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Optimization and performance of a rapid gas chromatography–mass spectrometry analysis for methylmalonic acid determination in serum and plasma

Mark M. Kushnir[a,*], Gabor Komaromy-Hiller[a,b]

[a]*ARUP Laboratories, Inc., 500 Chipeta Way, Salt Lake City, UT 84108, USA*
[b]*University of Utah School of Medicine, Department of Pathology, 50 North Medical Drive, Salt Lake City, UT 84132, USA*

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Abstract

We have developed a rapid and sensitive GC–MS assay for methylmalonic acid determination in serum and plasma utilizing an anion exchange solid-phase extraction and trimethylsilyl derivatization. Each step of the procedure was optimized by the experimental design methods to assure the assay reliable performance. The limit of detection and limit of quantitation were 0.025 and 0.1 μmol/l. The total coefficient of variation for the method was 9.8, 4.4, and 4.6% at the concentration of 0.2, 3.1, and 6.2 μmol/l methylmalonic acid concentration, respectively. The assay was linear up to 9.0 μmol/l, and showed good correlation with a reference method. The method has proven to be reliable in routine production, producing clean chromatography, unique ion fragments, and consistent ion mass ratio.

Keywords: Methylmalonic acid

1. Introduction

Methylmalonic acid (MMA) is a metabolic intermediate in the conversion of propionic acid to succinic acid. Cobalamin (vitamin B_{12}) is an essential cofactor of the enzymatic carbon rearrangement of methylmalonic acid to succinic acid, and the lack of it leads to elevated levels of MMA. Consequently, serum methylmalonic acid has been used to assess tissue vitamin B_{12} status [1]. Measurement of MMA has several advantages over cobalamin. First, serum or plasma vitamin B_{12} levels may not reflect adequately tissue cobalamin status [2,3]; second, serum methylmalonic acid level is about 1000-fold greater than serum cobalamin level, and an elevation rather than decrease indicates pathologic condition; third, MMA is more stable than cobalamin. The central 95-percentile reference interval for MMA concentration in serum and plasma for healthy individuals is 0.05–0.37 μmol/l [6]. Although serum methylmalonic acid reflects tissue vitamin B_{12} levels adequately in liver failure, it is not useful when renal function is decreased [2,4,5]. Chronic hemodialysis patients usually have elevated levels of serum MMA (0.16–1.87 μmol/l) [5], and patients with vitamin B_{12} deficiency may display levels from 0.47 to 190 μmol/l [7].

Due to its usefulness in assessing vitamin B_{12}

*Corresponding author.

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status, several methods for methylmalonic acid determination in serum and urine have been published, utilizing gas chromatography (GC), GC–MS, HPLC, capillary electrophoresis (CE), and LC–MS instruments [7–10]. All published HPLC procedures have long instrument run time, column switching, and utilize gradient elution [7–9]. Direct detection of organic acids by CE is still a problem, and the indirect detection methods do not provide adequate sensitivity and precision [10,11]. The primary choice of method for serum MMA analysis remains gas chromatography with mass spectrometric detection [12–16]. Earlier assays encountered problems related to losses of MMA during sample preparation [12,16], instability of MMA derivatives [12], and ion fragments crosscontribution between MMA and its deuterated internal standard [14]. Liquid/liquid extraction of MMA by organic solvents is widely used for sample preparation, however, it suffers from low extraction recovery (20–55%) [12,16]. In order to obtain adequate sensitivity, multiple extractions with subsequent mixing of the extracted fractions is required [12,14,15]. Solid-phase extraction (SPE) has been widely utilized for methylmalonic acid analysis, because it is potentially less labor intensive and provides cleaner extract compared to liquid/liquid extraction [13,17]. Following the sample cleanup step, carboxyl groups of extracted acids should be derivatized to obtain a nonpolar derivative. Various silyl (trimethylsilyl or *tert*.-butyldimethylsilyl) and alcohol (cyclohexyl or *n*-butyl) ester derivatives have been utilized for MMA analysis [13–17]. Under some derivatization conditions for the methods utilizing silyl derivative of MMA, authors experienced unreliable method performance, which they attributed to the formation of ester enols [13,14] or to the active hydrogen on C-2 [14]. Recently it was determined that MMA trimethylsilyl (TMS) derivative formation does not require incubation and takes place during the injection in a GC injection port [18]. On the other hand, derivatization of MMA by cyclohexanol or *n*-butanol requires incubation at temperature above 100°C to obtain adequate recovery [13,15].

The purpose of the study was to develop and characterize a rapid, reliable procedure for MMA analysis in serum and plasma.

2. Experimental

2.1. Instrumentation

The experiments were carried out using a Hewlett–Packard (HP) model 5890 gas chromatograph equipped with a HP 5970 mass selective detector, a 7673 autosampler, an Rtx^{R}-200 capillary column 20 m×0.18 mm I.D., 0.4 μm film thickness (Restek, Bellefonte, PA, USA), and DrugQuant™ software. The mass selective detector was used in the electron ionization mode at 70 eV. Helium was utilized as a carrier gas. The injection port temperature was 270°C, initial column temperature 100°C, ramped at 18°C/min to 160°C, ramped at 50°C/min to 300°C with a final hold of 2.5 min. The interface temperature was held at 300°C. The carrier gas pressure program was 275 kPa held for 0.2 min, ramped at 600 kPa/min to 150 kPa. A split injection mode was utilized with a ratio of 1:20 and injection volume 1 μl. The ions monitored for the derivatives were: *m/z* 247, 218 for MMA and *m/z* 250, 221 for d_3-MMA. Borosilicate glass culture tubes, 16 mm×100 mm, with Teflon™ lined plastic closures (Fisher Scientific, Pittsburgh, PA, USA) were used for the derivatization. The derivatization was carried out in a dry heating block (Fisher Scientific). Glass autosampler vials, vial inserts and crimp caps were obtained from Fisher Scientific. Miscellaneous supplies included a vortex mixer, adjustable pipets with disposable pipet tips.

2.2. Reagents, standards, and controls

Methylmalonic acid (MMA) was purchased from Sigma (St. Louis, MO, USA), d_3-MMA was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). The MMA and d_3-MMA stock standards were prepared in methanol at a concentration of 10 mmol/l and stored at −20°C. The MMA calibration standard and d_3-MMA internal standard were prepared in acetonitrile at concentration of 10 and 15 μmol/l, respectively. The standards for the method precision and linearity study were prepared in dialyzed human plasma and stored at 1–6°C.

Derivatizing reagents *N*-methyl-*N*-(trimethyl-

silyl)trifluoroacetamide (MSTFA), *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane (MSTFA+1% TMCS), and *N,O*-bis-[trimethylsilyl]trifluoroacetamide) with 1% trimethylchlorosilane (BSTFA+1% TMCS) were purchased from Pirce (Rockford, IL, USA). Methanol, acetonitrile and methyl-*tert*.-butyl ether (MTBE) were of analytical grade (Fisher Scientific).

2.3. Sample preparation and calibration

Separation of MMA from matrix was performed on solid-phase extraction columns CUQAX15Z (United Chemical Technologies, Inc., Bristol, PA, USA). The calibration standards were prepared at 0.2, 0.5, 1.0 and 2.0 μmol/l in dialyzed human plasma. To each calibrator, control and test sample 100 μl of the internal standard d_3-MMA and 1 ml of acetonitrile were added, the tubes were vortexed for 20 s and centrifuged for 5 min at 2000 *g*. The content of the tubes was transferred into the SPE columns, previously conditioned with 3 ml methanol and 5 ml distilled water. The columns were washed by sequential addition of 10 ml deionized water, 5 ml of methanol, 2 ml of MTBE, and dried after each wash with vacuum for 3 min. The analytes were eluted with 5 ml of elution solvent consisting of 3% formic acid in MTBE. The eluate was dried under a stream of nitrogen at 35°C, the residue was reconstituted with 25 μl of MSTFA +1% TMCS and 25 μl acetonitrile, and the tubes were incubated at 55±5°C for 5 min.

2.4. Optimization of extraction

Fractional factorial experimental design [20] was utilized for optimization of the extraction of MMA from serum by strong anion exchange (quaternary amine) SPE. Parameters evaluated in the experiment were: amount of the anion-exchange adsorbent, solvent utilized for MMA elution from the adsorbent, formic acid concentration in the elution solvent, and elution solvent volume relative to the amount of the adsorbent. The optimization experiment was performed as follows. A pool of human serum samples was spiked with MMA standard to a concentration of 10 μmol/l. Equal volume of acetonitrile was added to the sample, the tube was vortexed for 1 min, and centrifuged for 5 min at 2000 *g*. The supernatant was decanted from the precipitate and 2-ml aliquots of the obtained solution were transferred into conditioned SPE columns. The columns were washed as described in the sample preparation section. The MMA was eluted with variable volumes of the elution solvent prepared based on methanol or MTBE, with concentration of formic acid ranged from 1.5 to 5%. The eluate was dried and the residues were derivatized as described in the sample preparation section. Twenty microliters of the derivatized internal standard (d_3-MMA) was added to the tubes after derivatization to minimize the influence of variation during the GC–MS analysis. The peak area ratio of MMA to d_3-MMA was utilized as the optimization parameter. The derivatized internal standard was prepared as follows. Three milliliters of d_3-MMA internal standard was aliquoted into an empty tube. The solvent was evaporated and the residue was reconstituted with 25 μl of acetonitrile and 25 μl of MSTFA with 1% TMCS. The tube was incubated at 60°C for 5 min and 250 μl of acetonitrile was added to the solution. Each experiment in the matrix was performed in duplicate. Statistical analysis of the results was performed with the methods of factorial analysis [20].

2.5. Optimization of derivatization

A second order orthogonal experimental design [21] with parameters varied on five levels was utilized to determine a regression equation for the reaction recovery as a function of the derivatization solution composition (z_1), incubation temperature (z_2), and time (z_3). Ranges of variation for the parameters are presented in Table 1. The variables (z) were encoded by using the formula:

$$x_i = \frac{(z_i - z_i^0)}{\Delta z_i}, \quad (1)$$

where x_i is the coded value of a variable; z_i is the variable value in a real scale, z_i^0 is the zero level of a variable; Δz_i is the range of a variation; i is number of a variable.

Seventeen experiments were carried out in dupli-

Table 1
Variation ranges of the parameters for the experimental design on derivatization optimization

Parameter	Ratio acetonitrile: derivatizing reagent (total volume 50 μl), z_1	Incubation temperature (°C) z_2	Incubation time (min) z_3
Zero level, z_0	1:1	80	15
Variation step, Δz	0.5	15	8
+1.35	1.68:1	100	26
+1	1.5:1	95	23
−1	1:1.5	65	7
−1.35	1:1.68	60	4

cate (Table 2). The experiments were performed with a pool of human serum spiked with MMA to the final concentration of 10 μmol/l. Equal volume of acetonitrile was added to the aliquots, the tubes were vortexed and centrifuged. The solution was decanted from the precipitate and 2-ml aliquots were extracted as described in the sample preparation section. The MMA was eluted with 5 ml of 3% formic acid in MTBE. Effluent from all the columns was combined in a single pool, and spiked with 10 μl of internal standard (0.1 μg/μl of caffeine in methanol). The solution was mixed, 2-ml aliquots were transferred in separate tubes and the solvent was evaporated. Acetonitrile and MSTFA containing 1% TMCS were added to the tubes in the amount indicated in the experimental matrix (Table 2). The tubes were vortexed and incubated in a heating block at the temperature and time specified in the matrix. To stop the reaction the tubes were immersed into an isopropanol/dry ice bath for 30 s. Recovery of the derivative was calculated as the ratio of the peak area of MMA–TMS to the peak area of caffeine. Preferable conditions corresponded to a maximum conversion of a MMA to the derivative. The results of the experiments were utilized to calculate a second order polynomial for the recovery as a function of the

Table 2
Experimental matrix and optimization results for MMA derivatization

Exp	x_1	x_2	x_3	Relative recovery of the MMA–TMS derivative	
				Mean of two experiments	Calculated by Eq. (2)
1	−1	+1	+1	0.195	0.027
2	+1	+1	+1	0.280	0.383
3	−1	−1	+1	0.953	0.993
4	+1	−1	+1	1.095	0.993
5	−1	+1	−1	0.978	0.824
6	+1	+1	−1	0.920	0.824
7	−1	−1	−1	1.318	1.160
8	+1	−1	−1	0.890	0.803
9	0	0	0	0.800	0.873
10	0	0	0	1.030	0.873
11	0	0	0	0.905	0.873
12	1.35	0	0	0.795	0.752
13	−1.35	0	0	0.640	0.752
14	0	1.35	0	0.418	0.452
15	0	−1.35	0	1.063	1.091
16	0	0	1.35	0.890	0.668
17	0	0	−1.35	1.028	1.078

reaction parameters [21,22], and to make a plot for 3-dimensional graphical representation of the recovery surface.

3. Results and discussion

3.1. Solid-phase extraction

Statistical analysis of the results of the fractional experimental design (results not presented) showed that formic acid concentration and elution volume significantly affected the recovery. Although the MMA recovery was not affected by the solvent utilized for the elution, noticeable improvement in the extract cleanliness and eliminating unwanted peaks on chromatogram was observed with utilization of MTBE based elution solvent. The extraction appeared to be more selective as compared to methanol and produced much cleaner extract. The determined optimal concentration of formic acid in the elution solvent was 3%, and the volume of the elution solvent 0.9 ml per 100 mg of adsorbent. Amount of the adsorbent in the SPE column within range of 200–500 mg did not affect the recovery.

The mass spectra and chemical structure of the MMA–TMS ester presented in the Fig. 1. The major ion fragments m/z 247 for MMA and m/z 250 for d_3-MMA result from cleavage of a methyl group from the molecular ion. The qualitative ions for MMA and d_3-MMA were m/z 218 and m/z 221, respectively. Fig. 2 shows the selected ion chromatogram of a sample extracted by SPE and liquid/liquid extraction with ethyl acetate by the procedure described in [14]. Comparison of the SPE and liquid/liquid extraction proved that liquid/liquid extraction was less selective towards MMA. An unidentified compound was coextracted by ethyl acetate and interfered with MMA quantitation. The retention time and one of the fragments of the compound were identical with those of MMA. We did not attempt to characterize this compound, however, the interference was completely absent with sample preparation utilizing SPE. Significant background interference was also observed in the sample prepared by liquid/liquid extraction in the confirmation ions m/z 218 and m/z 221.

The experiments to evaluate extraction recovery were performed with spiked human plasma samples containing MMA at concentrations of 0.4 and 4 μmol/l. To the first group of samples the internal standard was added prior to acetonitrile precipitation, to the second group the internal standard was added into the SPE effluent. The extraction was performed as described in sample preparation section. The dried extracts were derivatized according to the procedure and analyzed at the same time. The percentage recovery was determined by comparing the MMA concentration in the samples to which the internal standard was added after the extraction to the results observed in the samples to which the internal standard was added before the extraction. The overall extraction yield of the acetonitrile precipitation and SPE was within 78±12%.

3.2. Derivatization

Trimethylsilyl esters are one of the most common types of derivative utilized to convert carboxyl group of organic acids to their nonpolar derivatives [13,14,16,23,24]. Such wide utilization of the TMS derivatives is due to fast derivatization, chemical stability, and good yield of the reaction. There are multiple reagents available to produce TMS diester of MMA. It was determined that the reaction between MMA and MSTFA is very fast and comes to completion within seconds while the sample is exposed to elevated temperature in the GC injection port [18]. In our experience of MMA analysis from serum and plasma samples, the derivatization by the procedure was not consistent. Approximately half of the patient samples were readily derivatized without incubation; however, the rest did not produce MMA–TMS derivative under the above conditions. We observed that the derivatization did not take place in the samples with elevated bilirubin concentration. A probable reason of the interference is bilirubin–MMA adduct formation in the acid containing solvent during the sample preparation, and insufficient silyl donor capacity of the reagent to break the adduct. The MMA–bilirubin complex prevents MMA–TMS derivative formation at the mild conditions suitable for the derivatization of MMA extracted from other matrixes.

Contrary to MSTFA when the derivative formation was inconsistent, BSTFA+1% TMCS was

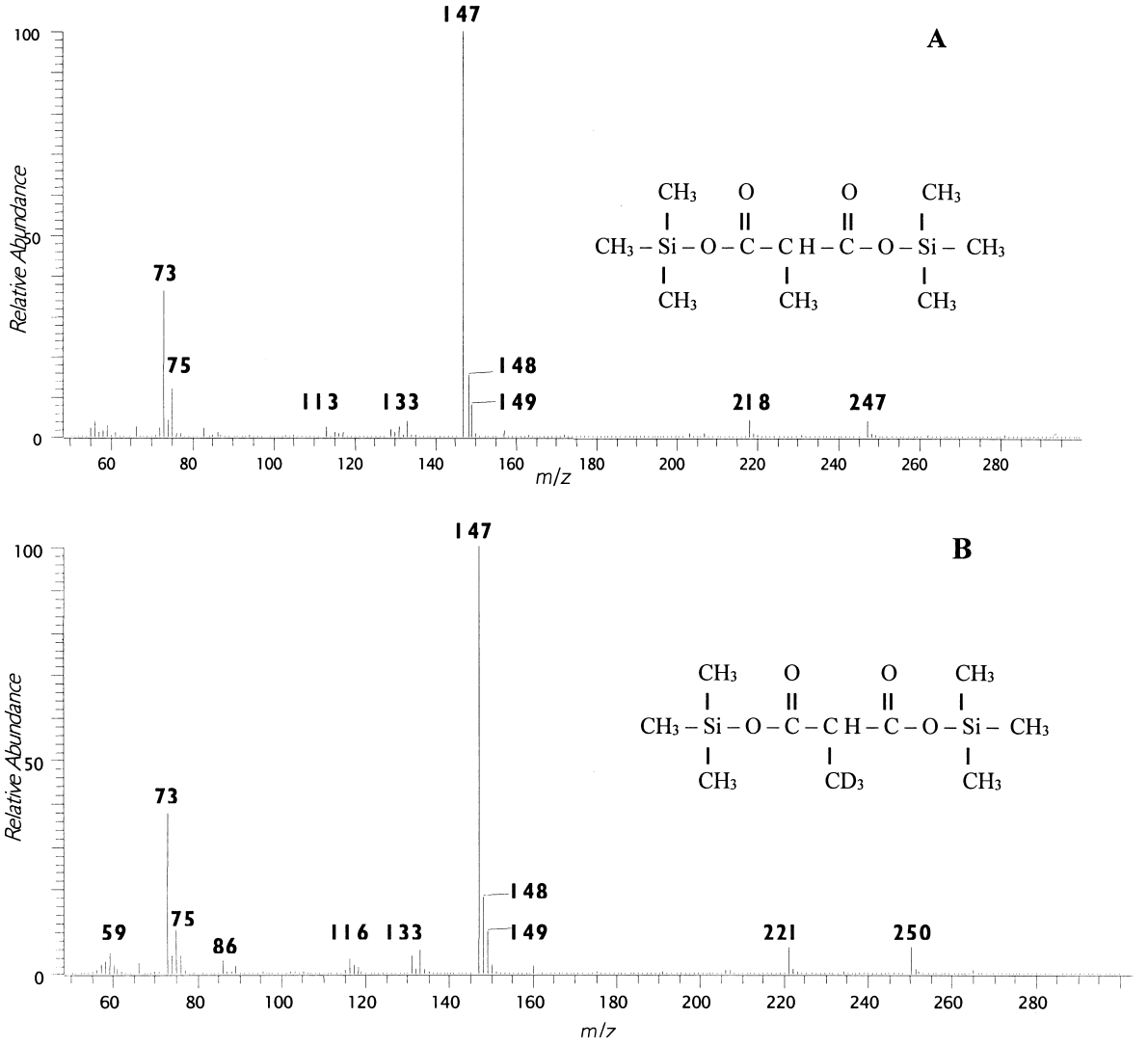

Fig. 1. Mass spectra and structure of TMS ester derivatives: (A) MMA–TMS and (B) d_3-MMA–TMS.

found to cause decomposition of the MMA–TMS ester and formation of multiple TMS derivatives of the coextracted from plasma compounds which interfered with the MMA–TMS peak. The increased interference potential can be attributed to high reactivity of the reagent, leading to formation of multiple TMS esters of the coextracted compounds, and their decomposition products. The interference potential can be minimized by utilizing milder reagents and reaction conditions. MSTFA with 1% TMCS, has silyl donor activity positioned between MSTFA and BSTFA containing 1% TMCS, and was selected for subsequent study as the most promising derivatizing reagent for MMA analysis. Preliminary experiments showed that the reaction conditions significantly affected the MMA–TMS ester recovery for the reaction of MMA with MSTFA containing 1% TMCS.

To optimize the derivatization, reaction between MMA and MSTFA containing 1% TMCS was studied by a second order orthogonal experimental design method. The coefficients of the second order

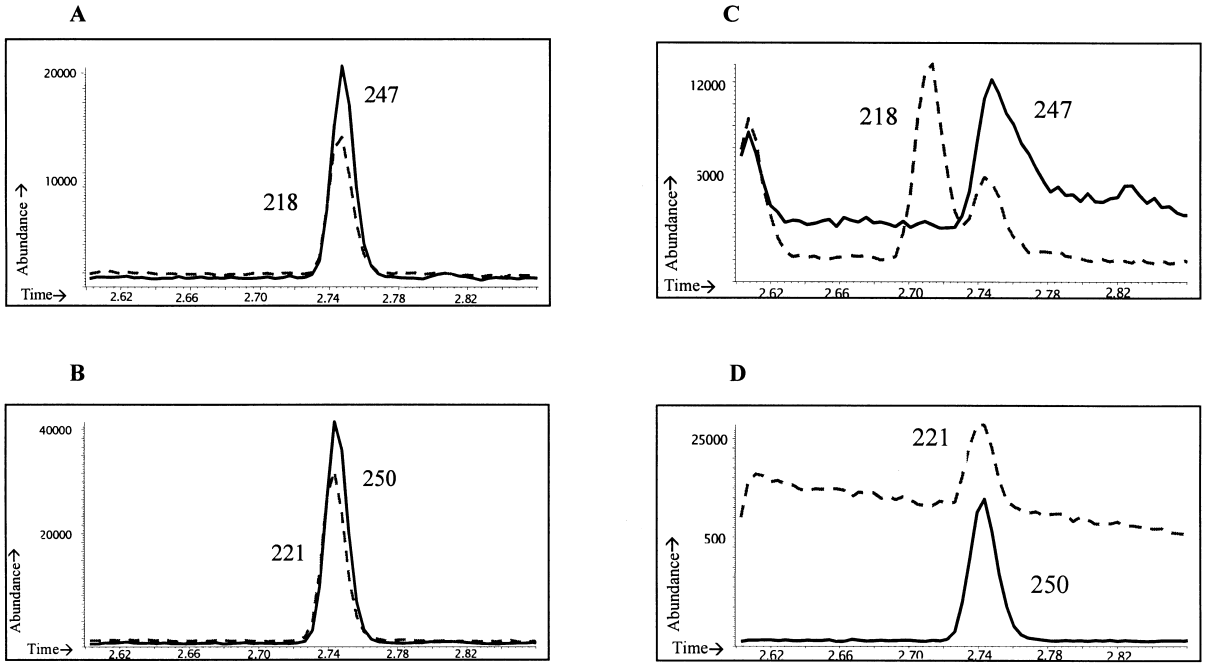

Fig. 2. Total ion chromatogram of a patient serum sample containing 1 μmol/l of methylmalonic acid extracted by SPE (A, B) and liquid/liquid extraction (C, D). MMA mass ion fragments *m/z* 247 and 218 (A, C), d_3-MMA mass ion fragments *m/z* 250 and 221 (B, D).

polynomial for the dependence of the derivative recovery on the reaction parameters were calculated from the experimental results (Table 2) utilizing an algorithm described in [21,22]. Significance of the polynomial coefficients was evaluated with a Student *t*-test. All the variables with corresponding *t*-test value less then the reference number ($t_{0.20(2)} = 1.89$) were eliminated from the polynomial. The obtained polynomial for the reaction recovery dependence on the experimental conditions is:

$$Y = 0.873 - 0.236x_2 - 0.152x_3 + 0.089x_1x_2 - 0.158x_2x_3 + 0.089x_1x_3 - 0.066x_1^2 - 0.056x_2^2 \quad (2)$$

The polynomial was assessed statistically with a Fisher test. The Fisher test value for the correspondence between the experimental recovery and the recovery calculated from the equation was 2.9. The obtained value is less than the reference number ($F_{0.20(9,\,2)} = 19.3$) that indicates validity of the equation.

Both terms at linear effects for the incubation temperature and time have negative signs that represent the advantage of utilizing milder conditions to improve the derivative recovery. A greater absolute value of the term corresponding to incubation temperature suggests its greater influence on the recovery. In addition to single parameter terms, the equation has second order terms, representing interrelationship between the parameters. The coefficient at the term corresponding to the mutual influence of the reaction temperature and time has the greatest absolute value among the second order terms, indicating its greatest effect. Composition of the derivatizing solution has less influence on the reaction yield compared to incubation temperature and time. The interrelationship terms and second order terms in the polynomial represent mutual influence of the parameters on each other and indicate a nonlinear dependence of the recovery on the reaction conditions.

In order to evaluate the interrelationship between the parameters the regression Eq. (2) was transformed to a canonical form:

$$R - 0.75 = 0.056\,X_2^2 - 0.112\,X_3^2. \quad (3)$$

The transformation was performed by methods of linear algebra. The canonical equation has one negative and one positive term and corresponds to the surface of a hyperbolic paraboloid with saddle point in the center. The surface within the experimental field is part of a branch of the hyperbolic paraboloid. Fig. 3 is a plot of the recovery surface for MMA–TMS ester depending on the incubation temperature and time. The equation predicts a major increase in the recovery with decrease of the incubation time, and to a smaller extent with increase of the incubation temperature. The recovery surface does not have a maximum within the experimental area, and suggests some additional gain in the recovery with further increase in the incubation temperature. Analysis of the recovery surface indicates that the optimal conditions correspond to incubation at the temperature of 60–100°C for 5 min. The improved recovery with short incubation time is probably related to minimizing side reactions leading to MMA–TMS ester decomposition.

3.3. Chromatographic separation

Capillary columns with different stationary phases have been utilized for MMA analysis. The majority of the published methods for MMA analysis have long MMA retention times, as well as total instrument run time. The long analysis time is required because of high potential for interference from the coextracted compounds. The extended total analysis time is necessary to elute all the coextracted compounds from the GC column prior to the following injection. Comparison of the chromatographic separation on capillary columns with different stationary phases showed significant improvement in separation of the TMS derivatives of the low-molecular-mass organic acids on the Rtx-200 capillary column. The column has unique selectivity allowing very effective separation of the TMS derivatives of organic acids. The retention time of the TMS derivatives of the clinically significant small organic acids eluted close to MMA–TMS is presented in Table 3.

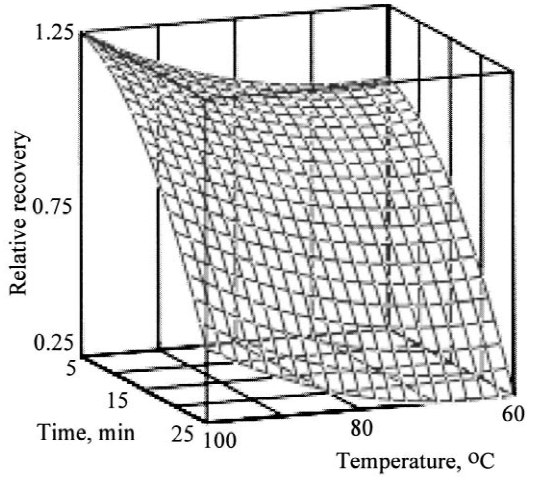

Fig. 3. MMA–TMS recovery dependence on incubation temperature and time.

The carrier gas pressure program utilized in the method is designed to improve the method sensitivity [25,26]. The method creates a pressure pulse at the time of injection, followed by a rapid reduction of the pressure to a value optimal for the capillary GC separation and MS performance. Thus under the pressure program conditions, sensitivity of the method was enhanced 2- to 3-fold compared to a constant flow mode. Increased method sensitivity allows monitoring MMA at clinically significant concentrations with relatively short instrument utilization time.

3.4. Analytical sensitivity

Method sensitivity was determined by analyzing standard solutions prepared in dialyzed plasma containing progressively lower concentration of

Table 3
Retention time of clinically relevant small organic acids potentially interfering with MMA

Compound	RT (min)	RRT
3-Hydroxybutyric acid	2.49	0.910
Glyoxylic acid	2.51	0.917
Caprylic acid	2.58	0.942
2-Oxohexanoic acid	2.62	0.957
Methylmalonic acid	2.74	1.000
Levulinic acid	2.90	1.058
Ethylmalonic acid	3.06	1.116
Phenylacetic acid	3.06	1.116
Succinic acid	3.29	1.199
Methylsuccinic acid	3.31	1.209

methylmalonic acid. Limit of quantitation (LOQ) was defined as the concentration at which the accuracy was maintained within ±20% of the expected value, and imprecision was less than 10%. The limit of detection (LOD) was determined as the lowest concentration that produced a peak with retention time and qualitative ion mass ratio consistent with values established by the calibration. Based on the criteria, LOQ for the method was 0.1 μmol/l, and LOD was 0.025 μmol/l.

3.5. Precision

The method precision was determined by analyzing three pools containing methylmalonic acid at low, medium and high concentrations for five consecutive days. Dialyzed plasma pools spiked at 0.2 μmol/l (low pool) and 6 μmol/l (high pool) of MMA were prepared. The medium pool of MMA was obtained by mixing the low and high pools in 1:1 ratio, and contained 3.1 μmol/l of MMA. Within-run, between-run and total precision are presented in the Table 4. GC–MS instrument precision was determined by repetitive injections of a 5 μmol/l MMA standard from the same vial. Coefficient of variation (CV%) for the area ratio MMA/d_3-MMA for 21 consecutive injections was 2.9%.

3.6. Linearity

Linearity of the method was evaluated by analyzing MMA standards prepared in dialyzed human plasma. The MMA concentration in the pools ranged from 0.2 to 15 μmol/l. Each standard was analyzed in duplicate in two runs and concentrations were calculated from a standard curve. Utilizing a criterion of maintaining accuracy of ±80% of a target value, the assay was found to be linear up to 9 μmol/l of MMA, well above the normal serum MMA level of 0.4 μmol/l [2,6]. A total of 99.7% of all the clinical specimens analyzed in the laboratory since the inception of the method had a serum MMA concentration within the linearity range.

3.7. Accuracy

Fifty-one patient specimens previously analyzed by a GC–MS reference method were analyzed by the evaluated method. The results were grouped in two sets according to the observed concentration. The first group contained samples with MMA concentration ranged from 0.1 to 9 μmol/l, the second group contained results obtained by analysis of all the available samples. To account for imprecision in both the reference and evaluated method the results were analyzed by Deming regression [19]. The correlation coefficient (r) and standard error of estimate ($S_{x,y}$) were 0.9889 and 0.146 μmol/l in the concentration range from 0.1 to 9 μmol/l, and 0.9941 and 1.315 μmol/l in the concentration range from 0.1 to 120 μmol/l, respectively. The linear regression equations were $y=1.138(\pm0.026)x+0.016(\pm0.042)$ for the range 0.1 to 9 μmol/l, and $y=1.056(\pm0.017)x+0.488(\pm0.289)$ for the range 0.1 to 120 μmol/l, respectively. The data indicated no significant constant bias between the methods, however the slope of the linear regression line shows positive proportional bias for the evaluated method. The bias may be causeed by a difference in the concentration of the calibration standards [17].

3.8. Interferences

None of the low-molecular-mass organic acids with relative retention time close to that of MMA interfered with the assay (Table 3). No difference in the method performance was observed for plasma

Table 4
Method accuracy and imprecision

Concentration (μmol/l)	Within-run CV (%)	Between-run[a] CV (%)	Total CV (%)	Accuracy (%)
0.2	6.0	7.8	9.8	115.8
3.1	3.6	2.4	4.4	98.6
6.0	2.6	3.8	4.6	90.7

[a] $n=5$.

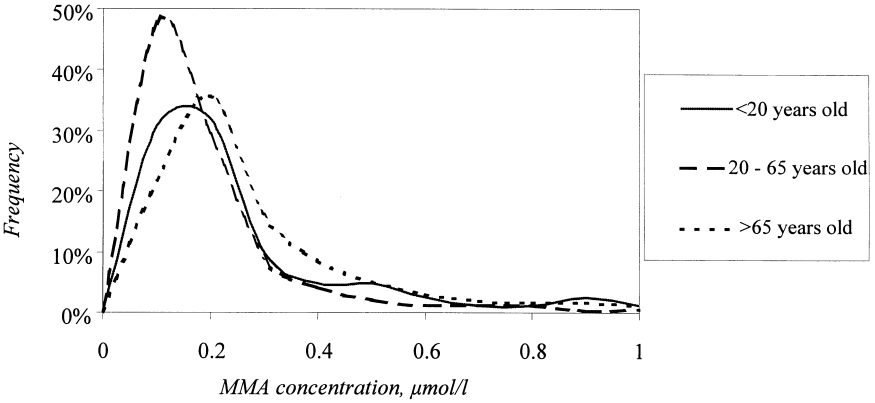

Fig. 4. Normalized distribution of serum MMA concentration for different age groups.

specimens collected in heparin, citrate, or EDTA tubes.

During four months of the method utilization, 2481 serum and plasma specimens have been analyzed for MMA. Only one specimen had an unidentified interference that prevented accurate quantitation of MMA. The majority of the specimens (57%) came from the elderly (>65 years of age), 40% from adults (20–65 years of age), and 3% from patients younger than 20 years. The distribution of MMA concentration for the three age groups is shown in Fig. 4. The serum MMA concentration (mean±SD) in individuals with observed MMA values below 0.4 μmol/l were 0.17±0.07, 0.16±0.07, and 0.19±0.07 μmol/l, respectively for the evaluated age groups. Frequency of observation elevated values of MMA (>0.4 μmol/l) was 9.9% for adults and 18.2% for elderly patients. Fig. 5 shows a distribution of serum MMA values for men and women. We found no significant gender bias for serum MMA concentration (Wilcoxon $P=0.134$) [27]. These findings agree with the results previously observed by Rasmunssen et al. [28], where with the exception of elderly women (>60 years old) there was no significant age and gender dependence for the serum MMA levels.

4. Conclusions

The MMA method employing an anion-exchange SPE in combination with MSTFA+1% TMCS derivatization is fast, reliable, efficient, and produces clean chromatography with unique ion fragments,

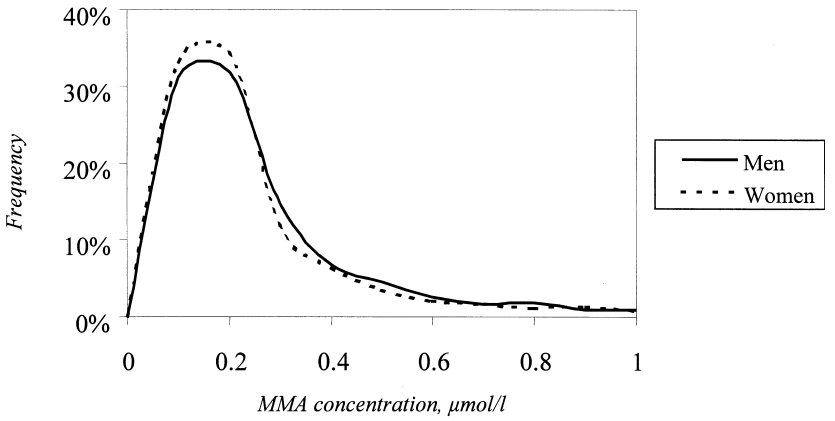

Fig. 5. Normalized distribution of serum MMA for men and women.

consistent ion mass ratios, and no observed interference. The two steps of sample preparation, i.e. solid-phase extraction and trimethylsilyl derivatization were optimized separately. Out of the evaluated parameters, statistically significant effect on the extraction recovery was observed from the acid concentration in the elution solvent and volume of the elution solvent. Comparison of the SPE and liquid/liquid extraction proved that liquid/liquid extraction was significantly less selective towards MMA and could not be utilized in combination with TMS derivatization without significant improvement in the cleanliness of the extract. The derivatization recovery was studied and optimized by a second order experimental design method. The results of the experiments showed that the recovery surface dependence on the incubation temperature and time corresponds to a hyperbolic paraboloid. The major increase in the recovery was observed with a decrease of the incubation time, and to a smaller extent with an increase of the incubation temperature. Capillary column Rtx-200 with trifluoropropyl stationary phase has superior separation power for TMS derivatives of low-molecular-mass organic acids. Statistical analysis of serum MMA concentration measured in over 2400 patients specimens showed no significant difference in the observed values for healthy individuals of different age groups and gender.

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