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Methylmalonic acid: a functional B12 biomarker in comparison with conventional B12 assays

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SUMMARY

Introduction

Vitamin B12, also known as Cobalamin, is a water-soluble vitamin. It belongs to the Corrin family, due to the its corrin nucleus. Vitamin B12 deficiency is a major public health problem: however, there is no consensus on how to diagnose this deficiency. Vitamin B12 deficiency can cause megaloblastic anaemia (MA) and degeneration of the myelin sheath. This can cause an array of physiological problems, including nerve damage, spinal cord deterioration, and development of pernicious anaemia (PA). There are several approaches to diagnosing vitamin B12 deficiency. Traditionally full blood count results and blood film morphology. Functional tests to assess the direct biochemical abnormalities linked to B12 deficiency methylmalonic acid (MMA) or total homocysteine levels. Increases in MMA is regarded as an early and specific indicator of functional vitamin B12 deficiency. Regardless of the numerous tests available to assist diagnosis of B12 deficiency, there is no "gold standard" for the diagnosis, and controversy surrounds the debate of the best diagnostic approach.

Aims

To compare different techniques of functional B12 testing and correlate results obtained from different techniques employed to reach the diagnosis of B12 deficiency, as a validation study for Heartlands Hospital.

Main Approaches

Methylmalonic acid (MMA) is determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS) stable isotope dilution analysis.

Expected Outcomes

A validation of MMA assay at Heartlands hospital. The determination of B12 deficiency detection ability of MMA assay.

Contribution to the Field of Research

A strong investigation into the relevance of current practices of diagnosis of B12 deficiency and a reliable assessment of MMA assay and its importance in the diagnosis of B12 deficiency.

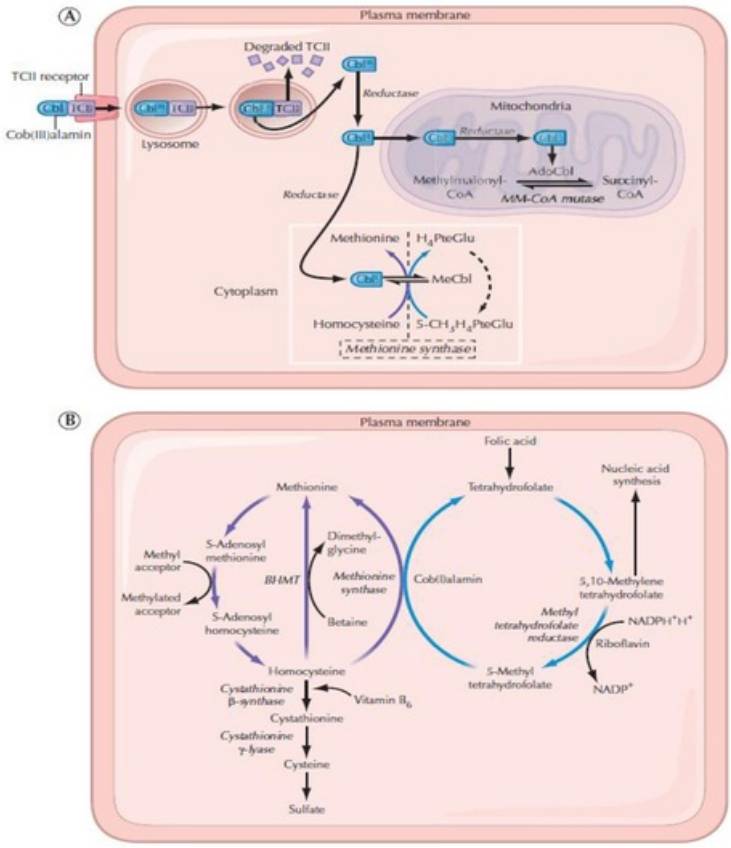
Introduction

Vitamin B12

Vitamin B12, also known as Cobalamin, is a water-soluble vitamin. It belongs to the Corrin family, due to the its corrin nucleus. The corrin group within vitamin B12 holds an atom of cobalt. The further attachments to this corrin group determines the function of the cobalamin. Only two of six cobalamins are active as coenzymes in vivo; methylcobalamin and adenosylcobalamin (Biolab Medical Unit 2017). The main sources of vitamin B12 enters the diet via food of animal origin, bound to animal proteins; this includes meat, dairy products, and foods consolidated with vitamin B12. After ingestion, dietary vitamin B12 is released within the stomach due to pepsin and hydrochloric acid. (Vashi *et al* 2016).

The free vitamin B12 is bound to two proteins; transcobalamin (TC) and haptocorrin (HC). The HC bound B12 is biologically unavailable and carries majority of the circulating B12. The TC bound B12 (holoTC) carries it to absorption sites of tissues in the body where it binds to a specific receptor with a high affinity for holoTC and releases vitamin B12 into the cells (Brady *et al* 2008). Released vitamin B12, functions as a cofactor for the conversion of methylmalonyl coenzyme-A to succinyl coenzyme-A, and the formation of methionine from homocysteine (Figure 1). Vitamin B12 is utilised in the synthesis of myelin, and alongside folate it is required for DNA synthesis (Hvas *et al* 2006).

Figure 1. Cellular uptake and processing of cobalamin (Andrès *et al* 2004).



Vitamin B12 Deficiency

The causes of vitamin B12 deficiency can be placed among three classes (Kuzminski *et al* 1998):

1) nutritional deficiency
2) malabsorption syndromes
3) gastrointestinal causes

The classic haematological manifestation of vitamin B12 deficiency is a megaloblastic macrocytic anaemia, which is illustrated by a high mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH), and a peripheral blood film containing macrocytic, oval red blood cells (RBCs) and hypersegmented neutrophils (Langan *et al* 2011).

Vitamin B12 deficiency can cause megaloblastic anaemia (MA) and degeneration of the myelin sheath. This can cause an array of physiological problems, including nerve damage, spinal cord deterioration, and development of pernicious anaemia (PA). Low serum B12 levels are associated with may conditions and lifestyle choices, including iron deficiency, pregnancy, vegetarianism, celiac disease, use of oral contraception, and age. An elevated serum B12 is associated with disorders including, renal failure, liver disease, and myeloproliferative diseases (Chromsystems 2016). B12 and folate deficiency leads to the compensatory increase in homocysteine, which is a significant risk factor for cardiovascular disease (Klee 2000). Often there are patients with low B12 levels whom have another cause to their macrocytosis (e.g. haemolysis with reticulocytes, drug induced, myelodysplasia and other neoplasms) (Berg *et al* 2013).

Pernicious anaemia is a cause for B12 deficiency, it is an autoimmune disorder which causes inflammation and consequent damage to the stomach lining, this results in the loss of parietal cells, digestive enzymes and mucus. Parietal cells produce intrinsic factor, a protein required in the absorption of vitamin B12 in the stomach. Destruction of parietal cells leads to a lack of intrinsic factor, which in hand leads to B12 deficiency (NICE 2017).

According to NICE, the clinical consequences of vitamin B12 deficiency include:

- anaemia
- loss of peripheral nerve function
- visual disturbance
- memory loss
- psychiatric abnormalities.

Testing and Diagnosis

There are several approaches to diagnosing vitamin B12 deficiency. Traditionally full blood count results and blood film morphology. Functional tests to assess the direct biochemical abnormalities linked to B12 deficiency, methylmalonic acid (MMA) or total homocysteine levels (Verhoef *et al* 1996). Total serum homocysteine is a late indicator of vitamin B12 deficiency. Direct testing of total vitamin B12 is the most commonly used test in the UK to measure vitamin B12 status (Stabler *et al* 1988). Serum holotranscobalamin (holoTC) measurement is another direct uptake test which is particularly an early B12 deficiency marker. There are pros and cons to every method utilised in the diagnosis of B12 deficiency. There are also a proportion of individuals with vitamin B12 levels in their serum that would be considered as deficient, however these individuals exhibit no clinical or biochemical evidence of deficiency (Brady *et al* 2008).

Total serum B12 measurement has several limitations. Most vitamin B12 measured is that bound to HC, and since holoTC has a shorter circulating half-life than holoHC, therefore a B12 deficiency may not decrease the total serum B12 concentration. Cells only uptake vitamin B12 in the form of holoTC, therefore the direct test of serum holoTC is more reflective of B12 status. (Brady *et al* 2008). Active B12 Enzyme Immunoassay is a novel monoclonal antibody that is highly specific for holoTC, this allows a direct immunoassay quantifying the active portion of B12. However, there may be false negative results obtained from patients with metabolic abnormalities (Axis-Shield 2015). Serum vitamin B12 level are sensitive to the concentrations of binding proteins. Falsely increased values of holoTC are caused by myeloproliferative disorders. Falsely depleted results are observed with folate deficiency, pregnancy, and transcobalamin deficiencies (Klee 2000).

Homocysteine and methylmalonic acid are better metabolic indicators of B12 deficiency at the tissue level. Homocysteine is a four-carbon amino acid [HS(CH2)2CHNH2COOH] which is created by the demethylation of methionine (Klee 2000).

Total serum homocysteine is an indicator of vitamin B12 deficiency. Vitamin B12 is required for the synthesis of methionine from homocysteine. Depletion in the levels of vitamin B12 lead to an increased total serum homocysteine. Homocysteine levels are also higher in individuals with conditions including; folate deficiency, vitamin B6 deficiency, renal failure and hypothyroidism. Increased homocysteine levels are also associated with increased risk for cardiovascular disease (Tungtrongchitr *et al* 2003).

Methylmalonic acid (MMA)

Methylmalonic acid (MMA) is a four-carbon molecule [COOH2CH(CH3)COOH] which is linked to the catabolism of valine, isoleucine, and propionic acid. The conversion of methylmalonyl coenzyme-A to succinyl coenzyme-A requires vitamin B12, therefore a B12 deficiency causes an increase in the concentration of cellular MMA. This increase is measurable in the early stages of B12 deficiency, even before measurable decreases in total serum vitamin B12. Increases in MMA is regarded as an early and specific indicator of functional vitamin B12 deficiency (Klee 2000).

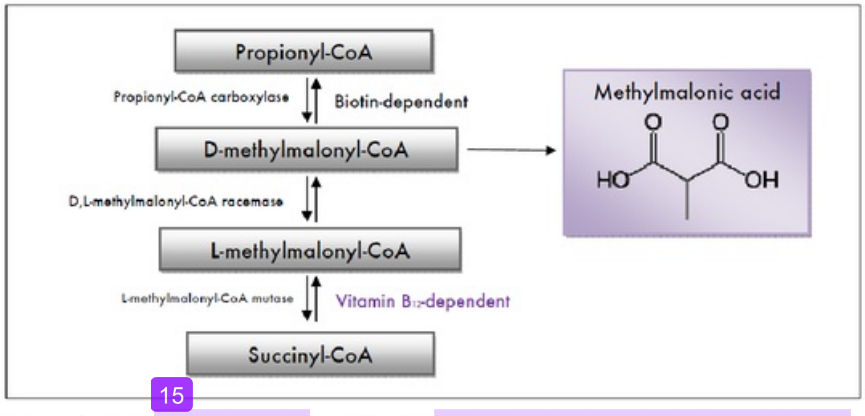


Figure 2. The conversion of propionyl-coenzyme A to succinyl-coenzyme A (Chromsystems 2016).

The measurement of MMA to determine vitamin B12 status is preferred to total serum B12 quantification because vitamin B12, in vivo, is bound to various transport proteins (transcobalamines) and total serum B12 level does not differentiate the functional B12 in the blood. Furthermore, MMA is markedly more stable in patient samples and the concentration of MMA in serum rises in a deficiency state, much before the vitamin B12 concentrations in serum decrease. MMA is a sensitive indicator, which displays existing functional vitamin B12 deficiency at an early stage. Electrospray tandem mass spectrometry is used to perform MMA and homocysteine assays (Klee 2000).

Regardless of the numerous tests available to assist diagnosis of B12 deficiency, there is no "gold standard" for the diagnosis, and controversy surrounds the debate of the best diagnostic approach.

Aims

To compare different techniques of functional B12 testing and correlate results obtained from different techniques employed to reach the diagnosis of B12 deficiency, as a validation study for Heartlands Hospital.

Objectives

- Training on the Mass Spectrometer.
- Training on Abbott Architects.
- Work with the clinical biochemistry team and learn to analyse results.
- To perform a small scale preliminary investigation testing different sample collection and retention strategies, and devise the most appropriate order of data collection.
- Utilise the appropriate sample collection method for the all the samples to be used in the investigation.
- Analyse samples for all tests for comparison.
- Data collection.
- Record data in appropriate tables.
- Conduct the data analysis and implement comparative and correlation statistical tests.
- Compile report

Experimental Design

Pre-Experimental Aspects

- Specimen Requirements: Serum sample from a SST tube.
- Special Considerations: Sample stability at +2 to +8 °C up to 14 days and below -18 °C up to 2 months.
- Health and Safety: Use correct personal protection equipment in the laboratory and when handling specimens.
- Equipment: Abbott Architect and Triple Quad Tandem Mass Spectrometer (Heartlands Hospital).
- Sample: The samples will be coming from the vast number of routine specimens processed by the blood sciences laboratory at Heartlands Hospital. Aiming to analyse 300+ samples to enable official validation of MMA testing.
- Ethics & Risk Assessment: New test validation in a hospital laboratory, therefore ethical approval waived. Risk assessments found on hospital quality management system.
- Method of Analysis: MassChroms MMA kit analysed with LC-MS/MS

Technique

Mass spectrometry is the study of systems causing the formation of gaseous ions, with or without fragmentation, which are then characterised by their mass to charge ratios (m/z) and relative abundances (Ho *et al* 2003). Methylmalonic acid (MMA) is determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS) stable isotope dilution analysis. As a prerequisite, the molecules must be charged therefore electrospray ionisation (ESI) in negative mode is utilised. The specimens will be mixed with an internal standard (methyl-d3-malonic acid). MMA and the internal standard will be isolated by the automated strong anion-exchange solid phase extraction(Chromsystems 2016).

Figure 3. Photograph of the Triple Quad Tandem Mass Spectrometer (Heartlands Hospital).

The MS/MS is operated in the multiple reaction monitoring (MRM) negative mode to filter out fragments which are not specified. The ratios of the extracted peak areas of MMA to internal standard determined by LC-MS/MS are used to calculate the concentration of MMA present in the sample. Disruptive substances such as isobaric compounds (e.g. succinic acid), are separated chromatographically via an analytical column, allowing false positive results to be avoided. The internal standard is used to ensure the precision and robustness of the assay (Chromsystems 2016).

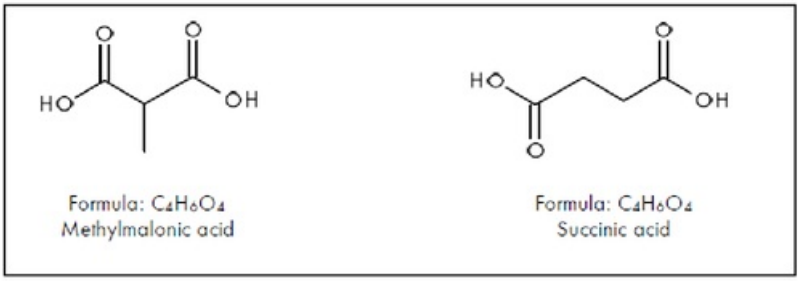


Figure 4. Chemical structures of the isobaric compounds (Chromsystems 2016)

Brief Method

- Pipette 100 µl sample/calibrator/control into Clean-Up Tubes
- Add 50 µl Internal Standard and mix (vortex) briefly
- Centrifuge 10 min at 14 000 x g
- Transfer filtrate into an autosampler vial
- Inject 10 – 20 µl of the filtrate into the LS-MS/MS system
- Analysis time 3.5min/sample

Milestones

Prospective Time Plan - GANNT chart

Activities		Duration (weeks)	Dec-Jan	Feb	Mar	Apr-May
Training		2				
Sample Collection		5				
Preliminary investigations		4				
INVESTIGATION	Data collection	6				
	Analysis	6				
	Interpretation	6				
Data analysis and Report completion		15				

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