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Quantification of methylmalonic acid in serum measured by capillary gas chromatography–mass spectrometry as *tert.*-butyldimethylsilyl derivatives

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ABSTRACT

Quantification of methylmalonic acid in serum is considered one of the most sensitive parameters for diagnosis of cobalamin deficiency. Methylmalonic acid was measured as *tert.*-butyldimethylsilyl derivatives formed in the presence of dimethylformamide. Under these conditions the derivative was quantified using gas chromatography–mass spectrometry with selected-ion monitoring at 403 and 406 for methylmalonic acid and methyl-d_3-malonic acid, respectively. The characteristics of the method were: linearity from 0.04 to 1.7 μmol/l with linear regression equation $y = -0.0199 + 0.727\,x$ ($r = 0.999$); recovery 95.5 ± 6.8%; detection threshold 17 fmol injected; within-day variation coefficient ranging from 3.10% to 5.6% according to sample concentration; and day-to-day coefficient of variation of 6.8% and 5.3% for methylmalonic acid concentrations of 0.103 μmol/l and 0.360 μmol/l, respectively. The normal range after log transformation was estimated at 0.03–0.26 μmol/l, 0.06–0.33 μmol/l and 0.02–0.40 μmol/l in children of 4–14 years (n = 39), in healthy subjects at 20–40 years (n = 70) and in healthy elderly persons older than 60 years (n = 14), respectively. The normal range in children was significantly lower than that in adults and, in contrast, normal values in the elderly were significantly higher.

INTRODUCTION

The serum cobalamin assay is a sensitive test for primary screening for cobalamin deficiency [1]. However, some patients with low levels of plasma cobalamin are not cobalamin deficient. This is the case in 8% of patients with low cobalamin level according to Carmel [2] and as many as 26% according to Moelby *et al.* [3]. Moreover, serum cobalamin concentration may be normal despite a tissue cobalamin deficiency [4].

Recently, methylmalonic acid (MMA) and homocysteine in serum have been proposed as indicators of cobalamin status [5,6]. L-Methylmalonyl coenzyme A mutase (EC 5.4.99.2) is a cobalamin-dependent enzyme involved in methylmalonic acid metabolism. An increased level of methylmalonic acid in serum or urine reflects tissue cobalamin deficiency. The rise in MMA is

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early, often preceding the decrease in serum cobalamin [7].

Several methods have been developed for determination of MMA in urine, such as enzymatic assay [8] or gas chromatography [9]. However, only gas chromatography–mass spectrometry (GC–MS) combined with selected-ion monitoring (SIM) mode is sufficiently sensitive and specific for quantifying MMA in cobalamin deficiency. At present, two GC–MS techniques are described for MMA determination in serum using either *tert.*-butyldimethylsilyl derivatives [10] or dicyclohexyl esters [11].

We have developed and validated a capillary GC–MS method using *tert.*-butyldimethylsilyl derivatives formed in the presence of dimethylformamide, which allowed us to obtain higher m/z fragmentometry than the methods previously described [10,11] in impact ion mode. High m/z has the advantage of decreasing the risk of interference with other organic acids. This method was used to determine normal values in healthy populations of children (4–14 years old), adults (20–40 years) and the eldery (> 60 years) and to diagnose mild cobalamin deficiencies [12].

EXPERIMENTAL

Apparatus

A Hewlett-Packard (Palo Alto, CA, USA) Model 5988 A gas chromatograph–mass spectrometer was equipped with a fused-capillary column (SPB, 30 m × 0.32 mm I.D., Supelco, St. Germain en Laye, France). The injection port, transfer line and source temperatures were maintained at 250°C, 270°C and 200°C, respectively.

The column head pressure of helium (carrier gas) was 9 p.s.i. Electron impact ionization was at 70 e.v. A temperature programme was used with oven initial and final temperatures of 135 and 230°C, respectively, increasing at a rate of 5°C/min.

Reagents

Methylmalonic acid (MMA) was purchased from Fluka (Buchs, Switzerland). Methyl-d_3-malonic acid (d_3-MMA) used as internal standard was supplied by MSD Isotopes (Montréal, Canada). N-Methyl-N-(*tert.*-butyldimethylsilyl) trifluoroacetamide (MTBSTFA) and dimethylformamide (DMF) were obtained from Pierce (Rockford, IL, USA). All HPLC-grade solvents were purchased from Merck (Darmstadt, Germany). Analytical-grade chemicals were obtained from Sigma (St. Louis, MO, USA).

Sample collection

Blood samples collected without anticoagulant were supplied by the Preventive Medicine Center (Vandoeuvre, France). Samples were obtained from children (4–8 and 8–14 years old, $n = 39$), adults (20–40 years, $n = 70$) and elderly subjects (> 60 years, $n = 14$). These subjects were apparently healthy after a medical and biological standard check-up. The serum was separated by centrifugation for 15 min at 1000 g at room temperature. Aliquots were stored at −20°C until assayed.

Procedure

Extraction. A 500-μl volume of serum was spiked with 200 ng of d_3-MMA (200 μl of a 1 μg/ml solution). The deproteinization step was performed by adding 50 μl of perchloric acid (4 *M*) to samples followed by vortex-mixing and centrifugation. A 500-μl volume of 0.2 *M* HCl and 500 μl of saturated solution of NaCl were added to the supernatant and the mixture was extracted three times with 3 ml of ethyl acetate and once with 3 ml of ethyl ether. Organic phases were pooled and evaporated to dryness under a gentle stream of nitrogen at room temperature and under a hood. The dry residue was dissolved in 2 × 200 μl of ethyl acetate and transferred into a mini reactivial (Pierce). The organic solvent was evaporated to dryness.

Derivatization

A 50-μl volume of MTBSTFA–DMF (v/v), prepared just before use, was added to dry residue, the vials were carefully capped and the derivatization reaction was performed at 75°C for 60 min. The derivative was extracted with 50 μl of hexane and, depending on MMA concentration, 1–2.5 μl were injected.

Statistical analysis

Since the distribution of values in healthy populations is non-Gaussian, the means and ranges were calculated in each group after log transformation.

The different groups were compared with one-factor analysis of variance (ANOVA) and with the non-parametric Fisher PLSD test and Scheffe *F*-test. The difference was significant for $P < 0.01$ (Program Statview II).

RESULTS AND DISCUSSION

Choice of derivative

Several methods of derivatization have been proposed in the literature for quantifying MMA in blood and urine. Rasmussen [11] described in detail and validated a technique using dicyclohexyl esters whose fragmentation pattern was studied by Norman *et al.* [13] for determining urinary dicarboxylic acids. These derivatives have the advantage of being very stable. In our hands and with our apparatus this method produced an interfering peak at mass 122 (characteristic mass of d_3 MMA).

Trimethylsilyl esters have been widely used for gas chromatography–mass spectrometry of organic acids as they are quantitatively formed [14]. *tert.*-Butyldimethylsilyl derivatives are more stable than trimethylsilyl derivatives and they often provide an intense base peak in their spectra of mass $[M - 57]^+$ [15].

We tested several mixtures to produce *tert.*-butyldimethylsilyl (*tert.*-BDMS) derivatives: MTBSTFA–CH_3CN (1:10, v/v) according to Marcell *et al.* [10] or MTBSTFA–DMF (50:50, v/v) (Pierce GC derivatization handbook and catalogue 1991). With this last mixture we obtained one main peak for MMA and the derivatives for MMA and d_3-MMA exhibited ions at *m/z* 403 and 406, respectively, which represented about 30% of total current ion (Fig. 1) and provided excellent sensitivity during SIM. A second small peak was seen on the chromatograms of the standard solutions. This peak was constant and was not a derivative of MMA. In fact, the principal ions and their relative abundance were: 312 (50), 270 (1448), 214 (104). So this peak does not represent a bis-*tert.*-BDMS derivative.

The derivative obtained bound three *tert.*-BDMS groups. This derivative was either an enol ether of the diester resulting from ketoenol tautomerism or the third *tert.*-BDMS group was bound to C-2, because DMF is a base that activates the mobile hydrogen on C-2 (Fig. 2).

The derivatives prepared under the described conditions were stable for several days. No hydrolysis was observed when the samples were re-extracted with hexane. The derivatization was reproducible. The derivative retention time was 4.81 min with a within-run and between-run coefficient of variation of 0.28% ($n = 16$) and 0.78% ($n = 7$), respectively.

The high mass of the peaks monitored in MMA analysis (403) diminished the risk of interference with other compounds extracted from plasma. We tested compounds with a structure close to that of MMA. Table I lists the products studied with their retention time and *m/z* of main fragments. Complete separation was achieved for all the derivatives tested. Using bis-*tert.*-BDMS derivatives, Marcell *et al.* [10] could not completely separate methylmalonic acid from dimethylmalonic acid.

Extraction procedure

Various sample preparations have been described for organic acids. Analytical solid phase extraction has recently been developed [16]. Extraction by organic solvents has been widely used and can be followed by an HPLC purification step [10]. This last technique is laborious and time-consuming. We chose solvent extraction for routine MMA assay because it is simple, cheap, fast and yields an adequate recovery [17]. We successively used ethyl acetate [18] and diethyl ether [14]. Under these conditions mean recovery of MMA from a serum spiked with 0.08 μmol of MMA was 95.5 ± 6.8% ($n = 6$).

This percentage recovery was much higher than that obtained by Marcell *et al.* [10], which was about 10–20%. These authors have recently omitted the HPLC step because of this low recovery [19,20]. Our results were close to those ob-

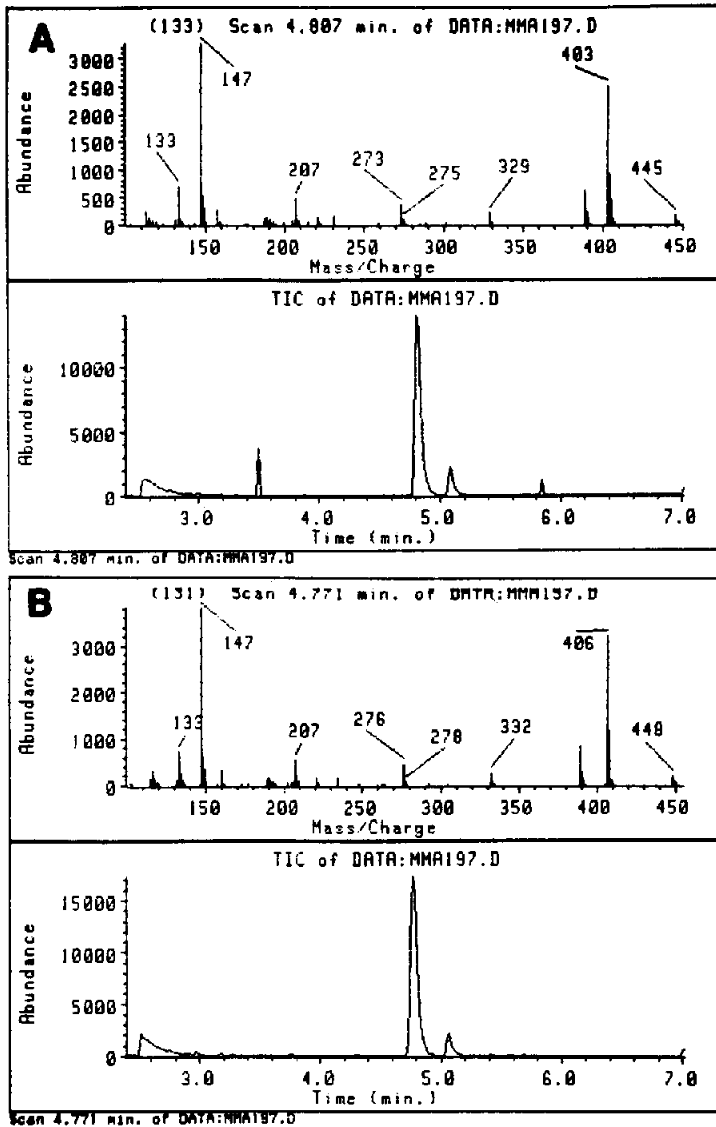

Fig. 1. Total ion and mass spectra data of methylmalonic acid (A) and methyl-d_3-malonic acid.

tained by Rasmussen [11] using solid phase extraction. Further, the addition of d_3-MMA to each sample before extraction overcame the recovery problem.

Evaluation of the method

Specifity. MMA was identified on SIM on the basis of retention time, the almost simultaneous appearance of d_3-MMA and the high mass of *m/z*. Other compounds with similar structures to MMA were tested, and had retention time and mass fragmentometry very different from those of MMA (Table I).

Linearity was tested by injecting increasing amounts of MMA (0.04–1.7 μmol/l) and a constant quantity of d_3-MMA (1.7 μmol/l). This range of values covered MMA concentration in normal subjects and almost all subjects with bor-

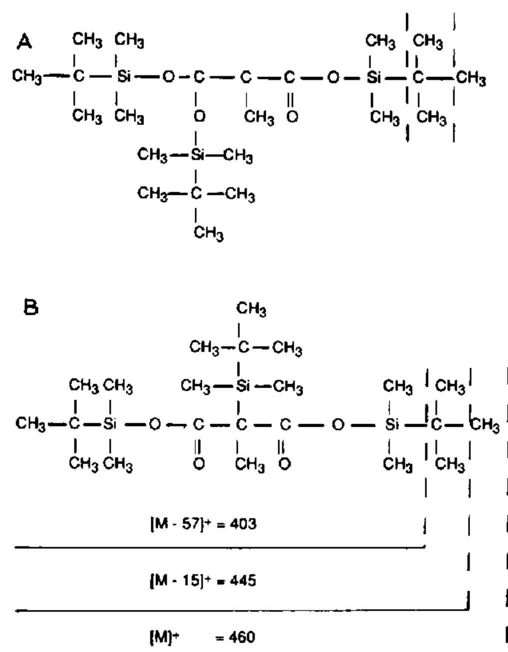

Fig. 2. Structure and *m/z* values of the *tert.*-butyldimethylsilyl derivative of methylmalonic acid and the fragmentation points.

derline cobalamin deficiency. Standard curves were established from the peak-area ratio MMA/d_3-MMA with linear regression equation $y = -0.0199 + 0.727$, $r = 0.999$ ($n = 6$).

The *limit of detection* of the described experimental procedure was 17 fmol injected with a signal-to-noise ratio of 4.

The *repeatability* of GC–MS analysis was evaluated from standard solutions. The repeatability of the method was determined from serum samples which were subjected to the routine procedure: extraction, derivatization and GC–MS analysis. Table II summarizes the results.

Reproducibility was determined from two serum samples that were tested in duplicate during routine analysis for 10 days. The means, standard deviations and coefficients of variation (C.V.) were 0.103 ± 0.007 µmol/l, C.V. = 6.8%, and 0.36 ± 0.019 µmol/l, C.V. = 5.3%, respectively.

Normal values were determined in children, adults and the elderly. This histogram of value

TABLE I

RETENTION TIME AND *m/z* OF MAIN FRAGMENTS OF SOME DICARBOXYLIC ACID *tert.*-BUTYLDIMETHYLSILYL DERVATIVES

The fragments were classified in decreasing order according to their intensity given in brackets.

Acids	Retention time	*m/z*			
		$[M]^+$	$[M-57]^+$	$[M-131]^+$	$[M-15]^+$
Succinic acid	2.363	346	289	215	326
MW = 118		ND[a]	(8996)	(504)	(326)
Methylsuccinic acid	2.473	360	303	229	345
MW = 132		ND	(9015)	(500)	(318)
Glutaric acid	2.948	360	303	229	345
MW = 132		ND	(9316)	(564)	(334)
Dimethylmalonic acid	–	No derivative obtained			
MW = 132					
Malonic acid	4.261	446	389	315	431
MW = 104		ND	(5733)	(1481)	(431)
Methyl-d_3-malonic acid	4.771	463	406	332	448
MW = 121		ND	(3237)	(269)	(197)
Methylmalonic acid	4.801	460	403	329	445
MW = 118		ND	(3420)	(268)	(223)
Ethylmalonic acid	5.119	474	417	343	459
MW = 132		ND	(799)	(63)	(57)

[a] Not detected.

TABLE II

REPEATABILITY OF METHYLMALONIC ACID MEASUREMENTS

Theoretical concentration (μmol/l)	n	Concentration found (mean ± S.D.) (μmol/l)		C.V. (%)
Standard solutions				
0.42	19	0.421	0.015	3.52
0.84	10	0.837	0.026	3.10
1.7	7	1.659	0.051	3.11
Serum 1	8	0.471	0.015	3.18
Serum 2	8	0.123	0.007	5.69

TABLE III

NORMAL VALUES (MEAN ± 1 S.E.M.) OF METHYLMALONIC ACID DETERMINED IN CHILDREN, ADULTS AND THE ELDERLY (μmol/l) AFTER LOG TRANSFORMATION

Group	n	Concentration (μmol/l)	
		Mean	S.E.M.
Children 4–8 years	19	0.12	0.06–0.24
Children 8–14 years	20	0.09	0.03–0.26
Adults 20–40 years	70	0.17	0.06–0.33
Elderly >60 years	14	0.25	0.02–0.40

distribution in children and in adults is given in Fig. 3. Normal ranges (Table III) were calculated as the mean ± 1 S.E.M. after log transformation.

No sex-related difference was observed. This was in agreement with the results of Rasmussen [11] and of Stabler *et al.* [21].

The values in healthy adults reported in this study were lower than those given by Lindenbaum *et al.* [20] of 0.16–0.64 μmol/l (n = 50), but agreed well with values determined by Rasmussen *et al.* in recent studies [22,23] of 0.05–0.28 μmol/l (n = 28) or 0.05–0.37 μmol/l (n = 58) or by Allen *et al.* [24], who reported normal values of 0.07–0.27 μmol/l (n = 50).

Few normal values have been reported for children in the literature. In the studied population there was no significant difference between girls and boys or between the 4–8 years group and the 8–14 years group. In contrast, the values for children were significantly different from those of adults ($P < 0.01$).

The MMA concentrations obtained in elderly people were significantly higher than those of younger adults. These subjects had a normal renal function as evaluated by serum creatinine, renal failure being known to increase serum MMA level [25]. These results need to be confirmed with more subjects, with a systematic exclusion of pa-

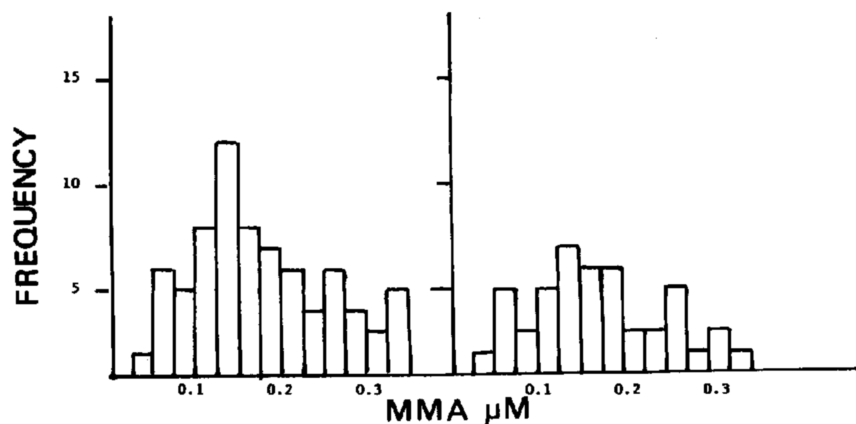

Fig. 3. Distribution histogram of serum MMA values obtained in two healthy groups. (A) Children 4–14 years. (B) Adults 20–40 years.

tients with mild cobalamin deficiency.

Rasmussen [11] did not observe any significant age-related differences in MMA level in a group of 50 subjects aged from 18 to 65 years, but she found a weak positive correlation in a second group of 28 subjects aged 22–86 years [22].

The determination of the serum MMA levels with the aforementioned technique can be used to demonstrate a mild blood cobalamin deficiency [12].

In conclusion, the described method has the advantage of selecting an ion of high mass for quantification of MMA. This method was sensitive, specific and sufficiently rapid for routine use in cobalamin deficiency diagnosis. The utility of the method for detecting mild cobalamin deficiency was demonstrated.

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