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Automated Assay of Methylmalonic Acid in Serum and Urine by Derivatization with 1-Pyrenyldiazomethane, Liquid Chromatography, and Fluorescence Detection

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Determination of methylmalonic acid (MMA) in serum has been established as an accurate test for the diagnosis of cobalamin deficiency. We describe here the development and performance of a liquid-chromatographic assay of MMA in blood and urine. The assay is based on our recent finding that one of the carboxylic acid moieties of some short-chain dicarboxylic acids reacts with the fluorogenic reagent 1-pyrenyldiazomethane in an aqueous medium, whereas the other remains underivatized (Anal Chem 1992;63:315–9). The pH-dependent ionization of the free carboxylic acid group of 1-pyrenylmethyl monoesters permits retention on anion-exchange columns, which are used for solid-phase extraction. The analysis is done with a cyanopropyl column coupled in series with an octadecyldimethylsilyl column. Solid-phase extraction and sample injection are carried out automatically by a Gilson ASPEC sample processor. The assay response varies linearly with MMA concentration in the range 0.1–1000 μmol/L in serum. The within-day and between-day CVs are 2.8–10.9%, and the detection limit of 5 fmol injected (~20 nmol/L in serum) is sufficiently low to determine MMA in serum (mean 0.187 μmol/L, SD 0.084, range 0.044–0.431, n = 44) and urine from healthy subjects.

Indexing Terms: *cobalamin · nutritional status · dicarboxylic acids*

Methylmalonic acid (MMA) is a saturated, short-chain dicarboxylic acid.[1] The intracellular precursor, D-methylmalonyl-CoA, is a point of convergence in the metabolic pathways for several amino acids (valine, isoleucine, methionine, threonine), odd-chain fatty acids, cholesterol, and thymidine. D-Methylmalonyl-CoA is racemized to the L-form, which is further metabolized to succinyl-CoA. The isomerization is catalyzed by the enzyme methylmalonyl-CoA mutase (EC 5.4.99.2), which requires adenosylcobalamin as cofactor (*1*).

In 1962, Cox and White (*2*) reported that urinary MMA was an index of cobalamin deficiency. The idea of MMA excretion as a marker of intracellular cobalamin function has been explored further because of problems with the traditional laboratory tests. Serum cobalamin assays do not adequately discriminate deficient patients from normal persons. Other procedures—e.g., the Schilling test, bone marrow examination, and the deoxyuridine suppression test—may also give false results or are rather cumbersome (*3, 4*). In addition, typical clinical signs—anemia and macrocytosis—are often lacking in cobalamin-deficient patients (*5*).

MMA in urine can be measured by several techniques based on gas chromatography–mass spectrometry (GC-MS) (*2, 6–8*), and a high accuracy of urinary MMA excretion as a diagnostic test for cobalamin deficiency has been demonstrated (*6, 9*). Determination of MMA in serum was motivated by the availability of serum samples in the clinical setting, and was first accomplished by Marcell et al. (*7*). Since then, two GC-MS assays of serum MMA have been described (*10, 11*), and the usefulness of this metabolite in diagnosing patients with cobalamin deficiency has been thoroughly documented (*12–15*).

The GC-MS assays of MMA in urine include time-consuming and tedious extraction procedures (*2, 7, 8*) and encounter problems related to loss of MMA during evaporation at 60 °C, instability of derivatives, and increase in blank values because of the presence of MMA in deuterated MMA (used as internal standard). The determination of MMA in serum is an even more cumbersome procedure because of the low concentration of MMA in ordinary serum (0.05–0.38 μmol/L) (*14, 16*). One assay involves acidification, extraction with ether, drying of sample, purification by HPLC, a second extraction step, and another drying before derivatization and GC-MS (*7*). In another serum assay, the samples are cleaned up by solid-phase extraction before derivatization and GC-MS (*10*). A GC-MS assay with only one extraction step has a lower limit of detection of 0.5 μmol/L (*11*), which is not sufficient for determining serum MMA in healthy humans.

We here describe the construction and performance of the first liquid-chromatographic method for MMA in serum, which also works for plasma anticoagulated with EDTA or heparin, and for urine. The assay is based on the fact that only one carboxylic moiety of MMA reacts with 1-pyrenyldiazomethane (PDAM). The unique product carries one free carboxylic acid group (*17*), and the ionization of this group is exploited in separation of the product by a procedure involving solid-phase extraction on ion-exchange columns followed by HPLC. Both steps are carried out with an automated sample processor.

Materials and Methods

Materials

PDAM was purchased from Molecular Probes, Inc. (Eugene, OR). Dissolved in ethylacetate (2.5 g/L) and stored at −20 °C, it is stable for 3–4 weeks. PDAM must be regarded as potentially hazardous, and skin and eye

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[1] Nonstandard abbreviations: MMA, methylmalonic acid; EMA, ethylmalonic acid; PDAM, 1-pyrenyldiazomethane; GC-MS, gas chromatography–mass spectrometry; CN, cyanopropyldimethylsilyl; and ODS, octadecyldimethylsilyl.

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contact should be avoided. Mechanical ventilation and respiratory protection are recommended.

MMA and ethylmalonic acid (EMA) were obtained from Aldrich Chemical Co., Milwaukee, WI. Methanol (HPLC-grade), acetonitrile (HPLC-grade), and ethylacetate were from Merck, Darmstadt, Germany; tetrahydrofuran (HPLC-grade) was from Rathburn Chemicals, Ltd., Walkerburn, Scotland. We used doubly distilled water, which was further purified on a Milli-Q-Plus ultra-pure water system (Millipore Corp., Bedford, MA). The cyanopropyldimethylsilyl column, 0.46 × 25 cm, 5-μm particle size (Supelcosil LC-CN, referred to as the CN column), was purchased from Supelco, Inc., Bellefonte, PA; the 0.46 × 15 cm octadecyldimethylsilyl column (referred to as the ODS column), packed with 3-μm-particle Hypersil, was obtained from Shandon Southern Products, Cheshire, UK. Bond Elut® SAX disposable extraction columns, 1-mL sample capacity and packed with 100 mg of strong anion-exchange sorbent (trimethylaminopropyl bonded to silica), were manufactured by Analytichem International, Harbor City, CA. Used extraction columns were regenerated by passing 5 mL of 1.75 mol/L HCl in methanol through them, then rinsing with 12 mL of water. The extraction columns were conditioned by rinsing them with 2 mL of methanol followed by 2 mL of water at a flow rate of 0.75 mL/min. Both regeneration and conditioning were carried out automatically by the sample processor (see below).

Instrumentation

A programmable sample processor (Gilson ASPEC; Gilson Medical Electronics, S.A., Villiers le Bel, France) was equipped with a Rheodyne (Cotati, CA) Model 7010 injector valve and a 20-μL sample loop, and coupled to a quarternary solvent-delivery system, a Series 410 BIO LC-pump (Perkin-Elmer, Norwalk, CT). The ODS column was mounted in a column heater (Model SP 8792; Spectra Physics, San Jose, CA). The fluorescence of the column effluent was monitored by a Shimadzu RF-535 fluorescence detector (Kyoto, Japan). The wavelength of the primary light path was routinely adjusted to 340 nm and the fluorescent emission was recorded at 376 nm with a Model SP 4290 integrator (Spectra Physics).

Procedures

Sample collection and storage. Blood was collected into evacuated tubes and allowed to clot at room temperature for 30 min before the serum fraction was transferred to an empty glass vial. Heparin-plasma and EDTA-plasma were obtained by collecting blood into Vacutainer Tubes (Becton Dickinson, Meylan Cedex, France) containing the respective anticoagulants. The final concentration of EDTA in the plasma phase was 4 mmol/L. Plasma was separated from the formed elements of blood within 10 min by centrifugation. Serum, plasma, and urine were stored at −20 °C until analysis.

Sample processing. Serum/plasma was deproteinized by mixing 100-μL samples with 300 μL of methanol/acetonitrile (2/1, by vol). The methanol/acetonitrile solution was supplemented with EMA, 13.33 μmol/L (internal standard), which corresponds to 40 μmol/L for EMA in serum/plasma. The mixture was left on ice for 10 min, after which the precipitated protein was removed by centrifugation. EDTA-plasma was processed similarly, except that the methanol/acetonitrile solution was supplemented with $ZnCl_2$ (3.33 mmol/L, unless otherwise indicated) to complex the EDTA. Urine samples were diluted with borate buffer (10 mmol/L, pH 8.0) to give a final creatinine concentration of 1 mmol/L. The diluted sample was mixed with methanol/acetonitrile, as described for serum.

Derivatization. To 300 μL of the solution obtained by mixing serum, plasma, or urine with methanol/acetonitrile we added 75 μL of PDAM (2.5 g/L) dissolved in ethylacetate. After mixing, the vials were capped. There was no phase separation of the final solution, which contained biological fluid, acetonitrile, methanol, and ethylacetate in the proportions 1/1/2/1 (by vol). Incubation was carried out at 25 °C for 12 h, with protection of the derivatization mixture from light to prevent photolysis (*18*) of the ester bonds of the PDAM-carboxylic acid adducts. The MMA and EMA derivatives were stable for ≥1 year when stored at −20 °C in the dark, or for several days at room temperature.

Solid-phase extraction. The solid-phase extraction was carried out by the Gilson ASPEC sample processor. After conditioning, the extraction columns were loaded with 250 μL of derivatized sample at a flow rate of 0.18 mL/min, and then washed with 250 μL of methanol followed by 350 μL of methanol in 83.3 mmol/L citrate buffer, pH 3.0 (40/60, by vol). After each step, the liquid surface was adjusted to the top of the column bed (packing material) by air pressurization. MMA and EMA derivatives were eluted at a flow rate of 0.18 mL/min with 250 μL of the methanol in 166.7 mmol/L citrate buffer, pH 3.0 (40/60, by vol). The columns were completely emptied by air pressurization, and the effluent was thoroughly mixed before injection into the HPLC column.

HPLC. Four solutions (A–D) were used for the gradient chromatography. Solution A was acetonitrile in 20 mmol/L phosphoric acid, pH 2.3 (70/30 by vol); solution B was water; solution C was tetrahydrofuran in water (60/40, by vol); and solution D was tetrahydrofuran in water (80/20, by vol).

Samples (20 μL) were injected into the CN column, which was coupled in series with the ODS column. Both columns were equilibrated with 40% A, 54% B, and 6% C, and were developed with a small linear gradient of tetrahydrofuran, obtained by changing the composition of the mobile phase to 40% A, 51% B, and 9% C at 22 min. At this time, a second linear gradient was started, to end at 0% A, 50% B, 0% C, and 50% D at 45 min. From 45 min on, both columns were washed with 40% A and 60% B for 5 min. The flow rate was 1 mL/min and the column temperature of the CN column was 25 °C; the ODS column was heated to 50 °C. The pressure range was 20–35 kPa during a chromatographic run.

Analytical variables. We prepared a standard curve by

supplementing serum or urine samples with various concentrations of MMA. The concentration of EMA (internal standard) was 40 μmol/L in all preparations. The MMA values were corrected for variations in the recovery of the internal standard.

To serum containing endogenous MMA at 0.12 μmol/L we added 0.5, 5, and 50 μmol of MMA per liter. To determine the within-run precision, we assayed 10 replicates of each serum sample in a single run. The between-run precision was determined by analyzing the same serum samples on 10 different days within 3 weeks.

Analytical recovery in serum and urine was assessed by adding different amounts of MMA to serum or urine from healthy subjects. Urine was diluted with borate buffer (see *Sample processing*) before MMA was added. Samples were assayed in 10 replicates, and the results were expressed as the percentage of the values obtained by adding the same amounts of MMA to borate buffer.

Other assays. Total plasma homocysteine was determined by a modification (*19*) of a fully automated assay described previously (*20*). Serum cobalamin was determined with a microparticle enzyme assay performed with an IMx system from Abbott (Abbott Park, IL).

Results

Assay Performance

Sample processing and derivatization. We deproteinized serum/plasma by mixing one volume of sample (usually 100 μL) with three volumes of methanol/acetonitrile. This ensured efficient protein precipitation at 0 °C within 30 min. Methanol was present to prevent phase separation after the addition of ethylacetate.

PDAM dissolved in ethylacetate was mixed with the deproteinized serum sample. The derivatization was carried out without addition of buffer or pH adjustment because the MMA and EMA derivatives are formed in high yield at neutral pH (*17*).

The time course for the formation of the MMA derivative in serum extract under these conditions is shown in Figure 1. The reaction rate was almost constant for 5 h and leveled off after 12 h. On the basis of these results, we derivatized the samples overnight at room temperature.

Figure 2 shows the formation of the MMA derivative as a function of the concentration of PDAM. Maximal fluorescence yield was obtained at a final PDAM concentration of 0.4–0.5 g/L.

We observed that the formation of PDAM derivatives of both MMA and EMA in EDTA-plasma was ~30% of that in serum, as shown by the fluorescence yield. In addition, material was formed that was eluted from the ODS column as two peaks. We regarded consumption of PDAM through reaction with EDTA as a possible explanation, and tried to complex EDTA by adding $ZnCl_2$. The yield of the MMA derivative in an extract of EDTA-plasma increased as a function of $ZnCl_2$ concentration until a maximum was reached. Further addition of $ZnCl_2$ caused the fluorescence yield to decrease. Similar results were obtained with serum supplemented with 4 mmol/L EDTA, whereas in nonsupplemented

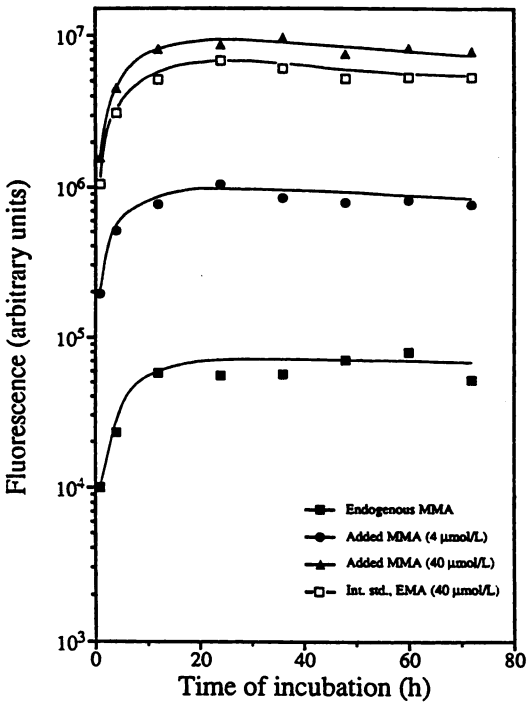

Fig. 1. Time course for formation of PDAM esters of MMA and EMA in extracts from serum

Untreated serum and serum supplemented with 4 μmol/L MMA, 40 μmol/L MMA, or 40 μmol/L EMA were extracted and derivatized with PDAM for up to 72 h at 25 °C

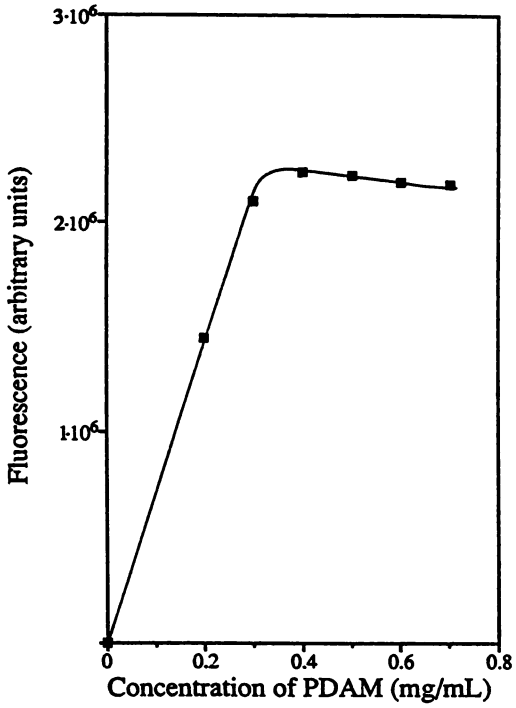

Fig. 2. Formation of PDAM ester of MMA in extracts from serum at various concentrations of PDAM

Serum with 10 μmol/L MMA added was derivatized at 25 °C for 24 h

serum, $ZnCl_2$ caused a progressive decrease in yield (Figure 3). This finding suggests that both free EDTA and $ZnCl_2$ influence the formation of the 1-pyrenylmethyl monoesters. When assaying EDTA-plasma, we deproteinized the samples with methanol/acetonitrile so-

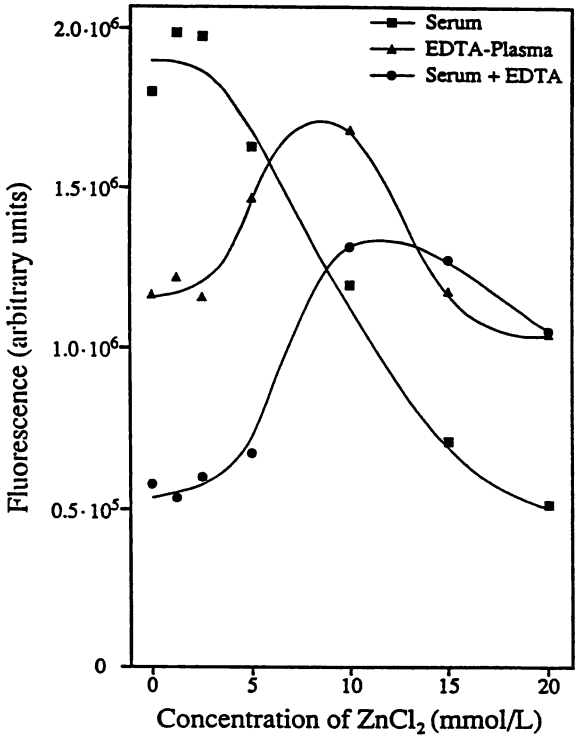

Fig. 3. Titration of EDTA with $ZnCl_2$ and the quenching effect of $ZnCl_2$ in EDTA-plasma and in serum

EDTA-plasma and serum were supplemented with MMA, 10 μmol/L. The fluorescence yield of MMA in EDTA-plasma (4 mmol/L EDTA), serum, and serum supplemented with EDTA (4 mmol/L) was determined in the presence of various concentrations of $ZnCl_2$. The $ZnCl_2$ concentrations refer to those in plasma/serum. The derivatization time was 20 h and the temperature 25 °C. Each point represents mean of triplicate determinations

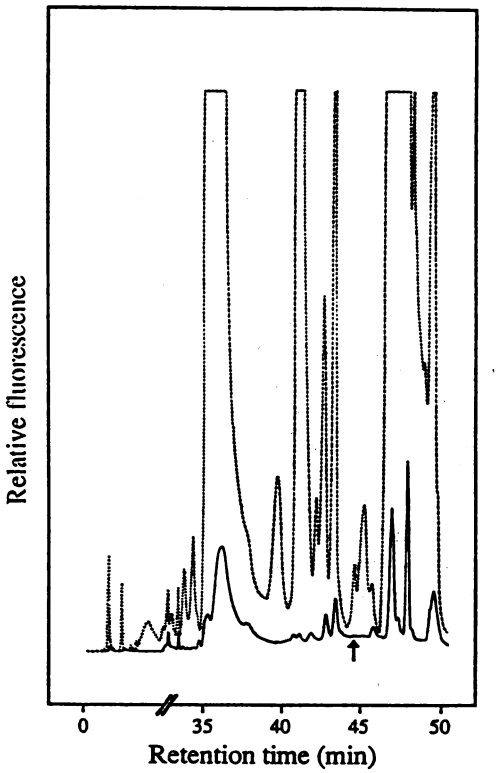

Fig. 4. Clean-up of serum sample on anion-exchange solid-phase extraction column

Serum (containing endogenous MMA, 0.122 μmol/L) was deproteinized and then derivatized with PDAM. One portion was subjected to solid-phase extraction on a SAX column, as described in *Materials and Methods*, and then subjected to HPLC (—). Another portion was chromatographed without solid-phase extraction (- - -). The attenuation of the recorder is reduced 16-fold compared with the experiments depicted in Figs. 5 and 7. The two profiles obtained before and after clean-up can be compared directly because the sample volume applied to the column equals the elution volume (250 μL). The *arrow* indicates the position for the elution of the MMA adduct

lution containing 3.33 mmol of $ZnCl_2$ per liter, which results in a molar ratio of EDTA to $ZnCl_2$ of 1:2.5.

Solid-phase extraction. We previously identified the PDAM derivative of MMA as 1-pyrenylmethyl methylmalonate monoester (*17*). This monoester and related compounds are retained on a strong anion-exchange resin probably through interaction with the free carboxylic acid moiety, which is in anionic form at neutral pH. We therefore developed optimal conditions for the purification of derivatized serum on a SAX extraction column. After sample loading, the column was washed first with methanol and then with dilute citric acid in methanol. Figure 4 demonstrates the high efficiency of this extraction step.

HPLC. We studied the chromatographic behavior of MMA and EMA derivatives on two reversed-phase columns, C_{18} and C_8, and on a CN column. In all systems, the retention time was highly dependent on the pH of the mobile phase, and decreasing the pH below pH 4.5 markedly increased the capacity factor. The MMA derivative was (almost) resolved from interfering peaks on an ODS column or on a C_8 column when eluted with an acetonitrile gradient in ammonium formate buffer (pH 4.56). However, the MMA derivative migrated close behind a large peak, which occasionally interfered. The order of elution of the PDAM derivatives from the CN column differed from that observed with the reversed-phase columns, and both the MMA and the EMA derivatives could be resolved on the CN column. One optimized system based on elution involving gradients of pH and acetonitrile content resulted in long retention times, broad peaks, low sensitivity, and occasional interference from closely eluting material. In another system, the CN column was developed isocratically at low pH (2.3), and the MMA derivative was eluted in a valley close to interfering material. Minor inaccuracies in pH adjustment or evaporation of the organic solvent from the mobile phase caused major changes in the elution profile and affected resolution.

We then developed a system based on column-switching, where a narrow zone of the effluent from the CN column containing the MMA and EMA derivatives was directed into an ODS column. The PDAM derivatives were eluted from the first column at low pH, and the derivatives in the appropriate segment of this effluent were stacked on the ODS column, which was then eluted with a sharp tetrahydrofuran gradient, with a gradual decrease in the concentration of phosphoric acid and acetonitrile (solution A). We used tetrahydrofuran because of its low fluorescence quenching. MMA and EMA derivatives were eluted as sharp peaks clearly separated from interfering material.

To investigate the dependence of the system on the performance of the CN column, we gradually widened the zone of effluent sent to the ODS column. No material

appeared in the regions for elution of MMA and EMA, and the chromatographic profile in this region did not change, even when all of the effluent from the CN column was directed into the ODS column. Thus, we decided to run the two columns permanently coupled in series without column switching. The gradient elution was started immediately after the MMA derivative had entered the ODS column.

Figure 5 shows the chromatographic profile of ordinary serum (containing MMA at 0.122 μmol/L) and serum supplemented with 0.5 and 5 μmol of MMA per liter. The MMA and EMA derivatives were eluted at 42 and 46 min, respectively.

This chromatographic system was very stable, with essentially identical elution profiles from one day to another and after preparation of new mobile phases. We observed no decrease in plating efficiency after analysis of several hundred samples, and saw no interference from components present in serum from healthy subjects or from patients with various diseases, including renal failure.

Evaluation of EMA as internal standard. We compared the reaction kinetics of PDAM with MMA and EMA in serum extracts at various PDAM concentrations and temperatures. The kinetics for the formation of these two derivatives were identical, and the ratio between the two peaks remained constant, regardless of derivatization conditions and reaction time (data not shown). The MMA and EMA derivatives also showed identical behavior on the SAX extraction column. Parallel elution was observed when the column was developed with large volumes of methanol (>2 mL) or with dilute acid in methanol (data not shown). These data verify the use of EMA as an internal standard for the MMA assay described here.

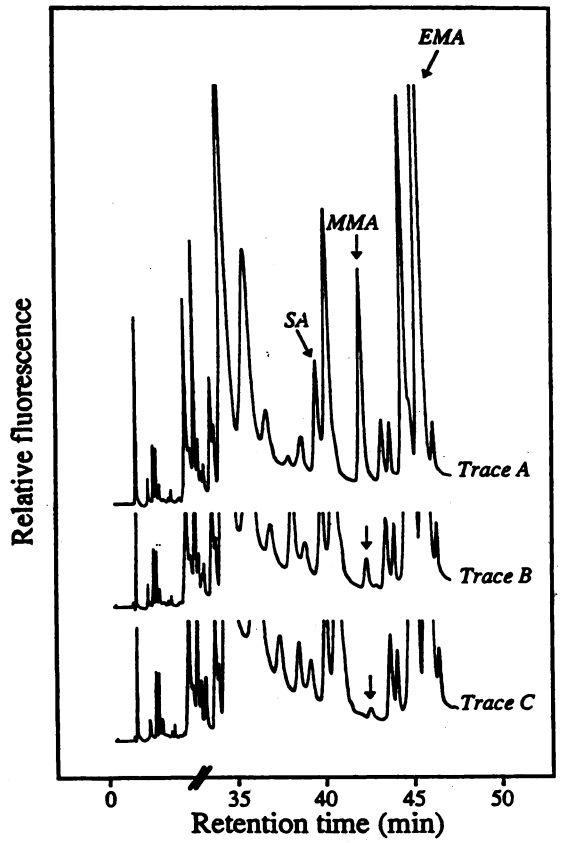

Fig. 5. Chromatographic profiles of serum containing different concentrations of MMA

Serum samples with 5 μmol/L added MMA (*A*), with 0.5 μmol/L added MMA (*B*), or with endogenous MMA (*C*) were extracted in organic solution containing EMA (internal standard; concentration corresponding to 40 μmol/L in serum). Positions of MMA, succinic acid (SA), and EMA indicated by *arrows*

Analytical Variables

We added MMA to serum and urine (diluted to a creatinine value of 1 mmol/L) to final concentrations of 0.125–1000 μmol/L. The standard curve was linear for MMA concentrations up to 1000 μmol/L in serum and to 300 mmol/mol creatinine in urine (Figure 6). In urine, the deviation above 300 mmol/mol creatinine may be explained by consumption of PDAM in the presence of large amounts of other carboxylic acids.

The detection limit (sensitivity) of the assay is ~0.02 μmol/L in serum, corresponding to 5 fmol of MMA in the 20-μL injection volume. The sensitivity is not limited by detector noise, but is determined by the magnitude of peaks eluting in the vicinity of MMA.

For the serum measurements, the within-day and between-day imprecision (CV) was <10.9% for MMA in the range 0.12–50 μmol/L. For the urine measurements, within-day imprecision was <2.5% for MMA in the range 1.86–53.20 μmol/L (Table 1). The analytical recovery of 0.5–50 μmol/L MMA added to either serum or urine exceeded 85% (Table 1).

MMA Concentrations in Serum and Plasma

We also determined MMA in 10 replicates of serum, EDTA-plasma, and heparin-plasma prepared from whole blood that had been supplemented with MMA, 0–50 μmol/L. The chromatographic profiles for serum and hep-

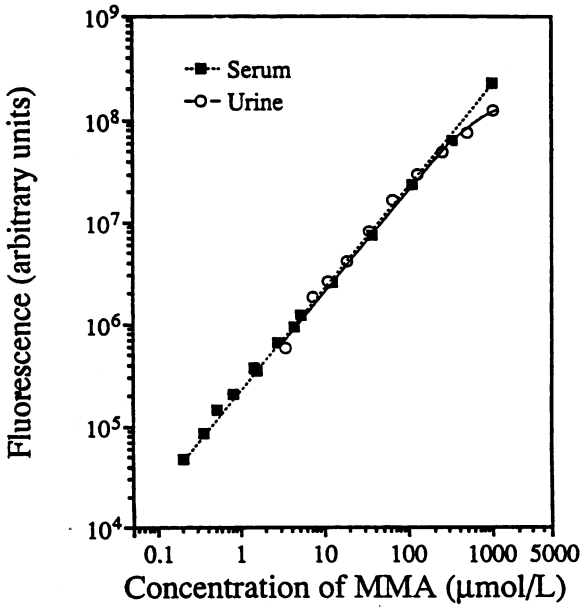

Fig. 6. Linearity of the assay for MMA in serum and urine

Urine samples were diluted with 10 mmol/L borate buffer, pH 8.0, to give a final creatinine concentration of 1 mmol/L. Serum and diluted urine were supplemented with increasing concentrations (0.156–1000 μmol/L) of MMA, then analyzed as described in *Materials and Methods*

Table 1. Precision and Recovery of the Assay

Within-day				Between-day: MMA, μmol/L measured
MMA, μmol/L				
Endogenous	Added	Measured	Recovered %	
Serum				
0.12 ± 0.01 (7.4)[a]	0.00	0.12 ± 0.01 (7.4)[a]	—	0.14 ± 0.01 (9.4)[a]
	0.50	0.55 ± 0.05 (8.4)	84.8	0.55 ± 0.06 (10.9)
	5.00	4.75 ± 0.15 (3.1)	92.5	5.33 ± 0.33 (6.2)
	50.00	49.60 ± 1.41 (2.8)	98.9	50.47 ± 3.50 (6.9)
Urine				
1.86 ± 0.05 (2.4)	0.00	1.86 ± 0.06 (2.4)	—	—
	5.32	7.20 ± 0.18 (2.5)	100.3	—
	53.20	48.25 ± 0.77 (1.6)	88.8	—

n = 10 each.
[a] Mean ± SD (and CV, %).

arin-plasma were similar, whereas recovery of MMA in EDTA-plasma was low and two peaks derived from EDTA were eluted ahead of MMA (Figure 7). The correlation between MMA measured in serum (y), heparin-plasma (x), and EDTA-plasma (x') was excellent: $y = 0.93x + 0.614$ ($r = 0.998$) and $y = 1.06x' - 0.764$ ($r = 0.994$).

The mean MMA concentration in serum from 44 healthy subjects was 0.187 (SD 0.084) μmol/L; the range was 0.04–0.43 μmol/L (Table 2). On the basis of these data, the calculated reference interval for MMA in serum (mean ± 1.96 SD) is 0.187 ± 0.164 μmol/L, and the upper reference limit is 0.35 μmol/L.

Serum MMA was also determined in 15 patients with cobalamin deficiency. The results, plus their serum cobalamin and plasma homocysteine concentrations, are listed in Table 2. The MMA concentrations (range 0.40–77.37 μmol/L) in most of these patients significantly exceeded the values in healthy subjects.

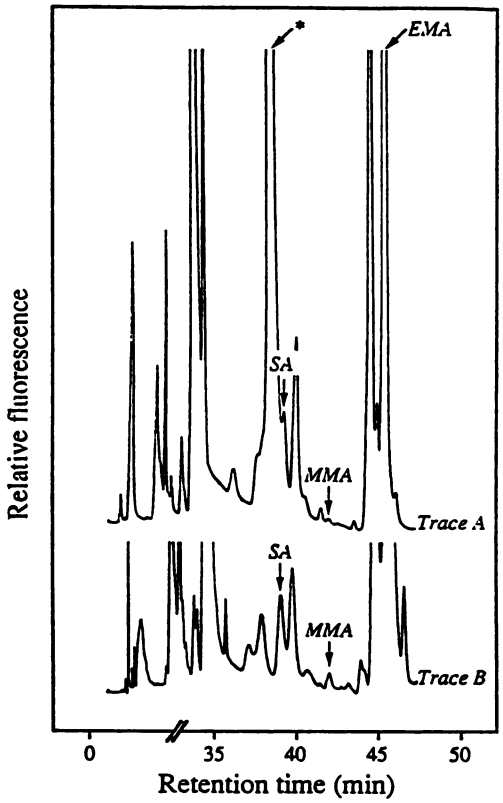

Fig. 7. Chromatograms of serum (*B*) and EDTA-plasma (*A*)
Serum and EDTA-plasma were prepared from blood from the same person, and the EDTA-plasma was extracted and assayed in the presence of $ZnCl_2$. The positions of MMA, succinic acid (SA), and EMA are indicated by *arrows*. The large peak (*), which interferes with succinic acid, is derived from EDTA. The fluorescence yield of both MMA and EMA is lower in EDTA-plasma than in serum

Discussion

Aryldiazomethanes are fluorogenic labeling reagents that react with carboxylic acids. 9-Anthryldiazo-

Table 2. Serum MMA, Serum Cobalamin, and Plasma Homocysteine in 44 Healthy Subjects and 15 Patients with Cobalamin Deficiency

	Age, years	Sex	Serum cobalamin, pmol/L	Plasma homocysteine, μmol/L	Serum MMA, μmol/L
Healthy subjects					
Mean	48		261	10.9	0.19
Minimum	29		120	6.0	0.04
Maximum	55		676	25.2	0.43
Mean ± 1.96 SD range			32–490	3.7–18	0.02–0.35
Cobalamin-deficient patients					
1	72	M	77	126.0	3.83
2	82	F	64	36.0	1.29
3	49	M	63	123.0	36.46
4	90	F	66	103.3	5.56
5	57	M	80	138.8	29.92
6	82	M	86	28.9	0.44
7	72	M	63	49.9	3.28
8	79	M	89	56.3	1.16
9	81	F	91	29.9	0.71
10	54	M	97	43.2	1.23
11	74	F	63	56.3	1.23
12	67	M	60	64.7	0.40
13[a]	85	F	180	51.7	3.28
14	73	F	31	144.3	77.37
15	68	M	40	30.7	0.70

[a] This patient showed typical clinical signs of cobalamin-deficiency with megaloblastic anemia, hypersegmentation of neutrophils, aphasia, and facial palsy.

methane has been widely used for determinations of fatty acids and prostaglandins (*21*). The newly developed aryldiazomethane PDAM has several attractive features: stability of the reagent and the 1-pyrenylmethyl ester derivatives, and high fluorescence intensity. It has also been used for determinations of fatty acids, prostaglandins (*22*), and short-chain carboxylic acids (*23*).

Determination of MMA in serum/plasma is difficult because of the low concentrations (0.05–0.56 μmol/L) in healthy subjects (*24*); moreover, the presence of high concentrations of other carboxylic acids may interfere. The high fluorescence intensity of the 1-pyrenylmethyl esters allows determination of prostaglandins in the femtomole range (*22*) and ensures sufficient sensitivity to measure MMA in serum. Problems related to chromatographic resolution, not sensitivity, are the main obstacle.

We observed that PDAM reacts with one carboxylic residue in MMA, EMA, and other short-chain dicarboxylic acids in an aqueous matrix. The other carboxylic group does not react, probably because of steric hindrance. Stable 1-pyrenylmethyl monoesters with one free carboxylic group are formed. The ionization of the free carboxylic group affects the chromatographic mobility (*17*).

We exploited the unique features of the pyrenylmethyl monoesters of MMA and EMA by purifying the derivatized samples on a strong anion-exchange (SAX) extraction column. The monoesters were retained at pH 7, whereas neutral and positively charged compounds were eluted with methanol and dilute acid. This step resulted in a substantial sample clean-up (Figure 4).

The 1-pyrenylmethyl monoesters were eluted from the SAX column with citric acid, pH 3.0. The capacity factor of these derivatives on a CN column is high for a mobile phase at low pH, as has previously been demonstrated for an ODS column (*17*). The low pH of the sample obtained after solid-phase extraction favored retention and thereby concentration of the analytes on the top of the CN column, which might become important if large volumes are injected.

The manual sample processing involved in this method is limited to deproteinizing the samples with organic solvent and adding the derivatizing reagent PDAM. The derivatization is done in an aqueous matrix at room temperature, and the subsequent chromatographic steps are carried out automatically by the sample processor. The automatization reduces the manual sample handling to <3 min but also confers reproducible sample handling and constant yield of the 1-pyrenylmethyl monoesters, as judged by recovery of the internal standard (not shown). Several cumbersome steps included in most GC-MS assays (*7, 8, 10, 11*) are avoided. No liquid sample extractions, manual solid-phase extractions, or evaporation or heating of samples is necessary.

Although solid-phase extraction and injection into the HPLC column are performed automatically in sequence, we occasionally carried out the method manually, using the same SAX columns. Several columns were eluted simultaneously with a vacuum manifold and the eluates were injected serially into the HPLC. The performance of the manual version seems to be comparable with that of the automated method, especially because we used the internal standard to correct for variations in sample extraction. However, we did not perform a systematic comparison between the manual and the automated procedure.

The eluent from the SAX column was chromatographed on a CN column coupled in series with an ODS column (Figure 5). Using a column-switching technique gave a much shorter retention time than did the serial elution of both columns, and the sample output was considerably higher because of simultaneous elution and equilibration of the columns. However, we preferred the method based on serial elution because this requires only one solvent-delivery system and no column-switching valve; moreover, columns are not subject to derangement from the pressure surge generated during column switching.

We found the present method to be reliable and adequately precise (Table 1), with a reproducible elution profile, long column life, and no significant decline in chromatographic resolution during column aging. There are several reasons for the presence of these attractive features. First, the samples injected into the HPLC column have been subjected to substantial clean-up. Second, the MMA and EMA derivatives eluted from the CN column are concentrated on the top of the ODS column, from which they are developed with a steep gradient of organic phase; thus, the plating efficiency and separation on the ODS column are essentially independent of the performance of the CN column. Third, the guard column and the CN column protect the analytical ODS column by serving as saturation columns and retaining injected material that may cause column deterioration.

The time required for the sample processing and the chromatographic run determines the sample output. Because the manual sample-preparation steps in our method are simple and rapid, these steps do not limit the number of analyses; in addition, the solid-phase extraction is carried out during the HPLC analysis of the preceding sample, for a total run time of ~50 min. To date, the output of our technique is ~30 samples per 24 h. The tedious sample processing required for the determination of serum MMA by GC-MS restricts the output of these methods to ~18 specimens during a working day (*10*), but this can obviously be increased to ~40, given the low retention times of the MMA derivative in GC-MS (*7, 10, 11*).

The sensitivity of our method (0.02 μmol/L) is sufficient to detect MMA in 50–100 μL of serum or plasma from healthy subjects and is similar to that obtained with GC-MS techniques (*7, 10, 11*). In the latter assays, the presence of unlabeled MMA in the deuterated internal standard may increase the background and decrease the sensitivity (*8*). No MMA contaminant is present in

the EMA used as internal standard in the present method.

The precision of the present assay corresponds to a CV of 9–10% at MMA concentrations <0.5 μmol/L. CVs decrease to ~6–7% at higher concentrations (Table 1) and are comparable with those of GC-MS methods (*10*).

We developed this method for the determination of MMA in serum, but it also measures MMA in heparin-plasma and in EDTA-plasma when EDTA is titrated with $ZnCl_2$. Free EDTA probably interferes with the method by consuming PDAM, but it may also affect the retention of the 1-pyrenylmethyl monoesters on the SAX columns.

The compatibility of the GC-MS techniques with EDTA-containing samples has not been documented (*7, 10*). The option of measuring MMA in EDTA-plasma or heparin-plasma may be advantageous when only these materials are available, e.g., in research projects involving stored plasma samples. Furthermore, given that homocysteine should be measured in rapidly prepared plasma to prevent artificial increases in plasma homocysteine (*25*), it is useful that MMA can be determined in such samples to verify the diagnosis of cobalamin deficiency.

Both the GC-MS assays (*7, 10*) and the methods developed by us measure MMA in urine.

The mean value for MMA in serum from 44 healthy subjects was 0.187 μmol/L (Table 2), which agrees with the normal values reported by others (*10, 12, 13, 24*). The concentrations of MMA in serum from 13 of 15 cobalamin-deficient patients were markedly increased (Table 2), which indicates that serum MMA is a marker of cobalamin deficiency. In addition, all cobalamin-deficient patients also showed a substantial increase in plasma homocysteine, emphasizing the value of simultaneous determination of both homocysteine and MMA.

In conclusion, the performance of this first liquid-chromatographic assay of MMA in serum is comparable with that of the previously described GC-MS assays. However, this assay has the advantage of simpler sample processing and can also measure MMA in EDTA- or heparin-treated plasma and urine. It represents an alternative to the established GC-MS techniques, especially in laboratories where the technician has no training in gas chromatography or mass spectrometry.

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