Anal. Chem. **1995,** *67,* 812–819

Application of Capillary Electrophoresis with Laser-Induced Fluorescence Detection for Routine Determination of Methylmalonic Acid in Human Serum

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Methylmalonic acid (MMA) in serum is an established marker of cobalamin deficiency. MMA and other short-chain dicarboxylic acids react with 1-pyrenyldiazomethane to form stable, highly fluorescent 1-pyrenylmethyl monoesters. We have analyzed these esters in human blood by capillary electrophoresis (CE) combined with laser-induced fluorescence detection, and here we describe our approach to achieve long-term reproducibility, which is a prerequisite for routine clinical application. To stabilize CE performance and to minimize solute adsorption to the capillary wall, we coated capillaries with linear polyacrylamide, used hydroxypropyl methylcellulose and dimethylformamide as buffer additives, and extensively diluted derivatized samples prior to injection. A discontinuous buffer system was used for sample stacking. Separation was performed in Tris–citrate buffer, pH 6.4, under reversed polarity conditions (negative potential at the inlet vial). The assay was linear for serum MMA concentrations in the range 0.1–200 μmol/L, the total run time was 26 min, the sample output was about 50 samples/24 h, and the coefficients of variation ranged between 3 and 12%, depending on the MMA concentration. Comparison of our assay with two established GC/MS methods demonstrated good correlation and measuring agreement.

The concentration of methylmalonic acid (MMA) in serum or urine is a useful marker for both the diagnosis and the follow-up of cobalamin deficiency. Increased MMA levels are a consequence of a reduced activity of the enzyme methylmalonyl-CoA mutase (EC 5.4.99.2), which converts D-methylmalonyl-CoA into succinyl-CoA[1] and requires cobalamin as cofactor.

Gas chromatography–mass spectrometry (GC/MS) has been widely used to measure MMA in serum as well as in urine. These techniques afford sufficient sensitivity and specificity to quantitate the low concentrations (<0.4 μmol/L) of MMA found in normal serum but include cumbersome procedures, like extraction, evaporation, reconstitution, and derivatization.[2–5]

We recently described a HPLC method for determination of MMA in serum and urine.[6] This technique included derivatization with the fluorescent labeling reagent 1-pyrenyldiazomethane (PDAM) to form stable 1-pyrenylmethyl monoesters with one free carboxylic acid residue. The ionization of this group allowed solid-phase extraction on strong anion-exchange columns and explained the pH-dependent mobility of these monoesters on reversed-phase chromatography.[7]

Capillary electrophoresis (CE) is an "instrumentalized" approach to electrophoretic separation. It has a remarkable resolving power and allows automation, on-line detection, and direct computer interfacing. When CE is used in combination with laser-induced fluorescence (LIF) detection, a high mass sensitivity is obtained.[8] The theories and fundamentals of capillary electrophoresis have been developed extensively for more than 10 years, whereas sparse data have been published on CE assays for routine analysis of real samples.[9,10]

We have previously shown that 1-pyrenylmethyl monoesters of MMA and related short-chain dicarboxylic acids can be analyzed in buffer solutions by free-zone CE and LIF detection. The 325 nm wavelength of the HeCd laser matches the excitation maximum of the 1-pyrenylmethyl monoesters, and a very high concentration sensitivity was achieved.[11] In an optimized system, we could also detect endogenous MMA in normal human serum. However, long-term stability and reproducibility required for a routine assay were unsatisfactory. Sample stacking was accomplished by field amplification, which occasionally resulted in excessive Joule heating or even boiling of the sample plug. More importantly, electroendosmotic flow (EOF) was difficult to control and progressively decreased after only a few injections. There was a concurrent decrease in apparent mobility, column efficiency, and resolution, and capillary replacement was necessary after only 20–30 analyses.

In the present paper we describe a robust CE assay for determination of MMA in human blood samples. Prevention of solute adsorption to the capillary surface, elimination of EOF, and a discontinuous buffer system for sample stacking were important elements to achieve long-term stability. Our solutions may also

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apply to other CE assays for hydrophobic analytes in complex biological matrices.

EXPERIMENTAL SECTION

Chemicals. PDAM was purchased from Molecular Probes, Inc. (Eugene, OR). It was dissolved in dimethylformamide (DMF, 2.5 mg/mL) and stored at −20 °C. Under these conditions, it was stable for at least 3−4 weeks. ***Caution:*** PDAM must be regarded as potentially hazardous, and skin and eye contact should be avoided. Mechanical ventilation and respiratory protection are recommended.

MMA, ethylmalonic acid (EMA), and dimethylmalonic acid (DMMA) were obtained from Aldrich Chemical Co. (Milwaukee, WI). Methanol (HPLC grade), DMF (analytical grade), and 2-propanol (analytical grade) were from Merck (Darmstadt, FRG). We used double-distilled water which was further purified on a Milli-Q-plus ultrapure water system (Millipore Corp., Bedford, MA). Citric acid monohydrate and tris(hydroxymethyl)aminomethane were from Merck, and hydroxypropyl methylcellulose (HPMC) was from Sigma (St. Louis, MO).

(γ-(Methacryloxy)propyl)trimethoxysilane (Bind-Silane), *NNN'N'*-tetramethylethylenediamine (TEMED), and ammonium persulfate were from Pharmacia (Uppsala, Sweden), and acrylamide was from Bio-Rad Laboratories (Richmond, CA). Bond Elut C18 bonded phase extraction columns (3 cm^3, 200 mg) were purchased from Varian (Harbor City, CA).

Instruments. A P/ACE 2210 capillary electrophoresis instrument equipped with a sample tray cooling option and a P/ACE LIF detector were used; both units were purchased from Beckman (Fullerton, CA). The LIF detector had an off-board 20 mW HeCd laser source, Model 3056-10M from Omnichrome (Chino, CA), operating at 325 nm. It was connected to a Model 100 power supply (Omnichrome). The laser excitation light was focused on the capillary window by means of a FVP 100/120/140 UV grade mm fiber cable with connectors SMA 905 and SMA 906 produced by Polymicron (Phoenix, AZ) and purchased from Composite Metal Services Ltd. (Worcestershire, U.K.). Fiber optical alignment was optimized using Optical Noise/Power Meter NP2 from Omnichrome. Two 383 nm band pass filters (Omega Optical, Brattleboro, VT), coupled in series, were used for control of fluorescence emission.

Outside polyimide-coated open fused silica capillaries (Polymicron) were mounted in a temperature-controlled cartridge at 20 °C. The total capillary length was 47 cm (40 cm to detector), and the inner and outer diameters were 75 and 375 μm, respectively. The inner surface of the fused silica capillaries was coated with linear polyacrylamide, according to the procedure described by Hjertén.[12]

Beckman System Gold software (version 7.11), run on an IBM PS/2 56 486 SLC 2 computer, was used for system control, data collection, and data processing. A Hewlett Packard Laser Jet II P plus was used for printouts of electropherograms and reports.

Sample Collection and Storage. Blood was collected into vacutainers 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 vacutainers containing heparin or EDTA, respectively. The final concentration of EDTA in the plasma fraction was about 4 mmol/L. Plasma was separated from the formed elements of blood within 10 min by centrifugation. Serum and plasma were stored at −20 °C until analysis.

Sample Processing and Derivatization. The serum/plasma sample was deproteinized by mixing 50 μL of sample with 150 μL of ice-cold methanol, containing 13.33 μmol/L EMA, which corresponds to 40 μmol/L in serum. Samples were then centrifuged for 5 min at 13 000 rpm, cooled for 30 min at −80 °C, and again centrifuged for 5 min. EDTA−plasma was processed according to the same procedure, except that the methanol solution (containing 13.33 μmol/L EMA) was supplemented with $ZnCl_2$ (3.33 mmol/L) to complex EDTA.

To 100 μL of deproteinized serum or plasma was added 25 μL of PDAM (2.5 mg/mL) dissolved in DMF. Incubation was routinely carried out at 25 °C for 12 h under light protection, unless indicated otherwise.

Prior to injection, 10 μL of derivatized serum/heparin−plasma solution were diluted 80-fold in a DMF/water (4/1 v/v) solution which contained 30 mM NaCl and was adjusted to pH 2.5 with 1 N HCl (referred to as sample dilution matrix). Diluted samples in 400 μL vials were placed in the inlet tray, thermostated at 4 °C.

Solid-Phase Extraction. Prior to injection, derivatized EDTA−plasma samples were subjected to solid-phase extraction on Bond Elut C18 columns. The columns were conditioned by successive rinsing with 2 mL of methanol and 2 mL of water. Samples of 50 μL were loaded on the columns, which were then developed with 1 mL of water followed by 1 mL of 30 mM Tris-HCl buffer, pH 7.5, containing 25% 2-propanol. MMA and EMA derivatives were eluted with 1 mL of 15 mM Tris-HCl buffer, pH 7.5, containing 35% 2-propanol. This eluate, containing the MMA and EMA derivatives, was diluted 4-fold with the sample dilution matrix prior to injection.

Capillary Electrophoresis. The running buffer was 30 mM Tris−citrate buffer, pH 6.4, containing 50% DMF and 0.1% HPMC. The pH was adjusted after the addition of DMF. All solutions used for sample dilution and buffer systems were sonicated and subjected to filtration through a 0.45 μm Gelman Acrodisc LC 13 PVDF syringe filter (Ann Arbor, MI).

Before injection, the capillary was flushed with running buffer for 1 min in reversed high-pressure mode, followed by introduction of a zone consisting of 30 mM phosphate buffer, pH 3.4, containing 40% DMF, by applying low pressure for 12 s at the capillary inlet. After hydrodynamic sample injection (20 s), the capillary inlet was positioned in a vial containing sample dilution matrix (capillary outlet in running buffer), and 10 kV were applied for 40 s. Subsequently, electrophoretic separation was carried out by using constant current (14.8−15.2 μA) and reversed polarity mode (negative potential at the source vial). The capillary was thermostated at 20 °C. After each run, the capillary was subjected to retrograde rinsing for 3 min with running buffer. For storage, capillaries were filled with running buffer and thermostated at 4 °C.

Linear Dynamic Range, Recovery, and Precision. In order to assess the range of linear response of the method, we spiked serum with various concentrations (0.2−200 μmol/L) of MMA. The area of the MMA peak vs MMA concentration in serum was recorded. Data were based on single MMA determinations without correction for the internal standard (IS) EMA, which was 40 μmol/L in all preparations.

Recovery and precision were determined with serum containing endogenous MMA (0.12 μmol/L) and serum spiked with 0.4,

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4, and 40 μmol/L of MMA. To obtain the within-day precision, we derivatized 10 replicates at each concentration level and assayed them in a single run. The between-run precision was determined by derivatizing and analyzing the same serum samples on 10 different days within 3 weeks.

Analytical recovery in serum was assessed by adding different amounts of MMA to serum from healthy subjects. Samples were assayed in 10 replicates, and the results are expressed as percent of theoretical values obtained by adding the known amounts of MMA to serum:

$$\% \text{ recovery} = \frac{\text{MMA value obtained } (\mu\text{mol/L}) - \text{endogenous level } (\mu\text{mol/L})}{\text{MMA added } (\mu\text{mol/L})} \times 100$$

Comparison of Methods. We validated the CE method by determining MMA in two sets of serum, termed sample sets 1 and 2. Set 1 ($n = 51$) was provided by Dr. Karsten Rasmussen at the University of Arhus, and MMA was determined in his laboratory by the GC/MS method 1.[5] Set 2 ($n = 64$) was provided by Dr. Robert H. Allen at the University of Colorado, and MMA was determined by another GC/MS technique, termed method 2.[13]

RESULTS

Sample Processing and Derivatization. The samples were deproteinized by mixing with three volumes of ice-cold methanol. We changed from acetonitrile[6] to methanol for deproteinization because the MMA and EMA monoesters were formed both at a higher rate and at a higher yield under this condition. Notably, in the absence of methanol (100% acetonitrile or 100% DMF), these derivatives were not formed (data not shown).

We investigated the kinetics for the formation of MMA monoester at various concentrations (0.12–40 μmol/L) of MMA. At room temperature (25 °C), the reaction proceeded almost linearly for the first 60 min and reached at plateau after 8–12 h. The reaction rate increased almost 4-fold at 50 °C, and under these conditions, maximal fluorescent yield was obtained within 2–3 h. At both temperatures, derivatization of MMA and EMA (40 μmol/L) showed similar reaction kinetics. MMA and EMA derivatives were stable for at least 72 h (Figure 1).

On line Sample Stacking and Cleanup. Before injection of the samples, we diluted the derivatized samples in a solution of 30 mM NaCl in 80% DMF, pH 2.5 (Figure 2). In the absence of NaCl, current error episodes were occasionally observed, probably related to boiling of the sample zone caused by excessive field amplification.

A plug containing phosphate buffer, pH 3.4, was introduced prior to sample injection. The presample plug and, in particular, the low pH of the sample dilution matrix resulted in sample stacking and removal of a large amount of interfering material (Figures 2 and 3).

Capillary Electrophoresis. We used fused silica capillaries coated with linear polyacrylamide according to the procedure of Hjertén and 0.1% HPMC as buffer additive to suppress residual EOF.[14] Under these conditions, electroendosmotic mobility was

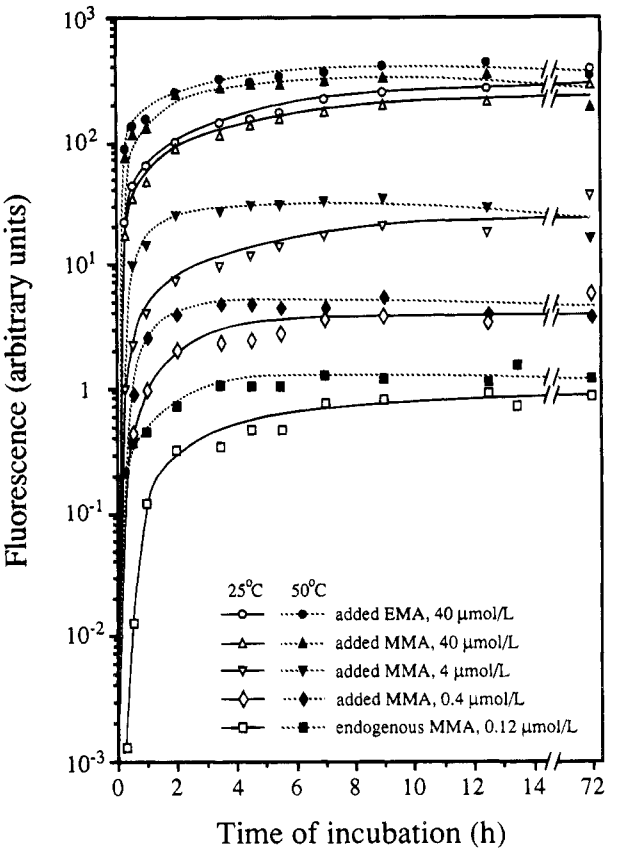

Figure 1. Time course for the formation of PDAM esters of MMA and EMA. Serum containing 0.12 μmol/L MMA and samples from the same serum supplemented with 0.4, 4, and 40 μmol/L MMA, together with 40 μmol/L EMA, were derivatized with PDAM for up to 72 h. Samples were incubated at 25 or 50 °C.

calculated to be less than 1.2×10^{-5} $cm^2 V^{-1} s^{-1}$ with use of underivatized PDAM as neutral marker (data not shown).

Capillary electrophoresis was performed in the reversed polarity mode (negative potential at the source vial, Figure 2). Resolution of the MMA and EMA peaks from peaks of interfering material was critically dependent on the pH (6.4) of the mobile phase, which was supplemented with 50% DMF in addition to HPMC to prevent solute absorption or precipitation and to increase selectivity.[15–19] The 1-pyrenylmethyl monoesters of MMA and EMA migrated toward the anode and showed migration times of about 22.0 and 22.8 min, respectively (Figure 4). The resolution (R_s) of the two components was about 2.4 at pH 6.4.

The electropherograms for derivatized serum and heparin–plasma were similar, and no sample cleanup was required before electrophoresis. In contrast, in EDTA–plasma, even when treated with $ZnCl_2$ to complex EDTA, fluorescent material comigrated with the MMA and EMA peaks. However, the MMA peak could be detected after reversed-phase solid-phase extraction of the derivatized sample (Figure 5).

Long-Term Stability. Running buffer was used for capillary rinse and storage (at 4 °C). The column performance was stable

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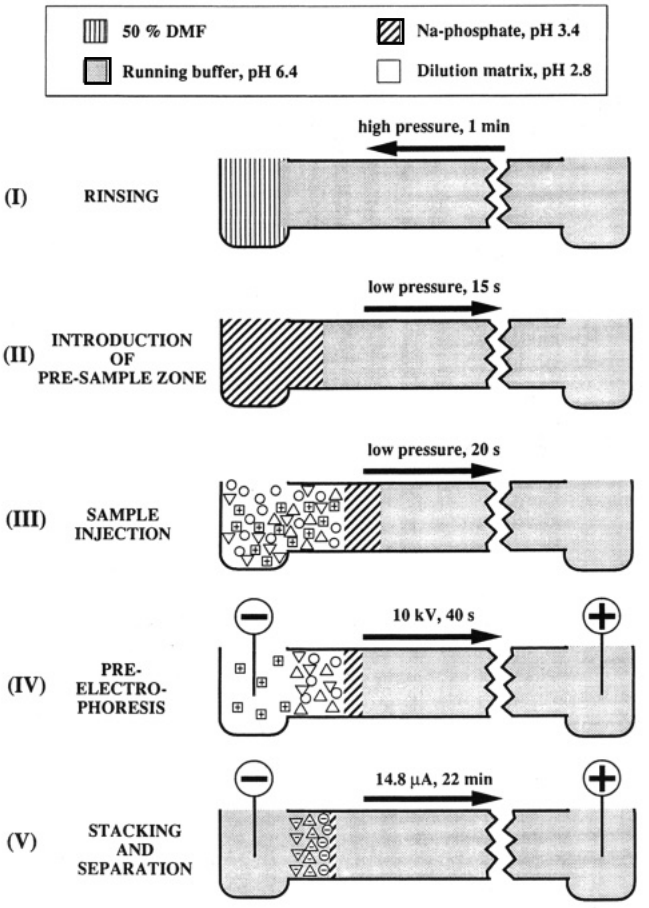

Figure 2. Schematic representation of the discontinuous buffer system. Step I, the capillary is rinsed with running buffer (30 mM Tris–citrate, pH 6.4, containing 50% DMF and 0.1% HPMC) for 1 min with capillary inlet positioned in 50% DMF in water. Step II, a phosphate buffer plug (30 mM sodium phosphate, pH 3.4, containing 40% DMF) is introduced ahead of the sample zone. Step III, derivatized sample dissolved in dilution matrix (30 mM NaCl, adjusted to pH 2.8 with 1 N HCl, containing 80% DMF) is pressure injected. Step IV, preelectrophoresis is done by applying 10 kV for 40 s with capillary inlet positioned in sample dilution matrix and capillary outlet in running buffer. Positively charged species migrate out of the capillary. Step V, sample stacking and separation are performed in constant current mode (14.8 μA), with both capillary ends in running buffer.

for several months, and more than 3000 analyses were carried out on the same capillary. The plate numbers were essentially the same throughout the column lifetime, as demonstrated by comparison of the electropherograms obtained with a new (Figure 6) and an old capillary after about 1500 injections (Figure 4). Precision and recovery values listed in Table 1 were obtained with a capillary that had been subjected to 1600–1800 serum analyses. Capillary replacement was required because of accidental capillary breakage or clogging.

Internal Standard. EMA is chemically closely related to MMA, elutes close to the MMA peak in our CE system (Figures 4 and 6), and shows similar reaction kinetics with PDAM compared to MMA (Figure 1). There are only small concentrations of endogenous EMA in normal serum.[2] However, in our CE system which was optimized for separation of MMA, there was an interfering peak eluting close behind the EMA peak (Figure 6). The amount of this material was essentially constant and constituted less than 10% of the EMA peak area in all serum samples we have analyzed so far (several thousand). The integration software separated this peak from EMA by baseline integration.

Notably, the within-day coefficient of variation (CV) of EMA (40 μmol/L) for analysis of 10 different serum samples prepared and derivatized the same day was 5% (data not shown). To further validate EMA as IS, we added an equimolar concentration of a second IS, DMMA, a constitutional isomer of EMA (Figure 6). The ratio between the peak areas of EMA and DMMA varied less than 10% in 80 serum samples.

Dynamic Range of the Assay. We spiked normal serum (containing 0.125 μmol/L MMA) with 0.2–200 μmol/L of MMA. The relationship between peak area (fluorescence yield) and MMA concentration in serum documents a linear dynamic range within this concentration interval ($y = 4.07x + 2.26$; $r = 0.9995$). The standard errors of the slope and the intercept were 0.048 and 3.317, respectively (Figure 7).

Sensitivity. The serum was diluted 5-fold during protein precipitation and derivatization, and further 80-fold prior to injection. The lower limit of detection (LOD, signal to noise ratio of 5:1) was about 0.04 μmol/L for both EMA and MMA in serum, which corresponds to about 40 attomol.

Precision and Recovery. For the serum measurements, the within-day precision and the between-day precision (CV) was <12% at MMA concentrations less than 0.4 μmol/L, and <5% at concentrations greater than 0.4 μmol/L (Table 1). The analytical recovery of 0.4–40 μmol/L MMA added to serum was higher than 90% (Table 1).

Comparison of Methods. MMA concentrations in serum sets 1 and 2 determined by GC/MS methods ranged from 0.05 to 2.50 and from 0.068 to 368 μmol/L, respectively. Linear regression analysis showed good correlation between the CE assay and both GC/MS methods 1 ($y_1 = 1.16x - 0.05$; $r = 0.994$) and 2 ($y_2 = 1.17x - 0.16$; $r = 0.999$) (Figure 8).

The between-methods differences for CE and GC/MS were assessed by the Bland–Altman procedure[20] (Figure 9). To be able to use linear scales, we included in the analysis only 57 samples from set 2, which had an average MMA concentration below 2.5 μmol/L, and all 51 samples from set 1. For both data sets, the (absolute) difference was less than 0.08 μmol/L at MMA levels below 0.4 μmol/L. There is an slight bias in our method toward measuring lower MMA values in the higher concentration range compared to the values obtained by the GC/MS assays. The overall measuring agreement between CE and GC/MS is acceptable for clinical purposes and can be improved by assay intercalibration.

DISCUSSION

General Considerations. Derivatization of MMA and other short-chain dicarboxylic acids with PDAM forms highly fluorescent 1-pyrenylmethyl monoesters with one free carboxylic acid residue carrying a negative charge at pH > 4. We have previously described a sensitive method for determination of MMA based on derivatization with PDAM, separation by free-zone CE and LIF detection.[11] The assay was applicable to complex biological matrices including human plasma but lacked the long-term reproducibility and stability required for automatization and routine application. These adversities were mostly related to variable EOF and deterioration of capillary performance due to solute adsorption.[11]

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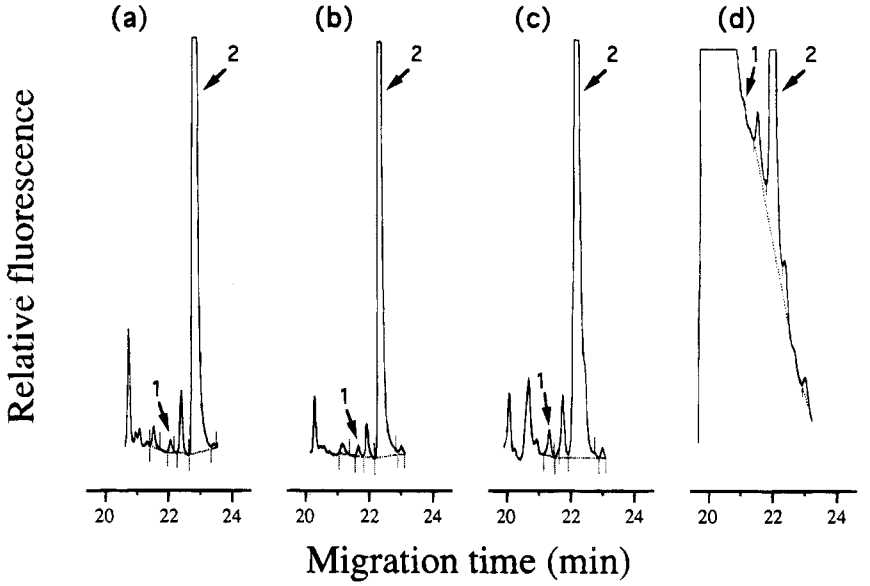

Figure 3. Electropherograms demonstrating the effects of various components of the discontinuous buffer system. (a) Electrophoretic profile of derivatized human serum containing 0.52 μmol/L MMA. The conditions for sample injection, preelectrophoresis, stacking, and separation were as depicted in Figure 2. (b) Conditions as in (a) except that the pH of the sample dilution matrix was not adjusted to 2.8. (c) Conditions as in (a) except that the presample phosphate zone (step II) was omitted. (d) Electrophoretic buffer as in (a), but the pH of the sample dilution matrix was not adjusted to 2.8, and both the presample zone and the preelectrophoresis were omitted. 1, MMA; 2, EMA.

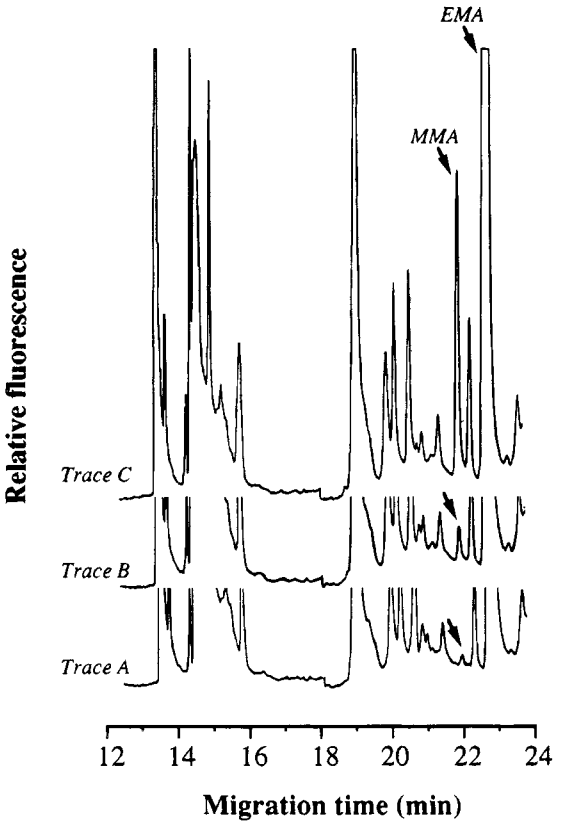

Figure 4. Electropherograms of human serum containing different concentrations of MMA. Trace A shows the electropherogram of serum containing 0.12 μmol/L MMA. Traces B and C show the same serum spiked with 0.4 and 4 μmol/L MMA, respectively. The concentration of EMA (internal standard) was 40 μmol/L in all three samples. The column had been used for about 1500 serum analyses.

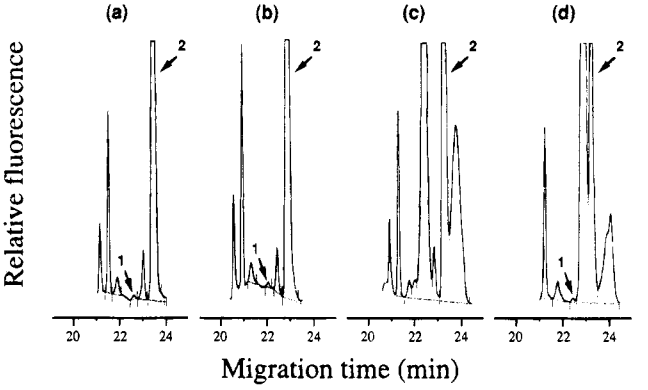

Figure 5. Electropherograms of serum (a), heparin–plasma (b), EDTA–plasma (c), and EDTA–plasma subjected to solid-phase extraction (d). The serum and plasma fractions were obtained from the same person. 1, MMA; 2, EMA.

Choice of Running Buffer. The analyte and running buffer background electrolytes should have similar electrophoretic mobilities to obtain optimum peak shape and separation efficiency.[21,22] We chose Tris–citrate as background electrolyte to match the relatively low mobility of 1-pyrenylmethyl monoesters. Tris–citrate has additional advantages, including high solubility in the presence of organic solvents, low specific conductivity (resulting in only moderate Joule heating), and high buffer capacity. The slow electrophoretic mobility reduces relative alterations in pH and composition of running buffer in the two buffer reservoirs used for electrophoretic separation (Figure 2, step V), allowing electrophoresis for 6 h without buffer replenishment. We found that pH was the most important factor for selectivity and resolution in our system. Efficient separation of the MMA peak from closely eluting material was obtained at pH 6.4 (Figure 3).

Suppression of EOF and Prevention of Solute Absorption. Variable EOF is related to nonequilibrium conditions and solute adsorption at the capillary surface. This results in unstable migration times and impairs column efficiency, and it often

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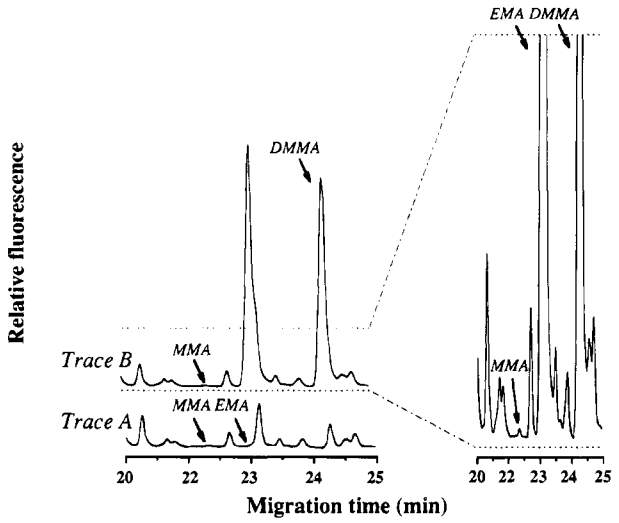

Figure 6. Electropherograms of serum with 0.125 μmol/L of endogenous MMA and without addition of internal standard (trace A), and the same serum spiked with 40 μmol/L of both EMA and DMMA (trace B). The graph to the right shows trace B in the same scale as used in Figure 4.

impedes routine application of CE.[23] Various procedures for static and dynamic coating of the inner capillary wall have been devised to prevent solute adsorption and to control or suppress EOF.[12,24]

We coated the inner capillary surface with linear polyacrylamide,[12] which almost completely eliminated EOF. Residual EOF was further suppressed by adding HPMC, a dynamic coating reagent, to the running buffer.[25]

The presence of an organic cosolvent in the sample dilution matrix and running buffer is required to keep hydrophobic compounds in solution and thereby reduces solute adsorption to the capillary surface. Organic cosolvents may also improve the resolution of highly hydrophobic compounds in CE.[17] We used DMF because it has superior solvent capabilities and a slow rate of evaporation (boiling point of 153 °C) and is recommended by the manufacturer as a solvent for PDAM. In addition, we found that the polyacrylamide coating was stable in 50% DMF.

Solute adsorption was further reduced by two measures. First, the high sensitivity afforded by LIF detection allowed extensive (80-fold) dilution of derivatized samples in a matrix containing 80% DMF. Second, CE separation was performed in the reversed-polarity mode (negative potential at the source vial), and only anionic species migrated through the capillary under the influence of the electric field. Uncharged (unreacted PDAM and PDAM derivatives of monocarboxylic acids) and positively charged species (including most derivatized amino acids) are excluded from the electrophoretic process. Noncharged species may even be removed from the injection site by residual EOF, which is directed toward the capillary inlet.

Discontinuous Buffer System and Analyte Stacking. In a previously published CE assay for determination of MMA,[11] sample stacking was achieved by field amplification. However, the resultant high field strengths over the injection zone, which is not thermostated, may cause excessive Joule heating or even boiling. This increases dispersion of the injection plug or may result in episodes of current error due to boiling.[26]

Another approach to achieve analyte focusing is transient on-line isotachophoretic preconcentration.[27-31] Conventional isotachophoresis applies a combination of an immobilized pH gradient and an amphoteric buffer component.[32] We used a discontinuous buffer system, forming a temporary pH gradient for analyte focusing (Figure 2). The sample zone is injected between the presample plug containing phosphate buffer, pH 3.4, and the running buffer at the rear end. To prevent Joule heating of the sample zone, the ionic strength of the sample dilution matrix was adjusted to that of the running buffer by adding NaCl. The electrolytes of the matrix (H^+, Na^+, and Cl^-) have high electrophoretic mobilities and will rapidly migrate out of the sample plug and thereby gradually increase the field strength over this zone. A pH gradient is built up over the sample zone with a low pH at the front (3.4) and approximately the pH of the running buffer (6.4) at the rear end. The 1-pyrenylmethyl monoesters ($pK_a \approx 5.6$) become stacked at the interface between the sample dilution matrix and the presample zone until the latter has disappeared. Analytes are then separated electrophoretically in the Tris–citrate buffer at pH 6.4. The effect from the separate components of the discontinuous buffer system on the electropherograms is shown in Figure 2.

Practicalities. The CE assay described here is characterized by long-term stability and reproducibility. More than 3000 analyses could be performed without capillary replacement.

As a consequence of excessive sample dilution, the serum requirement is only a few microliters, allowing us to perform studies even though only minimal amounts of stored samples were available. However, for more precise and convenient sample preparation, we routinely used 50 μL of serum. Serum or heparin–plasma is preferred, because EDTA–plasma caused interference with the assay. However, this problem can be overcome by subjecting the derivatized samples to solid-phase extraction (Figure 5). This option is valuable when only EDTA–plasma is available, as for example in research projects based on stored samples or when MMA is to be determined in samples collected for homocysteine analysis.

Sample processing (deproteinization and adding PDAM dissolved in DMF) in our CE assay is simpler than the procedures required by GC techniques.[2,5,13,33] The maximum sample output, which presently is about 50 samples/24 h, is not restricted by sample preparation but rather by the total run time (about 24 min, Figure 4). There is no need for equilibration, regeneration, or cleaning of the capillary between injections. For urgent analysis of single samples, results can be obtained within a few hours by increasing the derivatization temperature (Figure 1).

The prices of commercial instruments for CE–LIF and GC/MS are comparable, but the cost per analysis is negligible in our CE method because of long column lifetime and very low consumption of PDAM, organic solvents, and buffers. The HeCd laser head (lifetime of about 5000 h) is a main contributor to the operating costs.

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Table 1. Precision and Recovery of the Assay

within-day (n = 10) MMA, μmol/L				between-day (n = 10) MMA, μmol/L
endogenous	added	measured	recovered, %	measured
0.13 ± 0.015 (11.8)[a]	0.0	0.13 ± 0.015 (11.8)[a]		0.15 ± 0.018 (12.1)[a]
	0.40	0.50 ± 0.028 (5.6)	93.3	0.53 ± 0.043 (8.0)
	4.00	4.29 ± 0.197 (4.6)	104.2	4.14 ± 0.289 (7.0)
	40.00	39.33 ± 1.454 (3.7)	98.0	38.13 ± 2.150 (5.6)

[a] Mean ± SD (and CV, %).

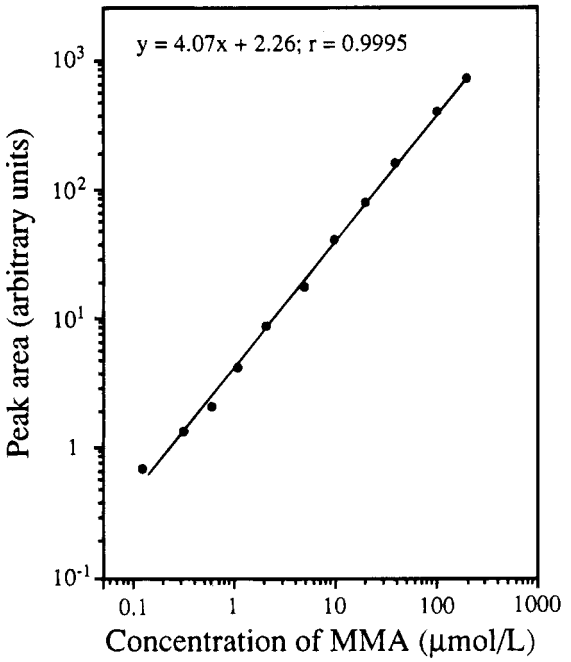

Figure 7. Linear dynamic range of the CE assay for MMA in serum. Relationship between peak area and MMA concentration. Serum containing 0.12 μmol/L of endogenous MMA was spiked with increasing amounts (0.2–200 μmol/L) of MMA. The linear regression curve ($y = 4.07x + 2.26$; $r = 0.9995$) is based on data points from single MMA determinations without correction for the internal standard EMA. The standard errors of the slope and intercept were 0.048 and 3.317, respectively.

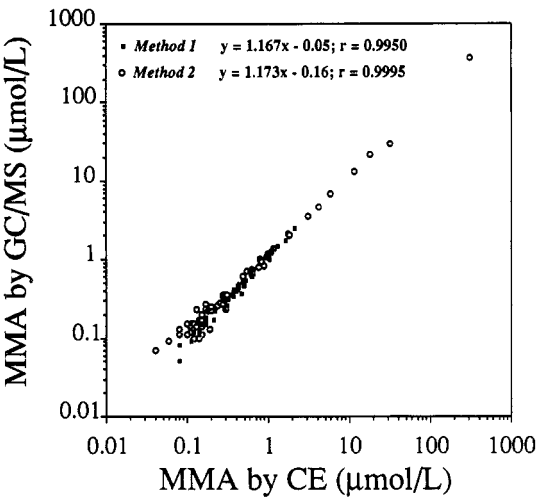

Figure 8. Comparison of serum MMA concentrations determined by CE and two GC/MS methods. MMA concentrations were determined by CE in two different sets of serum samples. Set 1 (n = 51, range 0.05–2.50 μmol/L) was analyzed by GC/MS method 1[5] (□), whereas set 2 (n = 64, range 0.07–368 μmol/L) was analyzed by GC/MS method 2[13] (○).

Compared with the previously described HPLC method,[6] the CE–LIF technique offers the advantage that there is no need for solid-phase extraction, only very small sample requirements, and low consumption of reagents and buffers. In addition, the method is faster and has better long-term stability, and column replacement is less often necessary.

Internal Standard, Assay Statistics, and Performance. EMA has several features that make it a useful IS in a CE assay for determination of MMA. It shows the same reaction kinetics with PDAM as MMA (Figure 1) and elutes close to MMA (Figure 4). Furthermore, only trace amounts have been found in human serum or urine.[2] EMA has therefore been used as IS in several assays for MMA in urine.[34–36] The concentration of EMA used in our assay (40 μmol/L) was selected to obviate interference from small amounts of coeluting material and to allow correction for a closely eluting peak (Figure 6).

Notably, we observed that the within-day precision for the determination of EMA was high (CV about 5%) and that the within-day CV for MMA was essentially the same with and without IS. This is explained by simple and reproducible sample preparation and derivatization. The IS is not required to correct for variable and low recovery during extraction steps. However, we observed a variable fluorescence yield from one day to another, which we believe is related to small variations in laser beam focusing on the capillary when the capillary cartridge is changed. Therefore, the addition of EMA as IS was necessary to achieve an acceptable between-day precision.

Generally, the use of an IS is important for precise quantitative determination by CE, especially when applying hydrodynamic injection.[37–41] The precision of the present assay corresponds to a CV of 6–12% at concentrations of MMA below 0.5 μmol/L and about 6–7% at higher concentrations (Table 1), which makes this method comparable to GC/MS methods.[3,5,33]

The mass sensitivity obtained by the present CE assay and by CE–LIF detection in general[42] is in the attomolar range, which is at least 3 orders of magnitude higher than that achieved with UV absorption[42] and exceeds that obtained with GC/MS. This allowed

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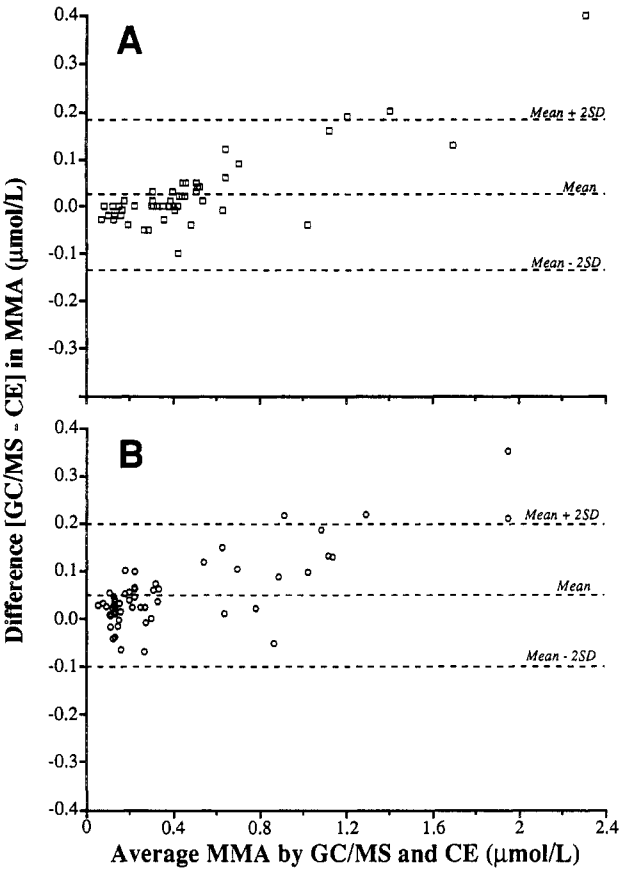

Figure 9. Assessment of measuring agreement between the CE method and the two GC/MS methods. Data from the sample sets 1 and 2 presented in Figure 7 were replotted according to the Bland-Altman procedure.[20] Only samples with MMA mean values less than 2.5 μmol/L of sample set 2 were included in the analysis. The mean of the differences and the 95% confidence interval of the differences are indicated by dashed lines.

us to dilute serum extensively (400-fold) prior to CE separation, and we could thereby reduce solute adsorption and increase assay stability. Dilution is ultimately restricted by the concentration sensitivity (about 0.04 μmol/L) required to detect MMA in the low normal range.[6,43-45] We also documented the linear dynamic range of the assay (Figure 7), which spanned from physiological concentrations to levels described in patients with severe cobalamin deficiency (>200 μmol/L[3]).

The Bland–Altman procedure[20] was applied to compare the CE assay with two established GC/MS techniques.[5,13] Good measuring agreement was observed, especially in the lower concentration range (<2.5 μmol/L) (Figure 9), which is clinically important for diagnosis of early cobalamin deficiency.[46]

Conclusion. Development of CE methods for routine clinical analysis of substances in real biological samples has been hampered by insufficient assay stability and reproducibility. Analysis of low concentrations of hydrophobic molecules, like the 1-pyrenylmethyl monoesters, in the presence of large amounts of interfering material poses additional demands on separation efficiency and prevention of solute adsorption. Our strategy to solve these problems included static and dynamic capillary coating, careful selection of pH, background electrolytes, and organic cosolvents, and application of a discontinuous buffer system for sample stacking. In this way we constructed a robust CE assay for the routine determination of MMA in serum. The assay was validated by comparison with two established GC/MS methods. Compared with GC/MS and HPLC techniques, this assay maintains the inherent advantages of CE, i.e., easy sample preparation, small sample requirements, low consumption of reagents and buffers, and high separation efficiency.

ACKNOWLEDGMENT

This work was supported by grants from the Nordic Insulin Foundation and the Norwegian Society for Science and the Humanities (NAVF). We are thankful to Drs. Karsten Rasmussen and Robert H. Allen for proving samples for comparison studies. The technical assistance provided by Else Leirnes is highly appreciated.

Received for review September 12, 1994. Accepted December 13, 1994.[⊗]

AC940912H

[⊗] Abstract published in *Advance ACS Abstracts*, January 15, 1995.

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