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Capillary zone electrophoresis with laser-induced fluorescence detection for analysis of methylmalonic acid and other short-chain dicarboxylic acids derivatized with 1-pyrenyldiazomethane

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Abstract

Methylmalonic acid (MMA) and other short-chain dicarboxylic acids react with 1-pyrenyldiazomethane (PDAM) in aqueous matrices and form stable, highly fluorescent 1-pyrenylmethyl monoesters [Schneede and Ueland, *Anal. Chem.*, 64 (1992) 315–319]. We investigated the migration behaviour of these derivatives in capillary zone electrophoresis in fused-silica capillaries. The 1-pyrenyldiazomethane derivatives were detected with a laser-induced fluorescence detector that was connected to a helium–cadmium laser, delivering light at a wavelength of 325 nm, which exactly matched an excitation maximum of the 1-pyrenylmethyl monoesters. These esters have one free carboxylic acid group, which carries negative charge at pH >4.0. The electrophoretic mobility varied according to pH, and the pH effect was most pronounced at values close to the pK_a value (5.2–5.8) of the esters. The effects of the composition of the running buffer (pH, organic modifier concentration, ionic strength) and some operational parameters (voltage, temperature and capillary length) were tested, and were largely found to comply with validated theoretical models for capillary zone electrophoresis. In an optimal system, as judged by high number of theoretical plates, high resolution and short run times, the MMA derivative was separated from related 1-pyrenylmethyl monoesters. The laser-induced fluorescence detection afforded a limit of detection of about 40 nmol/l (signal-to-noise ratio of 5) for the MMA derivative. Under optimal conditions, we were able to detect <1 μmol/l endogenous MMA in human serum.

1. Introduction

Short-chain dicarboxylic acids are important intermediates in the metabolism of diverse compounds like amino acids, fatty acids and pyrimidines, and their concentrations increase in some inborn metabolic disorders [1,2]. Among the dicarboxylic acids, methylmalonic acid (MMA) has gained much interest, because its levels in urine [3] and serum [4,5] have been established as a clinically useful indicator of cobalamin function.

GC–MS is the most widely adopted technique for the measurement of MMA in urine or serum. These methods are specific and sensitive, but require laborious sample processing prior to chromatography [6–8].

Recently, we developed an alternative method

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for analysis of MMA and other short-chain dicarboxylic acids based on HPLC [9,10]. In this method, short-chain dicarboxylic acids were derivatized with the fluorescent labelling reagent 1-pyrenyldiazomethane (PDAM) in aqueous media. Presumably due to steric hindrance, derivatization with PDAM results in 1-pyrenylmethyl monoesters with a free carboxylic acid residue. The ionisation of this group imposes unique chromatographic properties on the derivatives. They present a pH-dependent mobility on reversed-phase chromatography [9], and are retained on anion-exchange columns at pH >6. We exploited these features in a HPLC method combined with solid-phase extraction for the determination of MMA in biological fluids, such as serum, plasma and urine [10].

The analytical performance of capillary electrophoresis (CE) is regarded as complementary to that of HPLC [11,12]. In capillary zone electrophoresis (CZE), compounds migrate in open fused-silica capillaries in the presence of an electrical field and are separated on the basis of different electrophoretic mobilities. The technique is characterised by a short run time and a high number of theoretical plates. However, a major limitation of CZE is its low concentration sensitivity and, as a result, poor limit of detection (LOD). This is related to the low sample volume (usually a few nanoliters) injected into the capillary and the resultant small amounts of analyte presented to the detector [11,12].

High mass sensitivity in CZE is obtained with laser-induced fluorescence (LIF) detection, where a highly collimated laser beam is directed onto the capillary detection window. However, lasers emit at only a limited number of wavelengths, and therefore only a few fluorogenic reagents are appropriate for LIF detection [13].

The pyrenylmethyl monoesters are highly fluorescent compounds with an excitation maximum at 324 nm [9] which excellently matches one of the wavelengths (325 nm) emitted by the He–Cd laser. The esters are stable compounds carrying negative charge at pH above 4. These characteristics make pyrenylmethyl monoesters ideal candidates for CZE combined with LIF detection.

In the present work we investigated the migration behaviour of PDAM derivatives of MMA and other short-chain dicarboxylic acids in CZE using mobile phases with different pH values, ionic strengths and concentrations of organic cosolvent. We also evaluated different operational parameters, separation characteristics and LODs. On the basis of these data we were able to apply a CZE method for the detection of MMA in human serum.

2. Experimental

2.1. Chemicals

PDAM was obtained from Molecular Probes (Eugene, OR, USA). It was dissolved in ethyl acetate (2.5 mg/ml) and stored at −20°C. This solution was freshly prepared each fortnight. 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), malonic acid, maleic acid, fumaric acid, α-ketoglutaric acid and dimethylmalonic acid were purchased from Aldrich (Milwaukee, WI, USA), and succinic acid from Sigma (St. Louis, MO, USA). Methanol (HPLC grade), acetonitrile (HPLC grade) and ethyl acetate were from Merck (Darmstadt, Germany). We used double distilled water which was further purified on a Milli-Q-plus ultra-pure water system (Millipore, Bedford, MA, USA).

2.2. Instrumentation

A P/ACE 2100 CE instrument equipped with a P/ACE LIF detector was used; both units were purchased from Beckman (Fullerton, CA, USA). The LIF detector had an off-board 20-mW He–Cd laser source, Model 3056-10M from Omnichrome (Chino, CA, USA), connected to a Model 100 power supply (Omnichrome). The laser excitation light was focused on the capillary window by means of an 100/140 UV-grade fibre cable with connectors SMA 905 and SMA 906,

purchased from OZ Optics (Ontario, Canada). Fibre optical alignment was optimised using Optical Noise/Power Meter (NP2) from Omnichrome. The excitation light was at 325 nm, and a 383-nm band pass filter (Omega Optical, Brattleboro, VT, USA) mounted in the filter housing of the LIF detector was used for emission.

Open fused-silica capillaries, coated on the outer surface with polyimide, were obtained from Dionex (Sunnyvale, CA, USA). The column was mounted in a temperature-controlled cartridge specially designed for the LIF detector. The inner surface of the capillary was uncoated, and the inner and outer diameters were 75 and 375 μm, respectively. The total capillary length was 47 cm (40 cm to detector) when not indicated otherwise.

The Beckman System Gold software (version 7.11) run on an IBM PS/2 55 SX computer was used for system control, data collection and processing.

2.3. Derivatization of dicarboxylic acids

Dicarboxylic acids were dissolved in 10 m*M* sodium borate buffer pH 8.0. To 100 μl of this solution containing 100–200 μM of dicarboxylic acid, 100 μl of acetonitrile, 200 μl of methanol and 100 μl of PDAM in ethyl acetate were added. The solution was mixed and incubated overnight in the dark at room temperature. Serum samples were processed in the same way, except that precipitated protein was removed by centrifugation before the addition of PDAM.

2.4. Capillary electrophoresis

New capillaries were conditioned by rinsing in the high-pressure mode for 2 min with 0.5 *M* NaOH and 6 min with running buffer. Before each injection, the capillary was flushed twice for 1 min in the high-pressure mode with running buffer from two separate reservoirs. After each run, the capillary was flushed for 1 min with running buffer, and then for 2 × 3 min with 50 m*M* sodium dodecyl sulfate in water.

Solutions used for capillary conditioning and sample dilution, and running buffers were vacuum degassed and subjected to filtration through a 0.45-μm Gelman Acrodisc LC 13 PVDF syringe filter (Ann Arbor, MI, USA).

Before injection, the derivatized samples were diluted 1:15 (v/v) in a solution consisting of 54% acetonitrile, 16% methanol and 30% 2 m*M* Tris–HCl, pH 8.

If not indicated otherwise, the running buffer was composed of 30% acetate–phosphate buffer, pH 5.0, 54% acetonitrile and 16% methanol. The pH was adjusted to pH 5.0 after the addition of the organic co-solvent, and the final ionic strength of the acetate–phosphate buffer was 30 m*M*. Diluted samples were pressure-injected for 3 s, and electrophoresis was carried out at 30 kV constant voltage. The capillary was thermostated at 20°C.

2.5. Calculations

Electrophoretic mobility (μ) of solutes was calculated by the following equation:

$$\mu = \mu_{app} - \mu_{eo} = Ll(1/t_a - 1/t_{eo})/V \quad (1)$$

where μ_{app} is the apparent mobility of the analyte, μ_{eo} is the electroendosmotic mobility, t_a is the migration time of the analyte, t_{eo} is the migration time for an uncharged solute (neutral marker), L is the total capillary length, l is the capillary distance between the point of injection and the point of detection and V is the applied voltage.

The number of theoretical plates (N) was calculated by [14]:

$$N = 5.54t_a^2/w^2 \quad (2a)$$

or can be expressed by

$$N = \mu Vl/(2DL) \quad (2b)$$

where w is the peak width at the half-maximum peak height.

The resolution (R_s) was calculated according to the equation [15]:

$$R_s = N^{1/2}/4(\mu_1 - \mu_2)/\mu_{av} \quad (3)$$

where N is the plate number of the MMA peak,

μ_1 and μ_2 are the apparent mobilities of the derivatives of MMA and EMA, and μ_{av} is their average apparent mobility.

3. Results and discussion

The biological importance and diagnostic value of the determination of short-chain dicarboxylic acids, and MMA in particular, motivate the development of convenient analytical techniques. We found that the fluorescence labelling reagent PDAM reacts with short-chain dicarboxylic acids in aqueous matrices to form 1-pyrenylmethyl monoesters. These derivatives have physico-chemical properties that make them ideal candidates for CZE analysis combined with LIF detection. In the present work, we investigated in detail the migration and separation properties of 1-pyrenylmethyl monoesters in CZE with different compositions of electrophoresis buffer and variable operational parameters.

The physico-chemical properties of the 1-pyrenylmethyl monoesters determine to some extent the composition of the running buffer. These derivatives, and unreacted PDAM, are hydrophobic species which require the presence of a high percentage of organic co-solvent to stay in solution. This in turn prohibits the addition of high concentrations of phosphate to the running buffer, and limits the maximum ionic strength to about 35 m*M*.

Most studies on migration and resolution were done with MMA and EMA. A mixture of MMA, EMA and several other closely related short-chain dicarboxylic acids, and human serum was only analysed in optimised systems. Unreacted PDAM was used as a convenient neutral marker.

3.1. Effect of pH and determination of pK_a

PDAM derivatives of MMA and other short-chain dicarboxylic acids behave like weak acids upon reversed-phase liquid chromatography [9,10], which is in accordance with their chemical structure verified by MS [9]. They become negatively charged at pH > 4.0.

Smith [16] recently demonstrated that the electrophoretic mobility of weak acids in CZE can be described by the equation:

$$\mu = \mu_{A^-}(K_a/[H^+])/\{1 + (K_a/[H^+])\} \quad (4)$$

where μ is the electrophoretic mobility at a given pH, μ_{A^-} is the mobility of the acid in its anionic form, and K_a is the dissociation constant. The equation expresses a sigmoidal curve.

When we plotted the mobilities of the PDAM derivatives of MMA (Fig. 1) and EMA (data not shown) *vs.* pH, a sigmoidal curve was obtained, as predicted from Eq. 4. The anionic form of these species showed a negative mobility directed towards the anode (*i.e.* opposite to the

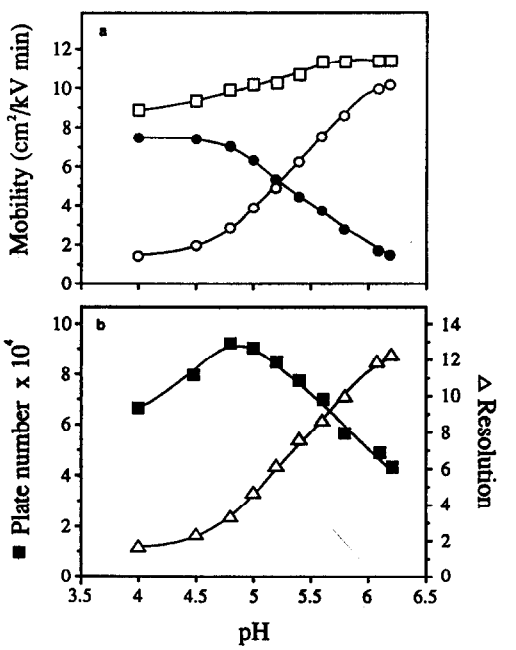

Fig. 1. Effect of pH on mobility and separation parameters. CZE was performed in 75 μm I.D. tubes of 47 cm total length (40 cm to detector) and thermostated at 20°C. The running buffer was 30 m*M* phosphate–acetate buffer containing 70% organic modifier. The applied voltage was 30 kV. Prior to injection, samples containing MMA and EMA were diluted 1:15 (v/v) in 2 m*M* Tris–HCl buffer, pH 8, containing 70% organic modifier. All mobilities are shown as absolute values. (a) □ = μ_{eo}; ● = μ_{app}; ○ = μ. (b) ■ = N; △ = R_s.

"positive" electroendosmotic flow). The inflection point gives, according to Eq. 4, the pK_a value, which was approximately 5.6 for the MMA derivative (Fig. 1) and 5.5 for the EMA derivative (data not shown).

The electroendosmotic flow (μ_{eo}) increased as a function of increasing pH (Fig. 1). Others have found that the plot of pH *vs.* electroendosmotic mobility exhibits an overall sigmoidal shape, and increases almost linearly between pH 4 and 6.5 [17–19].

The electrophoretic mobility of both MMA and EMA derivatives approached electroendosmotic mobility with increasing pH. The apparent mobility is the sum of the (positive) electroendosmotic and (negative) electrophoretic mobility. Thus, at pH >6, the apparent mobility of these analytes became very low, resulting in long migration times.

At pH >6.5, the electrophoretic mobility of the PDAM derivatives may equal or even exceed electroendosmotic mobility. Thus, the net mobility may approach zero or even be directed towards the anode, and the derivatives will not pass the detection window. Therefore, the pH of the running buffer in our system was restricted to the range between 4.0 and 6.4.

The number of theoretical plates showed a maximum at pH 4.8, *i.e.* somewhat below the pK_a values of the analytes, and then declined at higher pH (Fig. 1).

We observed that the increase in relative mobility difference was highest at pH values close to the pK_a of the MMA and EMA derivatives (data not shown). Similarly, others have suggested that best resolution is obtained with a pH of the running buffer close to the pK_a of the analytes [20].

3.2. *Ionic strength and organic modifier*

The electroendosmotic mobility in CZE is proportional to the ζ-potential of the capillary, which in turn is proportional to the reciprocal of the square root of the ionic strength [21]. This explains the observation that electroendosmotic mobility in our system decreased almost linearly as a function of the reciprocal of the square root

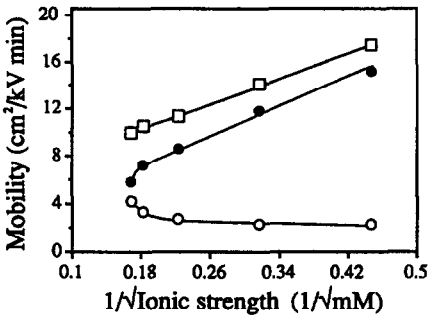

Fig. 2. Effect of ionic strength on mobility. The concentration of phosphate–acetate buffer varied between 5 and 35 m*M*, and the pH was 5.0. Other conditions as in Fig. 1. □ = μ_{eo}; ● = μ_{app}; ○ = μ.

of the buffer concentration (Fig. 2). Furthermore, the electrophoretic mobility increased at higher buffer concentrations, especially at concentrations of 30–35 m*M* (Fig. 2). These effects may be attributed to a higher current increasing Joule heating. The resulting temperature elevation inside the capillary decreases the viscosity of the running buffer, and thereby slightly enhances both the electroendosmotic and electrophoretic mobilities. This explanation is corroborated by the Ohm plot deviating from linearity at buffer concentrations higher than 30 m*M* (data not shown).

Organic additives were required in the present application to keep the analytes in solution. In preliminary experiments, we tested running buffers containing 70% organic solvent composed of either methanol or acetonitrile, and different mixtures of methanol and acetonitrile. The highest apparent mobility and number of theoretical plates were obtained with a buffer containing acetonitrile and methanol in the proportions 4:1. This mixture was used in all our experiments. We used, when not otherwise indicated, 70% of organic modifier mixture in the running buffer to prevent precipitation of the 1-pyrenylmethyl monoesters at low pH.

Organic modifiers effect physico-chemical properties of the running buffer (viscosity, conductivity and pH), the inner capillary surface (dielectric constant, ζ-potential) [22–24] and the

analyte (net charge, pK_a of weak acids) [25,26]. All these effects may contribute to changes in CE performance.

It has been reported [22,27] that organic modifier-induced reduction of viscosity was positively correlated to the observed migration times. This is in contrast to our findings. With acetonitrile as sole organic additive we observed that the electroendosmotic flow and electrophoretic mobility were reduced with increasing acetonitrile concentration (data not shown), even though viscosity was expected to decrease. Also, both parameters decreased when the portion of the mixture of organic modifier (acetonitrile–methanol, 4:1) was increased from 20 to 40%; essentially no effect was observed between 40 and 70% (Fig. 3). Likewise, others have found that organic modifiers reduce the electroendosmotic flow to a different extent. Methanol causes a pronounced reduction, whereas the influence of acetonitrile is moderate [24].

In our system organic additives cause a parallel decrease in both electroendosmotic and electrophoretic mobilities (Fig. 3). Increasing portions of organic solvents may reduce net charge of the capillary wall [28], of buffer electrolytes and of analytes. This may decrease the ζ-potential of the capillary wall and thereby the electroendosmotic mobility. Decreased net charge of the PDAM derivatives will reduce their electrophoretic mobility.

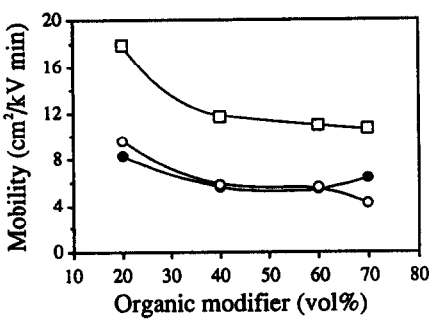

Fig. 3. Effect of organic modifier on mobility. Organic modifier is a mixture of 80% acetonitrile and 20% methanol, and it comprised from 20 and 70% of the mobile phase. pH was 5.0. Other conditions as in Fig. 1. □ = μ_{eo}; ● = μ_{app}; ○ = μ.

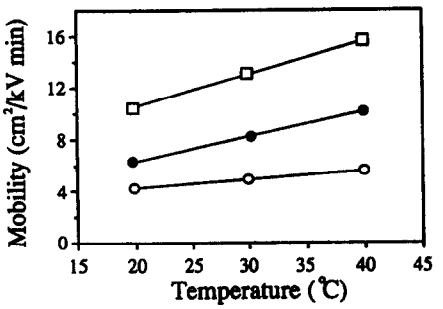

Fig. 4. Effect of column temperature on mobility. The pH of the running buffer was adjusted to 5.0 at 20°C. Capillaries were thermostated at temperatures between 20 and 40°C. Other conditions as in Fig. 1. □ = μ_{eo}; ● = μ_{app}; ○ = μ.

3.3. Temperature and capillary length

We found that the electroendosmotic and electrophoretic mobilities (Fig. 4) as well as current (data not shown) increased linearly as a function of capillary temperature. This may be explained by the effect of temperature on viscosity, pH and conductivity of the running buffer [22,29]. The number of theoretical plates showed a maximum at a temperature of about 30°C, and both plates and resolution decreased slightly when the temperature was raised to 40°C (data not shown). The temperature optimum may be explained by an initial temperature-induced reduction of viscosity leading to higher electrophoretic mobility, which in turn is positively correlated to plate number (Eq. 2b). At higher temperatures, thermal diffusion may predominate, leading to peak broadening and a reduction of plates and resolution.

Capillaries of 20, 30 and 40 cm length were tested at constant electric field strength. The number of theoretical plates and the resolution increased linearly as a function of capillary length. The plate number increased 4-fold and the resolution 1.6-fold when the capillary length was increased from 20 to 40 cm (data not shown). The increase is larger than predicted from a theoretical model [30,31] and may be explained by a larger contribution of the injection plug to total peak dispersion in short capillaries [31].

3.4. Separation and detection of 1-pyrenylmethyl monoesters

Fig. 5 shows the separation of PDAM derivatives of MMA, EMA, dimethylmalonic acid, malonic acid, succinic acid, maleic acid, fumaric acid and α-ketoglutaric acid by CZE in an optimised system. The running buffer was 30 m*M* phosphate–acetate buffer, pH 4.8, containing 50% organic modifier. The large peak (a) with a migration time of 3.4 min represented unreacted PDAM, which served as a neutral marker. The marked peak (b) migrating ahead of EMA is derived from the borate buffer present during derivatization.

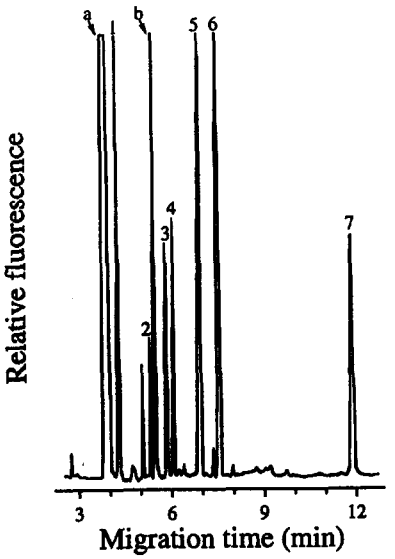

Fig. 5. Separation of the PDAM derivative of MMA from related 1-pyrenylmethyl monoesters by CZE. The dicarboxylic acids (100 μmol/l of each, except fumaric and maleic acid which were present at 200 μmol/l) were derivatized with PDAM. The derivatization mixture was diluted 1:30 (v/v) in 2 m*M* Tris–HCl buffer, pH 8, containing 70% organic modifier, prior to injection. The capillary had a total length of 47 cm, 50 μm I.D., was thermostated at 30°C, and the applied voltage was 30 kV. The running buffer was 30 m*M* phosphate–acetate buffer pH 4.8 containing 50% organic modifier. Peaks: a = unreacted PDAM; b = unidentified peak derived from the borate buffer; 1 = α-ketoglutaric acid + succinic acid (coeluting); 2 = dimethylmalonic acid; 3 = EMA; 4 = MMA; 5 = maleic acid; 6 = fumaric acid; 7 = malonic acid.

We evaluated the sensitivity afforded by the LIF detector by analysing diluted samples of a derivatization mixture containing 80 μmol/l of both EMA and MMA. The lower limit of detection, defined as a signal-to-noise ratio (*S/N*) of 5, was about 40 nmol/l for both EMA and MMA (Fig. 6). The high sensitivity of the LIF detector allows extensive dilution of derivatized sample before injection.

3.5. Dilution of sample

The derivatization solution contained 80% volatile organic solvent and had lower specific conductivity than the mobile phase. To avoid heating and boiling of the sample plug due to high electrical field strength [32], we had to dilute samples (1:15) prior to injection in low-concentration buffer. Especially when analyzing derivatized serum samples, we observed decreasing plate number and progressive reduction in electroendosmotic flow. This is probably due to interaction of unreacted PDAM, PDAM derivatives and serum constituents with the capillary surface. Dilution of derivatized samples delayed the column deterioration, but also enabled us to

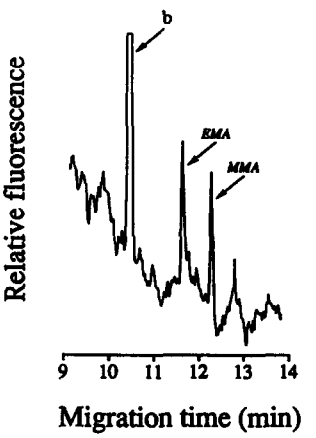

Fig. 6. Limit of detection of the PDAM derivatives of MMA and EMA in CZE with LIF detection. MMA and EMA were derivatized at 80 μmol/l, and then diluted to 40 nmol/l. The total capillary length was 57 cm (50 cm to detector), 75 μm I.D. The pH was 5.0. The sample was pressure-injected for 2 s. Other conditions as in Fig. 1. Peak b as in Fig. 5.

inject sample in low-concentration buffer, favouring sample stacking [33] and enhanced resolution.

3.6. Determination of MMA in human serum

The concentration of MMA in serum is low in healthy subjects (mean 0.19 μmol/l; range 0.04–0.43 μmol/l) [10], but often increases to 1 to 200 μmol/l in patients with cobalamin deficiency [10,34,35]. Since serum MMA is useful for the diagnosis and follow-up of cobalamin deficiency [4,5,34,35], we developed a CZE system to detect MMA in human serum. An electropherogram of normal serum containing 0.38 μmol/l and spiked with 5 μmol/l of MMA is shown in Fig. 7. The MMA derivative was baseline separated from interfering material and the derivative of EMA (used as internal standard) (Fig. 7).

We spiked normal serum with MMA at concentrations ranging from 0.1 to 500 μmol/l. The plot of the fluorescent 1-pyrenylmethyl methylmalonate monoester detected by CZE *vs.* MMA concentrations was linear (data not shown). These results agree with the recovery experiments performed with our HPLC assay [10], and are a prerequisite for a quantitative MMA assay based on derivatization of MMA with PDAM followed by CZE separation.

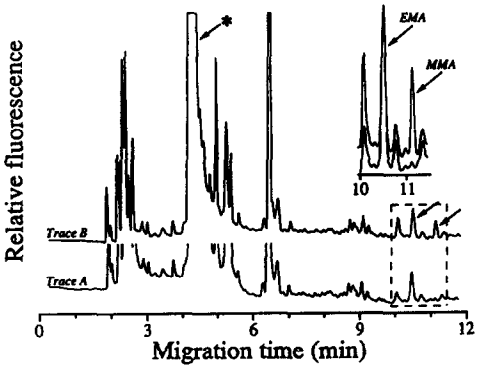

Fig. 7. Electropherogram of human serum. Serum samples (50 μl) were deproteinized and derivatized as described [10]. Samples were diluted 1:15 (v/v) prior to pressure injection for 4 s. The running buffer was 35 m*M* phosphate–acetate buffer, pH 5.4, containing 30% organic modifier. The total capillary length was 57 cm, 50 μm I.D. Trace A shows the profile of serum from a healthy subject, containing 0.38 μmol/l of MMA. Trace B shows the same serum spiked with 5 μmol/l of MMA. The concentration of EMA was the same in both samples and corresponded to 10 μmol/l EMA in serum. The inset shows the part of the traces containing the EMA and MMA peaks redrawn in an expanded scale. The peak marked with an asterisk shows the elution of unreacted PDAM and neutral derivatization products.

The column performance was impaired after injection of 10–30 serum samples, and could not be restored by rinsing for several minutes with 1 *M* NaOH. This impedes analysis stability required for a clinical laboratory method, and frequent capillary replacement was required. To develop a stable assay, we are currently evaluating separation of PDAM derivatives in capillaries which were coated to eliminate electroendosmosis [36,37].

4. Conclusions

The free carboxylic group of the 1-pyrenylmethyl monoesters of short-chain dicarboxylic acids explains their pH-dependent mobility in CZE (Fig. 1), as well as in HPLC [9]. For optimal separation in CZE, the pH of the running buffer should be close to the pK_a of the free carboxylic group. The PDAM derivatives are in anionic form at pH >4.0 and had a electrophoretic mobility opposite to the electroendosmotic flow. The effect of pH, ionic strength and some operational parameters on electroendosmosis and solute mobility could largely be predicted from validated theory of CZE. The negatively charged 1-pyrenylmethyl methylmalonate monoester was separated from neutral and positively charged PDAM derivatives that predominate in serum (Fig. 7), and could be detected in human serum without sample clean-up.

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6. References

[1] E. Jellum, *J. Chromatogr.*, 143 (1977) 427.
[2] V.E. Shih, *Clin. Biochem.*, 24 (1991) 301.
[3] E.J. Norman, O.J. Martelo and M.D. Denton, *Blood*, 59 (1982) 1128.
[4] K. Rasmussen, L. Moelby and M.K. Jensen, *Clin. Chem.*, 35 (1989) 2277.
[5] R.H. Allen, S.P. Stabler, D.G. Savage and J. Lindenbaum, *Am. J. Hematol.*, 34 (1990) 90.
[6] P.D. Marcell, S.P. Stabler, E.R. Podell and R.H. Allen, *Anal. Biochem.*, 150 (1985) 58.
[7] J.A. Montgomery and O.A. Mamer, *Methods Enzymol.*, 166 (1988) 47.
[8] K. Rasmussen, *Clin. Chem.*, 35 (1989) 260.
[9] J. Schneede and P.M. Ueland, *Anal. Chem.*, 64 (1992) 315.
[10] J. Schneede and P.M. Ueland, *Clin. Chem.*, 39 (1993) 392.
[11] I.S. Krull and J.R. Mazzeo, *Nature*, 357 (1992) 92.
[12] C.C. Campos and C.F. Simpson, *J. Chromatogr. Sci.*, 30 (1992) 53.
[13] L.N. Amankwa, M. Albin and W.G. Kuhr, *Trends Anal. Chem.*, 11 (1992) 114.
[14] B. Karger, A.S. Cohen and A. Guttman, *J. Chromatogr.*, 492 (1989) 585.
[15] J.C. Giddings, *Sep. Sci.*, 4 (1969) 181.
[16] S.C. Smith, *Anal. Chem.*, 65 (1993) 193.
[17] T. Tsuda, K. Nomura and G. Nakagawa, *J. Chromatogr.*, 385 (1983) 264.
[18] K.D. Altria and C.F. Simpson, *Chromatographia*, 24 (1987) 527.
[19] R.A. Wallingford and A.G. Ewing, *Adv. Chromatogr.*, 29 (1989) 1.
[20] H.-T. Chang and E.S. Yeung, *J. Chromatogr.*, 608 (1992) 65.
[21] R.-L. Chien and J.C. Helmer, *Anal. Chem.*, 63 (1991) 1354.
[22] G.M. Mc Laughlin, J.A. Nolan, J.L. Lindahl, R.H. Palmieri, K.W. Anderson, S.C. Morris, J.A. Morrison and T.J. Bronzert, *J. Liq. Chromatogr.*, 15 (1992) 961.
[23] K. Salomon, D.S. Burgi and J.C. Helmer, *J. Chromatogr.*, 559 (1991) 69.
[24] C. Schwer and E. Kenndler, *Anal. Chem.*, 63 (1991) 1801.
[25] E. Kenndler and P.J. Jenner, *J. Chromatogr.*, 390 (1987) 169.
[26] E. Kenndler, C. Schwer and P.J. Jenner, *J. Chromatogr.*, 470 (1989) 57.
[27] B.B. VanOrman, T.M. Olefirowicz, G.G. Liverside, A.G. Ewing and G.L. McIntire, *J. Microcol. Sep.*, 2 (1990) 176.
[28] R.J. Hunter, in R.J. Hunter (Editor), *Zeta Potential in Colloid Sciences: Principles and Applications*, Academic Press, New York, 1981, p. 386.
[29] C.-W. Whang and E.S. Yeung, *Anal. Chem.*, 64 (1992) 502.
[30] J.W. Jorgenson and K.D. Lukacs, *Anal. Chem.*, 53 (1981) 1298.
[31] S.L. Delinger and J.M. Davis, *Anal. Chem.*, 64 (1992) 1947.
[32] A. Vinther and H. Søeberg, *J. Chromatogr.*, 559 (1991) 27.
[33] D.S. Burgi and R.-L. Chien, *Anal. Chem.*, 63 (1991) 2042.
[34] S.P. Stabler, R.H. Allen, D.G. Savage and J. Lindenbaum, *Blood*, 76 (1990) 871.
[35] L. Moelby, K. Rasmussen, M.K. Jensen and K.O. Pedersen, *J. Int. Med.*, 228 (1990) 373.
[36] S. Hjertén, *J. Chromatogr.*, 347 (1985) 191.
[37] S. Hjertén and K. Kubo, *Electrophoresis*, 14 (1993) 390.