UNIVERSITÀ DEGLI STUDI DI ROMA "TOR VERGATA"

FACOLTA' DI MEDICINA

DOTTORATO DI RICERCA IN BIOTECNOLOGIE MEDICHE E MEDICINA MOLECOLARE

XXII° CICLO

Rapid and simple gas chromatography-mass spectrometry method for quantification of methylmalonic acid in plasma and urine. Its application to the diagnosis of methylmalonic acidurias.

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A.A. 2009/2010

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Acknowledgement

I wish to express my sincere appreciation and gratitude to Dr.Cristiano Rizzo, my tutor, for the dedicated guidance and encouragement given to me during the course of my research and in the preparation of this thesis.

I greatly indebted to my coordinator Prof.Giorgio Federici as well as Prof.Sergio Bernardini, Prof.Ambruzzi and Prof. Francesco Ciaraldi who gave me the opportunity to prosecute my research career.

I am also thankful for assistance given to me by Prof. Maurizio Muraca and the laboratory staff of the Analysis Laboratory of "Bambin Gesù" Hospital.

I am greatful to Dr.Federica Pavilli, Dr. Marta Scandurra, Dr. Capulli and the all the staff members of Comunità "Ripa Grande" assisting me to get out of difficulties

Finally I express my heartfelt gratitude to my mother, sister, brother in- law and nephew.

Abbreviations

GC/MS Gas chromatography mass spectrometry

MMCoA Methylmalonyl-CoA

MMA methylmalonic acid

MMAuria methylmalonic aciduria

Me-Cbl methylcobalamin

Ado-Cbl adenosylcobalamin

BSTFA N,O-bis (trimethylsylil)trifluoroacetamide

SUCL succinate-CoA ligase

Riassunto in Italiano

L'aciduria metilmalonica rappresenta un gruppo di acidurie organiche che comprendono principalmente difetti metabolici della metilmalonil-CoA mutasi e difetti del metabolismo della cobalamina.
Le acidurie metilmaloniche (MMAs) sono un gruppo eterogeneo di errori congeniti del metabolismo caratterizzate biochimicamente dall'accumulo di metilmalonato nelle urine e in altri fluidi corporei. Le MMAs che coinvolgono esclusivamente l'attività enzimatica della metilmalonil-CoA mutasi (mut^0, mut^-,cblA and cblB) sono definite MMAs isolate. I difetti che influiscono sul metabolismo della metilcobalamina (MeCbl), cofattore di più cicli metabolici, presentano un contemporaneo accumulo di acido metilmalonicico ed omocistinuria (cblC,cblD,cblF).
Sebbene l'eziologia delle acidurie metilmaloniche sia eterogena il quadro clinico dei pazienti é simile.
Nella maggior parte dei pazienti la sintomatologia si presenta, in età neonatale, con vomito ricorrente, disidratazione, epatomegalia, sofferenza respiratoria, ipotonia muscolare e progressiva alterazione dello stato di coscienza con probabile evoluzione in malattia ingravescente, coma profondo e morte.
Le alterazioni di laboratorio più importanti sono una severa cheto-lattico acidosi, un' ipo o iperglicemia, una neutropenia, un'iperglicinemia ed un'iperammoniemia che, se non corrette con un pronto e specifico intervento terapeutico possono portare a gravi handicap o morte.
Abbiamo sviluppato un metodo rapido e sensibile in cromatografia gassosa e spettrometria di massa utilizzando il metodo di diluizione isotopica per la quantificazione dell'acido metilmalonico nelle urine e nel plasma, utile per la diagnosi delle MMAs e nel follow up.

La diluizione isotopica con isotopi stabili è considerato il metodo gold standard per la misurazione dei metaboliti perché essendo l'isotopo lo standard ideale, risolve il problema dell' efficienza dell'estrazione nei metodi che si avvalgono dell' estrazione con solvente.

Il metodo richiede fasi successive: l'estrazione dalle urine e dal plasma con estrazione liquido-liquido, l'essiccazione dell'estratto e infine la derivatizzazione con BSTFA dei prodotti di estrazione per formare i trimetilsilil derivati prima di essere iniettati nel GC/MS.

Il metodo è stato validato seguendo un piano che comprende il campo di applicazione del metodo, le caratteristiche di efficacia del metodo e dei suoi limiti di accettazione.

I parametri esaminati nel processo di validazione sono stati: limite di rivelazione e quantificazione, accuratezza, precisione, linearità e il recupero del MMA.

Per il MMA plasmatico con l'aggiunta dell'analita, il limite di rilevazione o il limite del bianco (media più 3 SD del bianco) è stato di 0,1 μmol/l ed il limite di quantificazione (media più 10 SD del bianco) 0.8 μmol/l; per il MMA urinario con l'aggiunta dell'analita, il limite di rilevazione o limite del bianco (media più 3 SD del bianco) è stato di 0,1 μmol/l ed il limite di quantificazione (media più 10 SD del bianco) 0,7 μmol/l. Il range di linearità per le urine ed il plasma è compreso tra 0,1-400 μmol/l.

I valori normali di acido metilmalonico (plasma e urine) sono stati ottenuti da 50 pazienti sani di controllo. Il metodo è stato validato clinicamente analizzando 10 campioni prelevati da pazienti con tre varianti fenotipiche della aciduria metilmalonica. Il metodo oltre alla suo uso nella routine clinica potrebbe essere utilizzato come metodo nei programmi screening.

Abstract

Methylmalonic aciduria is a group of organic aciduria associated with methylmalonyl-CoA mutase and cobalamin metabolic defects. Methylmalonic acidurias (MMAuria) are a heterogeneous group of inborn metabolic errors biochemically characterized by the accumulation of methylmalonate in urine and other body fluids. MMAuria exclusively affecting MCM activity (mut^0,mut^-,cblA and cblB) are termed isolated MMAuria. Some of these defects additionally affect the metabolism of methylcobalamin (MeCbl) resulting in combined MMA and homocysteinuria (cblC,cblD,cblF).

Although the causes of Methylmalonic acidurias (MMAuria) are etiology, the clinical presentation of affected patients is similar. The majority of patients display early in life recurrent vomiting, dehydration, hepatomegaly, respiratory distress, muscular hypotonia and progressive alteration of consciousness, probably degenerating to overwhelming illness, deep coma and death. Severe combined keto- and lactic acidosis, hypo- or hyper-glycaemia, neutropenia, hyperglycinaemia and hyperammonaemia are the most important laboratory features. Without specific therapy these episodes result in severe handicap or death.

We developed a rapid and sensible GC/MS method using stable isotope dilution to diagnose and quantify methylmalonic acid in urine and plasma. Stable isotope dilution is an ideal internal standard and is considered the gold standard method for measuring metabolites because it obviates the problem of varying levels of extraction efficiency by solvent extraction methods.

Our methodology is capable of monitoring and quantifying MMA and it can follow up on and diagnose methylmalonic acidurias.

The method requires a sequence of steps: extraction from urine and plasma using liquid–liquid extraction, drying, and finally

derivatization by BSTFA of extraction products to form trimethylsilyl derivates prior to GC/MS injection.

The method was validated. The validation follows a plan that includes the scope of the method, the method performance characteristics and its acceptance limits. Parameters examined in the validation process were limit of detection and quantification, reproducibility, linearity and recovery. For blood MMA with added analyte, the limit of detection or limit of the blank (mean plus 3 SD of blank) for was 0.1 μmol/l and the limit of quantification (mean plus 10 SD of blank) 0.8 μmol/l; For urine MMA with added analyte, the limit of detection or limit of the blank (mean plus 3 SD of blank) for was 0.1 μmol/l and the limit of quantification (mean plus 10 SD of blank) 0.7 μmol/l. For urine and plasma the linearity range was from 0.1 μmol/l to 400 μmol/l.

Normal methylmalonic acid values were obtained from plasma and urine of 50 healthy control patients. The method was clinically validated by analyzed 10 samples from patients with three phenotypic variant of methylmalonic aciduria and therefore could be suitable for implementation in routine clinical screening programs and quantification environments.

Index

Rapid and simple gas chromatography-mass spectrometry method for quantification of methylmalonic acid in plasma and urine. Its application to the diagnosis of methylmalonic acidurias.

1.0 Introduction

We developed a rapid and sensitive method for the quantification of methylmalonic acid in urine and plasma that permits the diagnosis of methylmalonic acidurias.

Methylmalonic acidurias (MMAuria) are an heterogeneous group of inborn errors of metabolism which are biochemically characterized by the accumulation of methylmalonate in urine and other body fluids. They are caused by a defect of the mitochondrial enzyme methylmalonyl-CoA mutase (MCM) or by one of the many defects in the uptake, transport or synthesis of 5´-deoxyadenosylcobalamin (Ado-Cbl), the cofactor of MCM.[1] Some of these defects additionally affect the metabolism of methylcobalamin (MeCbl) resulting in combined MMA and homocysteinuria (cblC,cblD,cblF).

MMAs exclusively affecting MCM activity (mut^{0},mut $^{-}$,cblA and cblB) are termed isolated MMAs. The MCM deficiencies caused by mutations in the apomutase locus are further subdivided into defects without (mut^{0}**)** and with (mut $^{-}$) residual activity. MCM, a dimer of identical subunits situated in the mitochondrion, is part of the catabolic pathways of the amino acids isoleucine, valine, methionine, and threonine (50%) as well as of odd-chain fatty acids (30%), gut-flora derived (20%) and cholesterol side-chains linking the degradation of these metabolites to the tricarboxylic acid cycle.[2] Recently a few patients have been described with mild MMAuria associated with mutations of the MMCoA epimerase gene or with neurological symptoms due to *SUCL* mutations.

We have developed a new, rapid and simple method for the determination of methylmalonic acid in urine and plasma. The method requires a sequence of steps: extraction from urine and plasma using liquid–liquid extraction, drying, and finally derivatization by BSTFA of extraction products to form trimethylsilyl derivatives prior to GC/MS injection.

The method was validated. The validation followed a plan that included the method's aim, the method's performance characteristics

and its acceptance limits. The validation process parameters were limits of detection and quantification, reproducibility, linearity and recovery.

Normal methylmalonic acid values were obtained from 50 healthy control patients. The method was clinically validated by the analysis of 10 samples from patients with various methylmalonic acidurias.

2.0 Gas Chromatography/Mass spectrometry (GC/MS)

GC/MS is an analytical instrument technique using a gas chromatograph and a mass spectrometer. In general, the GC is used to separate complex chemical mixtures into individual components. Once separated, the chemicals can be identified and quantified by the mass spectrometer. Before analysis can occur a sample must be prepared, usually by extracting the analytes of interest in a liquid solvent phase. This extract is then injected into the GC where it is swept onto a separation column by an inert carrier gas such as hydrogen or helium. The analytes in the mixture are carried through the column by the carrier gas where they are separated from one another by their interaction between the coating (stationary phase) on the inside wall of the column and the carrier gas. Each analyte interacts with the stationary phase at different rates. Those that react very little move through the column quickly and will exit into the mass spectrometer before those analytes having longer interaction and retention times.

When the individual analytes exit the GC column they enter the ionization area (ion source) of the MS. Here they are bombarded with electrons, to form ionized fragments of the analyte. These ionized fragments are then accelerated into the quadrupole via a series of lenses and separated by their mass-to-charge ratio. This separation is accomplished by applying alternating RF frequency and DC voltage to

diagonally opposite ends of the quadrupole, which in turn allows a specific mass fragment to pass through the quadrupole filter. From here the fragments enter the mass detector (electron multiplier) and are recorded. The MS computer graphs a mass spectrum scan showing the amount of each type of ionized mass fragment.

Operation of a GC/MS in SIM mode allows for detection of specific analytes with increased sensitivity relative to full-scan mode. In SIM mode the MS gathers data for masses of interest rather than looking for all masses over a wide range. Because the instrument is set to look for only masses of interest it can be specific for a particular analyte of interest. Typically two to four ions per compound are monitored and the ratios of those ions will be unique to the analyte of interest. In order to increase sensitivity, the mass scan rate and dwell times (the time spent looking at each mass) are adjusted. When properly set up and calibrated, GC/MS-SIM can increase sensitivity by a factor of 10 to 100 times that of GC/MS-Full Scan. Because unwanted ions are being filtered, the selectivity is greatly enhanced providing an additional tool to eliminate difficult matrix interferences. The ability of the mass spectrometer to identify unknowns in the full-scan mode and quantify known target analytes in the SIM mode, makes it one of the most powerful tools available for trace level quantitative analysis in the lab today.

3.0 Methylmalonic acidurias

Although the causes of Methylmalonic acidurias (MMAuria) are various, the clinical presentation of affected patients is similar. The majority of patients present, early in life, recurrent vomiting, dehydration, hepatomegaly, respiratory distress, muscular hypotonia and progressive alteration of consciousness, probably degenerating to overwhelming illness, deep coma and death. Severe combined keto- and lactic acidosis, hypo- or hyper-glycaemia, neutropenia, hyperglycinaemia and hyperammonaemia are the most important laboratory features. Without specific therapy these episodes result in severe handicap or death.[3]

MMAS are classified into:

1) Isolated methylmalonic methylmalonic aciduria
2) Combined methylmalonic methylmalonic aciduria and homocystinuria

3.1 Isolated methylmalonic aciduria (MMAuria)

Isolated methylmalonic aciduria (MMAuria) is a heterogeneous group of rare autosomal recessive metabolic disorders caused by a severe deficiency or a lack of activity of the mitochondrial methylmalonyl-CoA mutase (MCM) enzyme which requires adenosylcobalamin (AdoCbl) as a cofactor. A defect in either mutase apoenzyme or AdoCbl synthesis results in an accumulation of methylmalonic acid in body tissues and body fluids without homocysteine accumulation. Methylmalonyl-CoA (the co-enzyme A ester of methylmalonic acid) is formed during the conversion of propionyl-CoA into succinyl-,CoA, a Kreb's cycle intermediate. There are three enzymatic reactions required to generate succinyl-CoA from propionyl-CoA. The first step converts propionyl-CoA to D- methylmalonyl-CoA by propionyl-CoA carboxylase, a biotin-containing enzyme. Methylmalonyl-CoA racemase is thought to act at the second step, the conversion of D-

methylmalonyl-CoA into L-Methylmalonyl-CoA. In the last reaction, methylmalonyl-CoA mutase catalyzes the isomerization of L-methylmalonyl-CoA to succinyl-CoA[4] **(Figure 1)**. All the above reactions are reversible but the pathway proceeds in the direction of succinyl-CoA formation[5].

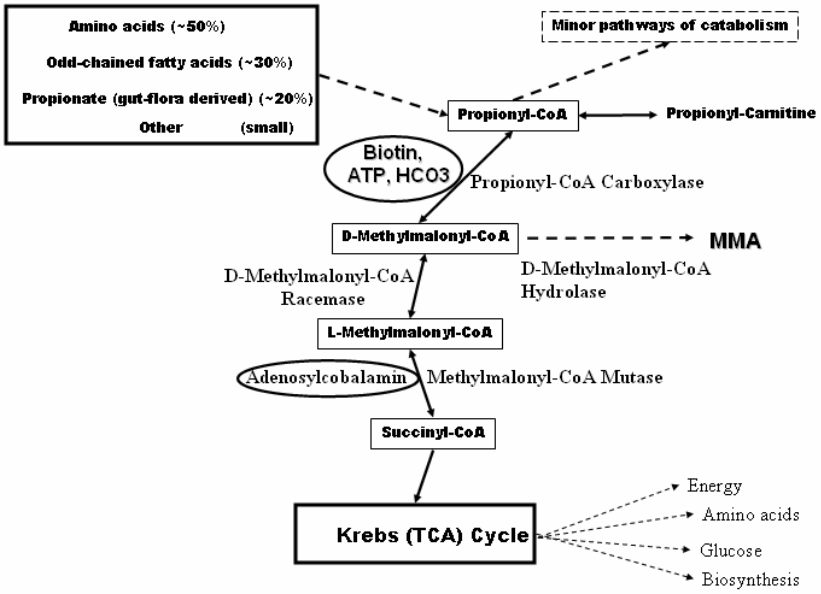


Figure 1. Major pathway of the conversion of propionyl-CoA into succinyl-CoA. The precursors are indicated with their approximated contribution to whole-body propionate metabolism in the fasting state [Thompson et al 1990][4]. The biotin-dependent enzyme propionyl-CoA carboxylase converts propionyl-CoA into D-methylmalonyl-CoA, which is then racemized into L-methylmalonyl-CoA and isomerized into succinyl-CoA, a Krebs cycle intermediate. The L-methylmalonyl-CoA mutase reaction requires adenosylcobalamin, an activated form of vitamin B_{12}.

Deficiency of MMCoA mutase apoenzyme due to mutations of the MUT gene can be differentiated into mut^0 and mut^- defects (OMIM 251000) belonging to a single genetic complementation class.

Differentiation of these defects is based on the presence (mut^-) or absence (mut^0) of residual enzyme activity in culture fibroblasts and response to vitamin B_{12} *in vitro* and *in vivo*. MMCoA mutase is a mitochondrial enzyme requiring 5´-deoxyadenosylcobalamin. The gene (MUT) maps to 6p21 and its full-length cDNA codes for a 750-aminoacid protein of 88.2 kDa. The gene contains 13 exons spanning more than 35 kb and so far approximately 200 mutations have been identified. The enzyme contains an N-terminal mitochondrial leader sequence of 32 amino acids and the mature form is a dimer of identical subunits of 78.5kDa. Each subunit contains two main functional domains, an N-terminal $(\beta\alpha)_8$ barrel (residues 88-422) with the substrate binding site and a C-terminal $(\beta\alpha)_5$ Ado-Cbl binding domain (residues 578-750). The two domains are connected by a long linking region (residues 423-577).[1]

The cblA (OMIM 251100) and cblB (OMIM 251110) complementation groups are linked to processes unique to Ado-Cbl synthesis and cause isolated MMAuria.

Genes for the cblA (*MMAA*) and cblB (*MMAB*) groups have recently been described [6,7].

The MMAA gene is located at 4q31.1-1-q31.2 and spans about 17.1kb containing 7 exons, but exon 1 is untranslated. The full-length cDNA codes for a 418-amino-acid protein of 46.5kDa. At least 28 mutations have been identified in 37 cblA patients. The cblA protein contains an N-terminal mitochondrial leader sequence and cleavage site as well as Walker A and B ATP-binding motifs, a Mg^{2+} binding site and a GTP-binding site, but its exact function is unclear. It was first thought to be responsible for the translocation of cobalamin into mitochondria prior to the final steps of AdoCbl synthesis.[6] More recent evidence, however, points to a role of the protein in the assembly and stabilization of holo-MMCoA mutase analogues to its bacterial homologue meaB. This enzyme forms a complex with MMCoA

mutase, stimulates its activity and protects the enzyme from irreversible deactivation.[5]

The MMAB genes for cobalamin adenosyltransferase map to 12q24; it consists of 9 exons extending over 18.87kb and its full-length cDNA codes for a 250-amino-acid protein (27.3kDa).[7] The cblB enzyme contains a leader sequence and signal cleavage site consistent with localization to the mitochondrion. At least 24 mutations associated with low Ado-Cbl synthesis and mainly involving amino acids from the active site have been found in patients in the cblB complementation group.[6]

Matsui, *et al.*[8] surveyed the histories of 45 patients with these etiologies: 15 mut^0, 5 mut^- 14 *cbl*A, and 11 *cbl*B. Equal numbers of males and females were in each group. The most common signs and symptoms at the onset of clinical difficulty are listed in **Table 1** and were similar in all etiologies. Patients in mut^0 class presented symptoms earlier than those in other groups. Whereas 80 percent of children in the mut^0 class fell ill in the first week of life, less than half the children in the three other groups were ill during this interval.[8] Furthermore, clinical onset occurred in 90 percent of the mut^0 patients before the end of the first month, whereas onset beyond the first month was observed in an appreciable fraction of patients of the other three groups. The laboratory findings in affected patients (cblA, cblB, mut^-, mut0) show marked similarity between the etiologies (metabolic acidosis, ketonemia/ketonuria, hyperammonemia, hyperglycinemia/glycinuria, leucopenia, anemia, thrombocytopenia, pancytopenia and normal serum cobalamin concentrations).

Table 1 Signs and symptoms at the onset

Signs and Symptoms at Onset	*Mutant Class*				
	cblA	*cblB*	*mut*$^{-}$	*mut*^{0}	*Total*
Lethargy	78	83	100	85	84
Failure to thrive	75	86	40	77	73
Recurrent vomiting	58	86	80	77	73
Dehydration	64	86	100	62	71
Respiratory distress	89	67	50	55	67
Muscular hypotonia	44	57	33	91	63
Developmental retardation	36	33	25	65	47
Hepatomegaly	11	67		57	41
Coma	50	29	40	38	40
Numerical values represent percentage of patients in each group.[8]					

A number of signs of pathology involving various organ systems have been documented and characterized to some degree: chronic renal insufficiency[9-10] may be associated with renal tubular acidosis and chronic tubulointerstitial nephritis[11-12], hepatic steatosis[9-12], ataxia and mental retardation possibly resulting from demyelination of subcortical and other neurons and characteristic lesions in the globus pallidus (evident from MR and CT imaging)[13-14], as with failure of linear growth (seen in some short and obese patients)[17,15]. These signs may be due to methylmalonate inhibition of marrow stem-cell growth in a concentration-dependent fashion[16], while other neurological consequences observed[17] (including some in animal models) may be due to excessive methylmalonate levels[15]or to the effects of hyperammonemia[17]

3.1.1 Physiological disturbances in isolated mutase deficiency (mut^0, mut^-, cblA, & cblB)

All studies *in vivo* and *in vitro* in patients with methylmalonic aciduria indicate that the primary block in the conversion of methylmalonyl-CoA to succinyl-CoA explains the methylmalonate accumulation and accompanying biochemical changes.[1] However, primary block does not explain several important physiological disturbances such as acidosis, hypoglycemia, hyperglycinemia, and hyperammonemia. Oberholzer, *et al*[8] suggested an explanation for the observed acidosis: that methylmalonyl-CoA might be "trapping" the cellular supply of coenzyme A, leading to impaired carbohydrate metabolism. Alternatively, methylmalonyl-CoA might interfere with gluconeogenesis,[19] leading directly to hypoglycemia, and the subsequent increase in lipid catabolism could cause ketoacidosis. Halperin, *et al.*[20] showed that methylmalonate inhibited the transmitochondrial shuttle of malate and argued that impairment of this key step in gluconeogenesis could lead to hypoglycemia. Treacy, *et al.*[21] have suggested that a deficiency of glutathione may also contribute to lactic acidosis in these patients.

Additionally, in methylmalonic aciduria the accumulated organic acids or their CoA esters inhibit intramitochondrial glycine cleavage and an enzyme associated with the urea cycle.[22-23] These are probable causes of hyperglycinemia and hyperammonemia in affected children. Carnitine deficiency results from decreased renal handling of filtered carnitine and the excretion of acylcarnitine derivatives formed from organic acids.[24,25,26] This deficiency may contribute to muscle hypotonia and other clinical findings **(Tables 1)**. Each of the four etiologies for isolated methylmalonyl-CoA mutase deficiency are inherited as autosomal-recessive traits. The prevalence of methylmalonic acidemia is difficult to define precisely. One survey in Massachusetts suggested an occurrence of 1:48,000 infants,[27] while

another in Quebec suggested 1:61,000 infants.[28] Others have suggested a figure of 1:29,000.[27,29]A greater prevalence between 1:1,000 and 1:2,000 has been reported in Middle Eastern populations.[30]

3.2 Combined methionine synthase and methylmalonyl-CoA mutase deficiency

Combined methylmalonic aciduria (MMAuria) and homocystinuria is a genetically heterogeneous disorder of cobalamin (cbl; vitamin B12) metabolism. The defect causes decreased levels of the coenzymes adenosylcobalamin (AdoCbl) and methylcobalamin (MeCbl), which results in decreased activity of the respective enzymes methylmalonyl-CoA mutase (MUT; 609058) and methyltetrahydrofolate:homocysteine methyltransferase, also known as methionine synthase (MTR; The defects in this category differ from isolated mutase deficiency in that they demonstrate both methylmalonic acidemia and homocystineuria.

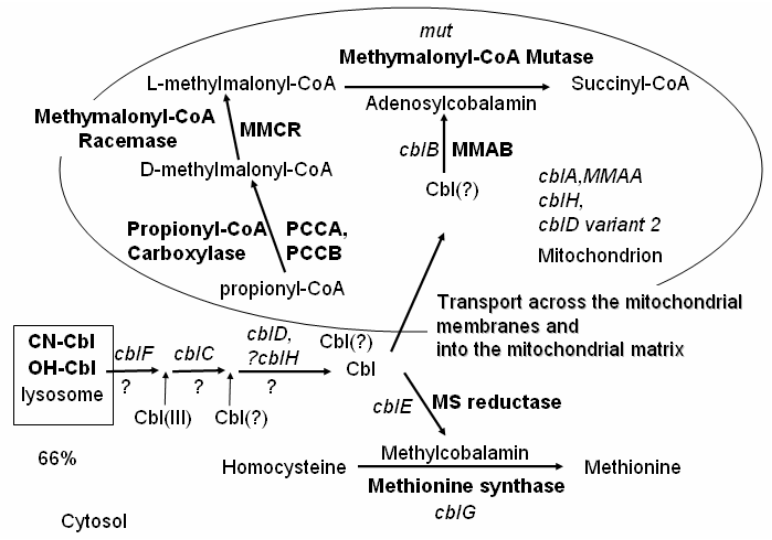


Figure 2. Pathway of cellular processing of cobalamin (OH-cbl). The class and genes associated with isolated methylmalonic acidemia are:
cblH, ? unknown gene [Watkins et al 2000][31]
cblA (MMAA)
cblB (MMAB)
cblD variant 2 [Suormala et al 2004][32]
mut (MCM)

Figure 2 describes the current understanding of cobalamin metabolism in cells. Cells from these children comprise three biochemically and genetically distinct complementation groups, designated *cbl*C, *cbl*D, and *cbl*F. [33,34,35, 36] Of these, the *cbl*C defect is inherited as an autosomal-recessive trait,[31] but the mode of inheritance of *cbl*D and *cbl*F defects is not yet known. Among more than 100 patients characterized by *cbl*C defect, clinical findings have varied widely and some cases were diagnosed only in adult life. In a review of 50 patients,[36] 44 had onset in the first year of life and 6 had onset after 6 years of age, and 13 early-onset patients died. The clinical presentation and laboratory findings of *cbl*C patients are given in **Table 2**.

Additionally, six patients have been reported in the *cbl*F group. The clinical and laboratory findings from patients in this category are presented in **Table 3**

The patient's cells were defective in the release of cobalamin from the lysosome into the cytoplasm. The cells also showed indirect evidence of methylmalonyl-CoA mutase and methionine synthase activity, as well as inability to synthesize AdoCbl and MeCbl. After incubation in labelled vitamin B12, accumulation was found in lysosomes (Vassiliadis et al., 1991)[37]. *In vitro* studies showed decreased synthesis of AdoCbl and MeCbl, and accumulation of unmetabolized free cobalamin.

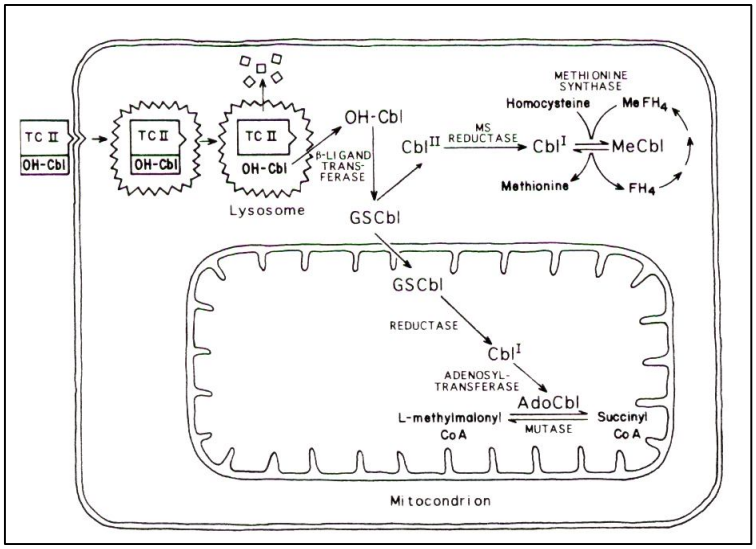


Figure 3. Pathway of cobablamin (vitamin B_{12}; Cbl) uptake. Dietary Cbl is endocytosed into a target cell as the TC II/OH-Cbl complex.[36] The free Cbl is released into the cytosol after lysozomal processing. From there it is reduced to either the MeCbl or AdoCbl coenzymes. [TC II = transcobalamin II; OH-Cbl = hydroxocobalamin; GSCbl = glutathionylcobalamin; MeCbl = methylcobalamin; $MeFH_4$ = methyltetrahydrofolate; AdoCbl = adenosylcobalamin; Cbl^{III}, Cbl^{II}, and Cbl^{I} = cobalamins with cobalt valence of 3^+, 2^+, and 1^+, respectively.]

Figure adapted from Fenton, WA, Gravel, RA, & Rosenblatt, DS. "Disorders of Propionate and Methylmalonate Metabolism," in Scriver, CR, *et al.* (editors) *The metabolic and molecular basis of inherited disease*. Volume II, 2001, p 2165.[1]

Table 2. Clinical presentation and laboratory findings in patientswith *cblC* type combined methylmalonyl CoA mutase and methionine synthase functional deficiencies

Early-Onset (≈88%)	Late-Onset (≈12%)
Eating difficulties	Decreased cognitive performance
Hypotonia	Confusion
Failure to thrive	Dementia
Seizures	Delirium
Microcephaly	Myelopathy
Develop-mental delay	Tremor
Cortical atrophy	Pigmentary retinopathy[c]
Hydrocephalus	Skin lesions[a]
Nystagmus	Megaloblastic[b] anemia
Pigmentary retinopathy	Thrombocytopenia[b]
Decreased visual acuity	Leukopenia[b]
Megaloblastic anemia	Neutropenia[b]
Thrombo-cytopenia	Cbl = Normal
Leukopenia	Folate = Normal
Neutropenia	
Renal failure[d]	
[a]reported by Howard, *et al.*[39,40]	
[b]hematological abnormalities observed in half the later-onset patients	
[c]only one case observed	
[d]only a few cases observed.	

Table 3. Clinical and Laboratory findings in *cblF* type combined deficiency disorder

Clinical signs and symptoms[41,42,43,44]	**Laboratory findings**
Small size for gestational age/Inadequate weight gain	Methylmalonic aciduria
Poor eating	Cbl malabsorption
Failure to thrive	Macrocytosis
Developmental delay	Homocysteinuria
Persistent stomatitis	Hypoglycemia
Growth retardation	Thrombocytopenia
Minor facial anomalies	Neutropenia
Dextrocardia	Anemia
Persistent rash	Low serum Cbl
Premature arthritis	
Confusion	
Disorientation	
Pigmentary dermatitis	
Aspiration pneumonia (at birth)	
Hypotonia	
Lethargy	
Recurrent infections	
Gastroesophageal reflux	

3.3 Miscellaneous disorders with mildly elevated MMA

3.3.1 Methylmalonyl-CoA epimerase deficiency

Bobik and Rasche (2001) [45]demonstrated that MCEE has DL-methylmalonyl-CoA racemase activity and determined that the MCEE gene contains 3 exons. The deduced 176-amino acid protein contains an N-terminal mitochondrial targeting sequence. Bikker et al. (2006)[46] suggested that deficiency of methylmalonyl-CoA epimerase does not have much clinical impact and could even be considered a non-disease.

3.3.2 Atypical MMA with neurological symptoms due to SUCL mutations

The succinate-CoA ligase (SUCL) enzyme complex catalyses the conversion of succinyl-CoA to succinate in the tricarboxylic acid cycle. Two forms of the complex exist, each comprising a common alpha unit and one of the two different beta subunits which impose specificity for either GDP or ADP in the reaction. Mutations of the ADP-forming beta subunits SUCLA2 are associated with depletion of mitochondrial DNA and deficiency of complexes I,III, IV of the respiratory chain.[47]

Fatal lactic acidosis of neonatal onset with mtDNA depletion was recently described in two patients with mutations in the alpha subunit, SUCLG1 with mildly increased urinary MMA (68mmol/mol creatinine) [48]

4.0 Materials and method

4.1 Solvents, standards and reagents

Ethylacetate, pyridine, hydroxyl chloride acid 30%, high-purity chromatography solvent grade were purchased (Sigma-Aldrich , St. Louis, MO). NaCl was purchased from Merck (Darmstadt, Germany). Hydroxylamine hydroxychloride and methylmalonic acid were obtained from Sigma (St.Luis MO, USA). Methylmalonic acid d3 was obtained from CDN Isotopes (Pointe-Claire, Quebec, Canada). Hydroxylamine (25 g/L) was dissolved in pyridine.

Stock solutions of 10 mmol/L of methylmalonic acid and labelled methylmalonic acid d3 (internal standard) were prepared in distilled water and stored at –40° C. (stable for 2 years).

Daily working solutions of methylmalonic and labelled methylmalonic acid d3 (internal standard) were prepared by diluting 1/10 of mother solution using methanol and stored at +4° C.

The chemical and internal standards storage temperature was -40°C. Under these conditions the stability of the standard solutions was at least 6 months.

Silylating reagents N,O-bis (trimethylsilyl)trifluoroacetamide (BSTFA) were obtained from Supelco (Park Bellefonte, PA, USA).

4.2 Glassware

Extraction tubes were 8 ml screw-capped Pyrex glass tubing (14x 100mm) with a Teflon-lined cap (Barcorword Scientific, Ltd, Stone Staff, UK) and the conical GC-MS vial was capped with siliconized rubber septum to avoid plastic and derivatization artefacts (Chromacol, Ltd. Welwyn Garden City, UK)

4.3 Columns and GC-MS instrument

Gas chromatography separation was carried out on Agilent J&W ULTRA2 (Methyl Siloxane 5% phenyl-) 25m x 0.20 mm x 0.33 µm fused silica capillary column. The gas chromatograph was a 6890 connected to 5973 or to 5975 single quadrupole mass detector both from Agilent (Palo Alto CA, USA).

The vial samples were kept at room temperature until analysis and were analyzed no later than 24 h after preparation.

4.4 Urine samples

Urine samples should be collected for 24 hours, when possible. But since prolonged urine collections are quite difficult to perform, especially in newborns or very young babies, a random sample, preferably the first morning voiding, is acceptable. Interpretation of results Similar to one-day collection is possible [49]although the amounts of many organic acids excreted per milligram of creatinine vary widely by age. Newborns and small babies have incompletely developed renal tubular function and normally excrete larger amounts of aliphatic acids. All urine samples have to be accompanied by a form containing information concerning clinical history, therapy and diet details of the last 48 hours. This is critical to correct interpretation of the data.

Urine should be collected in clean glass containers to avoid contamination of plastic compounds (e.g. phthalates) preferably sterile. Other protein-free physiological fluids should be collected in the same way. All patients undergoing organic acids analysis should cease any drug administration when possible to avoid artefacts due to drugs or drug metabolism. Since most patients are acutely ill, the latter procedure is often not practical.

4.5 Storage

The urine should be analyzed immediately or frozen at -70°C until analysis. If a -70°C freezer is not available, urine samples must be frozen at -20°C. In fact, especially if containers are not sterile, prolonged standing at room temperature enables bacterial growth, artifacts production and the formation of new organic acids.[50] Urine samples should be transported deep-frozen, preferably packed in solid carbon dioxide at -70°C.

Some authors suggested adding 2 ml of chloroform or merthiolate [o-(carboxyphenyl)thio]ethylmercury] sodium salt to the urine sample as preservative. .Merthiolate is toxic by inhalation, ingestion and in contact with skin , with a danger of cumulative effects. It is also very toxic to aquatic organisms and may cause long-term adverse effects in aquatic environments.

The procedure of adding chloroform or merthiolate as preservative is seldom used.

4.6 Creatinine assay

Creatinine (2-imino-1methyl-4-imidazolidinone) concentration is the reference for each organic acid concentration, for quantitative purposes.

This molecule has been declared unsuitable as reference standard due to its increasing excretion with age and to its wide variation among individuals, especially small babies and newborns. Many differences are moreover reported between males and females.

The accurate determination of creatinine prior to analysis is extremely important. Many analytical procedures are reported for the creatinine assay:

-alkaline-picrate-based method (a modified Jaffe method). This method has been reported as less susceptible than conventional methods to interference from non-creatinine Jaffe-positive compounds. Creatinine reacts with picrate (lithium picrate 25

mmol/l)in presence of a strong base, in this case sodium hydroxide (NaOH 100 mmol/l), forming a red chromophore (λ 510 nm). The rate of absorption is directly proportional to the creatinine concentration in the sample and it is measured using a bichromatic (λ 510 nm and λ 600 nm) rate technique (Siemens, Dimension®, clinical chemistry system or similar). Bilirubine, a potential interferon, is oxidized by using potassium ferricyanide $K_3Fe(CN)_6$ 0.13 mmol/l.

4.7 Extraction procedures

Solvent extraction: (Tanaka K clin chem. 1980)[51]

A volume of urine corresponding to 25 µl or 500 µl of plasma was placed in a 14 X 100 mm screw-cap culture tube, the pH was adjusted to 1 by drop-wise addition of 100µl 6 mol/L HC1 and 100µl of hydroxyl amine chloride in pyridine.

NaCl was added to the acidified sample until saturation and then was extracted successively three times with 2ml aliquots of ethyl acetate (or diethyl ether), with vigorous shaking.

After shaking the mixtures had to be centrifuged (3000 rpm, 10 minutes). The organic layers were combined in a second tube with glass pasture pipettes. The extract was evaporated under a nitrogen stream at 60°C. When the volume was about 1-1.5 ml, the solution was transferred to a 2ml glass vial and the remaining solvent was evaporated. In our personal experience, before the derivatization step, the samples, well capped with silicon caps, were stable for about 1 week at +4°C.

4.8 Derivatization procedures

After extraction procedures, when ready for analysis, in order to make the acids more volatile and stable for gas chromatography, a derivatization step is performed. Most labs commonly use *trimethylsylilation* while alkylation reactions such as methylation are not used. For the widely used trimethylsisylation step, 100 µL of(N,O-bis (trimethylsilyl)trifluoroacetamide) (BSTFA) was added to the

evaporated sample, vortex-mixed and incubated at 60°C for 30 min. The molecular weight of trimethylsilyl (TMS) derivate is determined from M-15 peak in the spectra, which should be a prominent high-mass ion in the spectrum. If two high-mass peaks separated by 15 mass units are observed in TMS derivate, then the highest mass peak is usually the molecular weight. If a high mass peak of odd mass is observed and a peak 15 mass units above is absent, then TMS derivate molecular is the odd mass plus 15 mass units. Identification of mass spectra could be easily obtained by comparison with an electronic spectra library database.

4.9 Gas chromatography/mass spectrometry analysis

The GC analyses were performed on a 6890N gas chromatograph with a single quadrupole mass spectrometer (5973 or 5975) as detector (Agilent Technologies, Palo Alto, CA, USA). The capillary column is typically non-polar-column such as J&W ULTRA2 or similar. The carrier gas is helium at 1ml/min as initial and constant flow, the column oven initial temperature is 70°C, a programmed ramp 7°C/min to 140°C and then ramp 40°C/min to 300°C for 2 minutes. The injection volume is 1 µl; the front inlet is at 250°C and works in split less mode. The detector temperature is typically about 280°C.

The retention times were obtained using a chemical methylmalonic acid and d_3-methylmalonic acid standards (8.60 and 8.64 min respectively). **(Figure 4)** The mass spectra were recorded in selected ion monitoring (SIM) and the selected ions were: 157 m/z, 218 m/z, 247 m/z for methylmalonic acid and 160 m/z, 221 m/z, 250 m/z. for labelled methylmalonic acid. **(Figure 5)** The quantifier ions are 247 m/z for methylmalonic acid and 250 m/z for labelled methylmalonic acid respectively.

Both standards and urine samples were analyzed under the same chromatographic conditions.

One of the most important steps is the calibration of the instrument by using methylmalonic acid at different concentrations.

Figure 4 . Methylmalonic and d_3-methylmalonic chromatogram with selected ions

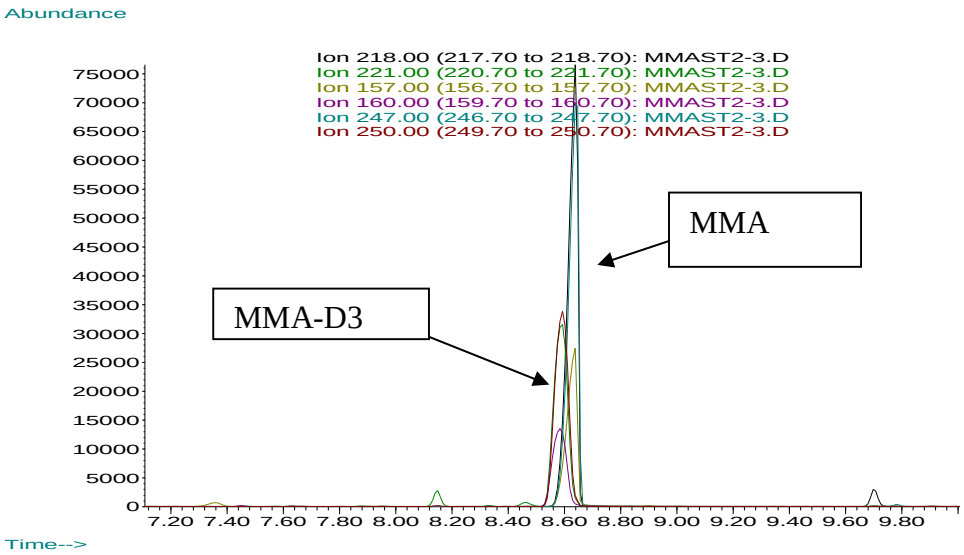
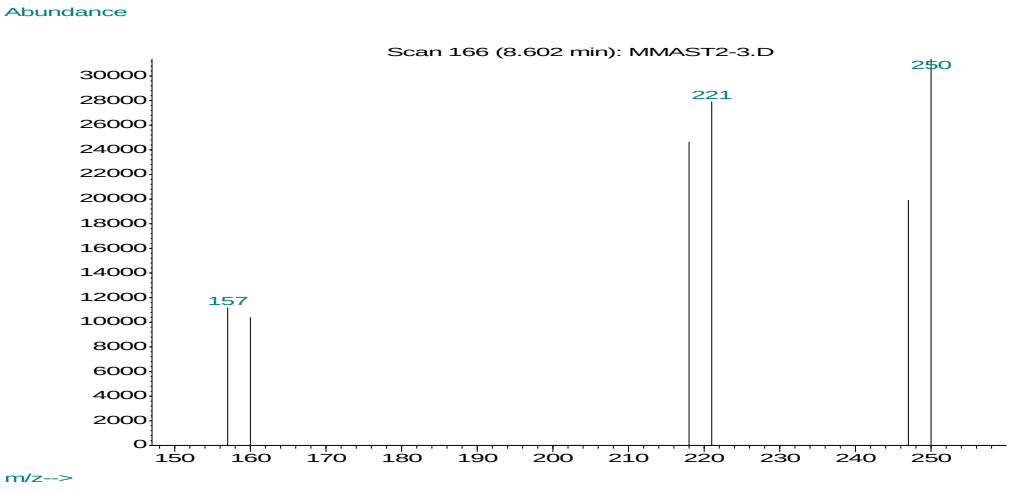


Figure 5 Methylmalonic and d_3-methylmalonic selected ions mass spectra



4.10 Calibration curves

Methylmalonic determination is performed using peak height or peak area in an internal-standard method.

Internal-Standard Method: prepare standard solutions, containing a constant amount of the specified internal standard (labelled methylmalonic acid) and graded amounts of the standard object component (methylmalonic acid). With each of the chromatograms obtained by injecting a constant volume of each standard solution, we calculate the ratio of the peak height or peak area of the standard object component to that of the internal standard. Then we prepare a calibration curve by plotting these ratios on the ordinate and the ratios of each amount of the standard-object component to the amount of the internal standard or the amounts of the standard-object component on the abscissa. The calibration curve is usually a straight line through the origin. After we have prepared the test solution containing the same amount of the internal standard as directed in the individual monograph, we record a chromatogram under the same conditions as for the preparation of the calibration curve, we calculate the ratio of the peak height or peak area of the object component to that of the internal standard, and perform the determination, using the calibration curve.

The peak height or peak area is generally measured by the method above.

We calibrated using a methylmalonic acid solution using a 5 point curve plasma and water containing 20 μmol/l, 50 μmol/l, 200 μmol/l ,400 μmol/l and blank.

Methylmalonic acid and internal standard were added into the pyrex tube as below.

For point one there were added 200μl of 1mmol/l methylmalonic acid standard; the final concentration is 400μmol/l when 500 μl of plasma or water were added.

For point two there was added 100µl of 1mmol/l methylmalonic acid standard; the final concentration is 200µmol/l when 500 of plasma or water was added.

For point three there was added 50µl of 1mmol/l methylmalonic acid standard; the final concentration is 100µmol/l when 500 of plasma or water was added.

For point four there was added 10µl of 1mmol/l methylmalonic acid standard; the final concentration is 20µmol/l when 500 of plasma or water was added.

For point five there was added only internal standard.

To each tube there were added 50µl of methylmalonic acid internal standard (MMA-D3) 1mmol/l and it was dried under a stream of nitrogen before adding methylmalonic acid.

Urine calibration curve. We followed the same method as described above for calibration curves, adding only 25 µl of urine.

The calibration must be performed monthly or more frequently. For the purposes of quantification a mixture containing 50 µmol/L of methylmalonic acid is analyzed weekly and if the quantification reported different from the expected values by more than 25%, a new calibration is made. The GC/MS system must have a correct maintenance to avoid artifacts and the generation of new compounds in organic acids profile.

5.0 Results

5.1 Linearity

To study the linearity we analyzed water, plasma and urine pool spiked with 400µmol/l, 200µmol/l, 100µmol/l, 20µmol/l and blank. Any calibration curves were repeated four times each day for 5 days. The average slope of the curve in water was 0.772±0.075, with an average intercept of 0.075±0.027 and an average R^2 of 0.994±0.003 **(Table 6);** the average slope of the curve in plasma was 0.784±0.015, with an average intercept of 0.102±0.013 and an average R^2 of 0.994±0.002 **(Table 7)**; the average slope of the curve in urine was 0.995±0.013, with an average intercept of 0.612±0.078 and average R^2 of 0.999±0.001 **(Table 8)**. All curves were linear from 0 to 400µmol/l. **(Figure 6)**

Table 6 Calibration curves studies (slope, intercept and R^2) in water

day	slope	intercept	R^2
1	0,626	0,111	0,987
1	0,626	0,109	0,988
1	0,666	0,0812	0,995
1	0,651	0,0934	0,993
2	0,675	0,0908	0,993
2	0,67	0,0924	0,992
2	0,675	0,0923	0,992
2	0,677	0,0886	0,993
3	0,738	0,0776	0,994
3	0,738	0,0779	0,994
3	0,738	0,0763	0,995
3	0,735	0,0759	0,994
4	0,846	0,0293	1
4	0,849	0,0325	1
4	0,856	0,023	1
4	0,855	0,0249	1
5	0,675	0,0908	0,993
5	0,67	0,0924	0,992
5	0,738	0,0776	0,994
5	0,738	0,0779	0,994
Average	**0,7221**	**0,07574**	**0,99415**
SD	**0,0758349**	**0,026704**	**0,003602**
%CV	**10,501999**	**35,25709**	**0,362346**

Table 7 Calibration curves studies (slope, intercept and R^2) in plasma

Day	Slope	Intercept	R^2
1	0,76	0,102	0,997
1	0,778	0,0911	0,998
1	0,778	0,0791	0,997
1	0,789	0,0828	0,999
2	0,781	0,105	0,996
2	0,806	0,091	0,998
2	0,794	0,096	0,997
2	0,807	0,0895	0,998
3	0,76	0,129	0,994
3	0,775	0,12	0,995
3	0,763	0,128	0,994
3	0,78	0,118	0,995
4	0,79	0,108	0,994
4	0,796	0,102	0,995
4	0,802	0,103	0,995
4	0,804	0,097	0,995
5	0,76	0,102	0,997
5	0,778	0,0911	0,998
5	0,79	0,108	0,994
5	0,796	0,102	0,995
Average	**0,784**	**0,102**	**0,996**
SD	**0,015**	**0,013**	**0,00168**
%CV	**1,984**	**13,342**	**0,164**

Table 8 Calibration curves studies (slope, intercept and R^2) in urine

Day	Slope	Intercept	R^2
1	0,9841	0.706	0,9992
1	1,0075	0.6	0,9989
1	0,9984	0.663	0,9985
1	1,0249	0.589	0,9961
2	1,0129	0.657	0,9979
2	1,007	0.615	0,9977
2	1,0129	0.657	0,9979
2	1,007	0.615	0,9977
3	0,9969	0.648	0,9998
3	0,9951	0.598	0,9998
3	0,9965	0.507	0,9998
3	0,9947	0.534	0,9998
4	1,0008	0.481	0,9997
4	0,979	0.657	0,9999
4	0,9894	0.42	0,9999
4	0,9929	0.599	0,9998
5	0,9737	0.606	0,9993
5	0,9779	0.721	0,9994
5	0,9824	0.669	0,9994
5	0,9807	0.715	0,9996
Average	**0,995**	**0.612**	**0,999**
SD	**0,013**	**0.078**	**0,001**
%CV	**1,370**	**12.776**	**0,104**

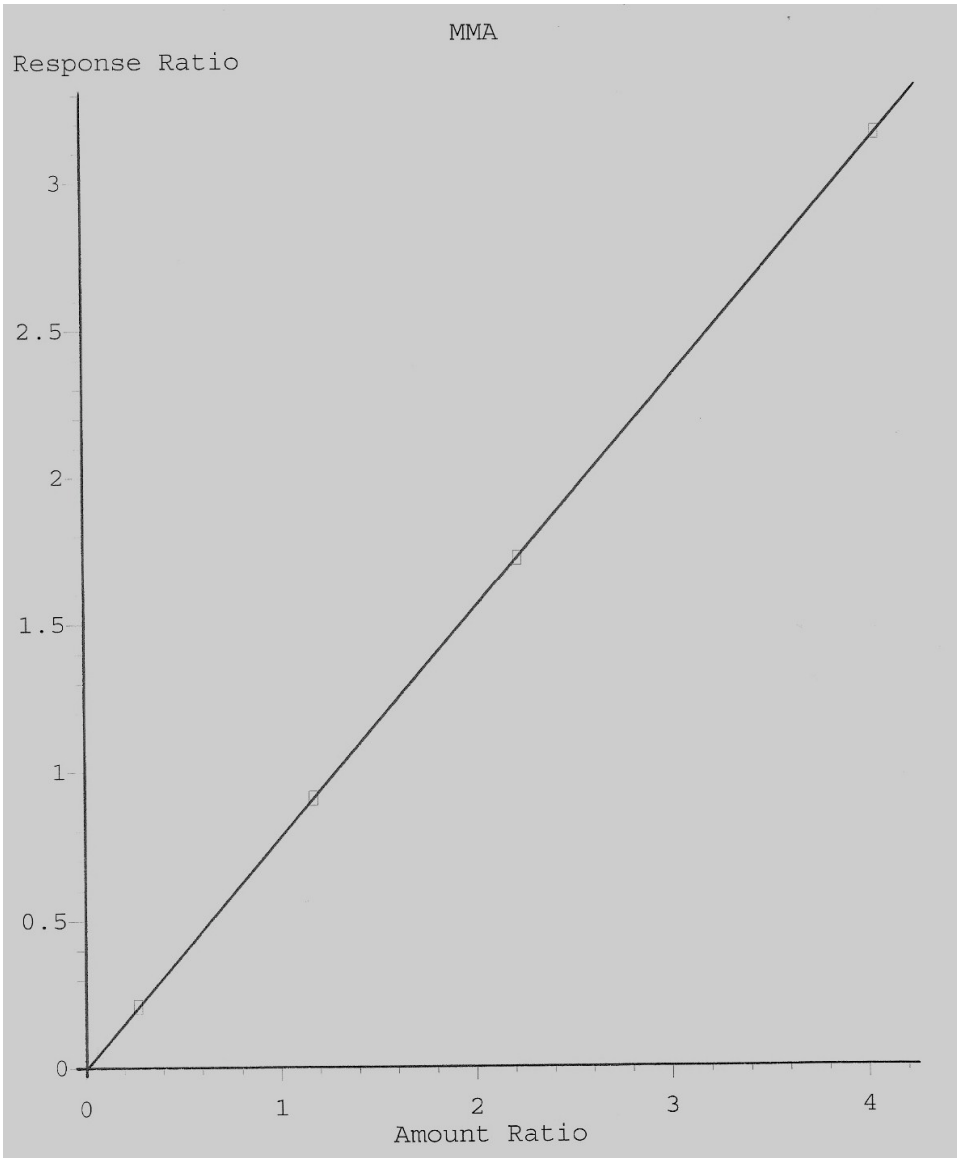


Figure 6. plasma calibration curve (5 points)**.**

5.2 Recovery

The recovery of an analyte in an assay is the detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared with the detector response obtained for the true concentration of the pure authentic standard. Recovery pertains to the extraction efficiency of an analytical method within the limits of variability.

Recovery of the analyte need not be 100%, but the extent of recovery of an analyte and of the internal standard should be consistent, precise and reproducible. Recovery experiments should be performed by comparing the analytical results for extracted samples at three concentrations (low, medium and high) with unextracted standards that represent 100% recovery. Our methylmalonic recovery in water, urine and plasma is shown in table **(9,10,11)**

Table 9. Water Recovery

Added concentration(µmol/L)	20	100	200
Recovery	103.8%	91.6%	106.23%

Table 10. Urine Recovery

Added concentration(µmol/L)	20	100	200
Recovery	108.7%	91.87%	186.5%

Table 11. Plasma Recovery

Added concentration(µmol/L)	20	100	200
Recovery	106.9%	91.75%	96.23%

5.3 Reproducibility

We added three levels of concentration: low, medium and high (20μmol/l, 100μmol/l, 200μmol/l) to validate reproducibility in plasma and urine. We assayed each level four times a day for 5 days to check intra-day variability and inter-days variability.

table 12. The inter-days plasma % of Coefficient of Variability (%CV) is 4.39% for 20μmol/l, 4.09 for 100 μmol/l and 2.5 for 200 μmol/l **table 12**.

The inter-day urine % of Coefficient of Variability (%CV) is less than 2.26% for 20μmol/l, 4.35% for 100 μmol/l and 3.08% for 200 μmol/l **table 13**.

Table 12. Plasma reproducibility

Concentration	20µmol/l	100µmol/l	200µmol/l
Day 1	22	96	191
Day 1	21	96	190
Day 1	22	96	190
Day 1	22	95	190
Average	**21,75**	**95,75**	**190,25**
SD intra	**0,5**	**0,5**	**0,5**
%CV intra	**2,29**	**0,52**	**0,26**
Day 2	22	99	185
Day 2	21	100	185
Day 2	23	98	185
Day 2	20	98	185
Average	**21,5**	**98,75**	**185**
SD intra	**1,29**	**0,95**	**0**
%CV intra	**6,00**	**0,96**	**0**
Day 3	22	95	188
Day 3	22	96	190
Day 3	19	96	189
Day 3	20	95	191
Average	**20,75**	**95,5**	**189,5**
SD intra	**1,5**	**0,57**	**1,29**
%CV intra	**7,22**	**0,60**	**0,68**
Day 4	22	91	196
Day 4	21	91	197
Day 4	22	90	196
Day 4	22	89	197
Average	**21,75**	**90,25**	**196,5**
SD intra	**0,5**	**0,95**	**0,57**
%CV intra	**2,29**	**1,06**	**0,29**
Day 5	22	97	183
Day 5	21	96	184
Day 5	22	97	183
Day 5	22	84	184
Average	**21,75**	**93,5**	**183,5**
SD intra	**0,5**	**6,35**	**0,57**
%CV intra	**2,29**	**6,79**	**0,31**
Inter-days average	**21.5**	**94.75**	**188.95**
Inter SD	**0.94**	**3.87**	**4.72**
Inter %CV	**4.39**	**4.09**	**2.50**

Table 13. Reproducibility urine

Concentration	20µmol/l	100µmol/l	200µmol/l
Day 1	22	97	196
Day 1	22	96	197
Day 1	21	97	190
Day 1	22	96	191
Average	**21,75**	**96,5**	**193,5**
SD intra	**0,5**	**0,57**	**3,51**
%CV intra	**2,29**	**0,59**	**1,81**
Day 2	22	84	176
Day 2	21	87	183
Day 2	22	88	180
Day 2	22	90	179
Average	**21,75**	**87,25**	**179,5**
SD intra	**0,5**	**2,5**	**2,88**
%CV intra	**2,29**	**2,86**	**1,60**
Day 3	22	91	197
Day 3	21	91	195
Day 3	22	90	190
Day 3	21	89	192
Average	**21,5**	**90,25**	**193,5**
SD intra	**0,57**	**0,95**	**3,10**
%CV intra	**2,68**	**1,06**	**1,60**
Day 4	21	96	191
Day 4	22	96	190
Day 4	22	96	190
Day 4	22	95	190
Average	**21,75**	**95,75**	**190,25**
SD intra	**0,5**	**0,5**	**0,5**
%CV intra	**2,29**	**0,52**	**0,26**
Day 5	21	96	193
Day 5	22	96	193
Day 5	21	97	192
Day 5	22	97	190
Average	**21,5**	**96,5**	**192**
SD intra	**0,57**	**0,57**	**1,41**
%CV intra	**2,68**	**0,59**	**0,73**
Inter-days average	**21.65**	**93.25**	**189.75**
Inter SD	**0.48**	**4.06**	**5.85**
Inter %CV	**2.26**	**4.35**	**3.08**

5.4 Limit of detection and limit of quantification.

The detection limit, lower limit of detection, or LOD (limit of detection), is the smallest quantity of a substance that can be distinguished from the absence of that substance (a *blank value*) within a stated confidence limit (generally 1%).[52,53] The detection limit is estimated from the mean of the blank, the standard deviation of the blank and some confidence factor. Another consideration that affects the detection limit is the accuracy of the model used to predict concentration from the raw analytical signal.

There are a number of different "detection limits" that are commonly used. These include the instrument detection limit (IDL), the method detection limit (MDL), the practical quantification limit (PQL) and the limit of quantification (LOQ). Even when the same terminology is used, there can be differences in the LOD according to the nuances of what definition is used and what type of noise contributes to the measurement and calibration.[54]

For normally distributed measurements at the blank the LOD is defined as 3 time the standard deviation of the blank and the LOQ defined as 10 time the standard deviation of the blank. For a signal at the LOD, the alpha error (probability of false positive) is small (1%). However, the beta error (probability of a false negative) is 50% for a sample that has a concentration at the LOD

This means a sample could contain an impurity at the LOD but there is a 50% chance that a measurement would give a result less than the LOD. At the LOQ there is minimal chance of a false negative.

For blood MMA with added analyte, the limit of detection or limit of the blank (mean plus 3 SD of blank) was 0.1 µmol/L and the limit of quantification (mean plus 10 SD of blank) was 0.8 µmol/L For urine MMA with added analyte, the limit of detection or limit of the blank (mean plus 3 SD of blank) was 0.1 µmol/L and the limit of quantification (mean plus 10 SD of blank) was 0.7 µmol/L.

5.5 Normal methylmalonic plasma and urine values

Normal urine and plasma methylmalonic acid values were obtained from 50 healthy control patients (age range: 1 week-18 years). The method was clinically validated by analysis of 10 samples from patients with various methylmalonic acidurias. As shown in **table 14**, Infantile/non-B_{12} responsive methylmalonic aciduria, B_{12} responsive methylmalonic aciduria, "Benign" /adult methylmalonic acidemia, have different ranges of methylmalonic values in blood and urine compared to control. However concordance between biochemical parameters and clinical phenotype does not always exist, partly because renal function can influence plasma MMA concentration. **Figure 7** and **8** show normal and methylmalonic aciduria GC-MS profiles.

Table.14 Methylmalonic Acid Concentration in Subtypes of Methylmalonic MMAuria.

Phenotypic variant	**N° patients**	**Urine**	**Blood**
Infantile/non-B_{12} responsive	3	1000-10,000 mmol/mol/Cr	100-1000 µM
B_{12} responsive	5	10 – 500 mmol/mol/Cr	5-100 µM
"Benign" /adult methylmalonic acidemia	2	10-100 mmol/mol/Cr	100 µM
Normal		<4 mmol/mol/Cr	<0.27 µM

Figure 7 . Methylmalonic acidemia GC-MS profile

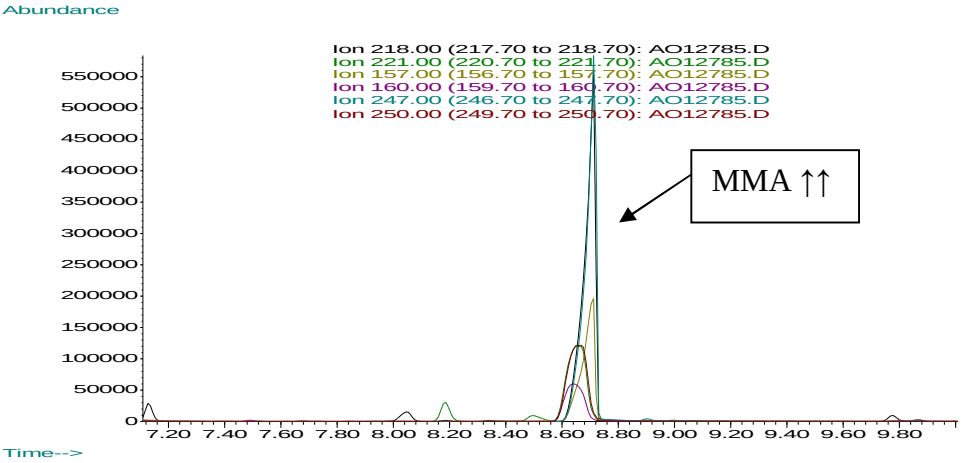
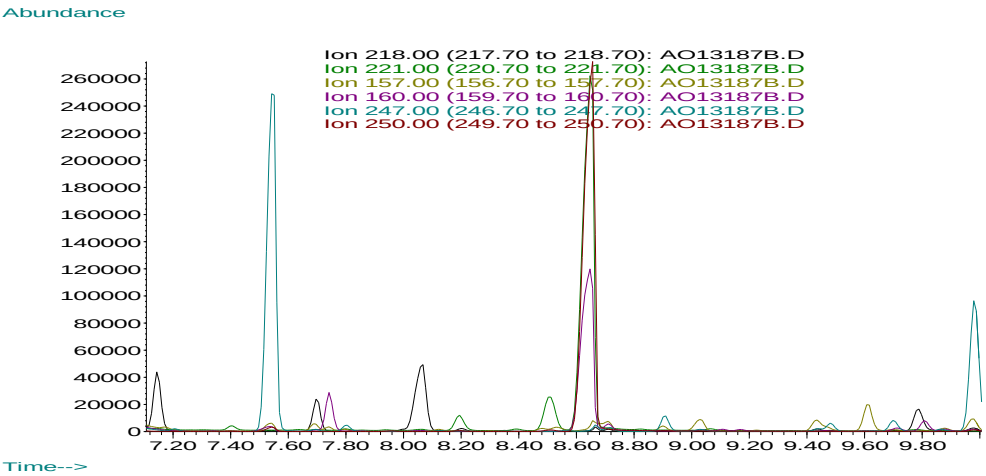


Figure 8 GC-MS plasma normal profile



6.0 Discussion and conclusions

MMAuria is a group of organic aciduria associated with methylmalonyl-CoA mutase and cobalamin metabolic defects. Dionisi-Vici et al. [55] estimated that the incidence of MMAuria detected after onset with symptoms was 1:61,775. Sniderman et al. reported in 1999 that 122 cases with high levels of methylmalonate excretion were found in a neonatal GC/MS screening program established in 1975 and only 13 of the 122 were symptomatic. Furthermore, 65 of 100 asymptomatic patients had transient high levels of MMA and responded to vitamin B12 therapy suggesting that there are a considerable number of asymptomatic patients with some degree of MMAuria. Based on the above data, the actual number of MMAuria patients is approximately 9.4 times the number of symptomatic MMA patients detected. The patients usually have markedly increased levels of urinary methylmalonic and methylcitric acids and blood propionylcarnitine. Significantly increased homocysteine concentrations can be found in the urine and serum of patients with combined MMA and homocysteinemia. Some MMAuria patients have severe neurological defects and multiple organ dysfunctions.

We developed a rapid and simple gas chromatography-mass spectrometry method for quantification of methylmalonic acid in plasma and urine and we evaluated its application to the diagnosis of methylmalonic acidurias.

In the past, mass spectrometry performance was noted for its sensitivity, resolution, and selectivity. Today, the clinical laboratory demands those attributes and also short- and long-term robustness. An analytical methodology cannot be exploited in the clinical domain if it is lacking robustness and requires labour-consuming sample preparation [57]. GC/MS is gaining increasing acceptance in clinical laboratories both for research and in routine procedures (through the quantification of targeted metabolites and markers). In addition to its benefits in terms of sensitivity and specificity, it enables multiple

compounds to be analyzed at one time. Routine analysis of multiple components in one step has been implemented in clinical laboratories for organic acids, steroid profiling and very-long-chain fatty acids and so on.

Many labs chose to perform a semi-quantitative determination of the urinary methylmalonic acid profile using a non-labelled internal standard. There are non-physiological compounds which are eluted in proximity to compounds to be quantified. In our own experience, Dimethylmalonic acid, Tropic Acid, Pentadecanoic acid, Tricarballylic Acid and 2-phenylbutyrric acid were routinely used as internal standards. This method has as its advantage lower cost per analysis.

The use of labelled internal standards and isotope dilution procedures makes it possible to obtain a correct and absolute quantification of organic acids but can be more expensive.

GC/MS with stable isotope dilution has been used to analyze MMA in body fluids. Having the advantage of an ideal internal standard, stable isotope dilution is considered the gold standard method for measuring these metabolites [58] because it obviates the problem of varying levels of extraction efficiency by solvent extraction methods. The MMA value is normalized to urine creatinine to correct for urine dilution. The urinary MMA/creatinine ratio test is more accurate than the blood test as it indicates tissue/cellular B12 deficiency.

Our methodology is capable of monitoring and quantifying MMA and it can follow up on and diagnose methylmalonic acidurias. We calculated normal values for methylmalonic acid in plasma and urine and we compared these with three phenotypic variants of methylmalonic aciduria. The method is precise and robust and therefore suitable for implementation in routine clinical screening programs and quantification environments.

Reference 7.0

1. Fenton, WA; Gravel, RA; Rosenblatt, DS: Disorders of Propionate and Methylmalonate Metabolism, in Charles R. Scriver, *et al*. (editors) *The metabolic and molecular basis of inherited disease.* Volume II, New York, McGraw-Hill, p 2165,2001

2 Chandler, RJ; Venditti,CP: Genetic and genomic system to study methylmalonic academia. *Molecular Genetics and Metabolism* 86:34,2005.

3.Zwickler,T;Linder,M;Aydin,HI;Baumgartner,MR;Bodamer,OA;Burlina,AB;Das,AM;deKlerk,JBC;Gokcay,G;Grunewald,S;Guffon,N;Maier,EM;Morava,E;Geb,S;Schwahn,B;Walter,JH;Wendel,U;Wijdurg,FA;Muller,E;Kolker,S;Horster,F: Diagnostic work-up and management of patients with isolated methylmalonic acidurias in European metabolic centres. *J.Inherit.Metab Dis* 31:361,2008.

4.0Thompson GN, Walter JH, Bresson JL, Ford GC, Lyonnet SL, Chalmers RA, Saudubray JM, Leonard JV, Halliday D. Sources of propionate in inborn errors of propionate metabolism. Metabolism. 1990; 39: 1133–7

5. IEM digest: *Molecular Genetics and Metabolism.*85:2,2005.

6.Dobson,MC;Wai,T;Leclerc,D: Identification of the gene responsible for the cblA
complementation groups of vitamin B_{12}-responsive methylmalonic acidemia base on analysis of prokaryotic gene arrangements. *PNAS*.99:15554,2002.

7.Dobson,MC;Wai,T;Leclerc,D: Identification of the gene responsible for the cblB complementation groups of vitamin B_{12}-dependent methylmalonic aciduria. *Hum Mol Genet.*11:3361,2002.

8 Matsui, SM; Mahoney, MJ; Rosenberg, LE: The natural history of the inherited methylmalonic acidemias. *New Engl J Med 308*: 857, 1983.

9.Broyer, M; Guesry, P; Burgers, EA; *et al*.: Acidemic methyl malonique avec nephropathic hyperuricenique. *Arch Fr Pediatr 31*: 543, 1974.

10.Molteni, KH; Oberly, TD; Wolff, JA; Friedman, AL: Progressive renal insufficiency in methylmalonic acidemia. *Pediatr Nephrol 5*: 323, 1991

11.Whelan, DT; Ryan, E; Spate, M; *et al.*: Methylmalonic acidemia: 6 years' clinical experience with two variants unresponsive to vitamin B_{12} therapy. *Can Med Assoc J 120*: 1230, 1979.

12.Rutledge, SL; Geraghty, M; Mroczek, E; Rosenblatt, D; Kohout, E: Tubulointerstitial nephritis in methylmalonic aciduria. *Pediatr Nephrol 7*: 81, 1993.

13.Trinh, B; Melhem, ER; Barker, PB: Multi-slice Proton MR Spectroscopy and Diffusion-weighted Imaging in Methylmalonic Acidemia: Report of Two Cases and Review of Literature. *AJNR Am J Neuroradiol 22*: 831, 2001.

14.Goetz: *Textbook of Clinical Neurology*, 1st ed., W. B. Saunders Company, 1999, p. 591.

15.Inoue, S; Krieger, I; Sarnaik, A; Ravindranath, Y; Fracassa, M; Ottenbreit, MJ: Inhibition of bone marrow stem cell growth *in vitro* by methylmalonic acid: A mechanism for pancytopenia in a patient with methylmalonic acidemia. *Pediatr Res 15*: 95, 1981.

16.Berry, GT: Inborn errors of amino acid and organic acid metabolism, in Cowett, RM (ed): *Principles of Perinatal and Neonatal Metabolism*, 2nd edition, New York, Springer 1998, p 799.

17.Msall, M; Batshaw, ML; Suss, R; Brusilow, SW; Mellits, ED: Neurologic Outcome in Children with Inborn Errors of Urea Synthesis: Outcome of Urea-Cycle Enzymopathies. *N Eng J Med 310*: 1500, 1984

18. Oberholzer, VC; Levin, B; Burgess, EA; Young, WF: Methylmalonic aciduria: An inborn error of metabolism leading to chronic metabolic acidosis. *Arch Dis Child 42*: 492, 1967

19.Utter, MF; Keech, DB; Scrutten, ML: A Possible role for acetyl CoA in the control of gluconeogenesis, in Webber G (ed): *Advances in Enzyme Regulation*, Vol. 2, New York, Pergaamon, 1964, p 49.

20.Halperin, ML, Schiller, CM; Friz, IB: The inhibition by methylmalonic acid of malate transport by the dicarboxylate carrier in rat liver mitochondria: A possible explanation for hypoglycemia in methyllmalonic aciduria. *J Clin Invest 50*: 2276, 1971.

21. Treacy, E; Arbour, L; Chessex, P; Graham, G; Kasprzak, L; Casey, K; Bell, L; Mamer, O; Scriver, CR: Glutathione deficiency as a complication of methylmalonic acidemia—response to high doses of ascorbate. *J Pediatr 129*: 445, 1996

22. Hillman, RE; Sowers, LH; Cohen JL: Inhibition of glycine oxidation in cultured fibroblasts by isoleucine. *Pediatr Res 7*: 945, 1973

23.Harris, DJ; Yang, BJ-Y; Snodgrass, PJ: Carbamyl phosphate synthetase deficiency: A possible transient phenocopy of dysautonomia. *Am J Hum Genet 29*: 52, 1977.

24.Berry, GT: Inborn errors of amino acid and organic acid metabolism, in Cowett, RM (ed): *Principles of Perinatal and Neonatal Metabolism*, 2nd edition, New York, Springer 1998, p 799

25.Chalmers, RA; Roe, CR; Stacey, TE; *et al.*: Urinary excretion of l-carnitine and acylcarnitines by patients with disorders of organic acid metabolism: evidence for secondary insufficiency of l-carnitine. *Pediatr Res 18*: 1325, 1984.

26.Libert, R; Van Hoof, F; Thillaye, M; Vincent, MF; Nassogne, MC; de Hoffmann, E; Schanck, A: Identification of undescribed medium-chain acylcarnitines present in urine of patients with propionic and methylmalonic acidemias. *Clinica Chimica Acta 295*: 87, 2000

27.Coulombe, JT; Shih, VE; Levy, HL: Massachusetts Metabolic Disorders Screening Program. II. Methylmalonic aciduria. *Pediatrics 67*: 26, 1981

28.MacMahon, M: Requesting vitamin B_{12} and folate assays. *Lancet 346*: 973, 1995.

29. Ledley, FD; Levy, HL; Shih, VE; Benjamin, R; Mahoney, MJ: Benign methylmalonic aciduria. *New Engl J Med 311*: 1015, 1984

30. Goetz: *Textbook of Clinical Neurology*, 1st ed., W. B. Saunders Company, 1999, p. 591

31.Watkins D, Matiaszuk N, Rosenblatt DS. Complementation studies in the cblA class of inborn error of cobalamin metabolism: evidence for interallelic complementation and for a new complementation class (cblH). J Med Genet. 2000; 37: 510–3.

32.Suormala T, Baumgartner MR, Coelho D, Zavadakova P, Kozich V, Koch HG, Berghauser M, Wraith JE, Burlina A, Sewell A, Herwig J, Fowler B. The cblD defect causes either isolated or combined deficiency of methylcobalamin and adenosylcobalamin synthesis. J Biol Chem. 2004; 279: 42742–9.

33. Gravel, RA; Mahoney, MJ; Ruddle, FH; Rosenberg, LE: Genetic complementation in heterokaryons of human fibroblasts defective in cobalamin metabolism. *Proc Natl Acad Sci USA 72*: 3181, 1975

34. Willard, HF; Mellman, IS; Rosenberg, LE: Genetic complementation among inherited deficiency of the methylmalonyl-CoA mutase activity: Evidence for a new class of human cobalamin mutants. *Am J Hum Genet 30*: 1, 1978.

35. Cooper, BA; Rosenblatt, DS; Watkins, D: Methylmalonic aciduria due to a new defect in adenosylcobalamin accumulation by cells. *Am J Hematol 34*: 115, 1990.

36. Rosenblatt, DS; Failure of lysosomal release of vitamin B_{12}: a new complementation group causing methylmalonic aciduria (cblF). *Am J Hum Genet 39*: 404, 1986

37.Vassiliadis, A.; Rosenblatt, D. S.; Cooper, B. A.; Bergeron, J. J. M. : Lysosomal cobalamin accumulation in fibroblasts from a patient with an inborn error of cobalamin metabolism (cblF complementation group): visualization by electron microscope radioautography.. *Exp. Cell Res.* 195: 295-302, 1991

38.Rosenblatt, DS; Aspler, AL; Shevell, MI; Pletcher, BA; Fenton, WA, Seashore, MR: Clinical heterogeneity and prognosis in combined methylmalonic aciduria and homocystinuria (cblC). *J Inherit Met Dis 20*: 528, 1997.

39. Howard, R; Frieden, IJ; Crawford, D; Mccalmont, T; Levy, ML; Rosenblatt, DS; Sweetman, L; Goodman, SI; Ohnstad, C; Hart, K; Berrios, M; Packman, S: Methylmalonic acidemia, cobalamin C type, presenting with cutaneous manifestations. *Arch Dermatol 133*: 1563, 1997.

40. Rosenblatt, DS; Aspler, AL; Shevell, MI; Pletcher, BA; Fenton, WA, Seashore, MR: Clinical heterogeneity and prognosis in combined methylmalonic aciduria and homocystinuria (cblC). *J Inherit Met Dis 20*: 528, 1997.

41.Rosenblatt, DS; Laframboise, R; Pichette, J; Langevin, P; Cooper, BA; Costa, T: New disorder of vitamin B_{12} metabolism (cobalamin F) presenting as methylalonic aciduria. *Pediatrics 78*: 51, 1986.

42.Shih, VE; Axel, SM; Tewksbury, JC; Watkins, D; Cooper, BA; Rsenblatt, DS: Defective lysosomal release of vitamin B_{12} (cblF): A hereditary metabolic disorder associated with sudden death. *Am J Med Genet 33*: 555, 1989.

43.Wong, LTK; Rosenblatt, DS; Applegarth, DA, Davidson, AGF: Diagnonsis and treatment of a child with cblF disease. *Clin Invest Med 15*(*suppl*): 111, 1992.

44.MacDonald, MR; Wiltse, HE; Bever, JL; Rosenblatt, DS; Clinical heterogeneity in two patients with cblF disease. *Clin Invest Med 51*: 353, 1992.

45. Bobik TA, Rasche ME *J. Biol. Chem.* 2001 Oct 5;276(40):37194-8

46.Bikker,H;Bakker,HD;Abeling,NGGM: A homozygous missense mutation in the methylmalonyl CoA epimerase gene (*MCEE*) results in mild methylmalonic aciduria.*Hum Mutat*.27:640,2006.

47.Elpeleg O,MillerC,Hershkovitz E; Deficiency of ADP-forming succinyl-CoA synthase activity is associated with encephalomyopathy and mitochondrial deletion. 2005*Am.J.Hum.Genet* 76:1081-1086.

48.Ostergaard,E;Hansen,FJ;Sorensen,N:Mitochondrial encephalomyophathy with elevated methylmalonic acid is caused by SUCLA2 mutations. 2007. Brain 130:853-861.

49. Chalmers, R.A., Healy, M.J., Lawson, A.M. and Watts, R.W. (1976) Urinary organic acids in man. II.

50.Hansen, S., Perry, T.L., Lesk, D. and Gibson, L. (1972) Urinary bacteria: potential source of some organic acidurias. *Clin Chim Acta.* 39(1), 71-4.

51.Tanaka, K., West-Dull, A., Hine. D.G., Lynn, T.B. and Lowe T. (1980) Gas-chromatographic method of analysis for urinary organic acids. II. Description of the procedure, and its application to diagnosis of patients with organic acidurias. *Clin Chem.* 26(13), 847-53.

52 International Union of Pure and Applied Chemistry. "detection limit". *Compendium of Chemical Terminology* Internet edition.

53."Guidelines for Data Acquisition and Data Quality Evaluation in Environmental Chemistry", *Anal. Chem.* 52: 2242–49, 1980

54.Long, Gary L.; Winefordner, J. D. (1983), *Anal. Chem.* 55 (7): 713A–724A

55. Inborn errors of metabolism in the Italian pediatric population: a national retrospective survey.Dionisi-Vici C, Rizzo C, Burlina AB, Caruso U, Sabetta G, Uziel G, Abeni D. J Pediatr. 2002 Mar;140(3):321-7.

56. Outcome of individuals with low-moderate methylmalonic aciduria detected through a neonatal screening program. Sniderman LC, Lambert M, Giguère R, Auray-Blais C, Lemieux B, Laframboise R, Rosenblatt DS, Treacy EP. J Pediatr. 1999 Jun;134(6):675-80.

57. Rapid 2nd-tier test for measurement of 3-OH-propionic and methylmalonic acids on dried blood spots: reducing the false-positive rate for propionylcarnitine during expanded newborn screening by liquid chromatography-tandem mass spectrometry. La Marca G, Malvagia S, Pasquini E, Innocenti M, Donati MA, Zammarchi E. Clin Chem. 2007 Jul;53(7):1364-9.

58. Detection of cobalamin deficiency using the urinary methylmalonic acid test by gas chromatography mass spectrometry. Norman EJ. J Clin Pathol. 1993 Apr;46(4):382.