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Automation and Analytical Techniques

Automated Assay for the Determination of Methylmalonic Acid, Total Homocysteine, and Related Amino Acids in Human Serum or Plasma by Means of Methylchloroformate Derivatization and Gas Chromatography–Mass Spectrometry

Amrei Windelberg,* Ove Årseth, Gry Kvalheim, and Per Magne Ueland

Background: **The combined measurement of methylmalonic acid (MMA) and total homocysteine (tHcy) in serum or plasma is useful in diagnosing and distinguishing between cobalamin and folate deficiencies. We developed and validated an isotope-dilution gas chromatography–mass spectrometry (GC-MS) method with automated sample workup for the determination of MMA, tHcy, and the related amino acids Met, total cysteine (tCys), Ser, and Gly in serum or plasma.**

Methods: **Serum or plasma samples (100 μL) were treated with a reductant (dithioerythritol), deproteinized with ethanol, and derivatized and extracted in a single step by the addition of methylchloroformate and toluene. All liquid handling was performed in 96-well (1 mL) microtiter plates by a robotic workstation. The *N*(*S*)-methoxycarbonyl ethyl ester derivatives were analyzed by GC-MS in the selected-ion monitoring mode.**

Results: **Detection limits (signal-to-noise ratio, 5:1) were between 0.03 μmol/L (MMA) and 10 μmol/L (Ser, tCys). The assay was linear to 100 μmol/L for MMA and tHcy and to 1000 μmol/L for Met, tCys, Ser, and Gly. The within-day CVs ranged from 0.7% to 3.6% (n = 20), and the between-day CVs from 2.1% to 8.1% (n = 20). The recovery was between 79% and 99% for the different analytes.**

Conclusion: **This assay combines a simple and automated sample preparation with selective and sensitive GC-MS analysis and is well suited for the combined measurement of MMA, tHcy, and the related amino acids.**

Methylmalonic acid (MMA)[1] and total homocysteine (tHcy) in serum or plasma increase in cobalamin-deficient individuals and are clinically useful indicators of cobalamin status. tHcy is also increased in folate deficiency and is used as an indicator of this deficiency *(1)*. tHcy is thus a less specific indicator of vitamin B_{12} function than MMA, also because the concentration of tHcy is influenced by diverse genetic and lifestyle factors and disease states *(2)*. In addition, an increased tHcy concentration increases the risk of cardiovascular disease *(3)*.

Numerous tHcy assays have been published, most of which are based on liquid or gas chromatography *(4–6)*. More recently, homogeneous immunologic tHcy assays adapted to commercial platforms have gained widespread use *(7, 8)*. The determination of MMA in serum or plasma has been accomplished by gas chromatography–mass spectrometry (GC-MS) *(9–13)*, capillary electrophoresis with laser-induced fluorescence detection *(14)*, and liquid chromatography–tandem mass spectrometry *(15, 16)*.

Simultaneous determination of MMA and tHcy in the same serum or plasma specimen is practical because of the complementarity of these 2 analytes in the diagnosis of cobalamin and folate deficiencies. The inclusion of other amino acids related to Hcy metabolism, such as Met, total cysteine (tCys), Ser, and Gly, in such an assay may

LOCUS for Homocysteine and Related Vitamins, and Section for Pharmacology, Institute of Medicine, University of Bergen, Bergen, Norway.

*Address correspondence to this author at: LOCUS for Homocysteine and Related Vitamins, Section for Pharmacology, Institute of Medicine, University of Bergen, Armauer Hansens Hus, N-5021 Bergen, Norway. Fax 47-55-97-4605; e-mail amrei.windelberg@farm.uib.no.

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[1] Nonstandard abbreviations: MMA, methylmalonic acid; tHcy, total homocysteine; GC-MS, gas chromatography–mass spectrometry; tCys, total cysteine; MCF, methylchloroformate; and LOD, limit of detection.

give additional information about metabolic abnormalities influencing the concentration of tHcy. Such multiplexing could be achieved by GC after alkylchloroformate derivatization, which allows simultaneous esterification of carboxylic groups and acylation of amino and thiol groups, creating *N*(*S*)-alkoxycarbonyl alkyl ester derivatives. This strategy was proposed by Husek *(17, 18)*, who introduced alkylchloroformates as general derivatizing reagents in GC.

Methods based on chloroformate derivatization for the determination of tHcy *(19–26)* or MMA *(27)* have been published. Most of them use ethylchloroformate *(20, 22, 23, 25–27)*, but other chloroformates have been used as well *(19, 21, 24)*. The advantages include fast and simple sample preparation in an aqueous medium at room temperature. The performance of the plasma tHcy methods based on chloroformate derivatization has been published previously *(19–26)*, whereas no such method has been optimized for the quantification of MMA in serum or plasma.

Here we describe an automated isotope-dilution GC-MS assay for the simultaneous determination of MMA, tHcy, and the related amino acids Met, tCys, Ser, and Gly based on derivatization with methylchloroformate (MCF). All liquid handling was performed in 96-well microtiter plates to enable high-throughput sample preparation.

Materials and Methods

MATERIALS

L-Cysteine (Cys), L-Ser, D,L-dithioerythritol, pyridine, and toluene were obtained from Fluka. Gly, L-homocystine, and L-Met were from Sigma Chemical Co. MMA and the derivatization reagent, MCF, were purchased from Aldrich. D,L-d_3-Ser and D,L-d_2-Cys were obtained from Medical Isotope; d_2-Gly, D,L-d_8-homocystine, and d_3-MMA were from Cambridge Isotope Laboratories; and D,L-d_4-Met was from C/D/N Isotopes. All other chemicals and solvents were of the highest purity available. Human serum for assay calibration and validation experiments was obtained by pooling surplus sera from routine testing. For precision studies, the pooled serum was treated with mercaptoethanol (20 mmol/L) and then dialyzed 4 times against 10 volumes of phosphate-buffered saline (137 mmol/L NaCl, 2.7 mmol/L KCl, 2.9 mmol/L KH_2PO_4, 14 mmol/L Na_2HPO_4) containing 4 mmol/L EDTA.

SAMPLE COLLECTION

EDTA-plasma was obtained by collecting blood into Vacutainer Tubes (Becton Dickinson); the final EDTA concentration in the samples was 4 mmol/L. The EDTA-blood samples were centrifuged within 60 min. Serum was obtained by collecting blood into Vacutainer Tubes with no additive. Blood was allowed to clot at room temperature for 30 min before isolation of the serum fraction. Plasma and serum were stored at −20 °C until use.

SAMPLE PREPARATION

For routine measurements, sample preparation and all liquid handling were performed in 96-well (1 mL) microtiter plates by a robotic workstation (ATplus 2; Hamilton). Serum or plasma samples (100 μL) were transferred from sample tubes into the microtiter plates, where they were mixed with 25 μL of D,L-dithioerythritol (200 mmol/L) containing d_8-homocystine and d_2-Cys (30 μmol/L and 1 mmol/L, respectively) and incubated at room temperature for 20 min to reduce the disulfide bonds of Hcy and Cys. Samples were then deproteinized by adding 450 μL of ethanol containing the remaining deuterated internal standards (2.6 μmol/L d_3-MMA, 5.6 μmol/L d_4-Met, 22 μmol/L d_2-Gly, and 44 μmol/L d_3-Ser). Aliquots (380 μL) of the supernatant obtained after centrifugation (3 min at 5800*g*) were transferred into an empty microtiter plate and mixed with 300 mL/L of water and 50 μL of pyridine; 250 μL of 200 mL/L in toluene was then added. Mixing was achieved by repeated pipetting. After incubation at room temperature for 6 min to obtain phase separation, 500 μL of the aqueous phase was replaced by water (450 μL), and the samples were mixed again. Most of the lower aqueous phase was removed before the plates were manually sealed and transferred to the sample tray of the autoinjector. An aliquot (1.5 μL) of the toluene layer was used for GC-MS analysis.

GC-MS

A Thermo Finnigan trace GC ultra system coupled to a Fisons MD800 mass spectrometer was used in the electron ionization mode. The analytes were separated on a CP Sil 24-CB low-bleed/MS capillary column from Varian [15 m × 0.25 mm (i.d.); film thickness, 0.25 μm]. Samples were injected in the splitless mode, and the oven temperature program was as follows. The initial temperature of 75 °C was increased at a rate of 45 °C/min to 85 °C, which was maintained for 1 min, and then increased at 30 °C/min to 125 °C and further at 120 °C/min to 290 °C. This temperature was maintained for 2 min. Helium was used as carrier gas and delivered at a flow rate that was increased from 1.1 to 2.2 mL/min during the run. The interface temperature was 250 °C, the source temperature was 200 °C, and the electron energy was 70 eV. The analytes were quantified in serum or plasma by measuring the area ratios of analyte vs deuterated internal standard and comparing these ratios with the area ratios obtained from external standards with known analyte concentrations.

ASSAY CALIBRATION

The assay was calibrated by replicate measurements of an external standard, which consisted of pooled serum with known concentrations of all analytes, and an assay blank of phosphate-buffered saline. Additional serum and plasma with known metabolite concentrations were used as quality controls. The concentrations of the calibrators were determined either by established methods [tHcy by

HPLC *(28)* and MMA by capillary electrophoresis *(14)*] or by an amino acid standard solution (containing 2.5 mmol/L Gly, 2.5 mmol/L Ser, 2.5 mmol/L Met, and 2.5 mmol/L Cys in 0.1 mol/L hydrochloric acid) obtained from Sigma.

LINEARITY AND LIMIT OF DETECTION

The linear range and the limit of detection (LOD) of the assay were determined by adding MMA, Hcy, Gly, Ser, Met, and Cys at concentrations from 0.003 to 1000 μmol/L to phosphate-buffered saline. The peak-area ratios of the analytes to their deuterated forms were plotted against their concentrations. The LOD was defined as a signal-to-noise ratio of 5:1.

RECOVERY

Pooled serum was divided into 3 portions, and 2 concentrations (medium and high; Table 1) of all analytes were added to 2 portions. At each analyte concentration, 10 replicates were analyzed in 1 run. The recovery (percentage) was calculated as:

$$\frac{\text{Measured concentration} - \text{Endogenous concentration}}{\text{Concentration added}} \times 100$$

PRECISION

Dialyzed serum was divided into 3 portions that were supplemented with low (0.05 μmol/L MMA, 3.3 μmol/L Hcy, 10 μmol/L Met, 40 μmol/L Ser, 60 μmol/L Gly, and 60 μmol/L Cys), medium (0.15 μmol/L MMA, 10 μmol/L Hcy, 30 μmol/L Met, 120 μmol/L Ser, 180 μmol/L Gly, and 180 μmol/L Cys), or high (0.45 μmol/L MMA, 30 μmol/L Hcy, 90 μmol/L Met, 360 μmol/L Ser, 540 μmol/L Gly, and 540 μmol/L Cys) concentrations of all analytes. Within-day precision was determined by assaying 20 replicates of each concentration on 1 day. Between-day precision was determined by assaying the same samples on 20 different days over a period of 6 weeks.

METHOD COMPARISON

Surplus plasmas from 50 routine determinations of tHcy by an immunologic assay (ADVIA Centaur®; Bayer) were reassayed by this GC-MS method. The tHcy concentrations ranged from 5 to 35 μmol/L in these samples.

The method described here was also validated as part of an external quality assessment program for MMA and tHcy determination *(29)*. During a period of 16 months, 16 serum samples covering the concentration range 0.17–1.20 μmol/L MMA and 16 EDTA-plasma samples covering the concentration range 4.9–64.5 μmol/L tHcy were analyzed, and the individual results were compared with the mean concentrations obtained by all participating laboratories.

METABOLITES IN PLASMA AND SERUM FROM HEALTHY INDIVIDUALS

Plasma and serum samples were obtained from 120 healthy blood donors (mean age, 43.3 years; range, 20–65 years). One half of the donors, 22 men and 38 women, were fasting; the other half, 42 men and 18 women, had eaten a light breakfast 2–3 h before blood sampling.

Results

GC-MS

The analytes showed retention times between 2.4 and 4.4 min and eluted in the following order: MMA (2.4 min), Gly (3.1 min), Ser (3.9 min), Met (4.0 min), tCys (4.2 min), and tHcy (4.4 min). The total run time was 6 min. The mass spectra of the MCF derivatives of all 6 analytes and their deuterated internal standards were obtained in the full-scan acquisition mode. For selected-ion monitoring analysis, the recorded ion pairs (labeled/unlabeled) were *m/z* 174/177 for MMA/d_3-MMA, 161/163 for Gly/d_2-Gly, 173/179 for Ser/d_3-Ser, 235/239 for Met/d_4-Met, 206/208 for Cys/d_2-Cys, and 233/237 for Hcy/d_4-Hcy. These ions were chosen on the basis of ion abundance, detection limit, and the absence of interfering material in biological matrices.

The high chromatographic resolution and separation from matrix components (Fig. 1) suggested no interference. Succinic acid had a retention time of 2.8 min and was clearly separated from MMA. We avoided peaks that interfere with the determination of Ser by monitoring different fragments for Ser (*m/z* 173) and its deuterated internal standard (*m/z* 179). Matrix effects were assessed by comparing the analytical recoveries of medium (0.15 μmol/L MMA, 10 μmol/L Hcy, 30 μmol/L Met, 120 μmol/L Ser, 180 μmol/L Gly, and 180 μmol/L Cys) analyte concentrations in phosphate-buffered saline, dialyzed serum, serum, and plasma. Recoveries were ≥80% for all analytes and all matrices, and the variation of recovery in the different matrices was between 6% and 15% for the different analytes.

To obtain adequate sensitivity, we divided the data acquisition into different retention windows so that only 2 ions were monitored simultaneously. A chromatogram of a plasma sample is shown in Fig. 1.

LINEARITY AND LIMIT OF DETECTION

The LOD (signal-to-noise ratio, 5:1) were 0.03 μmol/L for MMA, 0.1 μmol/L for tHcy, 3 μmol/L for Gly, 1 μmol/L for Met, 10 μmol/L for Ser, and 10 μmol/L for tCys. Least-squares linear regression analysis of the peak-area ratios vs analyte concentration indicated that the assay was linear from the LOD to 100 μmol/L for MMA and tHcy and to 1000 μmol/L for Ser, Gly, Met, and tCys. The equations for the regression lines were as follows: $y = 0.083x + 0.008$ ($r^2 = 0.9999$) for MMA; $y = 0.060x + 0.005$ for tHcy ($r^2 = 0.9999$); $y = 0.009x + 0.091$ ($r^2 = 0.9989$) for Gly; $y = 0.002x + 0.012$ ($r^2 = 0.9991$) for Ser; $y = 0.034x +$

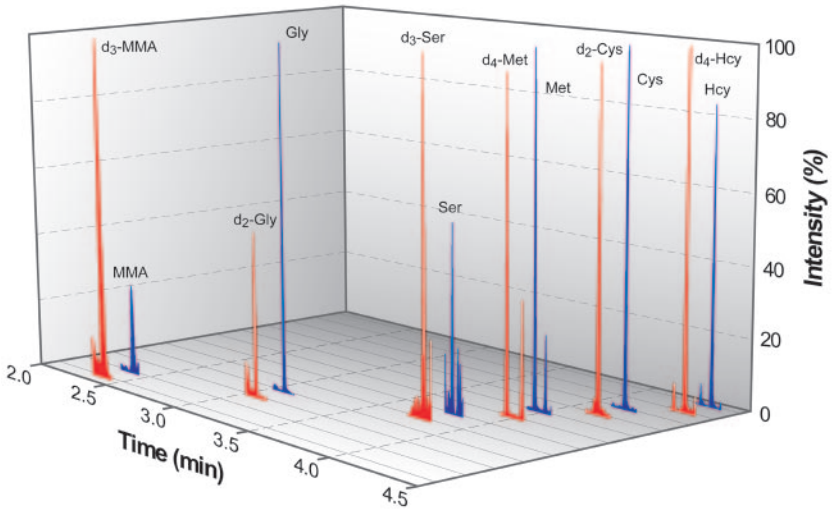

Fig. 1. GC-MS chromatogram for derivatized MMA, tHcy, and related amino acids and their internal standards in typical human plasma.

The selected ion traces for the analytes are shown in *blue*; the traces for the deuterated internal standards are shown in *red*. Different retention windows were used for each analyte and internal standard pair, and all peaks were normalized to the highest peak within each retention window.

0.317 ($r^2 = 0.9987$) for Met; and $y = 0.003x + 0.052$ for tCys ($r^2 = 0.9985$; Fig. 2).

RECOVERY AND PRECISION

Recoveries for all analytes were between 79% and 99% (Table 1). The results of the precision studies are shown in Table 2. Within-day CVs ranged from 0.7% to 3.6%, and between-day CVs ranged from 2.1% to 8.1%. At medium and high concentrations, the within-day CVs were <2.0% for all analytes, and the between-day CVs were <3.5% for all analytes except for tCys (6%).

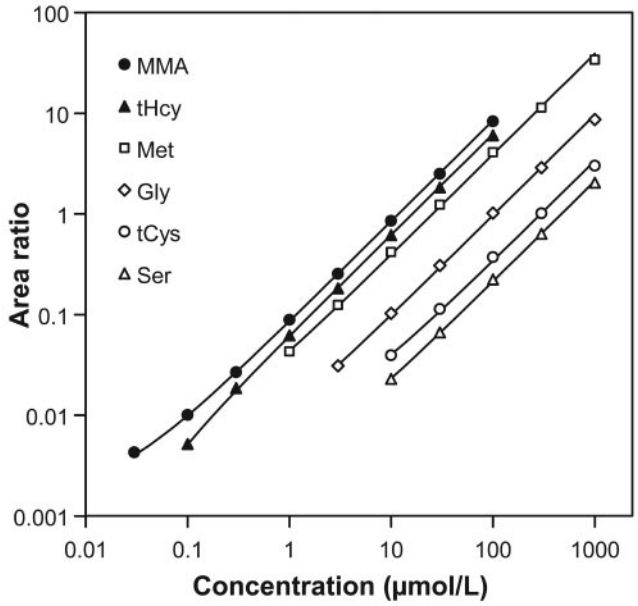

Fig. 2. Linear dynamic ranges for derivatized MMA, tHcy, Ser, Gly, Met, and tCys.

The axes were log-transformed to cover the broad concentration ranges in one figure. The regression lines are calculated by relative least-squares linear regression. They are slightly curved because of the logarithmic scaling.

METHOD COMPARISON

Comparison of tHcy determined in 50 plasma samples with this GC-MS method and an immunologic assay (ADVIA Centaur®) showed good correlation. The equation for the regression line was as follows: $y = 1.056x - 0.960$ ($r^2 = 0.990$; $S_{y|x} = 0.650$). Method comparison as part of the external quality assessment program gave a correlation of $y = 0.918x + 0.013$ ($r^2 = 0.998$; $S_{y|x} = 0.013$) for MMA and $y = 0.979x + 0.009$ ($r^2 = 1.000$; $S_{y|x} = 0.370$) for tHcy.

SAMPLE THROUGHPUT AND RUGGEDNESS

With automated sample preparation, 96 samples can be prepared in 1.5 h, and 288 samples can be prepared in 4 h. A single GC-MS instrument equipped with an autosampler can assay 130 samples in 24 h. The injection liner was changed every 1000 samples, and the column life exceeded 2000 injections. The ion sources of the mass spectrometers were cleaned every 2000 samples to maintain the sensitivity of the assay.

METABOLITES IN PLASMA AND SERUM FROM HEALTHY INDIVIDUALS

The concentrations of MMA, tHcy, Gly, Ser, Met, and tCys were measured in both serum and EDTA plasma from fasting and nonfasting individuals. Median concentrations and 25th to 75th percentiles are shown in Table 3. Median plasma concentrations in fasting individuals were 0.16 µmol/L for MMA, 9.2 µmol/L for tHcy, 230 µmol/L for Gly, 111 µmol/L for Ser, 26.5 µmol/L for Met, and 261 µmol/L for tCys. The serum concentrations of Ser and Gly were 21%–28% higher than those of plasma. For all other analytes, the serum samples contained only slightly higher amounts of metabolites (2%–7%) than the plasma samples. The difference between fasting and nonfasting metabolite concentrations was <10%. The measured concentrations were similar to those reported by others for

Table 1. Analytical recovery of the assay.[a]

	Concentration,[b] μmol/L						
		Added		Detected		Recovery, %	
Analyte	Endogenous	Medium	High	Medium	High	Medium	High
MMA	0.19	0.19	0.59	0.37 (0.01)	0.74 (0.02)	94 (6.1)	95 (3.6)
tHcy	13	9.5	28	23 (0.4)	41 (0.6)	99 (5.2)	98 (2.2)
Gly	373	202	575	553 (13)	886 (18)	89 (7.7)	89 (3.5)
Ser	182	135	404	305 (5.2)	551 (20)	91 (4.9)	91 (5.0)
Met	31	38	115	68 (1.6)	142 (2.7)	96 (4.6)	96 (2.4)
tCys	266	183	494	411 (5.6)	689 (8.5)	79 (4.4)	86 (2.1)

[a] n = 10 for all concentrations.
[b] Mean (SD).

MMA *(30)*, tHcy *(4)*, Gly *(31)*, Ser *(31)*, Met *(30)*, and tCys *(30)* in healthy individuals.

Discussion

This GC-MS method offers the combined measurement of MMA, tHcy, and the related amino acids Gly, Ser, Met, and tCys in plasma or serum. All sample preparation is adapted to 96-well microtiter plates, and the liquid handling is performed by a robotic workstation. The fast and simple derivatization with MCF combined with short retention times of the analytes ensures high sample throughput. The GC-MS analysis is characterized by selectivity, sufficient sensitivity, and good precision, with CVs often <3%.

In this assay, we derivatized the samples with MCF in an aqueous medium containing ethanol and measured the analytes as *N*(*S*)-methoxycarbonyl ethyl esters. Derivatization with ethylchloroformate in the presence of ethanol gave the same MMA derivative (*m/z* 174), whereas different derivatives were obtained for Hcy and the other amino acids. This is in agreement with the observation that the alkyl groups in the carbamate and thiocarbonate moieties of the derivatives are derived from the chloroformate, whereas the alkyl group in the ester moiety is derived from the alcohol. A suggested reaction mechanism for the ester formation is an alcohol exchange reaction between the alcohol and the mixed anhydride formed by a reaction between the alkylchloroformate and the carboxyl group *(32)*.

Most assays for tHcy based on chloroformate derivatization use chloroformate and an alcohol with the same alkyl group, with ethylchloroformate and ethanol being a common combination *(20, 23, 25, 26)*. We used the combination of MCF and ethanol because it gave superior assay performance in terms of retention times and resolution of the analytes. Ethanol was also used as a protein-precipitating agent in our method to avoid the addition of strong acids such as trichloroacetic acid, which may consume chloroformate and form pyridine salts, causing rapid deterioration of the column *(33)*.

Column life exceeded 2000 injections under the conditions that we used, which makes column stability another advantage of our method. With another assay based on ethylchloroformate derivatization and chloroform extraction, we observed a column life of ~600 injections. We have not systematically investigated the reasons for the extended column life, which could be caused by the different derivatizing agent, the extraction solvent (toluene vs chloroform), or the temperature programming.

Chloroformate derivatization is usually followed by liquid–liquid extraction of the derivatives into chloroform *(20, 21, 23, 24)* or, recently, chloroform/isooctane *(26)*; however, with the robotic pipetting device, the chloroform and aqueous phases were not mixed well enough in the microtiter plate to enable extraction of the derivatives into the organic solvent. This problem was solved by the use of toluene as the extraction solvent. We added toluene

Table 2. Precision of the assay.[a]

	Concentration,[b] μmol/L			Within-day CV, %			Between-day CV, %		
Analyte	Low	Medium	High	Low	Medium	High	Low	Medium	High
MMA	0.05	0.15	0.45	3.6	1.9	1.1	8.1	2.6	2.7
tHcy	3.3	10	30	2.2	0.9	1.2	2.2	2.1	2.2
Gly	60	180	540	1.7	1.2	1.2	3.3	2.5	3.1
Ser	40	120	360	2.4	1.7	1.8	5.7	2.9	3.1
Met	10	30	90	1.4	0.9	0.7	2.9	2.6	3.4
tCys	60	180	540	1.5	1.1	1.4	7.7	6.0	3.9

[a] n = 20 for all concentrations, for both the within-day and the between-day experiments.
[b] The concentrations low, medium, and high refer to the concentrations added to dialyzed serum.

Table 3. Concentrations of the analytes in plasma and serum from healthy donors.

	Fasting[a]		Nonfasting[a]	
Analyte	Plasma (n = 59)	Serum (n = 59)	Plasma (n = 59)	Serum (n = 47)
MMA, μmol/L	0.16 (0.14–0.20)	0.17 (0.14–0.21)	0.16 (0.14–0.20)	0.18 (0.16–0.20)
tHcy, μmol/L	9.2 (7.7–10.8)	9.8 (8.0–11.3)	9.0 (8.0–10.9)	8.9 (7.9–10.6)
Gly, μmol/L	230 (197–296)	292 (249–346)	221 (193–259)	281 (250–313)
Ser, μmol/L	111 (96–125)	138 (130–153)	118 (110–131)	149 (135–159)
Met, μmol/L	26.5 (23.8–28.6)	27.5 (25.6–30.3)	28.4 (24.2–32.2)	30.4 (26.0–33.6)
tCys, μmol/L	261 (241–277)	263 (249–285)	251 (239–264)	268 (245–278)

[a] Median (25th–75th).

together with MCF, thereby combining the derivatization and extraction steps.

Two features of the design of this GC-MS method caused some concern. First, to liberate Hcy and Cys from their disulfides, we used D,L-dithioerythritol as reductant, which may consume chloroformate and cause assay interference *(33)*; however, all selected-ion monitoring traces used to quantify the analytes were similar in the absence and presence of D,L-dithioerythritol. Furthermore, the use of deuterated internal standards in this method corrects for variable derivatization, which may occur if the amount of MCF becomes limiting. Second, for simplicity, our GC-MS method did not include a step for the removal of plasma lipids, which in other assays has been accomplished by hexane extraction *(21, 34)* or cation-exchange chromatography or solid-phase extraction *(20, 23, 24, 35)*. Injection of plasma lipids may cause rapid column deterioration *(33)*. In the present method, we increased the oven temperature (to 290 °C) at the end of each run to remove potentially adhesive material.

Despite the complementarity of tHcy and MMA determinations in the diagnosis of cobalamin and folate deficiencies, these 2 metabolites are usually measured by separate methods *(10, 16, 30, 36, 37)*. Our method is optimized for the combined measurement of these 2 markers. Met, tCys, Ser, and Gly were also included in the assay because these amino acids are related to Hcy metabolism *(38)*. Hcy is an intermediate in sulfur amino acid metabolism, and it is remethylated to Met by the folate- and cobalamin-dependent methionine synthase reaction or metabolized to Cys along the trans-sulfuration pathway *(38)*. The major source of the folate-linked 1-carbon units needed for Hcy remethylation is the conversion of Ser to Gly by serine hydroxymethyltransferase. Ser also acts as a substrate in the trans-sulfuration pathway *(39)*.

In conclusion, we have developed and validated a fully automated assay with high-throughput sample preparation for the combined determination of MMA, tHcy, and related amino acids in serum or plasma. Other attractive features of this method are low sample requirement (100 μL), simple derivatization in an aqueous medium at room temperature, selective and accurate quantification of the analytes with isotope dilution and selected-ion monitoring, and the adaptation of all steps of the method to a 96-well microtiter format. The method can easily be adapted to measure other carboxylic acids and amino acids. The present work may motivate further development of chloroformates as general derivatization agents.

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