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Formation in an Aqueous Matrix and Properties and Chromatographic Behavior of 1-Pyrenyldiazomethane Derivatives of Methylmalonic Acid and Other Short-Chain Dicarboxylic Acids

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Methylmalonic acid (MMA) and some short-chain dicarboxylic acids were derivatized with the fluorescent labeling reagent 1-pyrenyldiazomethane (PDAM) in an aqueous matrix. The derivatization of MMA and ethylmalonic acid (EMA) proceeded and leveled off rapidly at low pH, whereas at alkaline pH, the fluorescent derivative was progressively formed over a period of more than 24 h, and higher fluorescence yield was obtained. The PDAM esters of MMA and EMA had an excitation maximum at 340 nm and emission maxima at 376 and 395 nm. They were stable for days in aqueous media at room temperature. The MMA derivative was identified as 1-pyrenylmethyl methylmalonate monoester by mass spectrometry, and formation of 1-pyrenylmethyl methylmalonate diester could not be demonstrated. The free carboxylic acid moiety and the ionization of this group may explain the unique chromatographic properties of the MMA derivative, i.e. a marked increase in the capacity factor in a reversed-phase system by decreasing the pH of the mobile phase. The MMA derivative was separated from PDAM esters of several other short-chain dicarboxylic acids by a acetonitrile gradient in formate buffer, pH 2.5. These data may form the basis for the construction of automated MMA assays involving derivatization in aqueous media followed by liquid chromatography.

INTRODUCTION

Several chromatographic techniques have been developed for the determination of carboxylic acids because of the importance of these compounds in normal metabolism and cellular function, as well as their role in several diseases. Among the carboxylic acids, great interest has recently been focused on the short-chain dicarboxylic acid methylmalonic acid (MMA). Its concentration in extracellular media like plasma and urine may serve as an indicator of intracellular cobalamin function (*1*).

Carboxylic acids including MMA have been determined by GC or GC/MS (*2–4*). These methods often require extensive sample purification and derivatization prior to chromatography (*3, 4*). Recently, several liquid chromatographic procedures have been described, which involve precolumn derivatization with either a chromophor (*5, 6*) or fluorophor (*7*).

Fluorescence derivatization often creates a high sensitivity of the assay, and several fluorotags reacting with carboxylic groups have been developed. These include arylbromo- and arylchloromethanes, acylbromomethanes, fluorescent alcohol or amines, and aryldiazomethanes (*7*). These reactions are often performed in aprotic solvents because of the low reactivity of the carboxylic moiety in water. Therefore, tedious extraction procedures are often necessary, but this may be avoided by using phase-transfer techniques or more recently by micellar catalysis (*8*).

1-Pyrenyldiazomethane (PDAM) is a newly synthesized aryldiazomethane which offers several advantages as a fluorescent labeling reagent of carboxylic acids for liquid chromatography. Both PDAM and the reaction product are stable. PDAM readily reacts with monocarboxylic acids at room temperature without a catalyst. The reaction can take place in both protic and aprotic solvents, and the products are intensely fluorescent esters (*9*).

We studied the reaction of PDAM with MMA and some other saturated short-chain dicarboxylic acids ($C < 5$) in aqueous media. Under these conditions, only one carboxylic group of these acids reacts with PDAM, and the other remains underivatized. Stable fluorescent products are formed, and the free carboxyl group results in unique chromatographic properties of the monoesters. The retention of these compounds on reversed-phase and anion-exchange columns is influenced by the ionization of the free carboxyl group and thereby the pH of the mobile phase.

EXPERIMENTAL SECTION

Chemicals. PDAM was purchased from Molecular Probes, Inc. (Eugene, OR). It (2.5 mg/mL) was dissolved in ethyl acetate and stored at −20 °C. This solution was freshly prepared each 14 days. 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, fumaric acid, α-ketoglutatic acid, and dimethylmalonic acid were obtained from Aldrich Chemical Co. (Milwaukee, WI), and succinic acid was obtained from Sigma Chemical Co. (St. Louis, MO). Methanol (HPLC grade), acetonitrile (HPLC grade), and ethyl acetate were from Merck (Darmstadt, FRG). We used double-distilled water which was further purified on a Milli-Q-plus ultra-pure water system (Millipore Corp., Bedford, MA). Packing material for reversed-phase liquid chromatography, Hypersil (3 μm), was from

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Shandon Southern Ltd. (Chesire, U.K.), and the column (150 × 4.6 mm i.d) was packed with this material at 9000 psi using a Shandon column packer.

Instruments. A programmable sample injector, Gilson Model 232-Bio (Gilson Medical Electronics, S.A., Villiers le Bel, France) equipped with a Rheodyne Model 7010 injector valve and a 200-μL sample loop, together with a quarternary solvent delivery system, Series 410 BIO LC-pump from Perkin-Elmer (Norwalk, CT), was used. The column was mounted in a column heater, Model SP 8792 from Spectra Physics. The fluorescence of the column effluent was monitored by a Shimadzu RF-535 fluorescent detector (Kyoto, Japan). The wavelength of the primary light path was routinely adjusted to 340 nm and the fluorescent emission recorded at 376 nm, using an integrator, Model SP 4290 from Spectra Physics (San Jose, CA).

The gas chromatography/mass spectrometry (GC/MS) analysis was carried out on a HP 5890 A gas chromatograph coupled to a HP 5970 mass-selective detector, both from Hewlett Packard (Arondale, PA). The data were treated with Hewlett Packard MS PP 59970 C Chem Station software run on a HP 9133 computer.

The fluorescent excitation and emission spectra were recorded on a Perkin-Elmer LS-5 luminescence spectrometer connected to a Perkin-Elmer Model 7500 professional computer system.

Derivatization of Dicarboxylic Acids. Dicarboxylic acids were dissolved in aqueous solutions buffered to pH from 5.5 to 9 with 5 mM Tris–HCl or to pH from 8.0 to 10.3 with 10 mM sodium borate. To 500 μL of this solution containing 0.1–1000 μM dicarboxylic acid were added 500 μL of acetonitrile, 1000 μL of methanol, and 500 μL of PDAM in ethyl acetate. The solution was mixed and incubated in the dark at 25 °C or 50 °C for the time indicated.

Liquid Chromatography. One gradient system was developed for monitoring the reaction kinetics between MMA or EMA and PDAM (system 1), and another system for the separation of the derivatives of MMA and other dicarboxylic acids (system 2), whereas a set of isocratic conditions was set up to test the chromatographic behavior of 1-pyrenylmethyl methylmalonate monoester and 1-pyrenylmethyl ethylmalonate diester in a reversed-phase system.

System 1. This gradient system was composed of four solutions (A–D). Solution A was 50 mM Tris–maleate buffer, pH 7.4, solution B was 10% methanol in water, solution C is 65% acetonitrile in water, and solution D is 100% methanol. The column was equilibrated with 100% solution B and eluted with 50% A and 50% B from time 0 to 1 min after injection. At this time point, the mobile phase was changed to 50% A, 5% C, and 45% D and eluted with a linear gradient from 1 to 16 min to a final composition which was 30% A, 15% C, and 55% D. The column was then washed with 5% C and 95% D. The flow rate was 1.8 mL/min, and the temperature 60 °C. In this system, the MMA and EMA derivatives showed retention times of 13.5 and 15.5 min, respectively.

System 2. In this system the column was equilibrated with 40% acetonitrile and 5% methanol in 40 mM ammonium formate buffer, pH 2.5, and the mobile phase was changed as a linear gradient over 15 min to 60% acetonitrile and 5% methanol in the formate buffer. The column was washed with 100% methanol. The flow rate was 1.8 mL/min, and the column temperature 35 °C. The retention times of different dicarboxylic acid derivatives in this system are given in Figure 8.

A set of isocratic conditions was configured by varying the acetonitrile concentration (from 22.5 to 70%, in steps of 2.5%) at the expense of water. The pH of the mobile phase was 2.5, 3.0 (ammonium formate), 3.5, 4.0, 4.5 (ammonium acetate), 5.0, 6.0, 7.0, 8.0, and 9.0 (Tris–maleate). The buffer comprised 30% of the mobile phase, and the final concentration was 20 mM. The flow rate was 1.8 mL/min, and the column temperature 25 °C.

Gas Chromatography/Mass Spectrometry. The MMA derivative was analyzed either in the derivatization solution or after purification by HPLC (system 1). The samples were methylated with diazomethane prior to GC/MS analysis. Samples of 5 μL were subjected to analysis on a HP Ultra 2 methylsilicone capillary column (25 m, 0.32 mm i.d., 0.52 μm film thickness). Splitless injection was used, and the injector temperature was 250 °C. The oven was programmed from 60 to 150 °C (60 °C/min) and from 150 to 280 °C (5 °C/min). Helium (2 mL/min) was used as carrier gas.

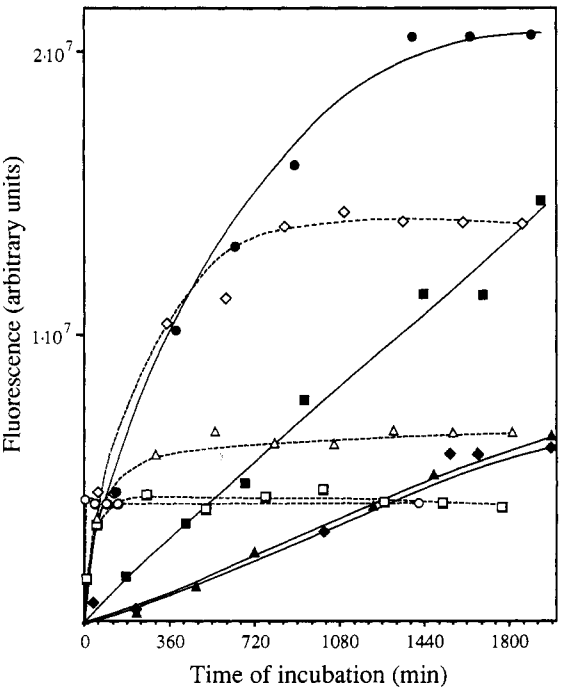

Figure 1. Time course for the formation of the PDAM ester of MMA in two buffer systems at different pH values. MMA (5 μM) was dissolved in 5 mM Tris–HCl buffer pH 5.5 (○), 7.4 (□), 8.5 (△), and 9.0 (◇) or 10 mM borate buffer pH 8.0 (●), 8.5 (■), 9.0 (▲), and 10.3 (◆). The temperature was 25 °C.

RESULTS AND DISCUSSION

General Considerations. Sensitive techniques are required for the detection of the low concentrations of MMA and related short-chain dicarboxylic acids in biological extracts under physiological conditions. Liquid chromatography and fluorescence detection after fluorescent labeling of the carboxylic moiety may represent an alternative to the laborious and complicated methods based on GC/MS (*2–4*). Assays without an extraction procedure seem attractive since such a step may prevent automatization. Micellar phase-transfer catalysis has been used for automated precolumn derivatization of free fatty acids, but the derivatization rate is low for short-chain carboxylic acids (*8*). PDAM was used in our study since it is stable; the derivatization occurs under mild conditions without heating (*9*) and in the presence of water, as previously demonstrated with the related agent, 9-anthryldiazomethane (*10*).

Derivatization Conditions and Reaction Kinetics. MMA and related short-chain dicarboxylic acids are soluble in aqueous solutions. In the present work we investigated the derivatization of MMA with PDAM in a homogeneous medium containing water, acetonitrile, and ethyl acetate. Water and acetonitrile were included since mixing plasma/serum with an equal volume of acetonitrile is a commonly used procedure for extraction and deproteinization prior to liquid chromatography. PDAM was dissolved in ethyl acetate since the reagent is most stable under this condition (*9*). Methanol was added to prevent phase separation. The reagents and reaction products were soluble and stable in a solution consisting of water, acetonitrile, ethyl acetate, and methanol in the proportions 1:1:1:2 (v:v:v:v).

Figure 1 shows the time course for the formation of the MMA derivative at various pH values. At pH 5.5, the reaction proceeded at a high rate but leveled off within about 30 min and the fluorescence yield was low under these conditions. By increasing the pH, the initial reaction rate was decreased

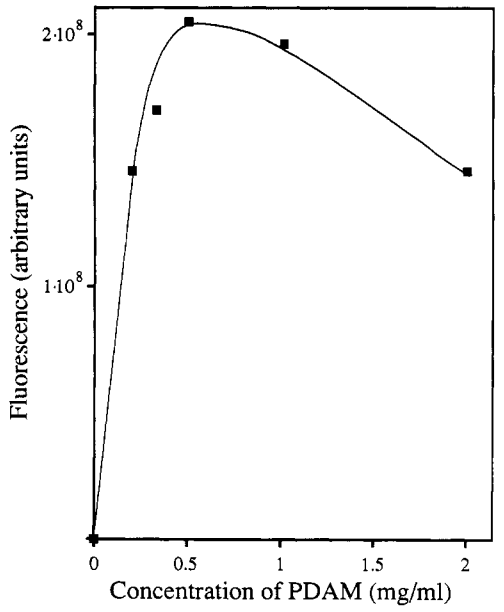

Figure 2. Formation of the PDAM ester of MMA at various concentrations of PDAM. The derivatization was performed at pH 8.0 (10 mM borate buffer), the temperature was 25 °C, and the reaction time 24 h.

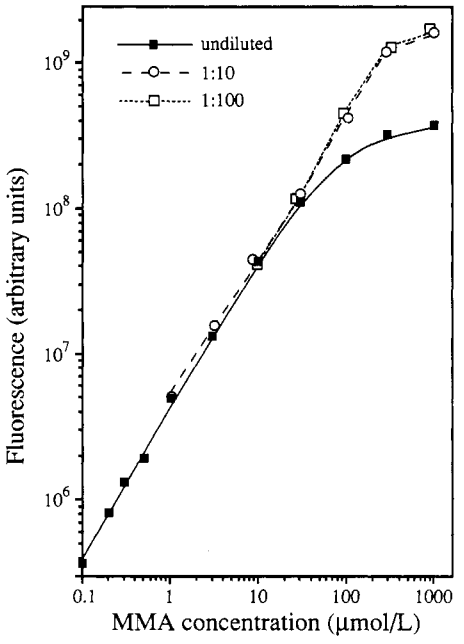

Figure 3. Formation of the PDAM ester of MMA as a function of MMA concentration. MMA, dissolved in 10 mM borate buffer pH 8.0 at a concentration of 0.1–1000 μmol/L, was derivatized with PDAM. The temperature was 25 °C and the reaction time 24 h. The derivatizing solution (20 μL) was injected into the HPLC column, either undiluted (■) or diluted 1:10 (●) or 1:100 (▲) with 50% acetonitrile in water. The column was eluted with the mobile phase of system 1.

but the reaction proceeded for hours, resulting in higher fluorescence yield. At pH 8 (10 mM borate buffer), maximal fluorescence yield was obtained within a reaction time of 24 h (Figure 1).

The fluorescence yield of the MMA derivative increased as a function of the concentration of PDAM, and a final concentration of 0.5 mg/mL gave maximal fluorescence (Figure 2).

The fluorescence increased in proportion to the concentration (0.1–300 μM) of MMA (Figure 3) or EMA (not shown), and a linear relationship was obtained. Above 300 μM, the curve leveled off. The coefficients of variation of repeated

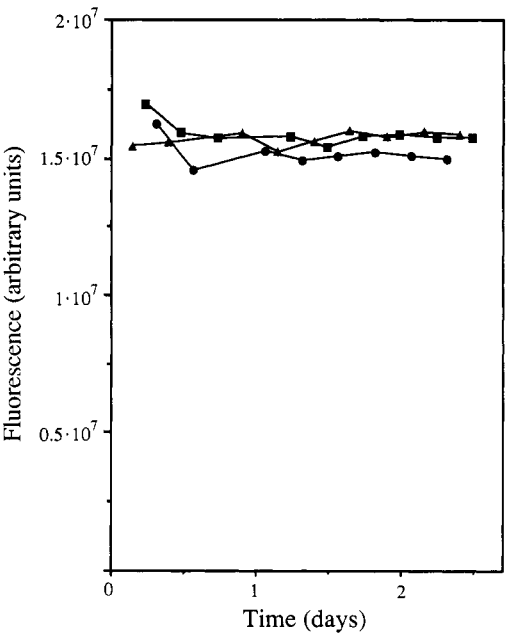

Figure 4. Stability of the PDAM ester of MMA in aqueous solution at various pH values. The MMA derivative was isolated by HPLC (system 1) and diluted in 25 mM buffer containing 40% acetonitrile. The pH was adjusted to 3.86 (●) (ammonium formate), 5.57 (▲) (ammonium acetate), and 8.98 (■) (Tris–maleate). The temperature was 25 °C, and the tubes were incubated in the dark.

(n = 13) analysis of 5 μM MMA and EMA were 2.0% and 2.8%, respectively.

We determined the fluorescent yield and reaction kinetics for the formation of the derivatives of MMA and EMA under various conditions, including different pH values (5.5–10.3) and temperatures (25 and 50 °C). The ratio between the amount of these derivatives remained constant under all conditions tested and equaled the ratio between the amount of MMA and EMA added to the reaction mixture (data not shown). These observations demonstrate equal reactivity of MMA and EMA toward PDAM but also indicate the feasibility of EMA as an internal standard in assays for MMA, based on derivatization with PDAM.

Stability and Fluorescent Properties. The PDAM ester of MMA was isolated by HPLC (system 1) and dissolved in buffers containing 40% acetonitrile. The stability was tested at pH 3.9, 5.6, and 9.9 at 25 °C. The derivative was stable under these conditions for at least 72 h (Figure 4). The same results were obtained with the EMA ester (data not shown).

The fluorescent excitation and emission spectra were recorded for purified PDAM esters of MMA and EMA dissolved in various buffers (ammonium formate, ammonium acetate, sodium acetate, Tris–HCl, and Tris–maleate) containing 20% acetonitrile and adjusted to different pH values (2.9–9.3). The fluorescence spectra were identical for the derivatives of MMA and EMA and were independent of buffer and pH.

The results for the PDAM ester of MMA are shown in Figure 5. The excitation spectrum showed three maxima (272, 324, and 340 nm) with the highest fluorescent yield at 340 nm. Maximal emission was observed at 376 nm, but a distinct peak was also observed at 395 nm. These spectra show similarities with those reported for 1-pyrenylmethyl palmitate by Nimura et al. (*9*).

Identification of the MMA Derivative. One or both carboxylic acid residues of MMA and other dicarboxylic acids may react with PDAM. We observed only a single peak after derivatization of MMA, EMA, malonic acid, succinic acid, α-ketoglutaric acid, and dimethylmalonic acid by reversed-phase liquid chromatography in different systems (1 and 2). The hypothetical products with two pyrenylmethyl residues

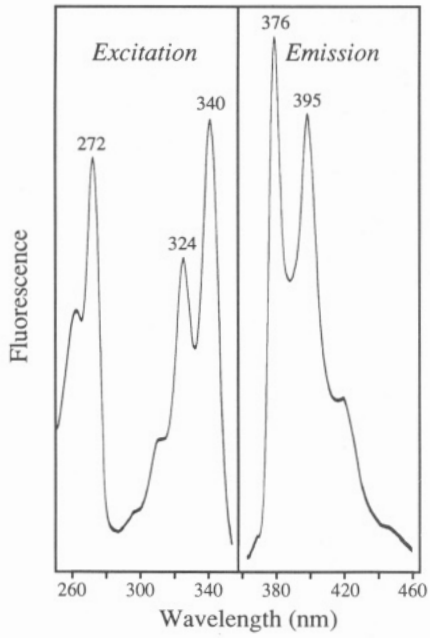

Figure 5. Excitation and emission spectra of the PDAM monoester of MMA. The MMA derivative was isolated by HPLC (system 1), and diluted in 25 mM ammonium formate buffer, pH 3.5 containing 20% acetonitrile.

would be more hydrophobic than those with one pyrenylmethyl residue and a free carboxylic moiety. We therefore eluted the column with the mobile phase with a high concentration of organic solvent (up to 100% acetonitrile), but a second peak could not be detected for these carboxylic acids (data not shown). This indicates that derivatization of MMA and other short-chain dicarboxylic acids with PDAM results in a single molecular species.

All samples subjected to GC/MS analysis were treated with diazomethane prior to injection. When the derivatization solution containing PDAM without MMA was subjected to gas chromatography, two main reagent peaks (retention times of 24 and 26 min) were observed. The sample containing *both* PDAM and MMA showed an additional peak with a retention time of 33.1 min. Further peaks were not detected within a total run time of 50 min (data not shown). These findings support the conclusion from the liquid chromatographic analysis, that the reaction of MMA with PDAM forms a single derivative.

The GC/MS analysis of the MMA peak (retention time of 33.1 min) showed a molecular ion at m/z 346 and a fragmented ion at m/z 215 (Figure 6). The latter fragment may result from cleavage of the ester bound between the pyrenylmethyl group and the methylmalonic acid methyl monoester.

MMA was derivatized with PDAM, and the derivative was isolated by reversed-phase liquid chromatography (system 1) and subjected to GC/MS analysis. A single peak with a retention time of 33.1 min was obtained, and the mass spectrum was essentially identical to that depicted in Figure 6, with an ion at m/z 346 and a fragment ion at m/z 215 (data not shown).

These data (M^+ ion = m/z 346 of the PDAM derivative of MMA after methylation of the free carboxylic acid with diazomethane) suggest that only one carboxyl moiety of MMA reacts with PDAM, giving 1-pyrenylmethyl methylmalonate monoester. The structure of this compound after methylation of the free carboxylic acid is shown in Figure 6.

Derivatization of only one carboxylic group with PDAM may be explained by steric hindrance imposed by the bulky 1-pyrenylmethyl residue and the short distance between the carboxylate moieties. This results in unique PDAM derivatives of MMA and other short-chain dicarboxylic acids, which

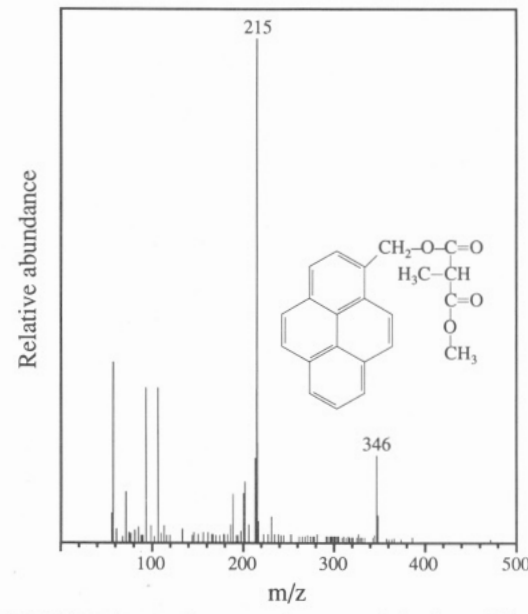

Figure 6. Mass fragmentogram and proposed structure of the PDAM ester of MMA. MMA (1 mM) was incubated with PDAM in ethyl acetate for 24 h at 25 °C. The sample was then treated with diazomethane to methylate free carboxylic acid residues and subjected to gas chromatography, as described in the Experimental Section. The figure shows the mass spectrum of the peak with a retention time of 33.1 min. The proposed chemical structure of the PDAM ester of MMA is shown.

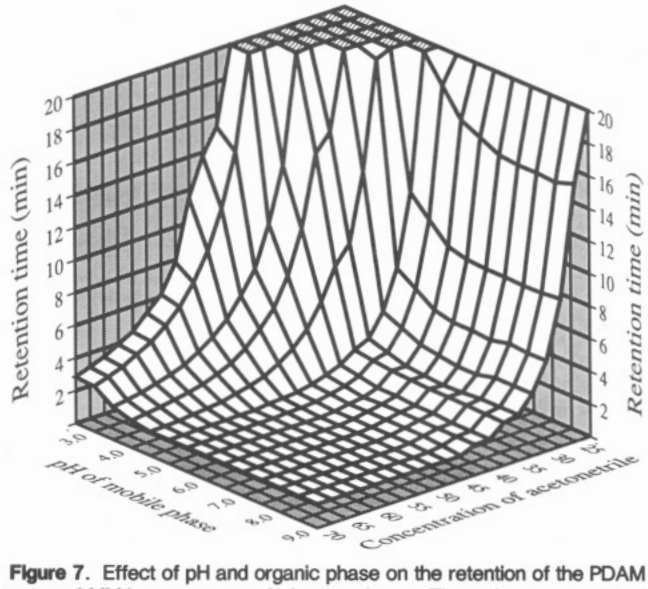

Figure 7. Effect of pH and organic phase on the retention of the PDAM ester of MMA on a reversed-phase column. The column was eluted isocratically at a flow rate of 1.8 mL/min. The temperature was 25 °C.

carry one free carboxylate group. The ionization of this group may affect the chromatographic properties of these derivatives.

Chromatographic Behavior. We investigated the effect of pH of the mobile phase on the retention of the PDAM esters of MMA and EMA on a reversed-phase column eluted isocratically with different concentrations of acetonitrile. Figure 7 shows the data for the MMA derivative and demonstrates that the capacity factor for this compound increased markedly when the pH was lowered. Drastic changes occurred between pH 3.5 and 4.5. At alkaline pH, this compound was poorly retained (Figure 7). Similar data were obtained with the PDAM ester of EMA, except that this monoester showed somewhat higher retention times. An increase in the capacity factor in a reversed-phase system by lowering the pH would be expected for a free carboxylic acid. The pronounced

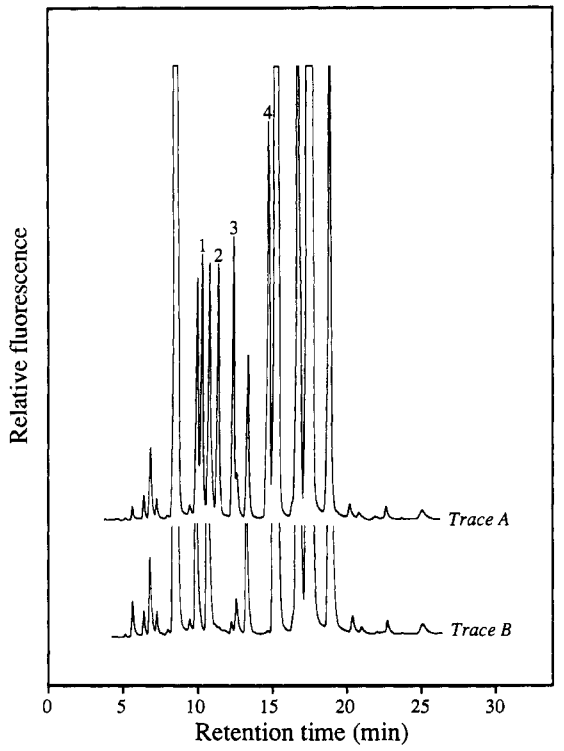

Figure 8. Separation of the PDAM ester of MMA from the derivatives of other dicarboxylic acids by reversed-phase liquid chromatography. The dicarboxylic acids (10 μM of each) were derivatized with PDAM, and the derivatization mixture was subjected to analysis on a reversed-phase column eluted with an acetonitrile/methanol gradient in formate buffer, pH 2.5 (system 2). Trace A shows the separation of MMA from the other dicarboxylic acids and the reagent peaks. Trace B shows the profile of the derivatizing mixture where the dicarboxylic acids have been omitted. Key: (1) malonic acid; (2) succinic acid and α-ketoglutaric acid; (3) MMA; (4) EMA and dimethylmalonic acid.

changes in the region pH 3.5–4.5 (Figure 7) may reflect the pK_a of the MMA derivative.

PDAM esters of malonic acid, succinic acid, α-ketoglutaric acid, MMA, EMA, and dimethylmalonic acid were eluted with relatively low concentrations of organic phase in a mobile phase at an alkaline pH. Under these conditions MMA was poorly separated from succinic acid and eluted ahead of the major reagent peaks. At low pH, elution of these acids required higher concentration of organic solvent. The dicarboxylic acids appeared now after the major reagent peak and were better resolved. Figure 8 demonstrates such a gradient system (system 2) with a mobile phase adjusted to pH 2.5. This system separates the esters of MMA (12.2 min) from the esters of malonic acid (retention time of 10.1 min), succinic acid/α-ketoglutaric acid (11.2 min), and EMA/dimethylmalonic acid (14.5 min).

Conclusion. MMA and some other short-chain dicarboxylic acids react with PDAM in an aqueous medium to form stable fluorescent monoesters with one free carboxylic residue. The PDAM esters are formed in high yield at alkaline pH. The pH of the mobile phase affects the ionization of the carboxylic group and thereby the chromatographic behavior of these derivatives on reversed-phase columns as well as on anion-exchange columns (unpublished). These findings suggest the possible construction of automated liquid chromatographic techniques for the determination of MMA and related compounds in aqueous matrices containing physiological media like plasma and urine.

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