B12 deficiency, because he was aware of the potential consequences of misdiagnosis. The author was motivated only by the desire to gain and share knowledge, to advance medical science, for the benefit of patients.

**Experiment design**
A human subject, initially replete in vitamin B12, consumed a low-cobalamin diet and gradually ceased taking vitamin B12 supplements to deplete the body of vitamin B12, culminating in significant metabolic disturbances. The responses of serum total vitamin B12 and HoloTC and the two metabolites, plasma MMA and tHcy, were monitored by routine blood tests. All tests were performed by pathology laboratories accredited by the Australian National Association of Testing Authorities (NATA). The experiment commenced on day 0 (5 March 2007) and ended on day 854 (6 July 2009).

**The subject**
The subject was this author, a 54 year old male non-drinker and non-smoker; he had for many years consumed a lacto-vegetarian diet. The subject was diagnosed with vitamin B12 deficiency in October 2005, based on low serum vitamin B12 concentration, chronic symptoms of abnormal sensations in the extremities and results of a neurological examination. Because of his history of vitamin B12 deficiency, possibly caused by a defect in the intracellular cobalamin metabolism, the subject had been taking 1000 μg oral cyanocobalamin daily for 12 weeks immediately prior to this study. Previous testing showed normal results for serum vitamin B12 and the two metabolites, homocysteine and MMA, at this level of supplementation.

**The low-vitamin B12 diet**
The low-vitamin B12 diet was the subject’s usual vegetarian diet. The only source of vitamin B12 in the diet was the estimated 1.8 μg/day contained in the 300 ml of milk added to his breakfast cereal (Food Standards Australia and New Zealand 2013). Although this dietary intake
would be adequate to maintain the vitamin B₁₂ store in a healthy individual (Herbert 1987b), extensive previous testing in his self-experiment of 2005 showed that this subject would become depleted without supplements. In that unpublished experiment, the subject’s serum total vitamin B₁₂ concentration decreased, and his MMA and tHcy concentrations increased, after ceasing oral cyanocobalamin treatment.

Nutritional precautions
Because the subject maintained his normal vegetarian diet, no special nutritional precautions were required. There were no confounding effects of deficiency of folate or other vitamins, minerals or protein. Previous testing showed normal electrolytes, liver function and red-cell haematology.

Changes in vitamin B₁₂ supplementation
At day 0 the subject had been consuming 1000 μg oral cyanocobalamin daily for 12 weeks. On day 7, the supplementation was reduced in steps to reach 10 μg on day 112 (Fig. 1a). The dose was then increased to 100 μg for 4 weeks, to test for the effect on the vitamin B₁₂ and metabolite assays, then reduced in steps to reach 0 μg on day 371. The dose was then increased to 100 μg for 10 weeks, to test for the effect on the vitamin B₁₂, HoloTC and metabolite assays, then reduced in a single step to 0 μg on day 497. The dose remained at 0 μg until the end of the vitamin B₁₂ depletion period on day 751.

Blood sampling
Timing of vitamin B₁₂ supplementation and blood sampling
Blood samples were collected 24 h after the last oral vitamin B₁₂ supplement was taken by the subject.

Blood sample collection, handling and transport
Precautions were taken to ensure that consistent and valid blood samples were received by the laboratories. The commercial clinical laboratory that performed the serum vitamin B₁₂, HoloTC and haematology tests collected their own samples, using professional phlebotomists at a government-approved collection centre. The same collection centre also collected and froze plasma samples for assay of the metabolites, homocysteine and MMA, by the NSW Biochemical Genetics Service; a specialised laboratory. The subject always fasted overnight, and was well hydrated, ensuring maximum possible consistency between samples. The phlebotomy technique was chosen to provide the highest quality samples; tourniquet application was carefully controlled, and discard tubes were used where required. Samples were promptly transported from the collection centre to the commercial clinical laboratory, cooled on ice, to avoid deterioration. The frozen plasma samples for metabolite assays were transported, packed in dry ice, by specialised courier.

Blood sampling frequency
Blood samples for the vitamin B₁₂ and HoloTC were collected weekly. The frequency of blood sampling for the metabolites was adapted according to the rate of change of the MMA and homocysteine responses. Blood samples were initially collected four-weekly; this was increased to fortnightly and then weekly as the rate of change increased.

Vitamin B₁₂ immunoassays

Serum total vitamin B₁₂ immunoassay
Vitamin B₁₂ was assayed weekly by the commercial clinical pathology laboratory, using the Siemens Advia Centaur immunoassay system with the ADVIA Centaur and ACS:180 Vitamin B12 Assay reagent kit (Siemens Healthcare Diagnostics 2007). The laboratory reference interval was given as 162–811 pmol/L and the quoted precision was 10 %.

Serum holotranscobalamin (HoloTC) immunoassay
HoloTC was assayed weekly by the commercial clinical pathology laboratory, using the Abbott AxSYM immunoassay analyser with the Axis-Shield AxSYM Active-B12 reagent kit (Axis-Shield and Abbott Laboratories 2007a). The laboratory reference interval was given as >35 pmol/L, with a maximum reportable concentration of 128 pmol/L; the quoted precision was 7 %.

Vitamin B₁₂ metabolite assays

Plasma methylmalonic acid (MMA)
Plasma MMA was assayed at intervals from four-weekly to weekly, by the NSW Biochemical Genetics Service, using tandem mass spectrometry. The measurement uncertainty was 12 % and reference interval was 0.06–0.34 μmol/L.

Plasma total homocysteine (tHcy)
Plasma tHcy was assayed at intervals from four-weekly to weekly, by the NSW Biochemical Genetics Service, using tandem mass spectrometry. The measurement uncertainty was 9 % and reference interval was 4.8–13.7 μmol/L.

Haematology
Cell counts were performed by the commercial clinical pathology laboratory using the Sysmex XE-2100 Automated Haematology System.
Results
Data availability
The data sets supporting all results are included in a Microsoft Excel spreadsheet file, Additional file 1: Table S1, containing charts and tables. High-resolution images for Figs. 1, 2, 3, 4, 5, 6 and 7 are included in a PDF file, Additional file 2: Figure S1, and a Microsoft PowerPoint file, Additional file 3: Figure S2.

Variation over time: vitamin B\textsubscript{12} immunoassays
Serum total vitamin B\textsubscript{12} immunoassay
The serum total vitamin B\textsubscript{12} concentration (Fig. 1a) was initially 606 pmol/L, well above the lower limit of the laboratory reference interval of 162 pmol/L, the Bates and Lewis (2012) minimum of 132 pmol/L and the Oh and Brown (2003) minimum of 295 pmol/L, indicating that the subject was replete in serum vitamin B\textsubscript{12}.

![Graph](image-url)
The concentration then fell as the oral cyanocobalamin supplement dose was reduced from 1000 μg/day, and rose again during the two short periods when the supplementation was increased to 100 μg/day. On day 728, 231 days after the supplementation was finally reduced to 0 μg/day, the total serum vitamin B12 concentration fell to a minimum of 171 pmol/L; this was slightly above the lower limit of the laboratory reference interval, and significantly above the Bates and Lewis minimum, but significantly below the Oh and Brown minimum.

**Serum holotranscobalamin (HoloTC) immunoassay**

Results for HoloTC (Figs. 1a, 2a, 3a) were not available until day 196 because of unexpected delays in the initial setting up for this test. The HoloTC concentration on day 196 was reported as >128 pmol/L, above the maximum assay limit, indicating that the subject was replete in serum vitamin B12. The concentration fell as the oral cyanocobalamin supplement level was reduced, then varied within a range of 60–91 pmol/L after the oral cyanocobalamin supplement dose was held at 10 μg/day.
concentration then fell to a first minimum, of 48 pmol/L on day 420, after the oral cyanocobalamin supplement dose was reduced to 0 μg/day. The concentration then rose to a peak, of 118 pmol/L on day 463, after the oral cyanocobalamin supplement dose was increased to 100 μg/day. The concentration then inexplicably rose, to a peak of 107 pmol/L on day 511, 14 days after the cyanocobalamin supplement dose was finally reduced to 0 μg/day. There were other unexplained variations in HoloTC concentration, over a range of 41–80 pmol/L, before it fell to a minimum of 33 pmol/L on day 742; this was the only time that the concentration fell below the lower limit of the laboratory reference interval of 35 pmol/L.

**Figure 3**: tHcy and HoloTC. 

**a** tHcy and HoloTC versus time. 

**b** tHcy versus HoloTC. Lab tHcy RI high = upper limit of plasma total homocysteine concentration reference interval defined by the testing laboratory. Lab HoloTC RI low = lower limit of serum holotranscobalamin concentration reference interval defined by the testing laboratory.

**Variation over time: vitamin B₁₂ metabolite assays**

**Plasma methylmalonic acid (MMA)**

Plasma MMA concentration (Figs. 2a, 4a) was not tested until day 112 because previous self-experimentation had shown that increases above baseline would not occur until after serum vitamin B₁₂ fell below about 300 pmol/L. The MMA concentration was initially 0.17 μmol/L, on day 112, well below the upper limit of
the laboratory reference interval of 0.34 μmol/L. The MMA concentration then increased, even during the first of the two short periods when the supplementation was increased to 100 μg/day, and continued to increase as the cyanocobalamin dose was decreased to 10 μg/day. The MMA concentration briefly and inexplicably fell to 0.20 μmol/L, on day 343, after reaching an initial peak of 0.32 μmol/L on day 330. The MMA concentration rose to a peak of 0.62 μmol/L on day 428, well above the upper limit of the laboratory reference interval, 58 days after the cyanocobalamin dose was initially decreased to 0 μg/day. The MMA concentration then fell to 0.20 μmol/L on day 483, during the second of the two short periods when the supplementation was increased to 100 μg/day. After the cyanocobalamin dose was finally decreased to 0 μg/day, the MMA concentration reached another peak, of 0.70 μmol/L on day 483, then inexplicably fell to 0.42 μmol/L on day 609 before reaching a maximum peak of 0.90 μmol/L on day 658. The MMA concentration then again inexplicably fell, to 0.22 μmol/L on day 672, before
increasing to remain within a range of 0.34–0.45 μmol/L, until the cyanocobalamin dose was increased to 1000 μg/day, then it finally fell to 0.12 μmol/L.

**Plasma total homocysteine (tHcy)**

As for the MMA, plasma tHcy concentration (Fig. 3a) was also not tested until day 112 because previous self-experimentation had shown that increases above baseline would not occur until after serum vitamin B12 fell below about 300 pmol/L. The tHcy concentration was initially 8.4 μmol/L, on day 112, well below the upper limit of the laboratory reference interval of 13.7 μmol/L. The tHcy concentration slowly increased, without any definite effect of the two short periods when
the supplementation was increased to 100 μg/day. The tHcy concentration reached a maximum of 14.2 μmol/L, on day 609, 112 days after the cyanocobalamin dose was finally decreased to 0 μg/day. The tHcy concentration then inexplicably gradually fell, reaching 11.4 μmol/L before the cyanocobalamin dose was increased to 1000 μg/day, then finally fell to 8.5 μmol/L.

Variation over time: derived data
Holotranscobalamin (HoloTC) as percentage of total serum vitamin B₁₂
The HoloTC concentration, calculated as a percentage of the total serum vitamin B₁₂ concentration (Fig. 5a), was initially 27.6 %, on day 196. The percentage HoloTC varied significantly, over a range of 16.3–38.6 % during the
vitamin B$_{12}$ depletion stage. During this time there were several distinct peaks and troughs, but these were not all clearly related to any change in the cyanocobalamin dose. One peak, of 37.6 % on day 434, occurred 6 days after commencement of the second of the two short periods when the supplementation was increased to 100 μg/day, and one trough, of 21.1 % occurred 7 days after the end of that period. Another inexplicable peak, of 35.1 %, occurred after the cyanocobalamin dose was finally decreased to 0 μg/day. The percentage HoloTC reached a maximum of 44.9 % on day 777, 26 days after the cyanocobalamin dose was increased to 1000 μg/day.

**Holohaptocorrin (HoloHC)**

The HoloHC concentration, calculated by subtracting the HoloTC concentration from the total vitamin B$_{12}$ (Fig. 5b), was initially 336 pmol/L, on day 196. The HoloHC concentration fell to 139 pmol/L on day 428, before the second of the two short periods when the supplementation was increased to 100 μg/day, rose to a

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**Fig. 7** Haematology. In a, Hgb low = low limit for haemoglobin concentration defined by Bates and Lewis (2012); RCC low = low limit for red-cell count defined by Bates and Lewis (2012). In b, Hct low = low limit for haematocrit defined by Bates and Lewis (2012); MCV high = upper limit for mean cell volume defined by Bates and Lewis (2012)
peak of 279 pmol/L on day 469 during that period, then fell to 151 pmol/L on day 518 after the cyanocobalamin dose was finally decreased to 0 μg/day. There were then two inexplicable peaks, the second reaching 256 pmol/L on day 694, while the cyanocobalamin dose remained at 0 μg/day. The HoloHC concentration increased rapidly, from 142 to 253 pmol/L, after the cyanocobalamin dose was increased to 1000 μg/day, then fell inexplicably to 150 pmol/L before rapidly rising again.

**Comparisons of analyte concentration variation over time: vitamin B₁₂ and metabolite assays**

**Cumulative change in analyte concentration as percentage of initial value**

The cumulative changes in total vitamin B₁₂, HoloTC, MMA and tHcy over time are shown in Fig. 6a. Day 203 was chosen as the reference point for the initial values because it was the first day on which concurrent values were obtained for all analytes. Plasma MMA was the most sensitive indicator of vitamin B₁₂ deficiency, increasing by a maximum of 309 % above the initial value on day 658 but, as noted above, there were unexplained anomalies. Plasma tHcy increased by a maximum of 43 % above the initial value on day 609 and, as noted above, was only this once above the upper limit of the laboratory reference interval value. Total vitamin B₁₂ fell by a maximum of 53 % below the initial value on day 728, and HoloTC fell by a maximum of 66 % below the initial value on day 742.

**Analyte concentration as percentage of cut-off value**

The analyte concentrations, as a percentage of the quoted cut-off values, of total vitamin B₁₂, HoloTC, MMA and tHcy over time are shown in Fig. 6b. Plasma MMA was the most sensitive indicator of vitamin B₁₂ deficiency, increasing by a maximum of 265 % of the cut-off value on day 658 but, as noted above, there were unexplained anomalies. Plasma tHcy increased to a maximum of 104 % of the cut-off value on day 609 and, as noted above, was only this once above the upper limit of the laboratory reference interval value. Total vitamin B₁₂ fell to a minimum of 106 % of the cut-off value on day 728, and HoloTC fell to a minimum of 89 % of the cut-off value on day 742.

**Variation over time: haematology**

**Haemoglobin and red-cell count**

Haemoglobin and red-cell count were not monitored until day 555 because previous self-experimentation had shown that this subject’s haematology was unaffected by this level of vitamin B₁₂ deficiency. The initial haemoglobin and red-cell count were 155 g/L and 5.1 × 10¹²/L respectively, both well within the reference normal ranges (Fig. 7a). Both fell slightly and insignificantly, to 149 g/L and 4.9 × 10¹²/L respectively, by the end of the vitamin B₁₂ depletion stage. Both haemoglobin and red-cell count rose and then fell slightly and insignificantly after the cyanocobalamin dose was increased to 1000 μg/day, with final values of 143 g/L and 4.8 × 10¹²/L respectively. The overall trend for haemoglobin and red-cell count was a slight fall, of 8 and 6 % respectively, not significantly affected by the final change in cyanocobalamin dose from 0 to 1000 μg/day.

**Analyte concentration correlations and significance: vitamin B₁₂ and metabolite assays**

Calculations of correlation and significance were performed using Microsoft Excel; complete details may be found in table T3 of Additional file 1: Table S1. Charts for analyte concentration correlations are shown in Figs. 1b, 2b, 3b and 4b. Results for Pearson’s correlation coefficient (r) and probability (p) are summarised in Table 1.

**Total serum vitamin B₁₂ versus holotranscobalamin (HoloTC)**

There was a very highly significant positive correlation, with r = 0.70, between total serum vitamin B₁₂ and HoloTC concentrations (Fig. 1b; Table 1). This result is consistent with no great difference in sensitivity of the total serum vitamin B₁₂, compared to HoloTC, to the onset of vitamin B₁₂ deficiency, but does not tell us which the most sensitive indicator is. This result is also consistent with the absence of any remarkable differences in response over time.

**Plasma methylmalonic acid (MMA) versus holotranscobalamin (HoloTC)**

The negative correlation between MMA and HoloTC concentrations was moderate, with r = −0.52 (Fig. 2b; Table 1). This result is consistent with the observed difference in sensitivity of the MMA, compared to HoloTC, to the onset of vitamin B₁₂ deficiency but does not itself tell us which the most sensitive indicator is.

**Plasma total homocysteine (tHcy) versus holotranscobalamin (HoloTC)**

The correlation between tHcy and HoloTC concentrations was weak to moderate, with r = 0.45 (Fig. 3b; Table 1). This result is also consistent with the low
sensitivity of both the HoloTC and tHcy to the onset of vitamin B\textsubscript{12} deficiency in this subject.

**Total serum vitamin B\textsubscript{12} versus plasma methylmalonic acid (MMA)**

There was a very highly significant negative correlation, with \( r = -0.67 \), between MMA and total serum vitamin B\textsubscript{12} concentrations (Fig. 4b; Table 1). This result could be consistent with no great difference in sensitivity of the MMA, compared to serum vitamin B\textsubscript{12}, to the onset of vitamin B\textsubscript{12} deficiency, but is not inconsistent with the observed higher sensitivity of the MMA to the onset of vitamin B\textsubscript{12} deficiency.

**Analyte sensitivity comparisons: vitamin B\textsubscript{12} and metabolite assays**

Analyte sensitivity comparisons were performed, using Microsoft Excel, by comparing the number of concurrent pairs of results in each chart quadrant; complete details may be found in table T3 of Additional file 1: Table S1. Results for counts, for the number of results for each analyte pair within each chart quadrant for charts shown in Figs. 1b, 2b, 3b and 4b, are summarised in Table 2.

**Total serum vitamin B\textsubscript{12} versus holotranscobalamin (HoloTC)**

The low sensitivity of the HoloTC and total serum vitamin B\textsubscript{12} concentrations to the onset of vitamin B\textsubscript{12} deficiency are illustrated in Fig. 1b and Table 2. Of the 95 concurrent test results, the 94 data points in the first quadrant show serum vitamin B\textsubscript{12} above the lower limit of the laboratory reference interval of 162 pmol/L (indicating vitamin B\textsubscript{12} adequacy) while HoloTC also is above the lower limit of the laboratory reference interval of 35 pmol/L (also indicating vitamin B\textsubscript{12} adequacy). The one remaining data point is in the second quadrant, showing HoloTC below the lower limit of the laboratory reference interval (indicating vitamin B\textsubscript{12} deficiency) while total serum vitamin B\textsubscript{12} is above the lower limit of the laboratory reference interval (indicating vitamin B\textsubscript{12} adequacy).

### Table 1 Results for correlation coefficient (r) and p value (p)

| Figure | Analytes | N  | r     | p            |
|--------|----------|----|-------|--------------|
| 1b     | Total B\textsubscript{12} and HoloTC | 95 | 0.70  | 2.9 \times 10^{-15} |
| 2b     | MMA and HoloTC | 52 | -0.52 | 7.6 \times 10^{-5}     |
| 3b     | tHcy and HoloTC | 52 | -0.45 | 9.2 \times 10^{-4}     |
| 4b     | Total B\textsubscript{12} and MMA | 55 | -0.67 | 2.9 \times 10^{-8}      |

- \( N = \) number of concurrent test results for each analyte pair
- \( r = \) Pearson’s correlation coefficient between analyte concentrations
- \( p = \) p value = probability of obtaining the result \( \geq r \), for \( N \) concurrent test results, if there is no actual linear correlation between analyte concentrations and that the apparent correlation is due to random chance

### Table 2 Calculated counts for analyte sensitivity comparisons

| Figure | Count criteria | Reference | Quadrant | Count |
|--------|----------------|-----------|----------|-------|
| 1b     | Concurrent B\textsubscript{12} and HoloTC tests (N) | 95 | B\textsubscript{12} \geq 162 pmol/L and HoloTC \geq 35 pmol/L | L 1 94 |
|        |                |           | B\textsubscript{12} \geq 295 pmol/L and HoloTC \geq 35 pmol/L | A 1 41 |
|        |                |           | B\textsubscript{12} \geq 133 pmol/L and HoloTC \geq 35 pmol/L | B 1 94 |
|        |                |           | B\textsubscript{12} \geq 162 pmol/L and HoloTC <35 pmol/L | L 2 1  |
|        |                |           | B\textsubscript{12} \geq 295 pmol/L and HoloTC <35 pmol/L | A 2 0  |
|        |                |           | B\textsubscript{12} \geq 133 pmol/L and HoloTC <35 pmol/L | B 2 1  |
|        |                |           | B\textsubscript{12} <162 pmol/L and HoloTC <35 pmol/L | L 3 0  |
|        |                |           | B\textsubscript{12} <295 pmol/L and HoloTC <35 pmol/L | A 3 1  |
|        |                |           | B\textsubscript{12} <133 pmol/L and HoloTC <35 pmol/L | B 3 0  |
|        |                |           | B\textsubscript{12} <162 pmol/L and HoloTC <35 pmol/L | L 4 28 |
| 2b     | MMA \geq 0.34 µmol/L and HoloTC \geq 35 pmol/L | 52 | MMA \geq 0.34 µmol/L and HoloTC \geq 35 pmol/L | 1 23 |
|        | MMA \geq 0.34 µmol/L and HoloTC <35 pmol/L | 2 1  |
|        | MMA <0.34 µmol/L and HoloTC \geq 35 pmol/L | 3 0  |
|        | MMA <0.34 µmol/L and HoloTC <35 pmol/L | 4 28 |
| 3b     | Concurrent tHcy and HoloTC tests (N) | 52 | tHcy \geq 13.7 µmol/L and HoloTC \geq 35 pmol/L | 1 1  |
|        | tHcy \geq 13.7 µmol/L and HoloTC <35 pmol/L | 2 0  |
|        | tHcy <13.7 µmol/L and HoloTC \geq 35 pmol/L | 3 1  |
|        | tHcy <13.7 µmol/L and HoloTC <35 pmol/L | 4 50 |
| 4b     | Concurrent MMA and B\textsubscript{12} tests (N) | 55 | MMA \geq 0.34 µmol/L and B\textsubscript{12} \geq 162 pmol/L | 1 24 |
|        | MMA \geq 0.34 µmol/L and B\textsubscript{12} \geq 162 pmol/L | 1 2  |
|        | MMA \geq 0.34 µmol/L and B\textsubscript{12} \geq 295 pmol/L | 1 24 |
|        | MMA <0.34 µmol/L and B\textsubscript{12} \geq 162 pmol/L | 2 31 |
The interpretation of this chart changes significantly if reference levels for total serum vitamin B$_{12}$ concentrations from Oh and Brown (2003) are used instead of those quoted by the testing laboratory. By increasing the minimum total serum vitamin B$_{12}$ concentration from the laboratory reference level of 162–295 pmol/L (Oh and Brown 2003), many data points move from the first to the fourth quadrant. Using the Bates and Lewis (2012) reference level of 133 pmol/L does not move any data points from the first to the fourth quadrant.

In the case of the Oh and Brown reference level, of the 95 concurrent test results, only the 41 data points now in the first quadrant show serum vitamin B$_{12}$ above the Oh and Brown reference minimum of 295 pmol/L (indicating vitamin B$_{12}$ adequacy) while HoloTC also is above the lower limit of the laboratory reference interval of 35 pmol/L (indicating vitamin B$_{12}$ adequacy). There are now 53 data points in the fourth quadrant, showing HoloTC above the lower limit of the laboratory reference interval (indicating vitamin B$_{12}$ adequacy) while total serum vitamin B$_{12}$ is below the Oh and Brown reference minimum (indicating vitamin B$_{12}$ deficiency).

### Plasma methylmalonic acid (MMA) versus holotranscobalamin (HoloTC)

The low sensitivity of the HoloTC concentration to the onset of vitamin B$_{12}$ deficiency, compared to MMA, is illustrated in Fig. 2b and Table 2. Of the 52 concurrent test results, the 23 data points in the first quadrant show MMA above the upper limit of the laboratory reference interval of 0.34 µmol/L (indicating vitamin B$_{12}$ deficiency) while HoloTC is above the lower limit of the laboratory reference interval of 35 pmol/L (indicating vitamin B$_{12}$ adequacy). Only one data point is in the second quadrant, showing MMA above the upper limit of the laboratory reference interval while HoloTC is below the lower limit of the laboratory reference interval (both indicating vitamin B$_{12}$ deficiency). The remaining 28 data points are in the fourth quadrant, showing MMA below the upper limit of the laboratory reference interval while HoloTC is above the lower limit of the laboratory reference interval (both indicating vitamin B$_{12}$ adequacy).

### Total serum vitamin B$_{12}$ versus plasma methylmalonic acid (MMA)

The high sensitivity of the MMA and low sensitivity of total serum vitamin B$_{12}$ concentrations to the onset of vitamin B$_{12}$ deficiency are illustrated in Fig. 4b and Table 2. Of the 55 concurrent test results, the 24 data points in the first quadrant show MMA above the upper limit of the laboratory reference interval 0.34 µmol/L (indicating vitamin B$_{12}$ deficiency) while total serum vitamin B$_{12}$ is above the lower limit of the laboratory reference interval of 35 pmol/L (indicating vitamin B$_{12}$ adequacy).
reference interval of 162 pmol/L (indicating vitamin B12 adequacy). The remaining 31 data points are in the second quadrant, showing MMA below the upper limit of the laboratory reference interval (indicating vitamin B12 adequacy) while total serum vitamin B12 also is above the lower limit of the laboratory reference interval (also indicating vitamin B12 adequacy).

As with the previous chart, the interpretation changes significantly if reference levels for total serum vitamin B12 concentrations from Oh and Brown (2003) are used instead of those quoted by the testing laboratory. By increasing the minimum total serum vitamin B12 concentration from the laboratory reference level of 162–295 pmol/L (Oh and Brown 2003), many data points move from the first and second quadrants to the fourth and third quadrants respectively. Using the Bates and Lewis (2012) reference level of 180 pmol/L does not move any data points between quadrants.

In the case of the Oh and Brown reference level, the single data point remaining in the first quadrant shows MMA above the upper limit of the laboratory reference interval 0.34 µmol/L (indicating vitamin B12 deficiency) while total serum vitamin B12 is above the lower limit of the laboratory reference interval of (indicating vitamin B12 adequacy). There are now 20 data points in the second quadrant, showing MMA below the upper limit of the laboratory reference interval (indicating vitamin B12 adequacy) while total serum vitamin B12 is above the Oh and Brown reference minimum (also indicating vitamin B12 adequacy). There are now 11 data points in the third quadrant, showing MMA below the upper limit of the laboratory reference interval (indicating vitamin B12 adequacy) while total serum vitamin B12 is above the Oh and Brown reference minimum (indicating vitamin B12 deficiency). The remaining 23 data points are now in the fourth quadrant, showing MMA above the upper limit of the laboratory reference interval (indicating vitamin B12 deficiency) while total serum vitamin B12 also is below the Oh and Brown reference minimum (also indicating vitamin B12 deficiency).

The subject
The subject’s weight did not change significantly during the course of the experiment. He observed worsening neurological symptoms including extreme mental tiredness, and unpleasant micro-dreams in the daytime. The subject also observed significant reduction in tactile sense in his extremities (peripheral neuropathy), and severe physical fatigue. Recovery following resumption of oral vitamin B12 treatment was very slow.

Discussion

Experimental findings

**Serum total vitamin B12 versus serum holotranscobalamin (HoloTC)**

Three graphical methods have shown that serum HoloTC was only slightly more sensitive to the onset of vitamin B12 deficiency than serum total vitamin B12, with HoloTC indicating vitamin B12 deficiency and serum total vitamin B12 indicating vitamin B12 adequacy for only 1 of the 95 concurrent tests. Firstly, there were no remarkable differences in the time charts for the two analyte concentrations (Figs. 1a, 6). Secondly, there was a very highly significant positive correlation between serum total vitamin B12 and HoloTC concentrations (Fig. 1b; Table 1), consistent with the similar responses over time. Thirdly, all except one data point were in the first quadrant of the analyte sensitivity comparison chart, indicating that both analytes were within the normal range for 94 of the 95 concurrent tests when using the laboratory reference level serum total vitamin B12 (Fig. 1b; Table 2).

It is important to note that the relative sensitivities can be very significantly altered by changing the reference level for either or both analytes. By using the Oh and Brown (2003) reference level (295 pmol/L) for serum total vitamin B12, instead of the lower limit of the laboratory reference interval for serum total vitamin B12 (162 pmol/L), HoloTC becomes far less sensitive than serum total vitamin B12 to the onset of vitamin B12 deficiency (Fig. 1a, b).

**Plasma methylmalonic acid (MMA) versus serum holotranscobalamin (HoloTC)**

Three graphical methods have shown that serum HoloTC was far less sensitive to the onset of vitamin B12 deficiency than plasma MMA, with serum HoloTC indicating vitamin B12 adequacy while MMA indicated vitamin B12 deficiency for 23 of the 52 concurrent tests. Firstly, there were very remarkable differences in the time charts for the two analyte concentrations (Figs. 2a, 6). Secondly, although there was a moderate correlation between MMA and HoloTC concentrations (Fig. 2b; Table 1), this is not inconsistent with the different responses over time. Thirdly, only one data point was in the second quadrant of the analyte sensitivity comparison chart, indicating that HoloTC was below the lower limit of the laboratory reference interval of 35 pmol/L only once, whereas 23 of the 52 data points were in the first quadrant, indicating that MMA was above the upper limit of the laboratory reference interval of 0.34 µmol/L while HoloTC was above the lower limit of the laboratory reference interval (Fig. 2b; Table 2).
Although plasma MMA showed the greatest sensitivity to the onset of vitamin B$_{12}$ deficiency, the results included several unexplained anomalies (Figs. 2a, 6).

Plasma total homocysteine (tHcy) versus serum holotranscobalamin (HoloTC)

Three graphical methods have shown that serum HoloTC was similarly insensitive to the onset of vitamin B$_{12}$ deficiency as plasma tHcy, with both indicating vitamin B$_{12}$ adequacy for 50 of the 52 concurrent tests. Firstly, there were no remarkable differences in the time charts for the two analyte concentrations except that, as expected, they tended to change in opposite directions (Figs. 3a, 6). Secondly, there was a weak to moderate correlation between tHcy and HoloTC concentrations (Fig. 3b; Table 1), not inconsistent with both having low sensitivity to the onset of vitamin B$_{12}$ deficiency. Thirdly, 50 of the 52 data points were in the fourth quadrant of the analyte sensitivity comparison chart, indicating that HoloTC was above the lower limit of the laboratory reference interval of 35 pmol/L while tHcy was below the upper limit of the laboratory reference interval of 13.7 µmol/L (Fig. 3b; Table 2).

The low sensitivity of the homocysteine is consistent with the absence of any significant haematological affects (Fig. 7a, b). The absence of anaemia does not rule out vitamin B$_{12}$ deficiency; as noted by Lindenbaum et al. (1988), 28 % of patients with neurological affects due to vitamin B$_{12}$ deficiency have no haematological abnormalities.

Comparison with previous experimental findings

The results of this experiment are inconsistent with the reported findings that HoloTC was the most sensitive and earliest marker of vitamin B$_{12}$ deficiency. Firstly, HoloTC was not significantly more sensitive than total vitamin B$_{12}$ to the depletion of the vitamin B$_{12}$ body store (Figs. 1, 6). Secondly, MMA overtly showed a disturbed metabolism well before HoloTC indicated any vitamin B$_{12}$ deficiency (Figs. 2, 6).

As noted earlier in this discussion, it is possible to alter the apparent relative sensitivity of any pair of analytes by selectively changing the cut-off value of one or both of them. As demonstrated in this experiment, selecting different cut-off values for total vitamin B$_{12}$ changed the relative sensitivities of HoloTC and total vitamin B$_{12}$ (Fig. 1a, b); the same applies to the cut-off value for HoloTC. When the HoloTC cut-off is increased, the sensitivity to vitamin B$_{12}$ deficiency is increased, but the specificity of the test is reduced, increasing the number of false positive results. Conversely, if the HoloTC cut-off is decreased, the specificity of the test is increased, but the sensitivity of the test is reduced, increasing the number of false negative results.

The results of this experiment are consistent with the findings of several researchers whose results do not support the claim that HoloTC is a significantly earlier and more sensitive indicator of vitamin B$_{12}$ deficiency than total vitamin B$_{12}$ and its metabolites (Miller et al. 2005; Clarke et al. 2007; Schrempf et al. 2011; Palacios et al. 2013; Remacha et al. 2014). These studies all involved aged patients whose vitamin B$_{12}$ deficiency was likely to have been caused by food-cobalamin malabsorption (Carmel 1995; Andrès et al. 2004). Miller et al. (2005) concluded that “HoloTC and total vitamin B12 have equal diagnostic accuracy in screening for metabolic vitamin B12 deficiency”. Clarke et al. (2007) reported “modest” superiority of the HoloTC immunoassay, but concluded that neither HoloTC nor total vitamin B$_{12}$ was suitable for screening for vitamin B$_{12}$ deficiency. Schrempf et al. (2011) reported that “holoTC does not show superior diagnostic accuracy compared to VitB12 for the detection of VitB12 deficiency in subjects with neuropsychiatric conditions”. Palacios et al. (2013) found that HoloTC sensitivity was only 44 %, and concluded that it was unsuitable for screening alone. Remacha et al. (2014) concluded that “These data do not support HoloTC as the earliest marker of Cbl deficiency and challenge the classification in stages of Cbl deficiency”.

Herbert’s model

The results of this experiment are inconsistent with Herbert’s model for sequential stages in the development of vitamin B$_{12}$ deficiency, in which HoloTC is the earliest marker of the change from Normal to Early Negative B$_{12}$ Balance (Herbert 1987a, 1994). Firstly, because the responses of HoloTC and total vitamin B$_{12}$ were so similar, these results do not support the hypothesis that HoloTC is an earlier or more sensitive indicator of a transition between normal and negative balance conditions (Figs. 1, 6). Secondly, as stated above, MMA overtly indicated a disturbed metabolism well before HoloTC indicated any vitamin B$_{12}$ deficiency (Figs. 2, 6).

Limitations of the experiment

Number of subjects

Because this subject has vitamin B$_{12}$ deficiency suspected of being caused by a defect in intracellular metabolism, a relatively rare condition, he might not represent a typical patient. The sensitivity of HoloTC to the onset of a deficiency condition, compared to total vitamin B$_{12}$ and the metabolites, is likely to depend on the specific cause of the deficiency.

For ethical reasons, a longitudinal experiment designed to produce vitamin B$_{12}$ deficiency can only be performed by means of self-experimentation on a single subject. It is therefore not possible to investigate the longitudinal
performance of the HoloTC immunoassay for a large group of subjects.

Even if there were no ethical objections to the performance of such an experiment, it would be of limited value. This is because a pure dietary vitamin B$_{12}$ deficiency, induced for the purpose of the experiment, would not necessarily produce the same effect on HoloTC as would other causes of deficiency such as malabsorption. In dietary deficiency, enterohepatic recycling tends to maintain the HoloTC concentration until the liver store of vitamin B$_{12}$ is exhausted; when deficiency develops due to the onset of severe malabsorption, the recycling would become ineffective and produce a faster reduction in HoloTC concentration because it has a very short half-life in serum. (Herbert 1994).

According to Allen B. Weisse (2012), "many self-experiments have proved invaluable to the medical community and to the patients we are seeking to help." There are numerous other examples of significant contributions made to medical science by single subject experiments (Altman 1998; Widdowson 1993).

**Number of laboratories**

To eliminate individual laboratory error, it would be desirable to have samples tested by more than one laboratory for each analyte. This was not practical because of the high cost, and logistical problems including the need to draw excessive volumes of blood from the subject on each sampling day. For the vitamin B$_{12}$ and HoloTC tests, with modern automated immunoassay systems using pre-packaged reagent kits, the likelihood of significant individual laboratory error has been reduced. For the metabolites, MMA and homocysteine, the specialised laboratory had very strict quality-control procedures. Taking these factors into account, and the very large number of sampling days over such a long time span, it is unlikely that the results can be explained by individual laboratory error.

**Conclusions**

The results of this experiment are inconsistent with Herbert’s hypothesis that HoloTC is the earliest marker of vitamin B$_{12}$ deficiency, and therefore do not support his model for the staged development of vitamin B$_{12}$ deficiency. MMA was the most sensitive indicator of vitamin B$_{12}$ deficiency but results contained significant unexplained anomalies. Self-experimentation has produced a detailed record of the response of HoloTC to experimental vitamin B$_{12}$ deficiency, whereas using patients or healthy volunteers as subjects would be unethical.

**Additional files**

**Additional file 1: Table S1.** Experimental Vitamin B12 Deficiency - for Figures 1 to 7, tables and charts.

**Additional file 2: Figure S1.** Figures 1 to 7, High-resolution images.

**Additional file 3: Figure S2.** Figures 1 to 7, High-resolution slides.

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**Competing interests**

The author declares that he has no competing interests.

**Primary data**

All primary data, as scanned PDF copies of pathology reports, are available from the author.

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