REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	5 Sep 02	THESIS

4. TITLE AND SUBTITLE
ESTABLISHING A METHOD FOR MEASURING SERUM METHYMALONIC ACID AND APPLICATION TO WOMEN WITH A HISTORY OF BREAST CANCER

5. FUNDING NUMBERS

6. AUTHOR(S)
CAPT HAUGE ILEANA

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
UNIVERSITY OF WASHINGTON

8. PERFORMING ORGANIZATION REPORT NUMBER
CI02-516

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
THE DEPARTMENT OF THE AIR FORCE
AFIT/CIA, BLDG 125
2950 P STREET
WPAFB OH 45433

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION AVAILABILITY STATEMENT
Unlimited distribution
In Accordance With AFI 35-205/AFIT Sup 1

DISTRIBUTION CODE

DISTRIBUTION STATEMENT A:
Approved for Public Release - Distribution Unlimited

13. ABSTRACT *(Maximum 200 words)*

14. SUBJECT TERMS

15. NUMBER OF PAGES
86

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT

THE VIEWS EXPRESSED IN THIS ARTICLE ARE THOSE OF THE AUTHOR AND DO NOT REFLECT THE OFFICIAL POLICY OR POSITION OF THE UNITED STATES AIR FORCE, DEPARTMENT OF DEFENSE, OR THE U.S. GOVERNMENT

University of Washington
Graduate School

This is to certify that I have examined this copy of a master's thesis by

Ileana Hauge

And have found that it is complete and satisfactory in all respects,
and that any and all revisions required by the final
examining committee have been made.

Committee Members:

Mark H. Wener

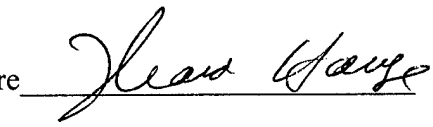
Robert F. Labbé

Wayne L. Chandler

Date: March 22nd, 2002

Master's Thesis

In presenting this thesis in partial fulfillment of the requirements for a Master's degree at The University of Washington, I agree that the Library shall make its copies freely available for inspection. I further agree that extensive copying of this thesis is allowable only for scholarly purposes, consistent with "fair use" as prescribed in the U.S. Copyright Law. Any other reproduction for any purposes or by any means shall not be allowed without my written permission.

Signature 

Date 22nd March, 2002

ESTABLISHING A METHOD FOR MEASURING SERUM METHYLMALONIC ACID AND APPLICATION TO WOMEN WITH A HISTORY OF BREAST CANCER

by

Ileana Hauge

A thesis submitted in partial fulfillment of the
requirements for the degree of

Master of Science

University of Washington

2002

Program Authorized to Offer Degree: Department of Laboratory Medicine

University of Washington

Abstract

ESTABLISHING A METHOD FOR MEASURING SERUM METHYLMALONIC ACID AND APPLICATION TO WOMEN WITH A HISTORY OF BREAST CANCER

Ileana Hauge

Mark H. Wener
Chair of the Supervisory Committee
Associate Professor
Department of Laboratory Medicine

Serum concentrations of methylmalonic acid (MMA), a dicarboxylic acid and intermediate in the conversion of propionic acid to succinic acid, are elevated if there is deficiency of cobalamin (vitamin B_{12}) at the tissue level. Measurement of serum MMA may be the best way to assess vitamin B_{12} status. Early diagnosis of vitamin B_{12} deficiency may improve patient treatment and prevent later sequelae.

A method of measuring serum MMA using gas chromatography/mass spectroscopy was developed in the laboratory. The new test method correlated well with values obtained in a reference laboratory assay. The method was used to determine the reference range in a normal female population. Frozen sera specimens from female patients with breast cancer were assayed for MMA, and compared with the normal population using the t-test statistic.

A statistically significant ($p<0.05$) difference was found between the normal and breast cancer group 2 (local/recurrent cancer) population, with increased levels of serum MMA in the breast cancer population. The breast cancer population was also stratified by the extent of cancer. No statistically significant differences were detected between the breast cancer subgroups, but the differences between the normal population and the breast cancer subgroups were most significant for the patients with recurrent disease.

The correlation between levels of the breast cancer tumor marker CA27.29 and serum MMA was highly significant.

Results of methylmalonic acid serum analysis suggests that vitamin B_{12} deficiency at the tissue level is common among patients with recurrent or local breast cancer.

TABLE OF CONTENTS

Page

List of Figures

Number **Page**

List of Tables

Number **Page**

List of Abbreviations

MMA.....methylmalonic acid

DNA.....deoxyribonucleic acid

Cbl.....cobalamin

GC-MS.....gas chromatographer-mass spectrometer

TIC.....Total ion chromatogram

SIM.....Selective ion chromatogram

Acknowledgements

I do wish to express sincere appreciation to the Department of Chemistry at Harborview Hospital and Medical Center for their moral long-term support, Agilent Technologies for technical support and instrument training, and ARUP Laboratories in Salt Lake City for their technical expertise advice. At the the UWMC I want to express my sincere respect for my committee members Dr. Wayne L. Chandler, Dr. Mark H. Wener, and Dr. Robert F. Labbé for supporting my thesis data and getting me up to the top of the project. At home, I want to say thank you to my family members who encouraged me through the tough times and showed me total unconditional devotion and love.

Dedication

To my family and friends

Introduction

Nutrition and Vitamins

Centuries ago it was recognized that symptoms of certain diseases could be eliminated or alleviated with various foods. One historical example was the story of British sailors who developed scurvy due to a diet low in fruits and vegetables. It was observed that eating citrus fruits eliminated the disease symptoms. A compound later named vitamin C was found to exist in very high concentration in citrus fruits like oranges and lemons. In 1906 F.G. Hopkins suggested that dietary requirements should include certain minimal factors present in the tissues of plants and animals. The same dietary factors were later involved in diseases known as scurvy and rickets.[1] Hopkins was the first scientist to present conclusive evidence about natural materials that contain these accessory nutritional elements with vital biochemical roles for body growth and metabolism.

Nutrition is the study of the substances (*nutrients*) that our body obtains from the foods we eat, to assure normal growth and metabolic maintenance. Among these biochemical substances are proteins, carbohydrates, lipids, inorganic elements (minerals), and vitamins. The study of human nutrition is very complex. Nutrition studies deal with required nutrients, foods where the nutrients are found, the necessary amount for each nutrient to maintain optimal growth, metabolism of each nutrient, and their biochemical and physiological function.

The human body requires thirteen vitamins and some fifteen minerals. The required amount of each nutrient can vary with gender, age, and individual. There are recommended dietary allowances (RDA), defined as the amount that should be ingested daily to maintain good nutrition and optimal health for the average healthy person.[2] Based on the U.S. guidelines, most of the RDAs are only estimations made according to clinical research and certified by nutritionists.

The term vitamin was derived from *vita (*meaning having life or vitality) and *amine,* since the first vitamin--*thiamine, or vitamin* B_1—was identified as an amine.[2] Vitamins constitute an important chapter in the human nutrition. Their complex biochemical roles are still under clinical investigation worldwide. Vitamins in generally act as catalysts or coenzymes, creating metabolically active enzymes for hundreds of important chemical reactions in our body. The thirteen well-identified vitamins are classified according to their solubility properties into fat-soluble and water-soluble vitamins.

The water-soluble vitamins are freely soluble in water, fragile, and include B complex vitamins and vitamin C. Because of their solubility characteristic, there are not

stored in the body, are easily excreted, and can be lost during extended cooking. For this reason, constant dietary intake is necessary to maintain the recommended functional level.

Fat-soluble vitamins are water insoluble, very hydrophobic, and include vitamins A, D, E, and K. They are absorbed in the intestine along with fats and later are stored in the body's tissue (fat). Because the fat-soluble vitamins are stored in the body, they can accumulate in excess quantities, causing a condition called hypervitaminosis. Depending on the vitamin involved, serious clinical problems can develop when hypervitaminosis goes on for a while.

Vitamins in generally act as catalysts, creating metabolically active enzymes for hundreds of important chemical reactions in our body. Lack of vitamins can trigger a wide range of metabolic dysfunctions and possible later irreversible sequels. Vitamin deficiencies can be grouped into two main categories: (a) *nutritional vitamin deficiency* and (b) *secondary vitamin deficiency* (not due to dietary insufficiency)[2]. A nutritional vitamin deficiency results from inadequate intake of one or more vitamins, and is in general easy to correct. It can be eliminated by a well balanced diet that contains food rich in the particular vitamin that is deficient. On the other side, biochemical or physiological - defects, like malabsorption can trigger secondary vitamin deficiencies. In this case, the body loses vitamins and other minerals in the feces. It is very important to distinguish the two categories. There are drugs that can affect vitamin absorption and metabolism in our body[2]. Drug interference can aggravate a preexisting nutritional vitamin deficiency.

Because some vitamin deficiencies are irreversible, early pathologic detection is absolutely necessary to prevent permanent health damage. Disease prevention becomes an effective clinical tool for physicians and other caregivers to improve patient care and reduce long-term hospital financial expenses.

CHAPTER 1

1. B VITAMINS

B vitamins belong to water-soluble vitamins group and are known as vitamin B complex. This group includes the following vitamins: B_1 known as thiamine; B_2 known as riboflavin; B_3 known as niacin or nicotinic acid; B_6 known as pyridoxine; B_{12} known as cobalamin; folic acid or folate, pantothenic acid, and biotin.

Thiamine (vitamin B_1) is found in cereal grains, legumes, nuts, milk, beef, and pork. The coenzyme form of thiamine is *thiamine pyrophosphate* (TTP), which participates in oxidative decarboxylation reactions. Excess thiamine produces no known toxic effects. Vitamin deficiency can produce the following pathologic symptoms: depression, irritability, failure to concentrate, muscular weakness, and altered reflexes. Vitamin B_1 is destroyed in foods that are baked, toasted, fried, or cooked for a long time at temperatures above 100 °C . Some diets can cause a thiamine-deficiency disease called *beriberi*, common in Southeast Asia where some diets contain antithiamine factors.[2]

Riboflavin (vitamin B_2) is found in egg whites, yeast, milk, red meat, green vegetables, and fish. *Flavin mononucleotide* (FMN) and *flavin adenine dinucleotide* (FAD are the two coenzyme forms of riboflavin. Both coenzymes participate in oxidation-reduction reactions catalyzed by flavin-linked dehydrogenases. Excess riboflavin causes no known toxicity. Deficiency can cause dermatittis, inflamation of the tongue, anemia, photophobia (intolerance to light), and loss of visual acuity. Ordinary cooking temperatures do not damage the concentration of riboflavin. Vitamin B_2 is light sensitive, and sunlight exposure causes 50 percent loss of its content in 2 hours.[2]

Niacin (Vitamin B_3) or nicotinic acid is found in red meat, liver, fish, eggs, green vegetables, and cereal grains. Its coenzyme form is *nicotinamide adenine dinucleotide* (NAD^+). $NADP^+$ is formed when a phosphate group is added to NAD^+. Both forms participate in oxidation-reduction reactions catalyzed by dehydrogenases. Excess of niacin is not toxic. A deficiency can lead to gastrointestinal tract disturbances, anorexia (loss of appetite), diarrhea, and red swollen tongue. The niacin-deficiency disease *pellagra* is common in regions where corn is a staple item of the diet.[2] The pathology of pellagra includes changes in the skin, diarrhea, and severe nerve dysfunction. Except for some niacin lost by leaching into the cooking water, at moderate cooking temperatures the vitamin is quite stable.

Pyridoxine (Vitamin B_6) or pyridoxal is found in meat, eggs, legumes, wheat, and corn. The coenzyme form is *pyridoxal phosphate*, known to participate in transamination and decarboxylation reactions. Excess of vitamin B_6 is not considered toxic. A deficiency can cause dermatitis, increased susceptibility to infection, insomnia, muscular

weakness, nervousness, and anemia. Pyridoxal is stable at normal cooking temperatures. Loss of vitamin B_6 is caused by exposure to light and complicated industrial food-processing procedures.[2]

Folic acid (folate) is rich in green vegetables, whole grain cereals, nuts, legumes like peas and beans, and liver. *Pteroylglutamic acid* is the coenzyme form. Reduced forms include *dihydrofolic acid* (DH_2) and *tetrahydrofolic acid* (TH_4). Folate is an active compound in amino acid metabolism (1-carbon groups). Folate excess is nontoxic. Deficiency can cause gastrointestinal problems, anemias, and mental deterioration. Normal cooking temperatures can cause up to 50 percent folic acid destruction.[2]

Pantothenic acid is abundant in liver, eggs, beef, wheat bran, and legumes. It is a part of Coenzyme A (CoA) which is known to be an acyl groups carrier. Coenzyme A is a component of acetyl-CoA and fatty acyl-CoA. Pantothenic acid excess is nontoxic. A deficiency can lead to gastrointestinal tract problems and depression. Moderate cooking temperatures do not affect the vitamin. High temperatures and prolonged cooking at moderate temperatures destroy the pantothenic acid.[2]

Biotin is found in milk, eggs, beef liver, and yeast. Biotin coenzyme form is named _-N-biotinyllysyl (biocytin). The coenzyme participates in carboxylation reactions (CO_2 group transfers). Excess biotin in considered nontoxic. Biotin deficiency can cause dermatitis, anorexia (loss of appetite), insomnia, muscle pains, nausea, and depression. Experimentally induced deficiency was caused experimentally in animals by feeding them large amounts of raw egg whites, known to contain the protein *avidin* that binds biotin. This vitamin is stable at normal cooking temperatures.[2]

Vitamin B_{12} and cobalamin are synonymous terms. Cobalamin is the overall group name. Plants and animals cannot synthesize vitamin B_{12}. Only microorganisms produce cobalamin, either free or in symbiotic relationship with other organisms. Food rich in vitamin B_{12} includes: fermented cheeses, organ meats (liver), and seafood such as clams, oysters, lobsters, scallops, and haddock. According to published literature, approximately 30 percent of vitamin B_{12} can be lost during prolonged cooking.[2] For this reason, boiled milk loses the vitamin B_{12} during the boiling process. In general, over processing food and excessive boiling can cause vitamin loss to different extents.

2. VITAMIN B_{12} -- BIOCHEMICAL FEATURES

The terminology of vitamin B_{12} has a complex meaning. It includes a family of substances that are formed of tetrapyrrole rings surrounding the central cobalt atom, as seen in figure 1, page 5. The term cobalamin is used as the overall group name. Different cobalt-linked ligands define the cobalamin name as follows: methyl (methylcobalamin), hydroxyl (hydroxycobalamin), H_20 (aquacobalamin), cyanide (cyanocobalamin), and tissue form 5-deoxyadenosine (deoxyadenosylcobalamin). Chemically, the term vitamin

B_{12} refers to cyanocobalamin or hydrocobalamin. Despite all these different forms, because of its long usage, vitamin B_{12} applies to all forms described above. For this particular research study, there are two critical terms to remember: methylcobalamin, the serum predominant vitamin B_{12} form; and deoxyadenosylcobalamin, the cytosol (tissue) form. Most immunoassays designed for vitamin B_{12} measure all of these forms, after conversion to the cyanocobalamin form.[3] Figure 1 depicts the basic structure of cobalamin main chemical frame.

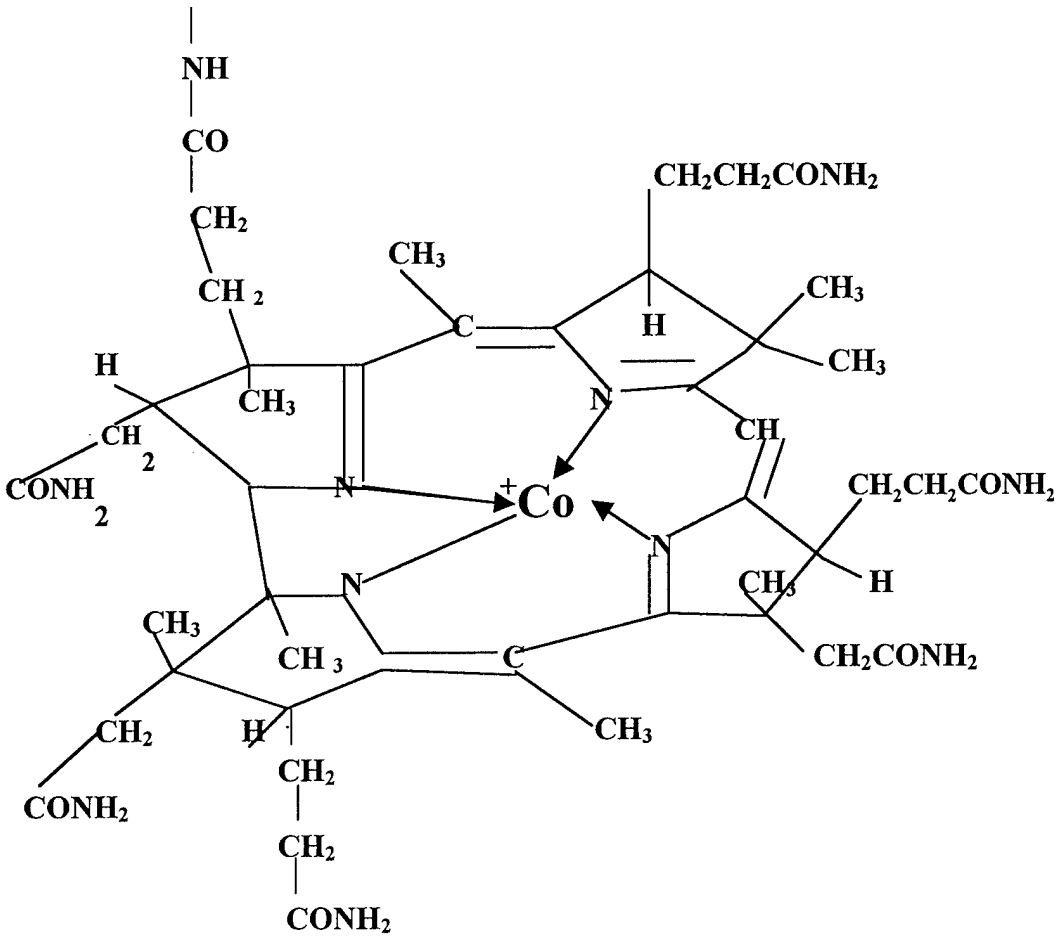


Figure 1. Vitamin B_{12} (Cobalamin) basic core chemical formula

An important physiological characteristic of vitamin B_{12} is its multiple binding proteins that facilitate both absorption and transport for the vitamin.[4,5] There are three binding proteins called transcobalamins (TC): I, II, III. Transcobalamin I is known as R or rapid protein. It is found in most body fluids (ubiquitous) and can cause falsely

increased vitamin B_{12} measurements. According to numerous published articles, impure sources of intrinsic factor used in automated competitive displacement assays may cause falsely elevated B_{12} results. This finding was of great diagnostic importance because approximately 20% of patients with clinically confirmed severe Cbl (cobalamin) deficiency had values for the total of Cbl and Cbl analogues that were within the normal range. After the nonspecific R protein was replaced with the specific intrinsic factor, only Cbl was measured, and lower values were obtained.[3] Intrinsic factor is secreted by the stomach's parietal cells, and is required for intestinal absorption of vitamin B_{12} in the distal part of the ileum. To avoid falsely elevated Cbl results, alkaline conditions or chemical reagents must be used to free the vitamin from binding proteins before quantification.[6]

Transcobalamin II is found in plasma and its role is to transport cobalamin to receptors located on cell membranes. Only the subcomponent of cobalamin bound to transcobalamin II is the biologically active form of vitamin B_{12}. This active form of cobalamin is called holo-transcobalamin II.

Transcobalamin III is produced by granulocytes. Increased concentrations of this protein, like in chronic myelogenous leukemia, may cause high blood concentrations of measured cobalamin, whereas the levels of the active form of the vitamin may be within the reference interval for healthy individuals.[7,8]

3. VITAMIN B_{12} -- METABOLIC FEATURES

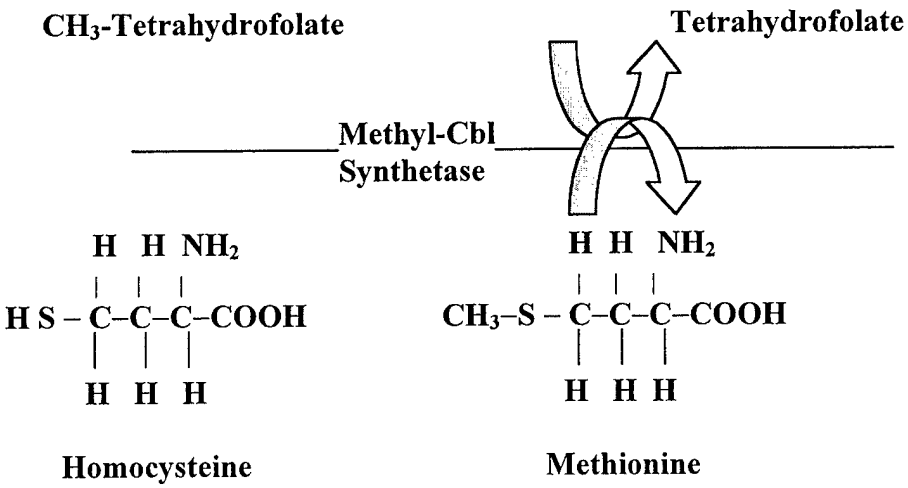


Figure 2. **Methyl-Cbl --Serum Form of Vitamin B_{12} -- and Enzyme Methionine Synthetase**

Vitamin B_{12} is a cofactor for two important enzymes, and has therefore two major metabolic roles. The concentration level for both cofactors is vital for the reactions to take place in the normal direction, and producing the desired metabolites.

First, when vitamin B_{12} has the form of methyl-cobalamin, it becomes the cofactor for the methionine synthetase enzyme. This cobalamin-dependent enzyme catalyzes the formation of methionine from homocysteine (Figure 2, page 6) Methyl-cobalamin catalyzes the methyl group (CH_3) transfer from a folic acid co-factor (CH_3-tetrahydrofolate) to form methionine. Methionine is an essential amino-acid, vital for nucleic acid synthesis. The new unmethylated folate, a co-factor named tetrahydrofolate, is ready now to participate further in single carbon reactions for nucleic acid synthesis.[40,42]

Second, when vitamin B_{12} (cobalamin) has an adenosyl group is called adenosyl-Cbl and acts as the cofactor for the enzyme L-methylmalonyl-CoA mutase. Adenosyl-Cbl is referred to as the tissue form of vitamin B_{12}. The B_{12} coenzyme deoxyadenosylcobalamin catalyses aminoacid, and fatty acid catabolic (breakdown) reactions. Vitamin B_{12} deficiency can shift the physiological reactions toward methylmalonic acid (MMA) formation (Figure 3, page 8). If the enzyme mutase has insufficient adenosyl-Cbl to catalyze the formation of succinyl-CoA, L-methylmalonyl-CoA accumulates at the tissue level. This left shift of reactions affects backwards the next compound in line, metabolite D-methylmalonyl-CoA. The racemase enzyme is now affected, and the reaction is shifted further left. The left shift chain of reactions ends in the hydrolysis of D-methylmalonyl-CoA to the metabolic final product, methylmalonic acid (MMA). This final reaction is irreversible (Figure 3, page 8). The last step is very important because the accumulation of MMA at the tissue level is an early indicator of vitamin B_{12} tissue deficiency.

From these biochemical reactions, we can see why some clinical symptoms of vitamin B_{12} and folic acid (folate) deficiencies are difficult to distinguish, just by judging the symptoms. Clinical observations suggested that elevated total homocysteine levels secondary to folate deficiency do not respond to vitamin B_{12} therapy. Patients in whom serum levels of both folate and cobalamin are low are frequently encountered clinically and often pose a diagnostic problem.[3] Laboratory analysis is a critical tool for correct deficiency diagnosis to avoid inappropriate therapy of folate deficiency with vitamin B_{12}.

Opposite, inappropriate therapy of cobalamin deficiency with folate may often result in hematologic responses and deterioration of neurologic functions.[3] There is evident proof that in treating vitamin deficiency, substituting one vitamin for the other is dangerous for patient's health. The appropriate vitamin treatment is once again clinically confirmed to be crucial for both patient and medical provider's interests.

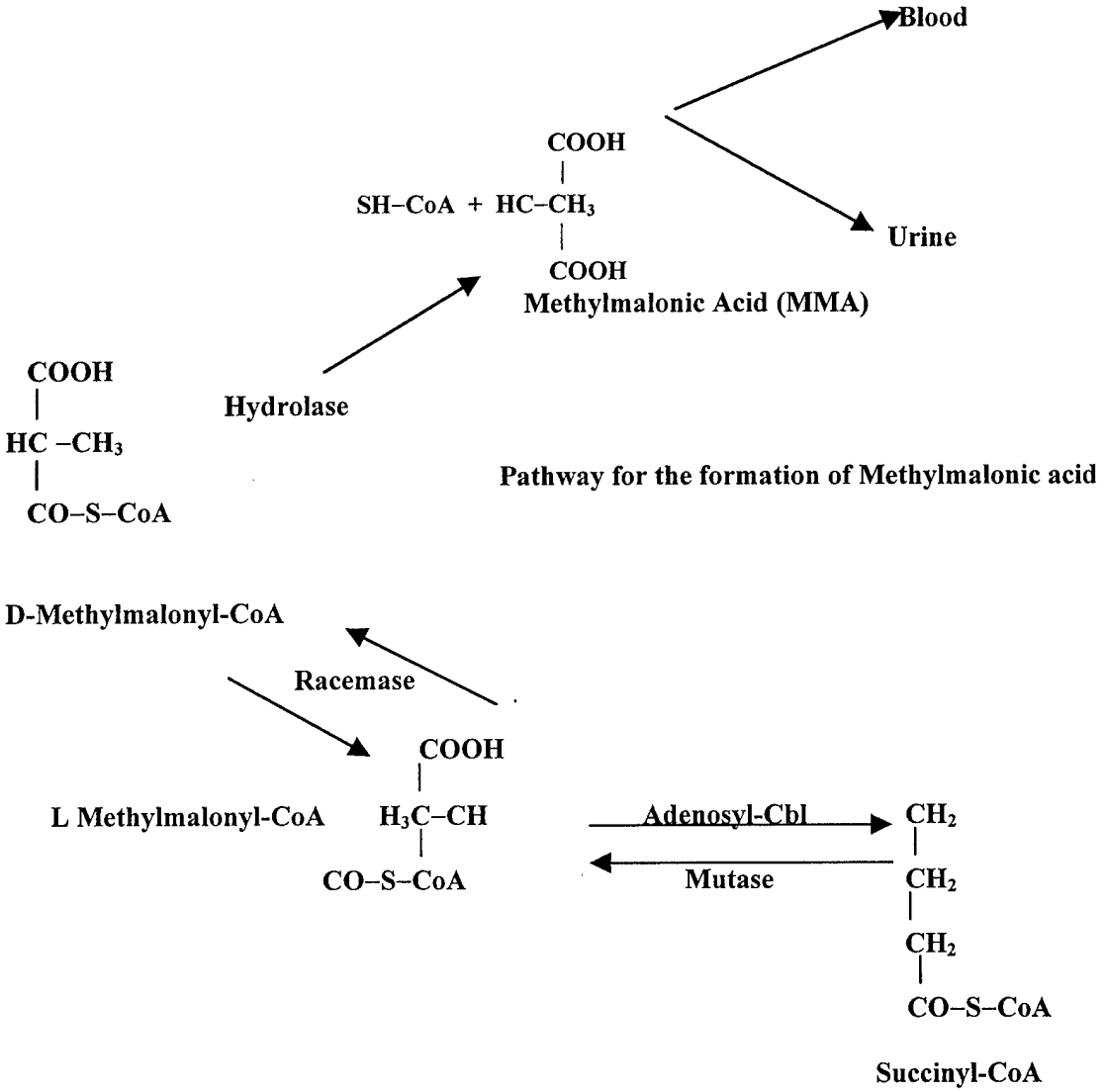


Figure 3. **Adenosyl-Cbl -- Tissue Form of Vitamin B_{12} -- and Enzyme L-methylmalonyl-CoA mutase. Methylmalonyl-CoA accumulates intracellular when the conversion of L-methylmalonyl-CoA to succinyl-CoA, catalyzed by L-methylmalonyl-CoA mutase and cofactor 5'-deoxyadenosylcobalamin, is shifted to the left. This impairment is due to low levels of the vitamin B_{12} tissue form, also called adenosylcobalamin (adenosyl-Cbl).**

4. VITAMIN B_{12} AND FOLATE INTERDEPENDENT ROLES IN NUCLEIC ACID SYNTHESIS--CONCLUSIONS

The reaction pathway for methionine systhesis (Fig.2, page 6), links cobalamin (vitamin B_{12}) and folate. A deficiency of either one can disrupt the metabolic pathway of methionine systhesis. Methionine is an essential aminoacid used for nucleic acid DNA (deoxyribonucleic acid) synthesis. Low levels of vitamin B_{12} affect folate (folic acid) level and later result in increased homocysteine level. High homocysteine levels pose a significant risk factor for cardiovascular disease. There are numerous published articles that relate to homocysteine and cardiovascular disease.[9,10]

Deficiency of either vitamin B_{12} or folate, can be clinically related to megaloblastic anemia. Vitamin B_{12} is stored in the liver and is involved in reticulocytes (young red blood cells) formation. As showed in Figure 2 (page 6), the serum form of vitamin B_{12} known as methyl-cobalamin (Cbl) is the cofactor for enzyme methyl-synthetase. Under normal methyl-Cbl levels this enzyme catalyses the formation of methionine from homocysteine. Under pathologic conditions, when methyl-Cbl is deficient, the reaction is impaired and methionine synthesis is compromised. Nucleic acid DNA synthesis is impaired when insufficient methionine production occurs. Consequently, cell production and reconstruction are negatively affected by low methionine levels. Red blood cells (reticulocytes) are one of the affected cells in the body.

Due to such a close relationship in the complex process of metabolism, deficiencies of either cobalamin or folate (folic acid) can simulate the same clinical symptoms. Deficiency of either vitamin B_{12} or folate can cause increased homocysteine levels.[3] For clinical workup purposes, it is recommended to measure both vitamins to fully investigate their differential for diagnosis.

5. VITAMIN B_{12} DEFICIENCIES AND CLINICAL IMPLICATIONS

The most common cause that triggers cobalamin deficiency is the defect of intrinsic factor secretion. Reduction in gastric parietal cells that secrete intrinsic factor leads to inadequate vitamin B_{12} absorption. Intrinsic factor is a protein that binds vitamin B_{12} and facilitates transport and absorption across the intestine, at ileum level. Inadequate intrinsic factor secretion associated with chronic (long term) gastric atrophy is the cause for pernicious anemia. This is a condition seen in people over 50 years of age, characterized by a reduction in the red blood cells number and increase in their size.[2]

Other causes for cobalamin deficiency are caused by gastrectomy leading to malabsorption, and small intestine bacterial or inflammatory diseases. Deficiency due to insufficient dietary intake is rare, usually involving the geriatric population. In all above situations, vitamin B_{12} absorption is impaired or is lacking from the diet. Rarely does

lack of dietary B_{12} cause a deficiency, except in total vegetarians.

According to published literature, there is no proven toxic effect to the body due to excess of vitamin B_{12}. On the other side, a deficiency in vitamin B_{12} can cause several symptoms with clinical implications. Summarized, clinical problems include: impaired cell division, demyelination and loss of neurons (nerve cells), abnormal carbohydrate and fat metabolism. Secondary vitamin deficiency is related to the lack of the intrinsic factor.

In the elderly, atrophic gastritis is commonly associated with vitamin B_{12} malabsorption and deficiency.[11] The absorbed vitamin B_{12} is secreted in bile and subsequently reabsorbed. Low vitamin intake can result in B_{12} deficiency that starts first at tissue level. Depressed vitamin serum levels are not evident for a long period of time, and clinical deficiency symptoms can take 20 years to develop. Studies show that in malabsorption, cobalamin deficiency can occur slowly, in months or even years. As patients age, absorption of food-bound vitamin B_{12} may be impaired by the age-associated loss of acid and pepsin.[12]

Deficiencies of both vitamin B_{12} and folate are involved in megaloblastic anemia, due to decreased (deoxyribonucleic acid) DNA synthesis. Folate and cobalamin metabolism is linked in transfer of a methyl group from N^5-methyltetrahydrofolate to cobalamin. In the absence of vitamin B_{12}, folate cannot be recycled back into the folate pool.[13] Megaloblastic anemia progresses in stages. In the late and the most severe stage, both megaloblastic and macrocytic anemias are evident. Ultimately, chronic deficiencies of cobalamin cause severe neurologic problems: poor attention span and depression, dementia; irreversible demyelination of spinal cord; brain, optic, peripheral nerves damage (peripheral neuropathy), and neuropsychiatric disorders.[13,14] Both vitamin B_{12} and folate are associated with neuropsychiatric disorders. Only cobalamin deficiency can cause demyelinating neuropathy. Some neural abnormalities are found more frequently in B_{12} deficiency. The mechanisms of these disturbances are not yet clearly known, and future directions for laboratory testing are recommended.[13]

Clinical implications of cobalamin deficiencies pose a major task for medical staff to target timely diagnosis and appropriate treatment. Studies indicate that the clinical picture of vitamin B_{12} deficiency is much more complex then previously believed.[3,13] The reason for the difficult diagnosis is also due to a slow deficiency process which starts at the tissue level. For this reason, new laboratory tests like serum methylmalonic acid, become important diagnostic tools to assess an early onset of cobalamin deficiency.

6. CLINICAL EVALUATION OF VITAMIN B_{12} STATUS

In general, serum cobalamin concentration is the main test used to assess vitamin B_{12} deficiency. Unfortunately, there are several technical method limitations that lower

and limits its usefulness. Mainly, serum vitamin B_{12} concentrations are directly altered by the concentration of binding proteins. Certain forms of myeloproliferative disorders display falsely increased cobalamin results. Falsely low values can be seen with folate deficiency, pregnancy, and transcobalamin deficiencies.[13]

Examination of peripheral blood smear, and complete blood count (CBC) is also commonly used to evaluate vitamin B_{12} status. In 1996, as part of a case-control research study, Metz et al. reported neutrophil hypersegmentation in two-thirds of the patients with low vitamin B_{12} compared with 4% of controls.[15]

The classical laboratory procedure to evaluate if the patient can absorb vitamin B_{12} is the Schilling test. This two-step test starts with measuring the percentage of administered radiolabel per 24-h urine volume after radioactive cobalamin is given by mouth, after flushing dose of nonradioactive cobalamin. In step two, patient receives intrinsic factor in addition to radiolabeled cobalamin. An abnormal stage one followed by a normal stage two is consistent with pernicious anemia.[14] If after given intrinsic factor (step two) the results remain still abnormal, there are other causes for the cobalamin deficiency that need to be investigated, including ileal malabsorption. Due to the fact that Schilling test is so cumbersome, involves radioactive materials, intensive laboratory labor and high costs, providers tried to avoid it as much as possible.[3,13]

Other evaluation methods for vitamin B_{12} status include: serum gastrin, serum pepsinogen, and upper gastrointestinal endoscopy to evaluate gastric atrophy; bone marrow examination by a hematopathologist; deoxyuridine supression, a sensitive indicator but is only rarely used because it requires bone marrow specimens, uses a radiolabel, and is difficult to control.[13,16]

Assessing the cause of vitamin B_{12} deficiency is a difficult and complex procedure. For this reason, many medical providers rely on different laboratory tests used in a cascade fashion. It is important to use different algorithms for each of the clinical presentations, like the one for pernicious anemia, depicted in figure 4. (page 12)

Mayo Medical Laboratories introduced this cascade of tests to aid physicians in diagnosing suspected pernicious anemia patients. It is recognized that the algorithms have many advantages for accelerating laboratory investigation. According to recent studies, today it is quite evident that there are some inadequacies with this approach. As shown in Figure 4 (page 12), the first test is vitamin B_{12}. Starting diagnosis process with serum cobalamin can be misleading. In this case, the physician could miss some patients with significant pathology that would be detected by other tests.[13] Another problem comes from the fact that the algorithm is mainly designed to detect the deficiency caused by pernicious anemia. It is important to remember that folate deficiency can also be implicated in similar pathologic circumstances. Adhering strictly to this pathway is one big concern among many clinical researchers.

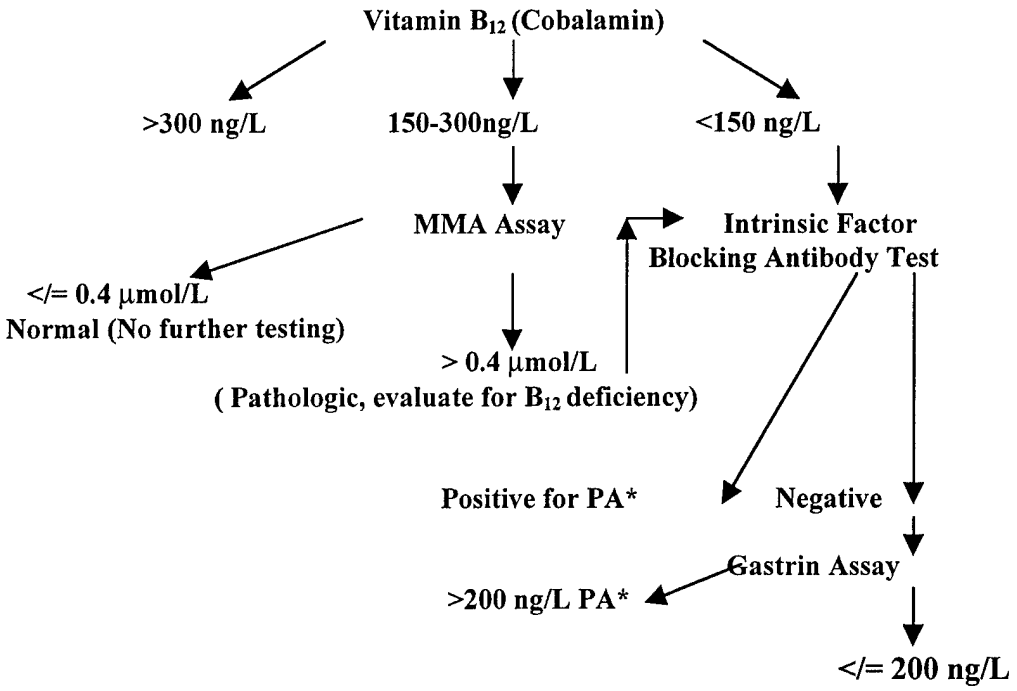


Figure 4. Laboratory algorithm for Pernicious Anemia (PA*)

7. Major Limitations of Serum Vitamin B_{12} Laboratory Measurements

In 1979, Food and Drug Administration asked the National Committee for Clinical Laboratory Standards (NCCLS) to appoint a panel team to investigate major problems reported with serum cobalamin available assays. Dr. Shilling was chosen to chair this panel and was the first to publish about differences in specificity of cobalamin binding proteins.[3] Clinical studies showed evidence that some patients with pernicious anemia are not recognized by radiodilution assays. In mid-1970, evidence of unknown cobalamin analogues were reported in human blood, called nonspecific R proteins. These proteins caused erroneously high cobalamin results. Imprecision problems were reported with the available vitamin B_{12} assays.[3] There were major concerns with respect to establishing normal ranges and diagnostic specificity using the available assays.

Even today, the poor stability of serum vitamin B_{12} is a daily issue for many clinical laboratories. This is a pre-analytical requisite that negatively affects both the results and the clinical interpretation. For this reason, laboratory scientists studied both vitamin B_{12} and folate stability. Behind the study was also the interest to find out the best transport conditions for the specimens that need to reach remote locations before being analyzed. Amazingly, serum cobalamin proved to be highly unstable, emphasizing that specimens should be frozen if not analyzed immediately.[17] The effect of storage on serum vitamin

B_{12} was studied under four well controlled conditions: frozen, refrigerated, light protected, and not light protected. This study demonstrates again cobalamin's poor stability. In refrigerated serum specimens, folate was stable up to 7 days of storage. Serum cobalamin, under all four storage conditions, followed similar patterns of an initial rise followed by a fall after 1-3 days of storage. Light protected specimens were less affected by storage temperatures (closer to the zero hour of tested values, being tested immediately right after drawing time). Frozen and light protected specimens were significantly closer to the zero hour values. The explanation for the rise of measured vitamin B_{12} serum specimens in short term storage (less than 1 to 3 days) was related to the possibility of degradation of vitamin B_{12} in serum specimens, which causes a rise in metabolites measured by the chemiluminescence immunoassay. Or, according to the same study, there is the possibility that B_{12} is slowly released from the binding proteins, and the vitamin B_{12} concentration increases with time.[17]

Another possible explanation for B_{12} short-term storage increase can be related to the fact that the assay in the above research used NaOH. This releasing agent has the ability to free all vitamin B_{12} from binding sites prior to analysis, and further implies the possibility that the releasing step is not complete.

For folate status was concluded that direct measurement of folate is better than homocysteine because folate is more stable.[17] Folate in serum is not affected by the light, and binding proteins may have a protective effect by preventing light degradation in serum.[33] Unlike vitamin B_{12}, temperature had only minor effect on folate stability. Serum folate proved to be amore stable analyte than serum B_{12}. In contrast to vitamin B_{12}, folate concentrations showed only small changes under all four storage conditions.

According to the stability study presented above, vitamin B_{12} proved to be quite stable in aqueous solution, stored at room temperature. Contrary, serum vitamin B_{12} showed poor stability. The available commercial assays use in general a releasing agent to free all the cobalamin from the binding proteins before the analysis. Because of the above findings, there could be the possibility of inaccurate serum B_{12} measurements, and furthermore an analytical problem for all clinical laboratories that currently do analyze serum vitamin B_{12}.

It was also demonstrated that serum samples for vitamin B_{12} testing that are frozen within 4 hours do not need to be light protected.[17] Frozen and light protected serum specimens proved to be stable up to a week. According to all these experiments, serum vitamin B_{12} indicated poor stability when refrigerated. Another draw back, was the short life span even when frozen, one week only. Additionally, it was mentioned the good stability of vitamin B_{12} in aqueous solution, versus serum specimens, very unstable at room temperature.

The experiment proved that serum specimens for cobalamin testing must be analyzed within first four hours, otherwise must be frozen and protected from light

exposure as soon as possible.[17] These data identifies the problem of transporting, storing, and testing serum B_{12} specimens received by remote clinical laboratories. Another drawback for serum cobalamin testing was exposed when falsely low serum vitamin B_{12} testing occurred consistently in other studies. Cobalamin sensitivity is less than perfect.[18] In some investigations, 11 of the consecutive series of 42 low serum cobalamin values did not reflect true cobalamin deficiency. These numbers translate to a incidence of falsely low serum cobalamin of almost 26%.[19,20] According to other articles, the incidence of false low serum cobalamin can start at 8 %.[19,21]

Serum Vitamin B_{12} (Cobalamin) Stability Research: Conclusions[17]

-- Serum vitamin B_{12} proved to be highly unstable.

-- Changes in vitamin B_{12} can be minimized by freezing at –20 C° (one week)

-- Light protection is necessary if the sample cannot be analyzed within 4 hours.

-- Refrigerated B_{12} specimens are not stable regardless of light exposure.

-- Storage temperatures had a higher effect than light protection.

-- The best conditions for the most accurate results for serum cobalamin laboratory testing were both frozen and light protection.

8. VITAMIN B_{12}: CLINICAL RESEARCH STUDIES

The interest for vitamin B_{12} research has grown international proportions due to its vast clinical applications. Below are presented a few of the main topics of interest.

Helicobacter Pylori bacterium and vitamin B_{12} deficiency. Helicobacter Pylori bacterium can cause stomach ulcers, indigestion (dyspepsia), gastritis (inflammation of stomach lining), stomach cancer, and MALT lymphoma. Researchers studied 138 patients with diagnosed vitamin B_{12} deficiency and anemia. At the same time, the patients tested positive for the presence of Helicobacter Pylori infection. Infection eradication successfully cured the anemia and reversed vitamin B_{12} deficiency in 31 (40 per cent) of the 77 infected patients.[22]

Chronic fatigue syndrome (CFS). A review of vitamin deficiencies that are involved in chronic fatigue syndrome (CFS) showed a slow down of the healing process.[23] Vitamin B_{12} and folic acid were some of them. Vitamin supplements under medical supervision was recommended as the basic treatment. Testing vitamin B_{12} level was one of the main laboratory tests used to monitor the patients.

Shaky-leg syndrome. Vitamin B_{12} injections resolved the shaky-leg syndrome.[24]
Plasma homocysteine. Homocysteine levels can be effectively lowered using vitamins B_6 and B_{12} supplements.[25]

Atherosclerosis. Low B vitamins levels were related to atherosclerosis. Studies showed that high levels of the amino acid homocysteine have been associated with a high risk of atherosclerosis.[26] Homocysteine is a metabolite of methionine, an essential amino acid found in proteins. This metabolic process can be blocked by folic acid (folate) and vitamin B_6 and B_{12}. Vitamin B_{12} level is also affected when vitamin E is low.

Tissue form of vitamin B_{12}, adenosylcobalamin. Adenosyl-cobalamin is an important coenzyme that is required in the metabolism of branched-chain amino acids, cholesterol, and odd-chain fatty acids. Adenosyl-cobalamin is synthesized in the cell nucleus from vitamin B_{12} (cobalamin). In 1993 it was demonstrated that the synthesis of adenosylcobalamin is impaired if the cell membranes have been subjected to peroxidative (free radical) attack. Using cell cultures, the researchers discovered also that vitamin E (alpha-tocopherol) effectively prevents the peroxidation and thereby allows the enzyme synthesis to take place.[27]

Geriatric population. Both in the United States and Europe, scientists paid a high interest to the elderly population. Numerous studies showed that elderly patients often suffer from a deficiency of vitamins B_6, B_{12} and folate. A high level of the metabolite methylmalonic acid, which indicates a vitamin B_{12} deficiency, was found in 23% of the healthy elderly people and in 39 % of the elderly hospitalized patients.[28]
Elderly people often suffer from a lack of vitamin B_{12} (cobalamin).[28,29,30] Studies showed that as people age, they are more and more less able to absorb vitamin B_{12} from food.

Accumulation of homocysteine. Another study in the same year indicated that vitamin B_{12} deficiency causes the accumulation of homocysteine in the blood.[31] Today it is widely recognized that an elevated level of homocysteine and its derivatives pose a major risk for heart disease and stroke.

Alzheimer's disease and Vitamin B_{12} deficiency. The genetic predisposition to Alzheimer disease is related to a genetic impairment in the ability to absorb vitamin B_{12}.[32]

Stomach surgery. The serious consequences of cobalamin deficiency were studied in patients that underwent stomach surgery.[33] Doctors noticed that a vitamin B_{12} deficiency can simulate Alzheimer's disease, amyotrophic lateral scerosis (Lou Gehrig's disease), spinal cord compression, alcoholic or diabetic peripheral neuropathy.

Neuropsychiatric abnormalities. Cobalamin deficiency was related to cause neuropsychiatric abnormalities.[14,34]

Folate deficiencies. Folate deficiencies can sometimes mask a vitamin B_{12} low level, especially in the elderly. Because vitamin B_{12} increases efficiency of folic acid, the addition of coablamin supplements to folic acid treatments can prevent the nerve damage and pernicious anemia, which may be not clinically evident. The capacity of folic acid to lower homocysteine level can be markedly increased by supplementing with vitamin B_{12}.[35]

Vitamin B_{12} levels in the vegetarian population. Research studies showed that almost 73 per cent of vegetarians are vitamin B_{12} deficient.[36] In this case, the strict vegetarian diet was the cause of deficiency.

Vitamin B_{12} level in postmenopausal women and breast cancer. *W*omen with breast cancer tend to have lower vitamin B_{12} levels in their blood than do women without breast cancer. Women with low vitamin B_{12} serum level a 2.5-4.0 times greater likelihood of being in the breast cancer group than did women with the highest levels.[37] These studies did not indicate what kind of diet was used in the study, if any.

Possible correlation between folate levels in breast tissue and breast cancer risk. It was speculated the theory of possible correlation between folate levels in breast tissue and the risk for breast cancer. The reasoning was based on the well-established information of vitamin B_{12} role in increasing folate efficiency and to ensure proper DNA replication and cell repair. Vitamin B_{12} deficiency can lead to breast cancer because of less folate available. Both the cell replication and repair processes are impaired.[37,38]

9. NEED FOR A NEW ASSAY

Serum vitamin B_{12} is still considered the primary diagnostic test to evaluate cobalamin deficiency. One reason is its long use over the years to test patients with clinically confirmed deficiency. There is also an in-house test performed by most of the clinical laboratories. So, serum vitamin B_{12} is easily available to provider's desk to order.

Recent studies indicate that the clinical picture of vitamin B_{12} deficiency is much more diverse than previously believed.[3] Also, serum or plasma vitaminB_{12} do not always reflect the adequate tissue cobalamin level. Problems with vitamin B_{12} stability and existing assays low specificity were well examined and researched since 1979. There is evidence that some patients with pernicious anemia are not recognized by radio-dilution assay. Cobalamin analogues present in human blood (nonspecific R protein) can yield erroneously high cobalamin results. Imprecision problems with respect to establishing normal ranges and diagnostic specificity are still lingering for some laboratory assays.

It looks like the need for a better assay that can overcome the laboratory analytical problems and detect early cobalamin deficiency is necessary. For this reason, scientists became interested in developing new laboratory methods that can determine more accurately a vitamin B_{12} deficiency, even before serum level can show it. Around 1985, the laboratory scientific literature became invaded by the new methods of measuring the metabolite methylmalonic acid (MMA), first in the urine, and later in serum. Both in the Unites States and Europe, numerous articles were published confirming the relationship between clinically confirmed vitamin B_{12} deficiency and urine/serum methylmalonic acid.

When vitamin B_{12} is low, the level of MMA is increased because the adenosyl-Cbl is low, and the reaction catalyzed by the mutase is reversed toward increased production of L-methylmalonyl-CoA. Clinical application of using methylmalonic Acid (MMA) as an indicator for evaluation of vitamin B_{12} (cobalamin) deficiency is today considered a future gold standard tool in clinical chemistry.

Just testing serum vitamin B_{12} to diagnose a vitamin deficiency is not sufficient. A long time lingering deficiency at tissue level is masked by a normal serum vitamin B_{12} level. Inappropriate treatment of folate and cobalamin deficiencies can lead to serious medical problems for the patient. Cobalamin deficiency is also a slow, progressive, and irreversible pathologic condition. Because the irreversible damages include nervous system, early diagnosis of the vitamin deficiency can warn doctors that patients need to receive immediately B_{12} supplements. Maintaining a normal level of vitamin B_{12} can assure adequate folic acid levels, and consequently a normal cells reproduction and repair, with a better healing prognosis and low medical costs in the long run.

CHAPTER 2

1. METHYLMALONIC ACID (MMA) BIOCHEMICAL BACKGROUND

Methylmalonic acid (MMA) is a very polar four-carbon dicarboxylic acid metabolite (Fig.5). Its molecular mass is 118 g/mol.[39]

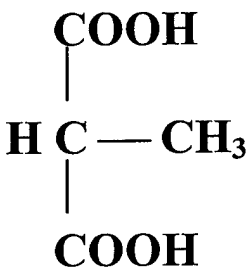


Figure 5. Methylmalonic acid (MMA) — Chemical Formula

Methylmalonic acid is related to the catabolism of aminoacids like valine, isoleucine, and propionic acid (Figures 6).

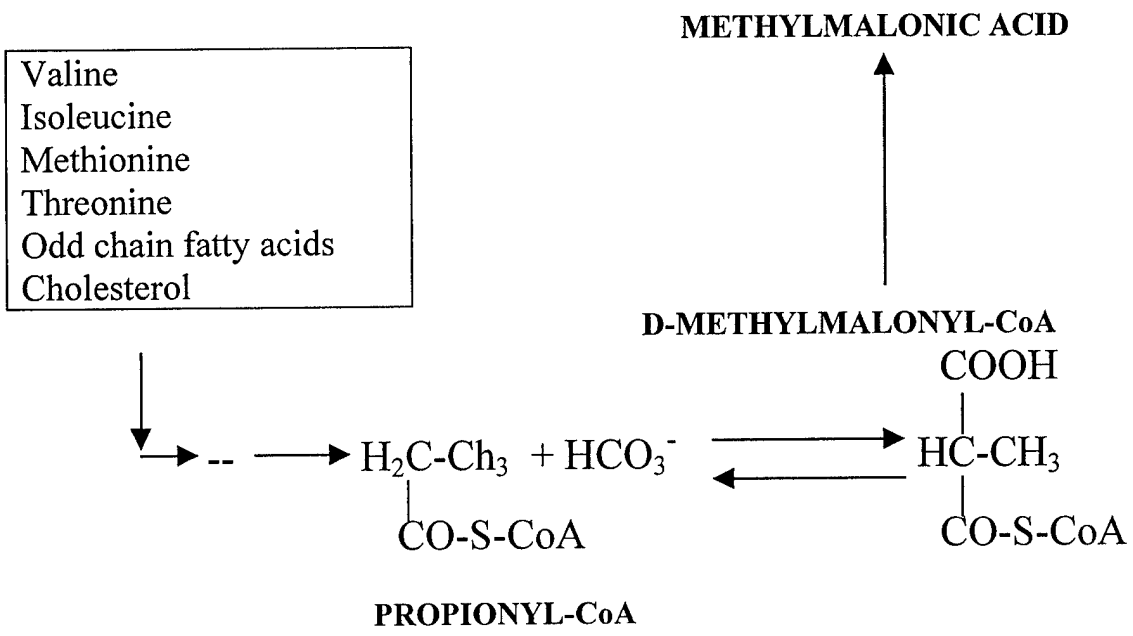


Figure 6. Methylmalonyl-CoA Intracellular Pathway Formation

Intracellular accumulation of methylmalonyl-CoA results when the conversion to succinyl-CoA by methylmalonyl-CoA mutase, mediated by 5'–deoxyadenosylcobalamin, becomes impaired due to low concentrations of cofactor adenosylcobalamin (tissue vitamin B_{12}). (Figure7)

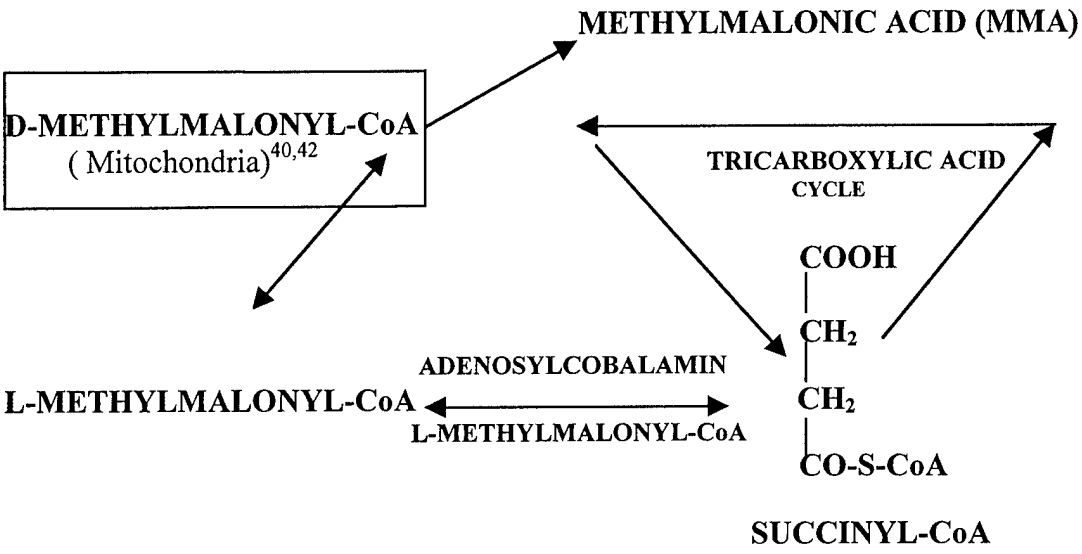


Figure 7. Intracellular Methylmalonic Acid Formation

MMA is excreted in the urine through the kidneys, and some is reabsorbed in miniscule amounts back into the blood. For this reason, healthy individuals have low concentrations of MMA in the serum or plasma. The fractional clearance ratio relative to creatinine is lower than 1, defining net reabsorption, with no apparent maximal reabsorption by the tubules, as proved by Karsten Rasmussen.[39]

2. METHYLMALONIC ACID (MMA) CLINICAL SIGNIFICANCE

The interest for measuring methylmalonic acid in humans has grown significantly in the last decade. Methylmalonic acid (MMA) has been recognized by numerous expert clinical chemistry research groups, as a better diagnostic tool for early detection of vitamin B_{12} deficiency at tissue level.[18,19,31,41,45] MMA concentrations often increase in the early stages of vitamin B_{12} deficiency, before serum B_{12} results are depressed. An increased concentration of MMA in serum and its excessive urinary excretion are believed to be direct measures of tissue stores of cobalamin.[40,45] As Figure 7 illustrates, adenosylcobalamin (the tissue form of vitamin B_{12}) is an essential cofactor for the enzymatic carbon rearrangement of MMA to succinic acid.[40,42] Lack of vitamin B_{12} leads to elevated levels of methylmalonic acid.[39,40,42] Studies suggest that in healthy persons only a tiny fraction (about $3X10^{-5}$) of the methylmalonyl-CoA molecules formed in the

mitochondria (Figure 7, page 19) escape conversion to succinyl-CoA. For this reason, amino acid-loading studies were performed to distinguish between cobalamin-deficient patients and healthy persons.[40]

The first attempt to assess the relative diagnostic usefulness of MMA in serum versus urine determination was done by Karsten Rasmussen in 1989 using data based on dietary influences.[39] Everyday meals caused an increase in urinary excretion, whereas the concentration in serum was not increased significantly.[40]

Normal urine concentrations of MMA are approximately 40-fold higher than serum MMA concentrations.[13,45] In one healthy individuals study, MMA in serum and plasma concentration normal reference range was 0.05-0.37 μmol/l.[41] Other studies showed a normal serum reference interval of 0.08 to 0.56 μmol/L, and for urine was established to be 0.58 to 3.56 mmol per mole of creatinine.[39,44]

A linear relationship between MMA concentration in serum and MMA in urine relative to creatinine clearance was proved by some investigators.[39,40,43] It was indicated that serum MMA has several advantages over the urine samples. The significant advantage of serum MMA compared to urine is that the former is routinely collected in the process of patient's evaluation for cobalamin deficiency. Serum specimens are usually available for additional studies in the clinical laboratory.[39] Changes in MMA metabolism, like effect of feeding that causes diurnal urine MMA increased concentrations, can affect random collected urine samples.

Urine MMA concentrations are affected by the kidneys function.[39,40,41] The effect of protein intake is long lasting and was still apparent 12 h later after food ingestion. Urinary excretion rate varies with urine flow rat, which further affects the creatinine clearance. For this reason, urinary MMA data can be ambiguously interpreted.[39]

Laboratory studies indicate that chronic hemodialysis patients have elevated levels of serum MMA (0.16-1.87 μmol/L), and patients with vitamin B_{12} deficiency may display levels from 0.47 to 190 μmol/L.[41] In patients with decreased renal function the MMA is not recommended and not useful, because the MMA is falsely elevated due to renal impairment, not vitamin deficiency. Serum MMA does not reflect adequately tissue cobalamin level when renal function was decreased, causing a modest result increment.[39] Disturbances in kidney excretory function caused more MMA to be reabsorbed back in to the serum.

Severe renal function impairment can increase serum creatinine. Marked intravascular volume depletion was related to renal retention of MMA.[39,40,44,46] Impaired renal function and hypovolemia appear to be the principal causes of false-positive increase in serum methylmalonic acid in patients who do not appear to be cobalamin deficient.[13,39] Only 24-h urine collection samples showed the linear relationship between serum and MMA urinary excretion.[40]

Although the MMA results in chronic hemodialysis and renal patients were affected by their medical status, patients with liver failure demonstrated no serum MMA interference.[41]

Serum methylmalonic acid evaluation diagnosed cobalamin (vitamin B_{12}) deficiency before depression of concentrations of serum B_{12}, showing that measurement of MMA was more sensitive than serum vitamin B_{12} in the diagnosis of early vitamin B_{12} eficiency. In 1989 Karsten Rasmussen reported for the first time the negative correlation between concentrations of cobalamin and MMA in serum.[39,40] Data were consistent with glomerular filtration and passive reabsorption of MMA by the tubules.

An increased concentration of MMA in serum and its excessive urinary excretion are believed to be direct measures of vitamin B_{12} tissue stores.[39,46] Patients with borderline, or even normal serum cobalamin results, could be vitamin B_{12} deficient at the tissue level. It was reported that of 434 episodes of cobalamin deficiency, 98.4% of serum MMA levels and 95.9% of serum homocysteine levels were elevated greater than 3 standard deviations above the mean in normal subjects. For the clinically confirmed cobalamin-deficient patients, measuring serum metabolites proved to be a highly sensitive test of deficiency.[46]

In other studies, serum MMA level was markedly elevated in most deficient patients more than three standard deviations (SD) above the mean of healthy control population, suggesting that this test is useful to establish a diagnosis of cobalamin deficiency.[39] Clinicians can use MMA results to treat patients suspected of being vitamin B_{12} deficient[13,39] by observing the effect on serum methylmalonic acid level after cobalamin supplementation.[39,40,46,49]

Normally, after parenteral injections of Cbl, serum Cbl will show elevated or close to normal Cbl levels whether or not the patient is Cbl-deficient at the tissue level. Serum methylmalonic acid level is about 1000-fold greater than serum cobalamin level, and an elevation rather than decrease indicates pathologic condition.[41]

As a new feature, methylmalonic acid is considered an important diagnostic procedure in clinical chemistry, especially in patients with neurological disorders with few or no hematological abnormalities, and normal or only slightly depressed serum cobalamin. Using MMA is very important since treating an undiagnosed cobalamin-deficient patient with folic acid alone will not prevent or correct the neuropsychiatric abnormality.[13,20,49]

Successful studies proved the increased sensitivity of serum MMA over serum vitamin B_{12} testing in patients with no clinical signs of Cbl deficiency, or with an underlying condition affecting cobalamin balance. While MMA and homocysteine were both increased in approximately 98% of cobalamin-deficient patients with megaloblastic anemia, the MMA level was more consistently elevated in nonanemic patients. These studies suggest that serum MMA had a greater sensitivity for detecting mild or early cobalamin deficiency. Serum MMA levels were elevated in 30 of 31 patients with true vitamin B_{12} deficiency.[39]

Serum vitamin B_{12} concentrations are directly altered by the concentrations of binding proteins. Patients with myeloproliferative disorders have usually falsely increased serum B_{12} values.[13]

Mean MMA values were higher when megaloblastic anemia was associated with renal failure (36, 527+/- 48,036 nmol/L versus 13,450+/- 29,0098 nmol/L, $p < 0.005$).[46]

Measuring serum metabolite concentrations proved to be a highly sensitive test of cobalamin deficiency when interpreting low or low-normal serum cobalamin level.

Because serum measurement does not necessarily reflect the true tissue vitamin B_{12} level, the clinical value of serum cobalamin estimation is insensitive for the diagnosis of cobalamin deficiency. By using serum MMA testing, medical providers can monitor more accurately the effectiveness of vitamin B_{12} deficiency treatments.

As discussed above, low levels of vitamin B_{12} in the tissue are reflected by a high level of methylmalonic acid in serum. Low levels of cobalamin impair folate activity, and further DNA synthesis and cell reconstruction. Vitamin B_{12} and folate (folic acid) are linked by the reaction pathway for methionine synthesis. A deficiency of either will disrupt this metabolic pathway or will cause the same clinical symptoms. A common clinical dilemma occurs when both vitamin B_{12} and folate concentrations are low and is not known whether a clinical deficiency is present for both vitamins, or for one or the other. An alternative diagnostic strategy would be to begin measuring all four components (vitamin B_{12} , folate, MMA and homocysteine), and then follow up with specific tests to subclassify the disorders in accordance with the clinical presentation (i.e., anemia, neurologic deficiency, neuropsychiatric disturbances, and cardiovascular risks).[13]

For a successful diagnosis and treatment, it is very important that clinicians do request both vitamins analysis, and interpret results against the clinical workup. Suspected or borderline level patients can be also assessed more accurately this way.[13]

Several studies done on patients that have folate deficiency and normal serum Cbl levels, indicated that the amount of Cbl in various tissues can become insufficient to saturate both Cbl-dependent enzymes.[13,37] Folate and vitamin B_{12} metabolism is linked in transfer of a methyl group from N^5-methyltetrahydrofolate to cobalamin. When cobalamin is absent, folate cannot be recycled back into the folate pool.[13]

In folate deficiency an attempt is made to increase levels of methionine synthetase activity by increasing the amount of Cbl bound to methionine synthetase with the result that the amount of Cbl bound to L-methylmalonyl-CoA mutase is decreased, and that in turn results in decreased formation of methylmalonic acid. [37,39,40] This theory could be plausible, taking in the consideration the biochemical pathways of both Cbl-dependent enzymes. At this time, there are beliefs that there must be other unknown metabolic and regulatory reactions between the Cbl-dependent enzymes L-methylmalonyl-CoA mutase, and methionine synthetase, besides of the theory that both are cobalamin dependent.

The complex interrelationships between the B vitamins choline, folic acid, B_{12} and methionine have been examined in detail with reference to effects on tumor induction by N-nitroso compounds, mycotoxins, and polycyclic aromatic hydrocarbons. Experiments were conducted on animals fed with diets low in lipotropic factors choline and methionine. The results showed an enhancement of cancer in the liver, the pancreas, and the colon, with the most consistent effect on the liver.[38]

An inadequate supply of methyl groups results in replication of defective cells and hypomethylation of DNA which can lead to deranged replication of cells and lack of growth control. It was estimated that in the United States 80-90 % of cancers may be attributable to environmental factors. Diet and nutrition accounted for approximately 30 % of the risk.[38]

Published micronutrients asociated with cancer susceptibility are showed in table 1.[38]

Amino Acids	Vitamins	Minerals
Methionine	Riboflavin	Calcium
Cysteine	**Folic Acid**	Zink
Tryptophan	Vitamin B_6	Copper
Arginine	Vitamin A	Iron
	Vitamin C	Selenium
	Vitamin D	
	Vitamin E	
	Choline	
	Carotine	

Table 1. Micronutrients Associated with Cancer Susceptibility.[38]

Because of the nucleic acid synthesis interrelationship with folate, choline, methionine, vitamin B_{12} and cancer susceptibility, measuring serum methylmalonic acid can be a more reliable, early, accurate, and sensitive evidence of vitamin B_{12} deficiency early diagnosis.

Clinical laboratory advantages of measuring serum methylmalonic acid (MMA) versus serum vitamin B_{12} are still under debate. When comparing serum cobalamin with serum MMA, laboratory management should consider whether the additional information obtained by measuring MMA will assist the clinicians in reaching the correct diagnosis and thereby the correct therapy.[41]

Diagnosis of vitamin B_{12} (cobalamin) deficiency is often difficult. Among the available tests, determination of serum MMA has received particular attention because of better serum stability, sample volume requirements and high diagnostic accuracy than measurement of cobalamin directly. The diagnostic performance of MMA assays was reviewed extensively.

The use of RIAs employing purified intrinsic factor to analyze cobalamin has reduced, but not eliminated, the incidence of falsely normal concentrations of Cbl measured in serum of subjects with cobalamin deficiency.[13] The assay of MMA in human serum is considered to be a sensitive measure of cobalamin deficiency. The tissue form of vitamin B_{12} deoxyadenosylcobalamin, in particular, is inversely proportional to methylmalonic acid level. This means that when the vitamin B_{12} cytosol

form is getting low, the serum or urine level of methylmalonic acid is rising considerably. Most immunoassays designed for vitamin B_{12} measure all of these forms, after conversion to cyanocobalamin. Increased MMA assay sensitivity leads to higher diagnostic efficiency.

Specimen stability is critical to determine appropriate conditions for transportation, especially for remote laboratory testing conditions. The goal is to ensure the least possible degradation of the analyte. One of the most striking advantages of measuring serum MMA is its indefinite stability, as long as 20 years. MMA and total homocysteine levels were measured in all patients seen between 1968 and 1981 who met the study's criteria for cobalamin and folate deficiency and from whom serum stored at -20°C was available. From 1982 to 1989, serum metabolite levels were measured in each of 267 consecutively seen patients who met the criteria for cobalamin deficiency (219 patients) or folate deficiency (48 patients).[46] During these studies, serum specimens had been stored at -20°C for 10 to 20 years was tested, frozen back again, and retested several times.[46] Methylmalonic acid was elevated in 12.2% of th folate-deficient patients; in all but one, the elevation was attributable to renal insufficiency or hypovolemia.
It was concluded that there was no indication that methylmalonic acid levels varied inversely with increased storage time.[46,48]

The easy availability of serum, already drawn for vitamin B_{12} measurements, is another advantage of MMA. Serum specimens also correlate with urinary samples. Linear relationships exist between MMA concentrations in serum (investigated range :0.05-34.2 μmol/L) and MMA concentrations in urine ($r = 0.74$),concentrations relative to creatinine ($r = 0.98$), and MMA excretions rates ($r = 0.97$) ($P < 0.001$ in each instance).[39] In most of the studied cases, patients testing low for vitamin B_{12} have elevated levels of methylmalonic acid in their urine.[39,40]

When cobalamin deficiency is suspected by clinicians, serum methylmalonic acid has been suggested as the first diagnostic test. Serum is the specimen of choice in the most laboratories. Leftovers from other tests can be used if serum was frozen at the appropriate temperature. Because only few labs do measure MMA, sending out specimens to these reference labs is a reasonable option because of analyte stability in frozen serum. Even more, the long-term stability of MMA in frozen serum makes it possible to retest, do batch analysis or analyze later patient samples as necessary.

As a final statement we can conclude that in situations where specimen stability is important, vitamin B_{12} status is better assessed with serum or urine methylmalonic (MMA) measurements. Measuring the end metabolite methylmalonic acid provides a more reliable, early, accurate, and sensitive measure of vitamin B_{12} deficiency at the tissue level.

Serum stability is summarized in table 2, page 24.

Table 2. COMPARED STABILITY

CONDITIONS	MMA Methylmalonic acid	VITAMIN B12 (COBALAMIN)	FOLATE (FOLIC ACID) ***
LIGHT SENSITIVITY	No information available in writing--assume is not light sensitive	Protect if not analyzed within 4 hours	In serum is not affected by the light*
REFRIGERATED	Stable for up to six hours	Not stable	Stable up to 3 days**
SERUM SEPARATION FROM CELLS	Consensus: separate after centrifugation	Consensus: Separate after centrifugation	Consensus: Separate
CNRU LAB DATA 2001-2002 RESEARCH DATA (CHAPTER 4)	**-20°C 1 YEAR STABLE During Method Correlation Studies**	**Separate after centrifugation**	**N/A**
FREEZING CONDITIONS **Consensus: multiple options**	-20°C Stable indefinitely in serum stored at -20°C[0] Stored for a month at -70°C After coagulation at RT for 1 h, serum separated by centrifugation and stored at -20°C	Consensus: Freeze Good only for one week	Consensus: Freeze

*Folate in serum is not affected by the light ; " binding proteins may have a protective effect by preventing light degradation in serum." [17]
**Unlike vitamin B_{12} , " temperature had only minor effect on folate stability." [17]
***Folate is a more" robust analyte than B_{12} regarding stability. In contrast to vitamin B_{12}, concentrations showed only minor changes under all four storage conditions" [17]

3. Methylmalonic Acid Urinary Studies in Humans, and the Relationship between Serum and Urinary Concentrations

Determination of methylmalonic acid in serum or urine is becoming an important diagnostic procedure in clinical chemistry. Compared to urine studies, serum studies are readily available due to collection convenience. Correlation studies, between the two specimens, were done by only a few research groups.

One study raised serious concerns about the reliability of urine MMA mesurements for diagnosis of vitamin B_{12} deficiency. In that study, serum MMA testing proved to be an appropriate means of discrimination between cobalamin deficiency and subjects without cobalamin deficiency (efficiency=0.95).[39] To investigate the relationship between serum and urine MMA, multiple and consecutive 3-h urine collection MMA studies were performed. The urine MMA excretion did not correlate with concentrations of MMA in serum, possibly because of variations in diuresis during the day.[39]

According to these studies, the wide use of random (untimed) collected samples for urinary MMA testing must be avoided. Data demonstrate that a complete 24-h urine collection is necessary for a valid estimation of MMA excretion. One of the experiments was related to the effect of feeding using both serum and urine samples. Serum specimens proved to stay fairly steady compared to urine ones. After the administered meals, urinary MMA excretion increased significantly, doubling after dinner.

The effect of fasting, feeding, and dietary loading with specific nutrients (fat, sugar, valine, isoleucine) in healthy subjects was investigated and demonstrated that MMA in the serum of normal subjects is not influenced by earlier food intake. In contrast, the urinary MMA/creatinine excretion rate was increased with food intake.[39]

A MMA equation for urinary clearance calculation, relative to that of creatinine, was recommended to use for the most accurate MMA calculations. To calculate urinary MMA clearance, the following formula[47] is recommended, which compensates MMA calculations for the diurnal urinary variation.

MMA/CREATININE CLEARANCE RATIO =

$$\frac{[\text{URINE MMA(NG/ML)} / \text{URINE CREATININE (NG/ML)}]}{[\text{SERUM MMA (NG/ML)} / \text{SERUM CREATININE (NG/ML)}]} \times 100$$

Figure 8. MMA Clearance Formula

Serum specimen is the recommended sample for analysis of tissue cobalamin assessment for daily testing requirements. If urinary MMA is requested for analysis, a complete 24-h UA collection is necessary for accurate results. In situations where patient's kidney function is under investigation, the methylmalonic acid (MMA) /creatinine clearance ratio is calculated according to the formula above (page 25).

There are not enough studies about the true diagnostic usefulness of measuring methylmalonic acid in serum/plasma versus urine. In healthy volunteers, normal serum MMA was relatively constant. In contrast, the MMA in timed urine samples showed quite significant concentration variation during the analysis. All the analyzed data suggests that when choosing urine samples for MMA analysis, a complete 24-h collection is necessary. However, due to the requirement of a prolonged collection time, this procedure will cause increased inconvenience to both the patient and the testing laboratory. Dietary influences must be also taken into consideration. In the same article, the authors made this powerful remark about urine MMA testing:

A complete 24-h collection is necessary for valid estimation of urinary excretion of MMA. In normomethylmalonic –acidemia, no significant correlationbetween serum cobalamin and MMA was found. In hypermethylmalonic-acidemia, a negative correlation existed between the concentrations of cobalamin and MMA in serum (in cobalamin deficiency there are perturbations of MMA metabolism).[39] Data compared also the concentrations of MMA in serum and its urinary excretion, using 24-h collected urine samples. Data showed a highly significant positive correlation in normomethylmalonic-acidemia as well as in normomethylmalonic-acidemia.[39] These findings suggest that the widespread use of determining the excretion of MMA in random (untimed) urine samples must be discouraged.[39]

4. Benefit Estimates of Serum MMA Measurements

For almost twenty years, clinical laboratory scientists have tried to design methods that accurately can measure vitamin B_{12} levels. MMA testing has been proposed as the method of choice. The main problem with MMA measurement is the cost involved with the instrumentation, reagents, and personnel training.

One study compared the costs and benefits of serum MMA in diagnosing vitamin B_{12} deficiency. The authors investigated the use of serum cobalamin requests. It was noticed that the test was requested on the basis of nonspecific indications or vague symptoms.[49] To supplement patients, most providers take in consideration the serum cobalamin value and if the patient is anemic.

Indeterminate serum vitamin B_{12} results sometimes cause a delay in the decision making process. The poor diagnostic utility for low and low-normal serum cobalamin results suggest that, in patients with B_{12} results <200-220 pmol/L, serum MMA is recommended.[49]

5. Comparison of Serum and Plasma MMA— 13 Clinical Laboratories International Study

Information on interlaboratory variation and on methodological differences for serum and plasma MMA testing is limited. At the invitation of CDC (Centers for Disease Control), 13 laboratories were invited to participate in a 2-day analysis study, to assess the compatibility of results among laboratories and within laboratories (among-run). The common method for testing was gas chromatography/mass spectrometry. A total of eight serum and eleven plasma specimens were analyzed for precision, recovery, and differences among laboratories and testing methods. The mean among-laboratory precision (CV) was 19% for serum and 21% for plasma. The mean within-laboratory (among-run) was 13% for both serum and plasma. The mean among-laboratory recovery of methylmalonic acid was 105% in serum and 95% in plasma.[50]

Participating laboratories were investigated and compared for extraction and derivatization reagents, calibrators, calibrator range, sample volume, and reference interval.

In conclusion, the international MMA study reported that no method differences were found, but some among-laboratory imprecision was discovered. To reduce the analytical imprecision, it was recommended that attention must be focused on calibration issues. Calibration range must be optimized to obtain the most accurate results for the clinically critical MMA concentrations. Too wide calibration ranges can underestimate low MMA concentrations, and too narrow ones can overestimate high MMA concentrations. As a general recommendation, the most critical level of accuracy was reported to be from low to slightly increased MMA concentrations (up to 1000 nmol/L), because cobalamin deficiency is more difficult to be determined at these levels. To achieve these testing requirements, high-quality reference materials and external quality assessment programs must be implemented to reduce the calibration differences among laboratories.[50]

CHAPTER 3

1. Methylmalonic Acid—Old and New Methods

Several methods for methylmalonic acid determination in serum and urine have been Published, using gas chromatography (GC), gas chromatography-mass spectrum (GC-MS), high performance liquid chromatography (HPLC), capillary electrophoresis (CE), and LC-MS instruments. HPLC procedures have several disadvantages, like long instrument run time, column switching, and need for gradient elution.[48] CE direct detection of organic acids and indirect detection assays do not provide adequate sensitivity and precision.[41,51] Methods for measuring serum methylmalonic acid (MMA) are quite costly and cumbersome. One big problem is the unavailability of commercial assays, little information about interlaboratory variations, and methodological differences.

Today's instrument of choice for most of clinical laboratories is the GC-MS, using gas chromatography with mass spectrometric detection.[41,39,40,50] Early assays encountered numerous problems related to MMA losses during sample preparation, MMA derivatives instability, and ion fragments crosscontribution between MMA and its deuterated internal standard.[41,50] Sample preparation can be performed using liquid/liquid extraction or by solid-phase extraction. MMA liquid/liquid extraction requires organic solvents and the extraction recovery is quite low, 20-55%. To increase sensitivity multiple extractions with subsequent mixing of the extracted fractions is required.[41] Solid-phase extraction (SPE) is one solution for increased serum MMA sensitivity with GC-MS.

The advantages of SPE are: one time extraction, cleaner extract, less labor intensive, shorter extraction time, and easy to train laboratory personel. SPE reported problems relate to ester enols formation due to active hydrogen on C-2.[41] This side products caused unreliable method performance for some assays utilizing silyl derivative of MMA.[50] New studies indicate that the derivatization recovery step can be optimized using short incubation time and optimizing the incubation temperature. The improved MMA recovery is attributed to the mild incubation conditions that minimize side reactions, leading to methylmalonic acid-trimethylsilyl (MMA-TMS) ester decomposition.[41]

2. Gas Chromatographer-Mass Spectrometer (GC-MS) GCD Instrumentation

The GCD is an integrated gas chromatography detector system that utilizes an electron-ionization detection to provide spectral identification. For the present research study the instrument used was Hewlett Packard (HP) G1800C gas chromatograph

detector (GCD) Series II. The instrument consists of a gas chromatograph (GC) and an electron ionization detector (EID). The detector generates mass spectral data for each component in the sample. The GCD is controlled by a data system. The data system consists of an HP personal computer (PC) running the GCD module of the HP G1701BA ChemStation software under Microsoft Windows NT.[52]

The gas chromatograph (GC) performs separations and is equipped with split/splitless capillary injection port. The GC also includes electronic pressure control (EPC). EPC automatically controls head pressure to maintain the specified carrier gas flow. It compensated for the vacuum at the detector end of the capillary column. The end of the capillary column passes through a heated detector interface. Compounds elute directly into the detector.

The electron ionization detector (EID) generates data like retention time, abundance, and mass spectral data. Main components of the detector are: analyzer, vacuum system, and the electronics.

The analyzer is the heart of the detector. It has three main components: ion source, mass filter, and electron multiplier. The analyzer receives the sample components as they elute from the capillary column. First, it ionizes and fragments the sample molecules. Next, it sorts the ions by their mass-to-charge ratio (*m/z*).[52]

The ion source generates the ions. It receives sample molecules from the GC capillary column. It bombards these molecules with electrons emitted by a hot filament. The sample molecules are ionized. Positively-charged ions are ejected from the ion source and enter the quadrupole mass filter.

The mass filter controls which ions are detected. It separates ions according to their mass-to-charge ratio *(m/z)*. At a given retention time, only ions of a selected mass-to-charge ratio can pass through. The mass filter can operate in two modes: scan and selected ion monitoring (SIM).

In scan mode, the mass filter scans a specified mass range (for example, *m/z* 10 to *m/z* 425). It does this many times in the time it takes a chromatographic peak to elute. Each scan generates a mass spectrum which can be compared to a library of mass spectra for identification.

In SIM mode, the mass filter passes only ions with a specific mass-to-charge ratio (or a few specific mass-to-charge ratios). SIM can be used to monitor for a chemical with a known fragmentation and ionization pattern. Sample components without the specified ions are ignored.

The electron multiplier detects the ions and generates an electrical signal proportional to the number of ions striking it.

The analyzer in the electron ionization detector must operate in a vacuum. It can only operate at very low pressure (high vacuum). Without an adequate vacuum, the mean-free-path is too short and the ions cannot traverse the distance from the ion source to the electron multiplier. The need for vacuum in the detector restricts the size and length of capillary columns that can be used in the GCD system. If there is too much flow into the detector, the vacuum system cannot maintain the necessary vacuum. In the EID, the analyzer is located in an air-tight chamber called the vacuum manifold.

Vacuum is created by two pumps: a vapor-diffusion, high vacuum pump and a mechanical, low vacuum pump called the foreline pump. The diffusion pump removes carrier gas and excess sample from the vacuum manifold. The foreline pump pulls away the diffusion pump exhaust. Both pumps must operate correctly or the required vacuum cannot be achieved.

Detector electronics control detector operation. They receive setpoints and operating instructions from the method specified by the operator through the data system. The electronics control the operation of the detector and data acquisition. The acquired data are processed by the electronics and returned to the data system for analysis.

The system uses the column dimensions to calculate the pressure required to obtain the flow rate for the method. To achieve constant flow, the column length and diameter must be accurately specified. Optimal flow rates are based on the column diameter. The following flow rates provide the linear velocity optimal for resolution:

-- 0.18 mm columns, use flow of 0.4-0.5 ml/min (helium) for splitless mode
-- 0.20 mm columns, use flow of 0.5 ml/min (helium) for splitless mode
-- 0.25 mm columns, use flow of 1.0 ml/min (helium) for splitless mode

Columns with a 0.32 mm diameter have an optimal flow rate of 1.6 ml/minute (helium). This is higher than the 1.0 ml/minute allowed by the EID. To use 0.32 mm columns, either set the flow to 1.0 ml/minute or install an optional post-column effluent splitter to use higher flows. For splitless injections, the split flow can be anywhere between 50 and 75 ml/minute.

For methods that require a split flow below 50 ml/minute use split injection mode. The correct split ratio is based on sample size and concentration. Higher sample volumes and concentrations usually require higher split ratios. The split ratio calculation is depicted in Figure 9, page 32. It is important to emphasize that the correct split flow measurement is necessary to achieve the desired split ratio. Total flow (split flow + column flow) must be greater than 20 ml/minute.[52]

$$\text{SPLIT RATIO} = \frac{\text{SPLIT FLOW + COLUMN FLOW}}{\text{COLUMN FLOW}}$$

Figure 9. Split Ratio Calculation[52]

Detector optimization is automatic. The GCD automatically tunes or optimizes itself as necessary to maintain optimal system performance. It consists of adjusting instrument parameters to yield predefined abundance and ratios between mass spectral fragments in the tuning compound perfluorotributylamine (PFTBA).

3. Data Acquisition Modes

In gas chromatography, the compounds in the sample are separated in the column, before reaching the detector. In the HP GCD Series II, once the compounds exit the column, they are bombarded with a stream of electrons, which causes reproducible fragmentation of the molecules. This process is called electron ionization. The detector on the GCD in an electron ionization detector (EID). Once the fragmentation has occurred, the mixture of ions created can be detected or monitored in two distinct modes:

-- Scan mode
-- Selected ion monitoring (SIM)

In Scan mode, the detector scans from high to low across a set range of atomic masses. When one scan is complete, the system resets and immediately scans the range again. This process is repeated continuously during the run. The only exception is during the solvent delay at the start when the detector is off. At the end of the run, the data are recorded in a file as scan numbers and the abundance of individual ions detected at each scan.

In SIM mode, only a few discrete ions are monitored. Because of the smaller number of ions monitored, each ion is monitored for longer than in Scan mode. For that reason, SIM mode is 5-100 times more sensitive than Scan mode. SIM mode is very useful in detecting low levels of known compounds in a mixture.[52] SIM mode is preferred over scan mode for sample quantitation, since it produces more reproducible peak areas.

The mass spectrum acquired at any scan in the run can be plotted with the mass-to-charge ratios (*m/z*) on the X-axis and abundance on the Y-axis (Figure 9, page 33).

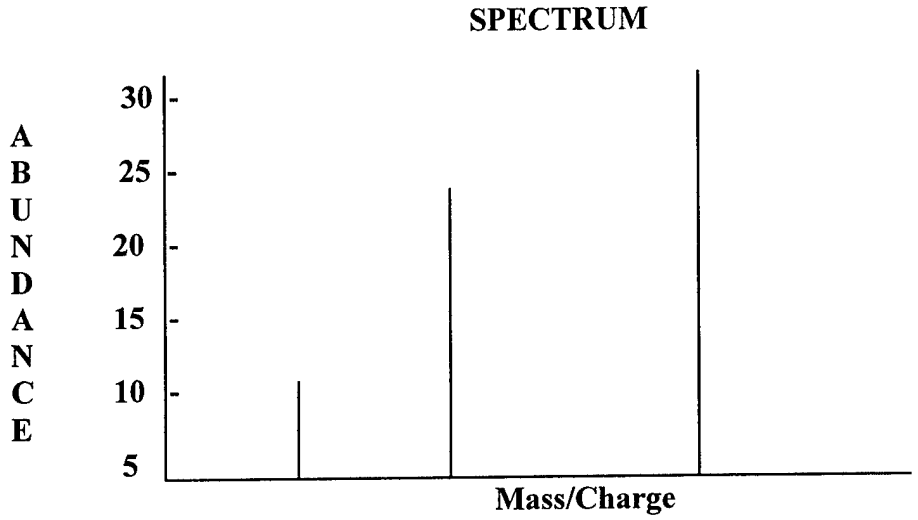


Figure 10. Mass Spectra

The data can also be plotted as a chromatogram, where time (scan numbers) are plotted on the X-axis and the abundance of ions are plotted on the Y-axis. (Figure 11).

When the abundances in the plot are the sum of all ions monitored, the chromatogram is known as a total ion chromatogram (TIC). When the abundance of a single ion is plotted, the chromatogram is called an extracted ion chromatogram (EIC).

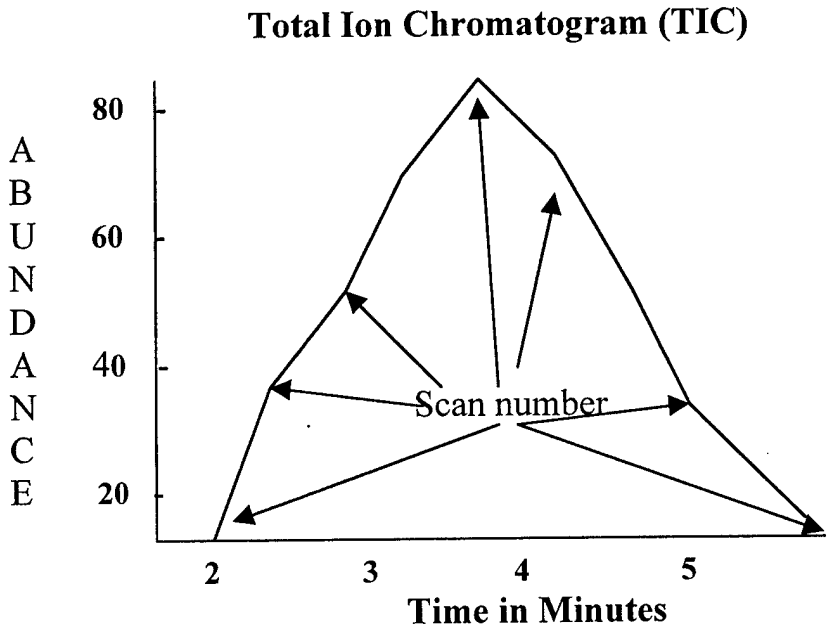


Figure 10. TIC

4. Quantitative Analysis

Quantitative analysis determines the presence of compounds, then calculates the quantities of those compounds. Identification is based on the retention times and specific ions present in the proper response ratio. The amount of each compound is determined by comparing the response in the sample to the response in one or more previously analyzed calibration samples (also called standards). The data for all calibration samples are stored in a quantitation database. Quantitative methods can be set up for Scan or SIM acquisition modes. The basic steps involved in quantitative analysis are:

a. Analyzing one or more calibration samples
b. Setting up the quantitation database using an automated procedure that selects ions and retention times from a calibration sample
c. Using the method containing the quantitation database to analyze abd generate quantitative reports for unknown samples
d. Running calibration samples intermittently to recalibrate (update) the quantitation database

SIM mode is preferred over Scan mode for quantitative analysis because it produces more reproducible peak areas. Quantitation is performed by measuring the peak area of the most unique and significant ion of the mass spectrum, called the quantitative ion. Measuring the quantitative ion with the qualitative ion helps minimize background and noise in the quantitation value and, since it is selective, adds identification confirmation to the detection signal.[52]

5. PROCEDURE: Serum Methylmalonic Acid Quantitative Analysis Date: March 2002

PRINCIPLE:

Methylmalonic acid (MMA) is a metabolite used to monitor tissue cobalamin level. When coenzyme adenosyl-Cbl becomes deficient, succinyl-CoA is decreased and MMA is increased. Vitamin B_{12} plays a key role in carbon rearrangement of methylmalonic acid to succinic acid.[41] There are several advantages of measuring methylmalonic acid instead of vitamin B_{12}: serum methylmalonic acid reflects more accurately tissue cobalamin status; serum methylmalonic acid level is 1000-fold higher than serum cobalamin level; an elevation rather than decreased concentration of MMA is measured in cobalamin deficiency; MMA is more stable than cobalamin[41,46] High MMA levels are considered pathologic and correspond to low tissue vitamin B_{12}. Although serum MMA reflects tissue vitamin B_{12} adequately in liver failure, it is not useful when renal function is decreased.[41]

The method for measuring serum methylmalonic acid is gas chromatography with mass spectrometric detection. The sample cleanup step is done using solid-phase extraction (SPE). Following the extraction, a trimethylsilyl (TMS) derivative will produce a volatile derivative of methylmalonic acid.

Internal standard and acetonitrile (ACN) are added to standards, controls and patient samples and then centrifuged. Centrifuged samples are transferred into ion exchange columns preconditioned with methanol (MeOH) and water. After several washings with different reagents, MMA is eluted. The eluent is evaporated under nitrogen, and the residues are derivatized with a trimethylsilyl derivatization agent. The TMS derivative of MMA is then incubated at 50 °C for five minutes. Next, the derivatives transferred into glass autosampler vials and injected into GCD Series II.

All specimens must be handled as potentially infectious material. To perform this procedure the medical technologist must wear the following: gloves, safety glasses, laboratory coat, mask (as necessary). All assay steps must be performed under the fume hood to protect testing personnel from exposure to possible chemical hazards. Additional information concerning the safe handling of the reagents and chemicals may be obtained from the Material Safety data Sheet (MSDS) and HAZ-COM manual, located in the CNRU laboratory.

NOTE: Pressurized Room and Ventilator are necessary for this assay due to hazardous fumes

SPECIMEN:

There is no special patient preparation for sample collection. Fresh serum or frozen specimens stored at –20°C from NaF/K or citrate in glass containers are acceptable. After collection, separate serum and store in the refrigerator at +4 °C up to two days (48 hours). If the MMA serum analysis is not performed within the 48h from collection, freeze the serum at –20 ° C until ready for analysis.[41,46]

Unacceptable specimens: Frozen whole blood samples are not acceptable. Total hemolysis compromises the results. Moderate hemolysis, icteric or lipemic samples are acceptable.[47]

REAGENTS AND MATERIALS:

I. Methylmalonic acid stock standard at 10 mmol/L prepared in methanol (MeOH). Stable for 2 years at –20 ° C.[41,53] Measure 0.0295g MMA powder for 25 mL MeOH. Use a volumetric flask and sonogram the mixture for 15 minutes. Vortex for 1-2 minutes, and aliquote in 1 mL glass vials suitable for freezing temperature.

II. Methylmalonic acid working calibration standard at 10 μmol/L. Stable for 1 year at -20° C. [41,53] In 50 mL volumetric flask add 40 mL of ACN and 50μL of MMA stock standard, add ACN up to the meniscus level, mix, sonogram, aliquote in glass vials and freeze.

III. Methylmalonic acid d_3 (deuterated) stock internal standard at 10 mmol/L prepared with MeOH. Stable for 2 years at -20° C.[41,53] Measure 0.0303 g of internal standard and add to a 25 mL volumetric flask. Fill with MeOH, mix, sonogram, vortex, aliquote in glass vials and freeze.

IV. Working internal standard (methylmalonic acid d_3) at concentration of 15 μmol/L. Stable for 1 year at -20° C.[41,53] In 50 mL volumetric flask add 40 mL of ACN and 75 μL of MMA d_3, fill up to the meniscus with CAN, mix, sonogram, vortex, aliquote in glass vials and freeze.

V. Solid phase extraction columns CUQAX15Z (United Chemical Technologies, Inc.).[41]

VI. Methyl-t-butyl ether (MTBE)[53]

VII. Acetonitrile (CAN)[41,53]

VIII. Formic Acid [41,53]

IX. Elution solvent: 3% formic acid in MTBE.[47] Add 3 mL of formic acid to 97 mL of MTBE and mix. Stable for 3 weeks at 4 °C.

X. Bovine albumin fraction V and saline solution. Prepare blank standard by mixing 4 g of albumin flakes with saline by using a 100 mL volumetric flask. Aliquote in glass vials after is stabilized, and freeze at -20°C. Stable for 3 months.

XI. Pool of serum using frozen – 20 °C serum from normal and high vitamin B_{12} pre-analyzed samples. Use as internal control.

XII. Derivatizing reagent: Methyl-silyl- trifluoroacetamide (MSTFA) with 1% trimethylchlorosilane (TMCS).

XIII. Column: RT_x200 (Restek) 20m X 0.18 mm X 0.4 μm[41,53]

XIV. Carrier gas: helium

METHOD NAME: CILEANAH.M

INSTRUMENT NAME: GC/MS Instrument # 1

ASSAY CONDITIONS: Split injection mode with split ratio of 20:1. Constant flow at 1 ml/min. injection volume 2 µL.

CHROMATOGRAPHIC PARAMETERS: Inlet: 220 °C; Detector: 280C; Split Injection

OVEN INFORMATION: Column maximum: 310 °C; Initial Temperature 75 °C; Initial time 0.2 min.

OVEN RAMP	RATE	FINAL TEMPERATURE	FINAL TIME
Level 1	30.00 C/min	185 °C	0.4 min
Level 2	70.00 C/min	300 °C	8.5 min

TOTAL TIME: 14.4 min

INJECTION PARAMETERS: Injection mode: Automatic; Sample Volume: 2 Stops; Solvent A (ACN) Washes: 8; Solvent B Washes (ACN): 8; Constant Flow 1ml/min; Solvent Delay: 3.5 min; Mass Range: Use SIM

INTEGRATOR: Chemstation

IONS MONITORED: Methylmalonic Acid 246.70 and 218.20. Internal Standard 250.10 and 220.70. Ions 246.70 and 250.10 are the quantitative ions. 218.20 and 220.70 are qualitative ions.

CALIBRATION: Prepare standard curve for methylmalonic acid with each run.

0.0 µmol/L: Use 1 mL of 4g/dL bovine albumin saline solution (Blank Serum Like)
0.2 µmol/L: Use 20 µL of 10 µmol/L working calibration standard
0.5 µmol/L: Use 50 µL of 10 µmol/L working calibration standard
1.0 µmol/L: Use 100 µL of 10 µmol/L working calibration standard
2.0 µmol/L: Use 200 µL of 10 µmol/L working calibration standard
16 µmol/L: Use 200 µL of 80 µmol/L working calibration standard
32 µmol/L: Use 400 µL of 80 µmol/L working calibration standard

QUALITY CONTROL: Control A: Internal Quality Control (IQC): Pool frozen serum from pre-tested vitamin B_{12} normal and above range. Mean value: 0.25 µmol/L.
Control B: External Quality Control (EQC) : Use pre-tested serum samples from ARUP Reference Laboratories.

PRE-EXTRACTION SAMPLE PREPARATION:

- Add 1 mL of serum to a disposable glass tube
- Add 100 µL of working internal standard
- Add 1 mL of ACN, vortes for 13 seconds, and centrifuge in precooled at 4 ° C centrifuge at 2000 g for 7 minutes

SOLID PHASE EXTRACTION (SPE): [41,53]

a. Label the SPE columns with sample ID.
b. Condition the columns with 3 mL of MeOH. Drain until is about 1 mL of MeOH left in column. Add 5 mL water or up to _ of column volume.
c. Drain the water to _ column volume and add processed samples to the columns.
d. Add 3 mL water or up to _ column volume. Let samples drain by gravity.
e. Add 6 mL of water and open full vacuum. Let it drain and dry the columns for 5 minutes.
f. Open vacuum and add 3 mL of methanol (MeOH), let it drain, close vacuum, and let columns dry for 3 minutes.
g. Add 2 mL of MTBE, open full vacuum, let it drain and dry the columns for 3 minutes.
h. Discard vacuum manifold content in the MMA solvent waste labeled container.
i. Label a new set of glass tubes with samples ID, tech ID and date.
j. Elute methylmalonic acid with 5 mL of the pre-cooled elution solvent.
k. Evaporate the solvent using nitrogen.
l. Add to each tube 30 µL of acetonitrile (ACN) and 20 µL of MSTFA with 1 % TMCS.
m. Cover glass tubes to avoid evaporation.
n. Incubate for 5 minutes at 50-52 ° C.
o. WARNING: INCUBATION TEMPERATURE AND TIME ARE CRITICAL FOR METHOD PERFORMANCE. The heating block should not exceed 52° C.

DERIVATIZED SAMPLE ANALYSIS: Label auto sampler vials. Transfer incubated sample into the glass vials. Use glass microinserts.

RUN ACCEPTABILITY CHECK LIST:

- Evaluate chromatogram for acceptable peak shape and MMA peak area
- Check calibration curve for linearity
- Internal and External Controls must be within +/- 2sd of the expected concentration
- Background check with MeOH and ACN mix must be free of any MMA traces
- Tuning must be within acceptable limits
- Patient samples should not be tested if calibrators and controls are not within acceptable limits.

CHAPTER 4

1. METHOD EVALUATION BACKGROUND

The process of selecting a method for measurement of serum methylmalonic acid (MMA) included the following steps: a) defining the method requirements, 2) searching the available technical literature to find what methods are available, 3) selecting the method whose characteristics best fulfilled our laboratory requirements, and 4) method performance evaluation. We term the new assay a test method since it was being evaluated to demonstrate performance prior to being used as on clinical samples.[54] The goal of test method evaluation is the discovery of analytical errors that may effect the interpretation of clinical test results. All method evaluation experiments including intra-assay precision, recovery, inter-assay precision, correlation studies used to validate the new serum MMA Assay used split injection with a split ratio of 20:1 and constant 1ml/minute flow.

2. SENSITIVITY OR DETECTION LIMIT

The objective was to determine the smallest MMA level that can be distinguished from zero or is the smallest quantity of MMA that can be measured. Two types of samples were used to evaluate the detection limit: a sample with an MMA level close to the anticipated detection limit and a zero standard or blank matrix. As recommended 20 replicates[55] were tested for each sample type, the same as for the precision study. The blank sample was made from fraction V bovine albumin and saline: 4 grams of albumin per 100 mL of saline. This concentration of 4 g/dl is close to the human albumin in serum. The purpose was to keep the matrix as close as possible to the patient serum samples. The low sample level concentration target was at 0.05 μmol/L. To achieve low target sample 10 μL of 5 μmol/L MMA solution was added to 1 mL of bovine albumin-saline blank target. The mean, SD and CV were calculated as shown in Table 3.

The detection limit was defined as 3 SD above the zero blank level. Using the 0.05 μmol/L target the SD was 0.011 μmol/L, equivalent to a detection limit of approximately 0.03 μmol/L. Using the zero blank the SD was 0.0062 μmol/L, equivalent to a detection limit of approximately 0.02 μmol/L. The final detection limit was assumed to be 0.03 μmol/L.

Table 3. Detection limit or analytic sensitivity study

DETECTION LIMIT STUDIES			
DATA 1	Target 0.05 (umol/L)	DATA 2	TARGET 0.00 (umol/L)
1	0.05	1	0
2	0.03	2	0
3	0.05	3	0
4	0.05	4	0
5	0.05	5	0.01
6	0.04	6	0
7	0.06	7	0
8	0.05	8	0
9	0.07	9	0
10	0.05	10	0.02
11	0.04	11	0
12	0.05	12	0
13	0.05	13	0
14	0.06	14	0.01
15	0.02	15	0
16	0.04	16	0
17	0.05	17	0
18	0.05	18	0
19	0.03	19	0
20	0.06	20	0.02
21	0.05	21	0
MEAN	**0.0476**	**MEAN**	**0.0028**
MEDIAN	**0.05**	**MEDIAN**	**0**
SD	**0.0108**	**SD**	**0.0061**

3. CARRYOVER STUDIES

Carryover can alter the results of low concentration samples following a very high sample during a sequence run. Carryover can occur under different circumstances, such as sample derivatives adhering due to incomplete residue burning. Carryover analysis was performed by analyzing in sequence a high concentration sample followed by a blank bovine-albumin sample. The carryover test high concentration sample contained

16 μmol/L MMA. Carryover was 0.02 μmol/L in the blank sample after 16 μmol/L sample. This is less than or equal to the detection limit of the assay and so does not represent a problem. To reduce carryover effect, the oven temperature is kept at 300°C for 6 minutes as part of the procedure. Total running time is close to 15 minutes per sample.

4. ANALYTICAL SPECIFICITY AND INTERFERENCE

Interference studies determine the error caused by other materials present in the patient sample. No hemolyzed or icteric samples were analyzed during this study. All serum samples were clear except for three of the correlation samples. No interference was observed in the134 serum samples runs between November 2001 and March 2002. Lipemia observed in 3 serum samples from method correlation studies did not interfere with the method. Serum was the only specimen type analyzed.

5. PRECISION STUDIES

Two types of samples were used for the precision studies. Standards, which did not undergo the serum extraction procedure, and serum pools that were extracted.

Intra-assay precision consists of running the same sample repeatedly on the same run. Intra-assay precision samples from the same serum pool used in the inter-assay analysis were analyzed for nine consecutive times.

Table 4. Intra-Assay precision Study

Measurement number	μmol/L
1	0.25
2	0.24
3	0.25
4	0.24
5	0.24
6	0.24
7	0.25
8	0.25
9	0.25
MEAN	0.246
MEDIAN	0.248
SD	0.005

Results were evaluated for mean, SD and CV. If the CV is < 3% it is generally considered acceptable performance.[55] Intra-assay precision results are depicted in Table 4. The intra-assay CV was 2%.

Inter-assay imprecision was evaluated in two ways. First, standards at seven concentrations were analyzed in duplicate over a period of eight weeks.
Standard I: 0..2 μmol/L Standard II: 0.5 μmol/L Standard III: 1.0 μmol/L
Standard IV: 2.0 μmol/L Standard V: 8.0 μmol/L Standard VI: 16.0 μmol/L
Standard VII: 32 μmol/L
Standard levels were chosen at random and run in different combinations, not more than four levels at a time. Inter-assay precision data based on unextracted standards is shown in Table 5. Average inter-assay CV on unextracted standards was about 2%.

Table 5. Inter-assay precision study using unextracted standards

	STD I	STD II	STD III	STD IV	STD V	STD VI	STD VII
	0.2	0.5	1	2	8	16	32
	0.19	0.49	0.99	2	8.1	16	32
	0.22	0.5	1	2.1	7.98	15.9	31.7
	0.2	0.5	1.1	1.98	8	16.1	32
	0.2	0.5	1	2	8	16.1	32.2
	0.2	0.5	1	2	7.99	15.9	32
	0.2	0.49	0.99	2	8	16	32.2
	0.2	0.5	1	2	8	16	32
	0.21	0.5	1	2	8	15.7	32
	0.2	0.48	1.1	2.1	8	16	32
	0.2	0.5	1	2	8.1	16	31.8
	0.2	0.5	1	2	8	16	32
	0.2	0.49	1.1	2	8	15.8	32
	0.21	0.5	1	2	8	15.7	32
	0.2	0.5	1	2	7.98	16	32
		0.5	1	2	7.99	16	32.1
		0.2	1	1.98	8	16	32
	0.21	0.2	1	1.99	8	16	32
	0.18	0.51	1	2	8	15.99	32
	0.2	0.5	1.2	2	8	16	32
Mean	0.201	0.4965	1.023	2.0075	8.007	15.9595	32
Median	0.201	0.5	1	2	8	15.9595	32
SD	0.007880	0.00745	0.05557	0.032261	0.03246	0.055574	0.10760
CV	3.9%	1.5%	5.4%	1.6%	0.4%	0.3%	0.3%

Extracted precision studies were done using a pool of serum samples from patients tested for serum vitamin B_{12} level. Serum leftover from 50 adult men and women that tested normal or above the normal cobalamin range in serum were pooled , aliquoted, and stored at - 20°C until the extraction procedure. After extraction, the MMA levels in the samples was stable at - 20°C for up to seven days, making batch sample analysis possible after earlier extraction. For the serum MMA inter-assay precision study an extracted serum pool was analyzed over a period of 25 days. Data were evaluated for consistency, shifts or trends. After 25 consecutive runs pool serum can be used as the internal control if data results are acceptable under the quality control implemented standards.

Table 6. Inter-Assay Precision Studies on extracted, pooled serum

DAY	umol/L	DAY	umol/L
1	0.24	15	0.25
2	0.24	16	0.24
3	0.24	17	0.23
4	0.25	18	0.22
5	0.25	19	0.26
6	0.24	20	0.26
7	0.21	21	0.22
8	0.21	22	0.26
9	0.21	23	0.29
10	0.21	24	0.23
11	0.24	25	0.22
12	0.26		
13	0.24		
14	0.25		
	MEAN	0.239	
	MEDIAN	0.24	
	SD	0.020	
	CV	8.3%	

Figure 12 depicts one run on an extracted pooled serum (day 12) from the inter-assay precision analysis.

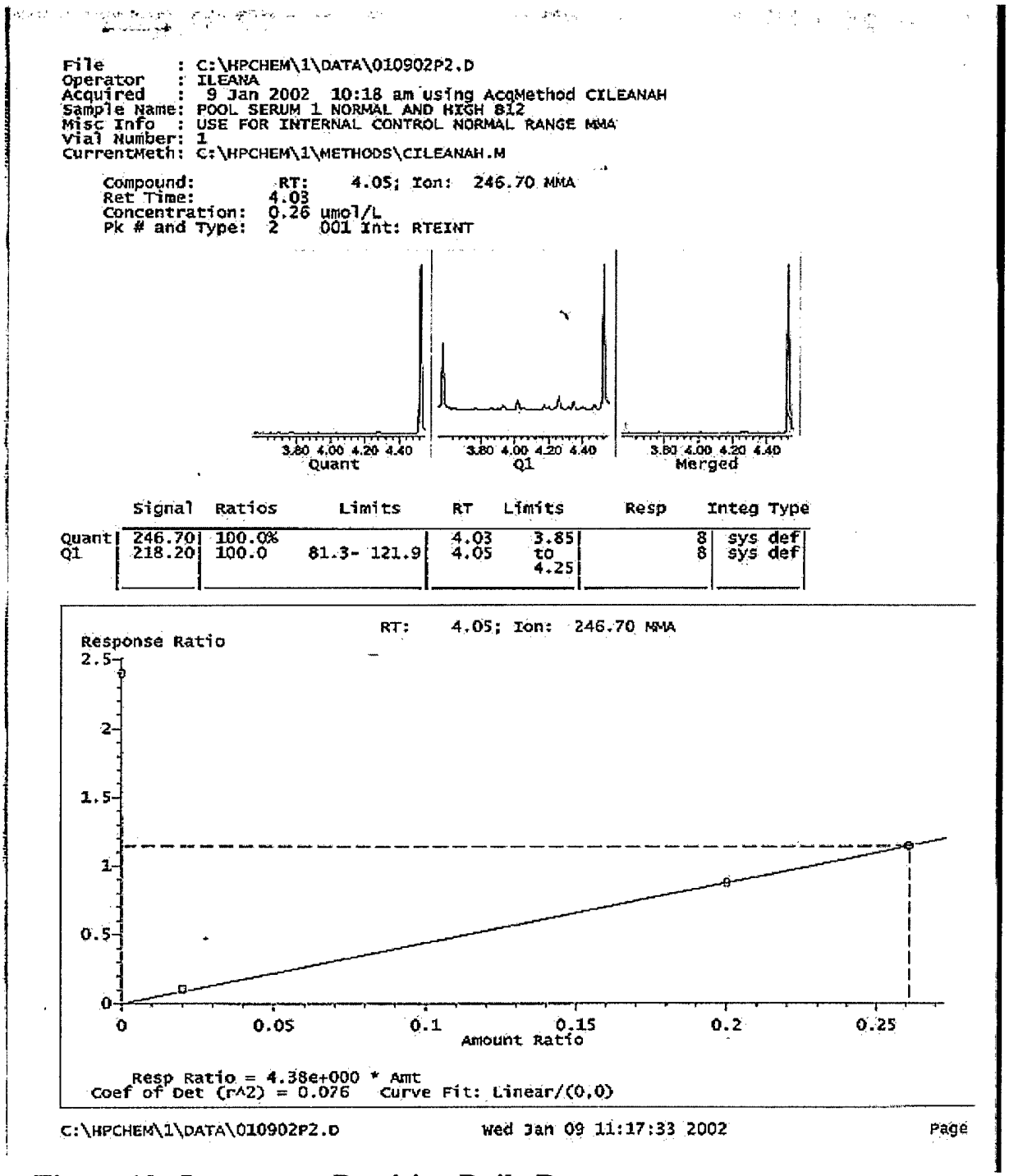


Figure 12. Inter-assay Precision Daily Run.

6. LINEARITY STUDIES

Linearity of the method was evaluated by analyzing MMA standard (Table 6 and Figure 13 below). The method was linear to 32 umol/L. A separate linearity was run with day to evaluate instrument performance.

Table 7. Instrument setup for linearity study

Method Name : C:\HPCHEM\1\METHODS\CILEANAH.M
Instrument Name : GC/MS Instrument #1
Last Calibration : Tue Dec 04 10:41:21 2001

2) RT: 4.05; Ion: 246.70 MMA
Retention Time: 4.06 minutes
Retention Time Window: 0.50 minutes
Concentration Units: umol/L

	Signal	Rel Resp	Level ID	Conc	Response
Quant	246.70		1	0.100	70
Q1	218.20	121	2	0.400	227
			3	16.000	15407
			4	32.000	30844

Curve Fit : Linear

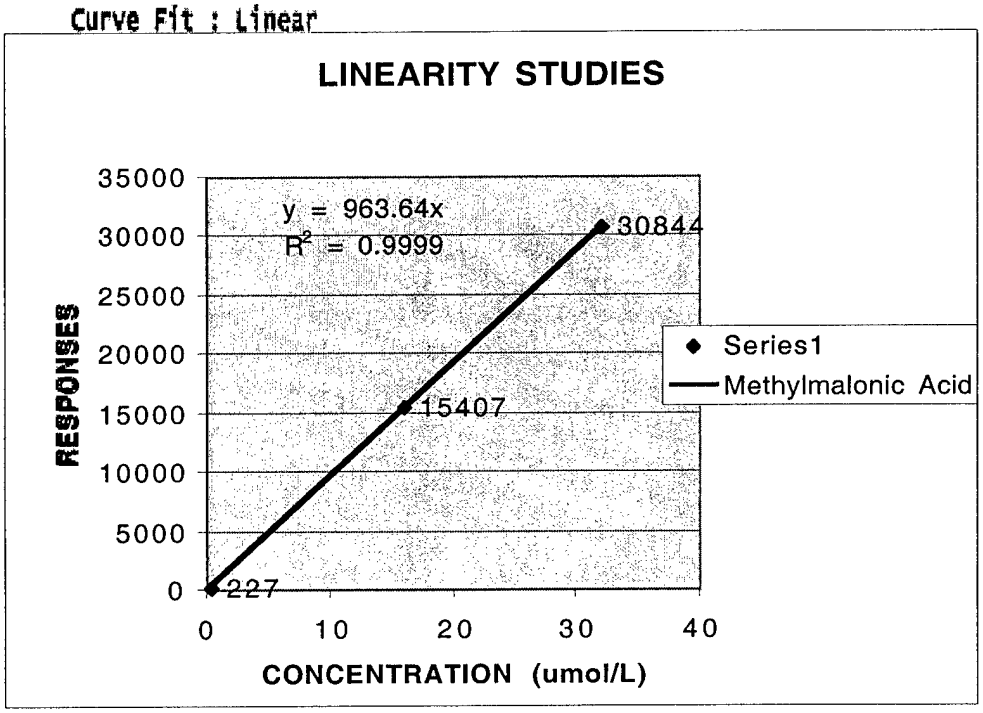


Figure 13. Linearity studies

7. RECOVERY STUDIES

Recovery experiments are most useful in evaluating methods for which there is no comparative method. If correlation to a comparative method is good, the recovery probably does not need to be done.[55] Recovery studies were performed by spiking 0.2 umol/L of MMA into buffer or a serum-like (albumin containing) matrix. The buffer was run unextracted while the serum-like matrix was extracted to evaluate recovery of MMA by the extraction process. The concentration 0.2 µmol/L was selected because most of the correlation samples were running around this range. The extracted versus unextracted repeat analysis was performed for seven consecutive runs. Results are depicted in Table 7. Average recovery was 94%.

Table 8. Serum MMA Recovery Studies

Run	Extracted Matrix	Buffer	Recovery
1	0.2	0.2	100%
2	0.18	0.2	90%
3	0.17	0.19	90%
4	0.19	0.2	95%
5	0.17	0.2	85%
6	0.2	0.2	100%
7	0.2	0.2	100%
MEAN	0.187	0.199	94%
MEDIAN	0.19	0.2	
SD	0.014	0.004	

8. CORRELATION STUDIES AND METHOD ACCURACY

A correlation study was performed using split frozen serum samples tested at ARUP Laboratories in Salt Lake City, Utah. A total of 30 serum samples ranging from < 0.1 to 13.57 µmol /L were analyzed in duplicate analysis.

Table 9. Method Correlation Studies.

ARUP MMA	UW1	UW2	Mean UW MMA	Diff UW1vs2	Diff UWmean-ARUP
0.05	0.05	0.04	0.045	-0.01	-0.005
13.57	13.9	13.91	13.905	0.01	0.335
0.04	0.04	0.05	0.045	0.01	0.005
0.5	0.45	0.46	0.455	0.01	-0.045
0.5	0.45	0.5	0.475	0.05	-0.025
0.5	0.49	0.46	0.475	-0.03	-0.025
0.1	0.12	0.12	0.12	0	0.02
0.1	0.06	0.06	0.06	0	-0.04
0.12	0.12	0.12	0.12	0	0
0.3	0.3	0.3	0.3	0	0
0.3	0.29	0.29	0.29	0	-0.01
0.3	0.29	0.3	0.295	0.01	-0.005
0.1	0.1	0.12	0.11	0.02	0.01
0.1	0.1	0.1	0.1	0	0
0.3	0.29	0.3	0.295	0.01	-0.005
0.3	0.29	0.29	0.29	0	-0.01
0.23	0.23	0.27	0.25	0.04	0.02
0.7	0.66	0.8	0.73	0.14	0.03
0.45	0.44	0.45	0.445	0.01	-0.005
0.1	0.13	0.13	0.13	0	0.03
0.1	0.13	0.13	0.13	0	0.03
0.64	0.66	0.66	0.66	0	0.02
0.91	0.93	0.9	0.915	-0.03	0.005
0.6	0.66	0.66	0.66	0	0.06
0.1	0.09	0.1	0.095	0.01	-0.005
0.26	0.23	0.26	0.245	0.03	-0.015
0.2	0.2		0.2	-0.2	0
0.2	0.25	0.25	0.25	0	0.05
0.5	0.45	0.46	0.455	0.01	-0.045
0.2	0.15	0.15	0.15	0	-0.05

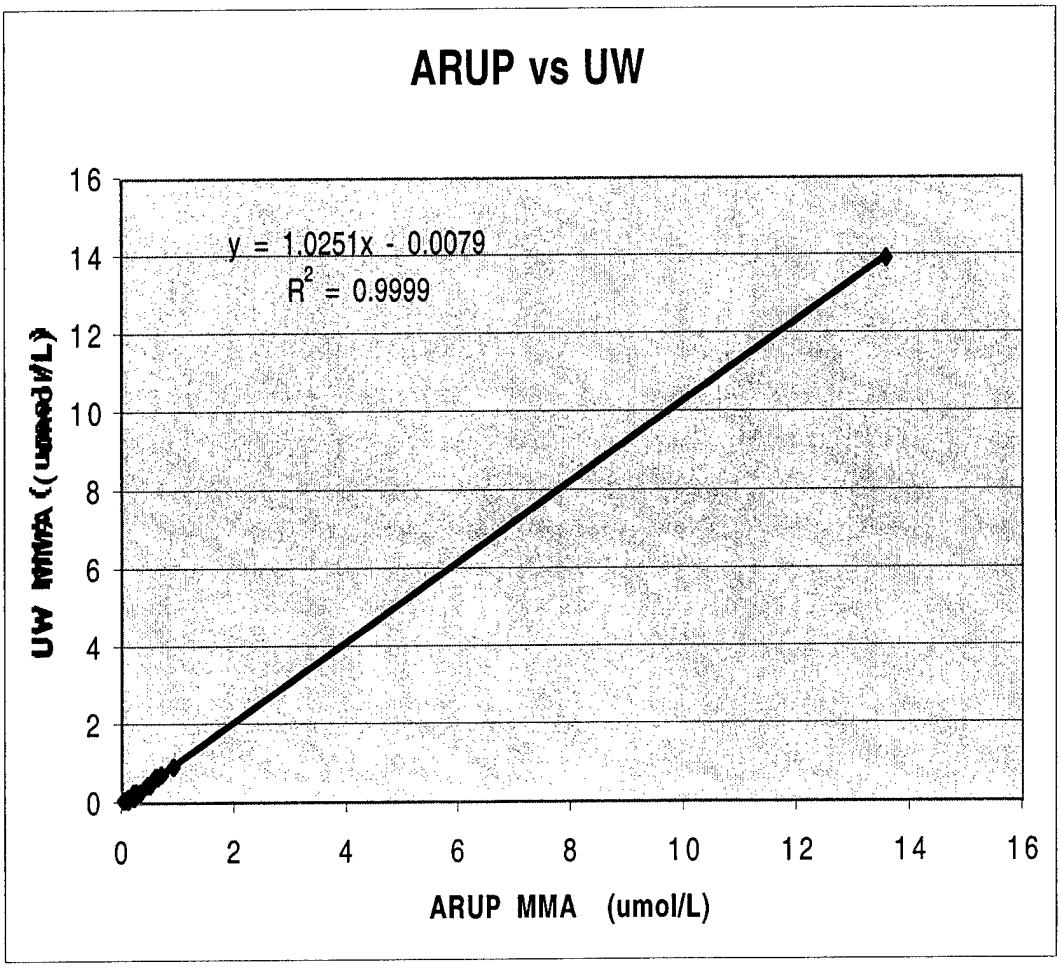


Figure 14. Correlation studies including the highest result (13.57 μmol/L).

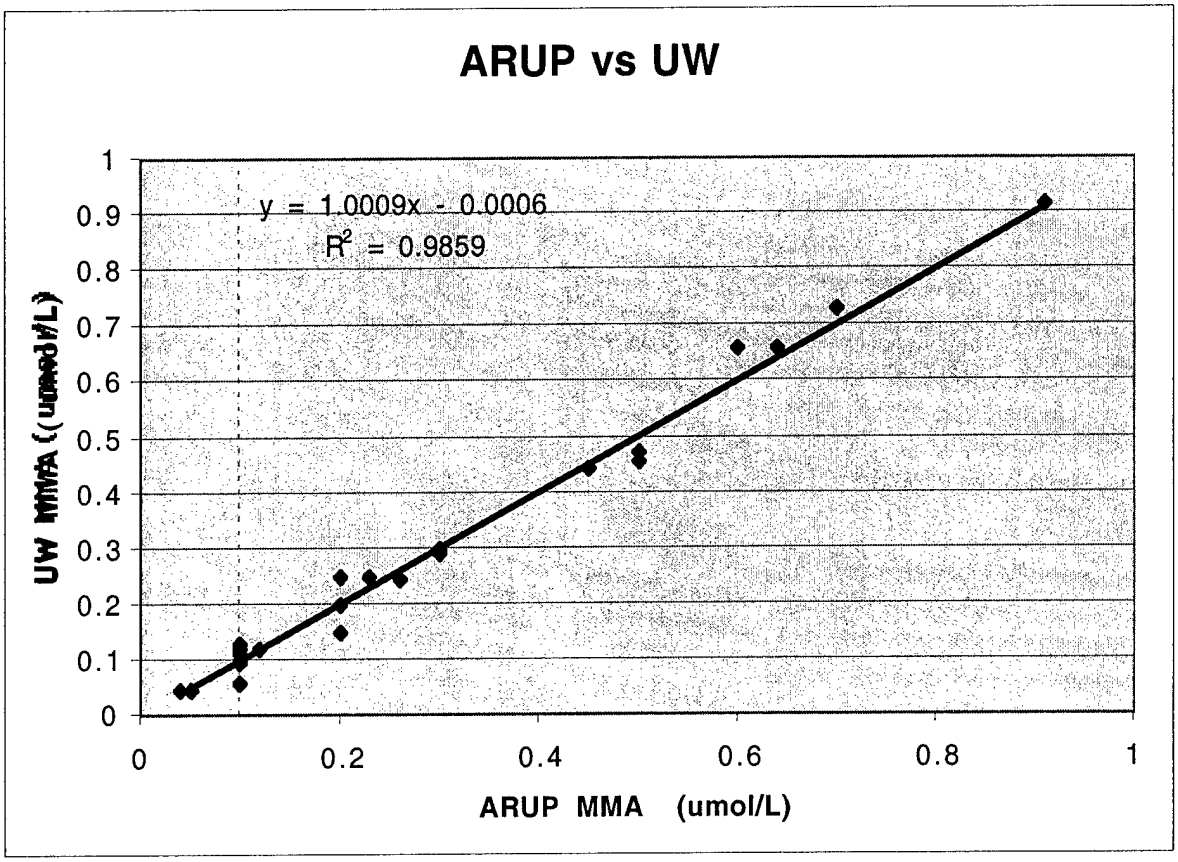


Figure 15. Correlation results without the highest result

NOTE: Correlation (excluding the 13.57 umol/L point) was greater than 98.59% between the UW method and the ARUP method. The method was considered to be valid between 0.3 and 13.6 umol/L.

9. REFERENCE RANGE STUDIES

Table 10. Normal Reference Range Studies

FIRST RUN	SECOND RUN	AVERAGE (umol/L)
0.3	0.29	0.295
0.26	0.26	0.26
0.26	0.26	0.26
0.05	0.05	0.05
0.05	0.05	0.05
0.25	0.29	0.27
0.29	0.26	0.275
0.25	0.25	0.25
0.29	0.3	0.295
0.25	0.29	0.27
0.29	0.3	0.295
0.42	0.4	0.41
0.3	0.29	0.295
0.26	0.25	0.255
0.05	0.05	0.05
0.08	0.1	0.09
0.3	0.26	0.28
0.46	0.42	0.44
0.25	0.25	0.25
0.46	0.46	0.46
0.08	0.1	0.09
0.26	0.26	0.26
0.26	0.3	0.28
0.37	0.37	0.37
0.24	0.26	0.25
0.19	0.19	0.19
0.22	0.26	0.24
0.29	0.29	0.29
0.4	0.4	0.4
0.19	0.23	0.21

0.25	0.29	0.27
0.4	0.4	0.4
0.42	0.4	0.41
0.26	0.23	0.245
0.42	0.4	0.40
0.26	0.23	0.245
0.17	0.2	0.185
0.19	0.19	0.19
0.43	0.4	0.415
0.2	0.19	0.195
0.4	0.36	0.38
0.36	0.36	0.36
0.23	0.23	0.23
0.29	0.29	0.29
0.36	0.4	0.38
0.44	0.4	0.42
0.47	0.44	0.455
0.3	0.29	0.295
0.3	0.27	0.285
0.1	0.12	0.11
0.13	0.13	0.13
0.27	0.3	0.285
Mean		**0.27**
SD		**0.11**
Median		**0.27**
Max		**0.46**

Normal population reference range was established using frozen serum samples from Madigan Army Medical Center, Fort Lewis, WA. A total of 50 serum samples from healthy women were run in duplicate. The age range was 17 to 51 years old and the serum samples were drawn in January 2002, and kept frozen at - 20° C up to the testing date. The normal serum reference range for woman was estimated to be 0.05 –0.46 μmol/L. (Table 8).

CHAPTER 5

1. HYPOTHESIS

THE HYPOTHESIS IS THAT, BREAST CANCER IS ASSOCIATED WITH AN INCREASED RISK OF VITAMIN B_{12} DEFICIENCY. FUNCTIONAL VITAMIN B_{12} DEFICIENCY CAN BE DETECTED WITH HIGH SENSITIVITY BY MEASUREMENT OF SERUM METHYLMALONIC ACID (MMA). THE EXPERIMENTAL APPROACH WILL INVOLVE DETERMINING THE PREVALENCE OF ELEVATED METHYLMALONIC ACID (MMA) IN SERUM OF WOMEN DIAGNOSED WITH BREAST CANCER, WITH BREAST CANCER SERUM OBTAINED USING SERUM SPECIMENS ALREADY TESTED FOR THE BREAST CANCER TUMOR MARKER CA27.29.

2. CA27.29 TUMOR MARKER

The tumor marker CA27.29 is considered a valuable marker for breast cancer management. One comparison study involving tumor markers CA27.29 and CA153 in a wide variety of patients affected with breast cancer, showed that the marker CA27.29 was more sensitive than CA15.3 in detecting low antigen concentrations (below the cut-off point).[56] Both markers showed comparable results in healthy controls, with higher levels in post-menopausal women than pre-menopausal ones. Tumor marker CA27.29 proved to be more sensitive than CA15.3 in monitoring limited variations of tumor extension.[56]

Elevation of blood tumor markers such as CA27.29 generally correlates with the stage of breast cancer. The major role of current blood markers is in monitoring of metastatic disease. Tumor marker measurement is now used as a complimentary test in the diagnosis of symptomatic metastases.[57] Clinical studies are performed to optimize the use of blood markers in both advanced disease and early primary breast cancer.

Another tumor marker study suggested that CA27.29 is more sensitive and more specific than CEA, but that it is similar to CA15-3 for metastatic breast cancer detection and monitoring.[58]

Tumor marker CA27.29 was compared for diagnostic accuracy with CA15.3 in regard to discrimination between primary breast cancer versus healthy subjects.[59] Investigators claim that more prospective studies are necessary to confirm the above conclusion. CA27.29 is considered to have prognostic significance for predicting recurrent breast cancer patients with stage II and III disease.[60]

3. SERUM MMA MEASUREMENTS IN SERA OF BREAST CANCER PATIENTS - METHODS

To verify the proposed hypothesis the following analysis was performed:

a. Using a protocol approved by the University of Washington Human Subjects Committee, serum from patients having measurement of the breast cancer marker CA27.29 was obtained, and the concentration of MMA was measured in those specimens. All of the patients whose specimens were analyzed had a diagnosis of breast cancer. The breast cancer patients were divided into four groups according to the disease status, as assessed clinically. Patients were classified as indicated in Table 12, page 54.

Table 11. Classification criteria for breast cancer population

0 remission, no evidence of disease
1 localized disease, primary diagnosis
2 local, recurrent disease
3 regional , recurrent, disease
4 metastatic (wide spread , for example .liver, lung, bone)

b. Different groups of breast cancer patients were compared with each other and with the normal female population. All data were analyzed using the t-test two-sample analysis assuming unequal variances (Microsoft Excel, Redmond, WA). Tables below show the results of the compared data.

The CA27.29 tumor marker population samples were obtained from the Immunology Division at the UWMC after completion of testing for the tumor marker. Random selection was applied in selecting the serum frozen samples. The lowest CA27.29 level was < 7 and the highest tested was 2860. Table 13 includes all 45 CA27.29 tested samples. Female age range was from 31 to 80 years old.

Table 12. Ca27.29 Serum MMA Measurement in Breast Cancer Sera

CA27.29 Results	AGE	FIRST RUN	SECOND RUN	AVERAGE (umol/L)
22	52	0.45	0.45	0.45
23	61	0.46	0.45	0.455
11	42	0.35	0.38	0.365
18	45	0.23	0.2	0.215
18	44	0.33	0.27	0.3
26	49	0.25	0.31	0.28

25	70	1.33	1.4	1.365
17	43	1.7	1.66	1.68
34	41	1.14	1.03	1.085
6	35	0.34	0.37	0.355
35	77	0.34	0.36	0.35
47	48	0.8	0.91	0.855
36	48	0.92	0.84	0.88
595	49	0.68	0.64	0.66
780	73	0.6	0.6	0.6
76	64	1	0.8	0.9
560	69	0.6	0.6	0.6
30	69	0.25	0.3	0.275
11	50	0.45	0.4	0.425
16	47	2.4	2.1	2.55
20	39	0.47	0.47	0.47
23	41	0.45	0.48	0.465
26	58	0.73	0.6	0.665
26	56	0.24	0.25	0.245
26	64	0.04	0.01	0.025
14	45	0.59	0.58	0.585
23	57	0.4	0.47	0.435
22	49	0.64	0.6	0.62
910	45	13.3	13.31	13.305
2860	57	18.63	18.6	18.615
7	43	0.8	0.8	0.8
343	80	0.8	0.8	0.8
29	48	0.47	0.4	0.435
505	72	1.46	1.26	1.36
30	73	0.33	0.4	0.365
38	84	0.87	0.74	0.805
946	53	1.27	1.19	1.23
520	80	0.53	0.6	0.565
797	62	0.73	0.6	0.665
28	31	0.33	0.27	0.3
50	55	1.6	1.6	1.6
34	59	0.8	0.8	0.8
10	33	0.13	0.13	0.13
28	69	0.44	0.47	0.435
9	41	3.2	3.2	3.2

Mean	1.44331852
STD	3.2414606
Median	0.6
Min	0.025
Max	18.615

Table 13 shows the CA27.29 population grouped according to the classification of the extent of breast cancer.

Table 13. CA27.29 population classification

MMA FIRST RUN Results	MMA SECOND RUN Results	MMA CONCENTRATION (AVERAGE OF 2 RUNS) µmol/L	Classification of Breast Cancer
0.46	0.45	0.46	0
0.35	0.38	0.37	0
0.33	0.27	0.30	0
0.25	0.31	0.28	0
0.34	0.37	0.36	0
0.34	0.36	0.35	0
0.25	0.3	0.28	0
0.45	0.48	0.47	0
0.24	0.25	0.25	0
0.04	0.01	0.03	0
0.4	0.47	0.44	0
0.33	0.27	0.30	0
0.44	0.47	0.44	0
3.2	3.2	3.20	0
0.45	0.45	0.45	1
1.14	1.03	1.09	1
0.45	0.4	0.43	1
0.59	0.58	0.59	1
1.27	1.19	1.23	1
0.13	0.13	0.13	1
0.23	0.2	0.22	2
1.7	1.66	1.68	2
0.8	0.91	0.86	2
0.92	0.84	0.88	2
0.6	0.6	0.60	2
2.4	2.1	2.55	2
0.47	0.47	0.47	2
0.8	0.8	0.80	2
0.47	0.4	0.44	2
0.33	0.4	0.37	2
0.87	0.74	0.81	2
0.68	0.64	0.66	4
0.6	0.6	0.60	4
0.64	0.6	0.62	4

13.3	13.31	13.31	4
18.63	18.6	18.62	4
1.46	1.26	1.36	4
0.53	0.6	0.57	4
0.73	0.6	0.67	4
1.6	1.6	1.60	4
0.8	0.8	0.80	4
0.8	0.8	0.80	4

Mean MMA	**1.44**
STD	**3.24**
Median	**0.60**
Min	**0.03**
Max	**18.62**

Note: The range of measured MMA in the breast cancer population was from 0.03 to 18.62 μmol/L

3. WITHIN-POPULATION ANALYSIS.

CA27.29 group 0 was compared with the group 4. CA27.29 group 0 included patients in remission or free of disease symptoms. The mean MMA in sera of patients in group 0 (0.53) was lower than the mean serum MMA in patients in group 4 (3.42), however the t-test shows no statistically significant difference between the mean of the two subgroups.

Table 14. t-Test of Difference Between Mean Serum MMA in Breast Cancer group 0 (remission, no evidence of disease) vs group 4 (metastatic disease)

t-Test: Two-Sample Assuming Unequal Variances
v1= MMA in group 0 vs v2=MMA in group 4

	Variable 1	*Variable 2*
Mean	0.53464286	3.42125
Variance	0.60125563	35.70419 15
Observations	14	12
Hypothesized Mean Difference	0	
df	11	
t Stat	-1.6615245	
P(T<=t) one-tail	0.06240582	
t Critical one-tail	1.79588369	
P(T<=t) two-tail	0.12481164	
t Critical two-tail	2.20098627	

5. COMPARISON OF MMA MEASUREMENTS IN SERA FROM A NORMAL REFERENCE POPULATION VERSUS UWMC BREAST CANCER PATIENTS

The mean serum MMA in the normal female population was 0.27 μmol/L, with variance 0.011, and the mean serum MMA in the breast cancer patient population was 1.41 μmol/L, with variance 10.75. The difference between these groups was statistically significant by the t-test, with p=0.024. For additional data analysis, the mean serum MMA concentration results were compared against the MMA in different subgroups of the breast cancer population.

Table 15.
t-Test: Two-Sample Assuming Unequal Variances
v1=nl, v2 = group 0 cancer

	Variable 1	*Variable 2*
Mean	0.2722	0.53464286
Variance	0.01125935	0.60125563
Observations	50	14
Hypothesized Mean Difference	0	
df	13	
t Stat	-1.2630879	
P(T<=t) one-tail	0.11437111	
t Critical one-tail	1.7709317	
P(T<=t) two-tail	0.22874222	
t Critical two-tail	2.16036824	

Table 16 shows data comparison between the normal population and group 0 CA27.29. The difference was not statistically significant.

Table 16. Comparison of the normal female population and breast cancer group 4 (patients with metastatic disease).

t-Test: Two-Sample Assuming Unequal Variances
v1=nl, v2=group 4 ca

	Variable 1	*Variable 2*
Mean	0.2722	3.037142 86
Variance	0.01125935	31.16526 43
Observations	50	14
Hypothesized Mean Difference	0	
df	13	
t Stat	-1.8530742	
P(T<=t) one-tail	0.04335103	
t Critical one-tail	1.7709317	
P(T<=t) two-tail	**0.086702**	
t Critical two-tail	2.16036824	

The statistical analysis using the t-test shows that there is no significant difference between the normal population and the class 4 cancer patients with metastases. However, group 4 MMA mean is quite higher than the normal population. There are different possibilities that can cause an increase in serum methylmalonic acid, i.e. low levels of the tissue form of vitamin B_{12}. Some of the patients underwent radiation and chemotherapy for extensive periods of time. Also, some patients are on tumor marker cancer suppressors like metrotrexate that can cause impaired folate metabolism. Under these circumstances it is possible that the folate/vitamin B_{12} and methionine common pathway can be disrupted due to low

availability of the enzyme cofactors. It is also possible that enhanced use of vitamin B12 by the tumor leads to low levels of cobalamin for other tissues. Furthermore, altered metabolism by malignant cells could directly lead to elevated production of MMA, which would be reflected by elevated serum levels of MMA. However, statistically the difference is not significant. for the few numbers of studied samples.

Table 17. Normal population vs cancer group 1 comparison.

t-Test: Two-Sample Assuming Unequal Variances v1=nl, v2 =group 1 cancer		
	Variable 1	*Variable 2*
Mean	0.2722	0.5925
Variance	0.01125935	0.21614167
Observations	50	4
Hypothesized Mean Difference	0	
df	3	
t Stat	-1.3750378	
P(T<=t) one-tail	0.13140781	
t Critical one-tail	2.35336302	
P(T<=t) two-tail	0.26281562	
t Critical two-tail	3.18244929	

No significant statistical difference was found.

Table 18. Normal population MMA study vs CA27.29 group 2 (local, recurrent disease breast cancer patients).

t-Test: Two-Sample Assuming Unequal Variances
v1=nl, v2=group 2
CA27.29

	Variable 1	*Variable 2*
Mean	0.2722	0.87772727
Variance	0.01125935	0.45934682
Observations	50	11
Hypothesized Mean Difference	0	
df	10	
t Stat	-2.9552339	
P(T<=t) one-tail	0.00720254	
t Critical one-tail	1.81246151	
P(T<=t) two-tail	**0.014405**	
t Critical two-tail	2.22813924	

Data analysis shows significant difference between the two populations.

Since the breast cancer sera had previously been tested for levels of the tumor marker CA27.29, the correlation between serum MMA concentrations and concentrations of CA27.29 could be determined. The two analyzed values correlated well, with a high statistical significance in the breast cancer population. Correlation coefficient was r=0.80, $p<10^{-10}$. The high correlation was largely accounted for by two specimens with high values of both MMA and CA27.29.

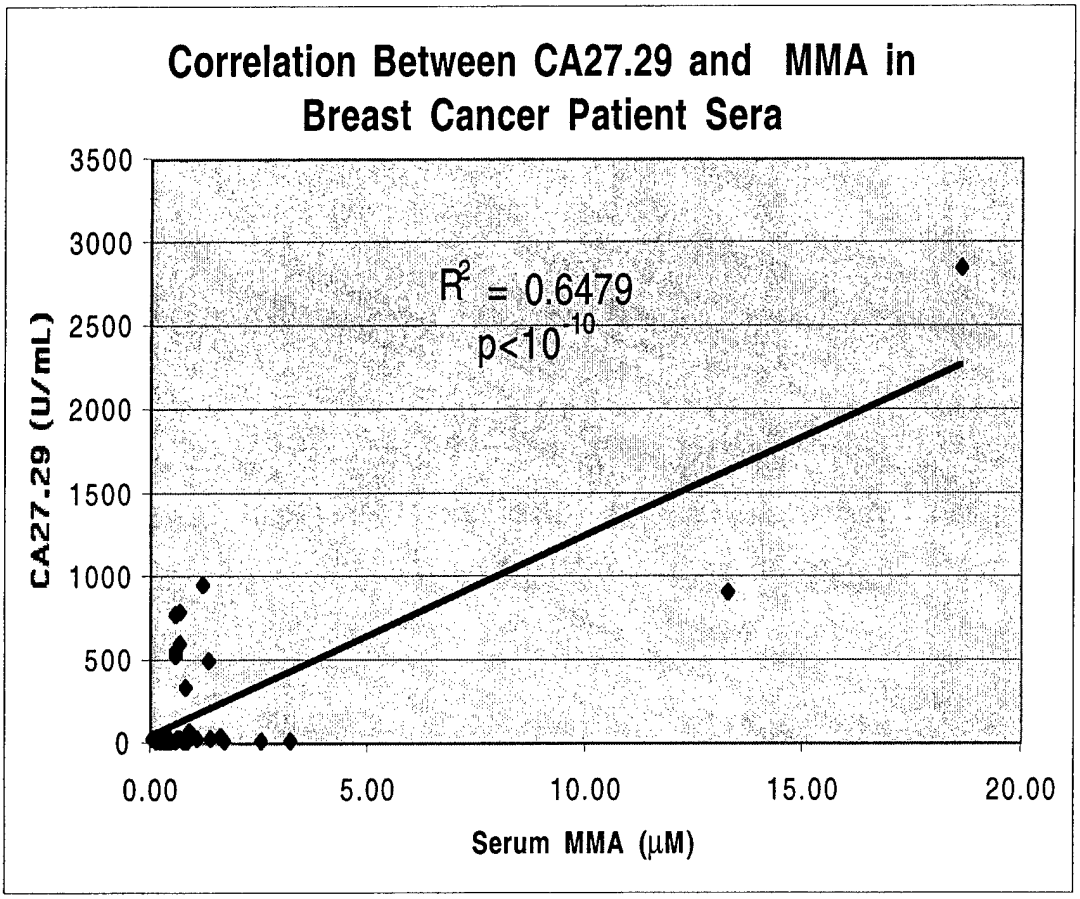


Figure 16. Serum MMA versus CA27.29 tumor marker

6. DATA ANALYSIS CONCLUSIONS.

No statistically significant difference was discovered between the normal population and the breast cancer group 4, except between the normal population and breast cancer group 2 (local, recurrent). These data suggest that the advanced breast cancer patients, especially patients with recurrent disease, exhibit low tissue cobalamin levels. The subgroups of patients with breast cancer studied showed no statistical significan between-group difference in serum MMA concentrations.

There was substantial variation in the serum MMA concentrations in the same breast cancer group. Even among patients with the same classification of the extent of breast cancer, the variation in serum MMA concentrations was substantial. The concentration of serum MMA showed good correlation with the CA27.29 level.

CHAPTER 6

1. RESEARCH SUMMARY

To analyze the level of methylmalonic acid level in the frozen serum samples from females tested for breast cancer the following steps were taken:

a. A new serum assay was developed to detect serum methylmalonic acid.
b. The MMA test method was evaluated for precision, accuracy, sensitivity, recovery, linearity and carry-over phenomenon.
c. Method correlation studies were performed using split serum samples versus the reference ARUP laboratories in Salt Lake City.
d. Normal serum samples were collected at Madigan Army Hospital in Tacoma and the normal reference range was calculated using duplicate analysis. A total of 50 serum samples were used to establish the normal range.
e. Frozen leftover CA27.29 tested serum samples were collected randomly from UWMC Immunology Lab. All samples were tested in duplicates and the CA27.29 population reference range was calculated using 45 patient samples.
f. A t-test statistical comparison was done between the both normal and CA27.29 populations.
g. Several t-test data analysis was done within the CA27.29 population after staging in four groups.

2. DISCUSSION

For the normal reference range analysis only serum for females were used during this research. The scope of this was to keep all data within the same gender category for better and closer comparison.

The results of this study showed that serum methylmalonic acid is statistically higher in the recurrent breast cancer population (group 2) tested against a normal adult female population. A high serum MMA indicates a low tissue vitamin B_{12} level. Most of CA27.29 patients tested in the lower end of the pathologic MMA range that starts above the 0.46 μmol/L. These data indicates that some of the patients can still test normal for serum cobalamin, despite the slight low tissue cobalamin level. We can conclude that for

some breast cancer patients a serum MMA analysis will be more appropriate to detect an early stage of vitamin B_{12} deficiency.

By comparing serum MMA between the normal population and the stage two recurrent CA27.29 group was detected a high statistical difference in the analyzed data. It looks that recurrent/local cancer patients, grouped under stage 2 CA27.29 population, showed the highest serum MMA at group level, and consequently a lower tissue cobalamin level.

The final correlation was done between the serum MMA concentrations and the CA27.29 for breast cancer population. The serum MMA and CA27.29 levels correlated with high statistical significance with a correlation coefficienct r=0.80. The results suggest that there is a significant potential risk of developing tissue cobalamin deficiency in breast cancer patients with increased tumor marker Ca27.29.

In conclusion, the research study done on the breast cancer patients demonstrated that serum methylmalonic acid was significantly higher in recurrent /local breast cancer population than serum MMA in the normal female population. Serum methylmalonic acid can be used to identify cancer patients that are at risk of developing tissue cobalamin deficiency before serum vitamin B_{12} can do it.

CITED REFERENCES

1. Fruton Simmonds, Yale University. Vitamins and Growth Factors, *Gen Biochem* 2nd ed 1958. p. 976-1002.

2. Larry G. Scheve, California State University. The Vitamins, *Elements of Biochemistry* 1984. p. 394-402.

3. Robert H. Allen, Sally P. Stabler, David G. Savage, John Lindenbaum. Diagnosis of Cobalamin Deficiency I: Usefulness of Serum Methylmalonic Acid and Total Homocysteine Concentrations. *Am J Hematol* 1990(34):90-98.

4. Fairbanks VF, Klee GG. Burtis CA, Ashwood ER. Biochemical Aspects of Hematology. *Tietz, Textbook of Clin Chem* 3rd ed 1999. p.1690-1698.

5. Snow CF. Laboratory diagnosis of vitamin B_{12} and folate deficiency: a guide for the primary care physician [Review]. *Arch Intern Med* 1999(159):1289-1298.

6. Chad DA, ed. Immunoassay automation: an updated guide to systems. New York: Academic Press, 1996.

7. Lindgren A, Kilander A, Bagge A, Nexo E. Holotranscobalamin- a sesitive marker of cobalamin malabsorption. *Eur J Clin Investig* 1999(29):321-329.

8. Wickramasinghe SN, Ratnayaka ID. Limited value of serum holo-transcobalamin II measurements in the differential diagnosis of macrocytosis. *J Clin Pathol* 1996(49):755-758.

9. Refsum H, Ueland PM, Nygård O, Vollset SE. Homocysteine and cardiovascular disease. Annual Review Med 1998(49):31-62.

10. Donner MG, Klein GK, Mathes PB, Schwandt P, Richter WO. Plasma total homocysteine levels in patients with early-onset coronary heart disease and a low cardiovascular risk profile. *J Clin Pathol* 1998;47(3):273-9.

11. Allen, L.H. & Casterline, J. Vitamin B_{12} deficiency in the elderly; diagnosis and requirements. *Am J Clin Nutrition* 1994(60): 12-14.

12. Carmel R, Sinow RM, Siegel ME, Samloff IM. Food cobalamin malabsorption occurs frequently in patients with unexplained low serum cobalamin levels. *Arch Intern Med* 1988(148):1715-9.

13. George G. Klee; Cobalamin and Folate Evaluation: Measurement of Methylmalonic Acid and Homocysteine vs Vitamin B_{12} and Folate. *Clin* Chem 2000;46(8B):1277-1283.

14. Bottiglieri T. Folate, vitamin B_{12} , and neuropsychiatric disorders. *Nutr Rev* 1996(54):382-390.

15. Metz J, Bell AH, Flicker L, Bottiglieri T, Ibrahim J, Seal E, Schultz D. The significance of subnormal serum vitamin B_{12} , concentration in older people: a case control study. *J Am Geriatr Soc* 1996(44):1355-1361.

16. Carmel R, Rasmussen K, Jacobsen DW, Green R. Comparison of the deoxyuridine test with serum levels of methylmalonic acid and homocysteine in mild cobalamin deficiency. *Br J Haematol* 1996(93):311-318.

17. Gabor Komaromy-Hiller, Kern L. Nuttall, Edward R. Ashwood. Effect of Storage on Serum Vitamin B_{12} and Folate Stability. *Annals Clin Lab Sci* 1997(27):249-253.

18. Lindenbaum J, Savage D, Stabler SO, Allen RH. Diagnosis of cobalamin deficiency. II. Relative sensitivities of serum cobalamin, methylmalonic acid and total homocysteine concentrations. *Am J Hematol* 1990(34):99-107.

19. L. Moelby, K. Rasmussen, M.K. Jensen , K. O. Pedersen. The relationship between clinically confirmed cobalamin deficiency and serum methylmalonic acid. *J Intern Med* 1990(228):373-378.

20. Lindenbaum J, Healton EB, Savage DG et al. Neuropsychiatriac disorders caused by cobalamin deficiency in the absence of anemia or macrocytosis. *N Engl J Med* 1988(318):1720-8.

21. Carmel R. Pernicious anemia, the expected findings of very low serum cobalamin levels, anemia and macrocytosis are often lacking. *Arch Intern Med* 1998(148): 1712-4.

22. Stopeck, Alison. Links between Helicobacter pylori infection, cobalamin deficiency, and pernicious anemia. *Arch of Internal Med* 2000(160):1220-30.

23. Werbach, Melvyn R. Nutritional strategies for treating chronic fatigue syndrome. *Alternative Medicine Review* 2000;2(5):93-108.

24. Benito-Leon, Julian and Porta-Etessam, Jesus. Shaky-leg syndrome and Vitamin B_{12} deficiency. *New Engl J Med* 2000;342(13):981.

25. Garg, Rekha, et al. *Niacin treatment decreases plasma homocysteine levels.* Am Heart J, Vol. 138, December 1999, pp. 1082-87.

26. Chao, Chia-Lun, et al. Effect of short-term vitamin (folic acid, vitamins B_6 and B_{12}) administration on endothelial dysfunction induced by post-methionine load hyperhomocysteinemia. *Am J Cardio* 1999(84):1359-61.

27. Turley, Charles P. and Brewster, Marge A. Alpha-tocopherol protects against a reduction in adenosylcobalamin in oxidatively stressed human cells. *J Nutr* 1993(123):1305-12.

28. Norman EJ, Morrison JA. Screening elderly populations for cobalamin (vitamin B_{12}) deficiency using the urinary methylmalonic acid assay by gas chromatography mass spectrometry. *Am J Med* 1993(94):589-594.

29. Lindenbaum, John, et al. Prevalence of cobalamin deficiency in the Framingham elderly population. *Am J of Clin Nutr* 1994(60):2-11.

30. Allen, Lindsay H. and Casterline, Jennifer. Vitamin B_{12} deficiency in elderly individuals: diagnosis and requirements. *Am J Clin Nutr* 1994(60):12-14.

31. Sumner, Anne E., et al. Elevated methylmalonic acid and total homocysteine levels show high prevalence of vitamin B-12 deficiency after gastric surgery. Ann Int Med 1996;124(5):469-76.

32. McCaddon, A. and Kelly, C.L. Familial Alzheimer's disease and vitamin B_{12} deficiency. *Age and Aging* 1994(23):334-37.

33. Anne E. Sumner, Margaret M. Chin, Janet L. Abrahm, Gerard T. Berry, Edward J. Gracely, Robert H. Allen, Sally P. Stabler. Elevated methylmalonic acid and total homocysteine levels show high prevalence of vitamin B_{12} deficiency after gastric surgery. *Ann Int Med* 1996;(124):469-476.

34. Herbert V. Cobalamin deficiency and neuropsychiatric disorders (letter). *N Engl J Med* 1988;319:1733

35. Bronstrup, Anja, et al. Effects of folic acid and combinations of folic acid and vitamin B-12 on plasma homocysteine concentrations in healthy, young women. *Am J Clin Nutr* 1998(68):1104-10.

36. Hokin, Bevan D., Butler Terry. Cyanocobalamin (vitamin B-12) status in Seventh-day Adventist ministers in Australia. *Am J Clin Nutr* 1999(70): 576S-78S.

37. Wu, K., et al. A prospective study of folate, B12, and pyridoxal 5'-phosphate (B6) and breast cancer. *Cancer Epidemiol. Biomarkers Prev.* 1999:209-17.

38. Paul M. Newberne, Mary Locniskar. Roles of Micronutrients in Cancer Prevention: Recent Evidence from the Laboratory. *Recent Progress in Research on Nutrition and Cancer*, Wiley-Liss,Inc.1990:119-134.

39. Rasmussen K, Moelby L, Jensen MK. Studies on methylmalonic acid in humans. II. Relationship between concentrations in serum and urinary excretion, and the correlation between serum cobalamin and accumulation of methylmalonic acid. *Clin Chem* 1989: 2277-80.

40. Karsten Rasmussen. Studies on Methylmalonic Acid in humans. I. Concentrations in Serum and Urinary Excretion in Normal Subjects after Feeding and during Fasting, and after Loading with Protein, Fat, Sugar, Isoleucine, and Valine. *Clin Chem* 1989;35(12):2271-2276.

41. Mark M. Kushnir, Gabor Komaromy-Hiller. Optimization and performance of a Rapid gas chromatography-mass spectrometry analysis for methylmalonic acid determination in serum and plasma. J of Chromatography 2000;741(B): 231-241.

42. Kolhouse, J. F., and R. H. Allen. Recognition of two intracellular cobalamin-binding proteins and their identification as methylmalonyl-CoA mutase and methionine synthetase. *Proc. Natl. Acad. Sci. USA* 1997(74):921-925.

43. K. Rasmussen, J. Moller, K. Ostergaard, M.O. Kristensen, J. Jensen, *Clin Chem* 1990(36):1295.

44. Rasmussen K. Solid-phase sample extraction for rapid determination of methylmalonic acid in serum and urine by a stable-isotope-dilution method. *Clin Chem* 1989(35):260-4.

45. Norman EJ. Urinary methylmalonic acid/creatinine ratio defines tissue Cobalamin Deficiency [Letter]. *Br J Haematol* 1998(100):614-5.

46. David G. Savage, M.D., John Lindenbaum, M.D., New York, New York, Sally P. Stabler, M.D., Robert H. Allen, M.D., Denver, Colorado. Sensitivity of Serum Methylmalonic Acid and Total Homocysteine Determinations for Diagnosing Cobalamin and Folate Deficiencies. *Am J of Med* 1994(96).

47. Paul D. Marcell, Sally P. Stabler, Elaine R. Podell, and Robert H Allen. Quantitation of Methylmalonic Acid and Other Dicarboxylic Acids in Normal Serum and Urine Using Capillary Gas Chromatography-Mass Spectrometry. *Analytical Biochem* 1985(150): 58-66.

48. Sally P. Stabler, Paul D. Marcell, Elaine R. Podell, Robert H. Allen, and John Lindenbaum. Assay of Methylmalonic Acid in the Serum of Patients with Cobalamin Deficiency Using Capillary Gas Chromatography-Mass Spectrometry. *J. Clin. Invest* 1986(77):1606-1612.

49. Hølleland G., Schneede J., Ueland P.M.,Lund P. K., Refsum H., Sandberg S. Cobalamin deficiency in General Practice. Assessment of the Diagnostic Utility and Cost-Benefit Analysis of Methylmalonic Acid Determination in relation to Current Diagnostic Strategies; *Clin Chem* 1999;45(2);189-198.

50. Pfeiffer C. M., Smith S. J., Miller D. T., Gunter E. W. Comparison of Serum and Plasma Methylmalonic Acid Measurements in 13 Laboratories: An International Study. *Clin Chem* 1999;45(12): 2236-2242.

51. Schneede J,Ueland PM. Application of capillary electrophoresis with laser-induced fluorescence detection for routine determination of methylmalonic acid in human serum. *Anal Chem* 1995(67):812-9.

52. Analytical Biochem Hewlett Packard. How to start up the GCD system and verify performance. *HP G1800C GCD Series II User's Guide* 1997:14-398.

53. ARUP Laboratories. Methylmalonic Acid in Serum or Plasma by GC/MS: *Procedure Number META001* 1999.

54. Saria E, Roth T, Ball R, Haver V., Prunty J., Delaney C. Method Evaluation Manual. VA Medical Center, *Clin Chem Lab* 1985.

55. University of Washington Medical Center, Chemistry Div. *Method Evaluation Procedures*; Rev Feb-2000;1-13.

55. Gion M, Mione R, Leon AE, Luftner D, Molina R, Possinger K, Robertson JF. CA27.29 : a valuable marker for breast cancer management. A confirmatory multicentric study on 603 cases. *Eur J Cancer* 2001 Feb;37(3):355-63.

56. Cheung KL, Graves CR, Robertson JF. Tumor marker measurements in the Diagnosis and monitoring of breast cancer. *Cancer Treat Rev* 2000 Apr;26(2):91-102.

57. Hou MF, chen YL, tseng TF, Lin CM, Chen MS, Huang CJ, Huang YS, Hsieh JS, Huang TJ, Jong SB, Huang YF. Evaluation of Serum CA27.29, CA15-3 and CEA in patients with breast cancer. *Kaohsiung J Med Sci* 1999 Sep;15(9):520-8.

58. Gion M, Mione R, Leon AE, Dittadi R. Comparison of the diagnostic accuracy of CA27.29 and CA15.3 in primary breast cancer. *Clin Chem* May1999 Jan;45(5):630-7.

59. MacLean GD, Reddish MA, Longenecker BM. Prognostic significance of Preimmunotherapy serum CA27.29 (MUC-1) mucin level after active specific immunotherapy of metastatic adenocarcinoma patients. *J Immunother* 1997 Jan;20(1):70-8.