1-Pyrenyldiazomethane as a Fluorescent Labeling Reagent for Liquid Chromatographic Determination of Carboxylic Acids

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1-Pyrenyldiazomethane (PDAM), a new fluorescent labeling reagent, was developed for liquid chromatographic determination of carboxylic acid. PDAM was more stable than the other previous diazoalkane type reagents. The reagent readily reacted with carboxylic acids to give stable and strongly fluorescent 1-pyrenylmethyl ester derivatives without a catalyst and heating. PDAM derivatives of short- and long-chain fatty acids and prostaglandins were satisfactorily separated on a reversed-phase column and were sensitively detected fluorometrically at 395 nm with excitation at 340 nm. Detection limits of PDAM derivatives of both fatty acids and prostaglandins were 20-30 fmol.

Many kinds of precolumn fluorescent derivatization reagents (*1-14*) have been developed for the determination of carboxylic acids such as fatty acids and prostaglandins by high-performance liquid chromatography (HPLC). Halogenomethyl (*1-5*) or halogenoacyl (*6, 7*) aromatic compounds such as 4-bromomethyl-7-methoxycoumarin (*1, 2*) and 1-bromoacetylpyrene (*6*) have extensively been examined. The reactions of these reagents with carboxylic acids, however, proceeded only at elevated temperature in the presence of base catalysts. Aryldiazoalkanes (*8-10*) have therefore been developed because they react readily with carboxylic acids at room temperature without catalysts. These are generally unstable against heating and light and cannot therefore be well-purified by recrystallization. 9-Anthryldiazomethane (ADAM), previously developed in our laboratory (*9*), has been widely used (*15-24*) for HPLC of various biologically important carboxylic acids. However, ADAM was unstable in solution and could not be stored for long, even in the solid state. The reagent solution must be prepared freshly before use.

In the present study, a more stable aryldiazoalkane, 1-pyrenyldiazomethane (PDAM), was prepared as a new fluorescent labeling agent for carboxylic acids. PDAM readily reacted with carboxylic acids at room temperature without a catalyst to give an intensely fluorescent ester.

EXPERIMENTAL SECTION

Materials. 1-Pyrenecarboxaldehyde was purchased from Aldrich Chemical Co. (Milwaukee, WI). Hydrazine monohydrate, fatty acids, and other reagents were of special grade (Wako Pure Chemical Industries, Tokyo). Solvents for chromatography were obtained from Wako, and other solvents were purchased from Kokusan Kagaku Co. (Tokyo). Water was purified with a Milli-Q water purification unit (Millipore Corp., Bedford, MA). Sep-PAK C-18 extraction cartridges were purchased from Waters Assoc. (Milford, MA).

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Preparation of PDAM. A 5-g sample of 1-pyrenecarboxaldehyde was suspended in 80 mL of ethanol, to which was added 3.4 g of hydrazine monohydrate, and the mixture was stirred at room temperature for 3 h. During the reaction, the color of the reaction mixture changed from yellow to dark yellow. The solid product was then filtered off on a G-3 glass filter and was washed with 50 mL of cold ethanol. Recrystallization from ethanol gave yellow crystals of 1-pyrenecarboxaldehyde hydrazone. (Yield: 5.0 g, 94%; mp 186-194 °C (dec). Anal. Calcd for $C_{17}H_{12}N_2$: C, 83.58; H, 4.95, N, 11.47. Found: C, 83.66; H, 4.91; N, 11.43.)

To 2 g of the hydrazone suspended in 300 mL of diethyl ether, 6.55 g of activated manganese dioxide (*25*) was added, and the resulting mixture was allowed to react in a water bath of a Branson Model B-521 ultrasonic cleaner at room temperature. The residual hydrazone in the reaction mixture was monitored by HPLC during the oxidation. After about 80 min, the hydrazone completely disappeared as the chromatographic peak. The manganese dioxide was then filtered off on a G-3 glass filter to terminate the reaction and was washed with a small amount of ether on the filter. The obtained reddish brown solution was evaporated to dryness to give red crystalline PDAM (yield, 1.7 g, 85%). The PDAM was usable without recrystallization.

Derivatization of Fatty Acids. To 100 μL of 0.01-10 μg/mL fatty acid solution in methanol was added 100 μL of 1 mg/mL solution of PDAM in ethyl acetate. The resulting mixture was allowed to stand for 90 min at room temperature, and 5 μL of the mixture was injected into the column.

Preparation of PDAM Derivative of Palmitic Acid. To 5 mL of a methanolic solution containing 10 mg of palmitic acid was added 5 mL of a 0.4% (w/v) solution of PDAM in ethyl acetate, and the resulting mixture was allowed to stand for 90 min at room temperature. The mixture was then applied on a Sep-PAK C-18, which was previously washed with 20 mL of the mixture of methanol and water (7:3). After the Sep-PAK was washed with 10 mL of the methanol/water mixture, the product was eluted with 10 mL of methanol and the effluent was evaporated to give the ester. The product showed a single peak on a chromatogram under conditions for the separation of PDAM derivatives of long-chain fatty acids, as will be described later. The structure of the palmitate ester was confirmed by mass spectrometry using a mass analyzer Model QP-1000 (Shimadzu Seisakusyo, Kyoto, Japan).

Excitation and fluorescence spectra of the pure palmitate ester were recorded with a Shimadzu Model RF-500 spectrofluorophotometer (Shimadsu Seisakusyo).

Examination of the Stability of PDAM. The stability of PDAM was examined both in the solid state and as the 0.1% (w/v) solution in ethyl acetate at room temperature, 5 °C, and -20 °C. The PDAM in each sample was determined as follows. To 200 μL of 5 mg/mL palmitic acid solution in methanol was added 200 μL of the PDAM solution. The resulting mixture was allowed to stand for 90 min at room temperature, and a 5-μL aliquot of the mixture was submitted to HPLC as described in Derivatization of Fatty Acids.

Chromatographic Procedure. The HPLC system consisted of a Model Trirotar VI HPLC (Japan Spectroscopic Co., Ltd., Tokyo) with a Model WISP 710B automatic sampler (Waters) and a Model F-1000 spectrofluoromonitor (Hitachi Co., Ltd., Tokyo) or a Model UVIDEC-100-VI ultraviolet spectrophotometer

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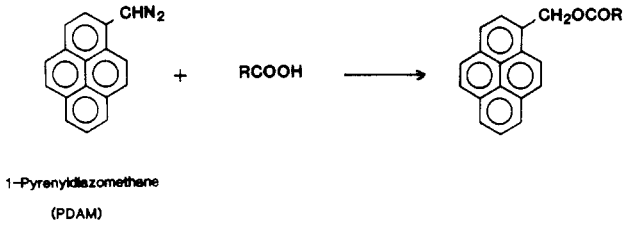

Figure 1. Reaction path of PDAM with carboxylic acid.

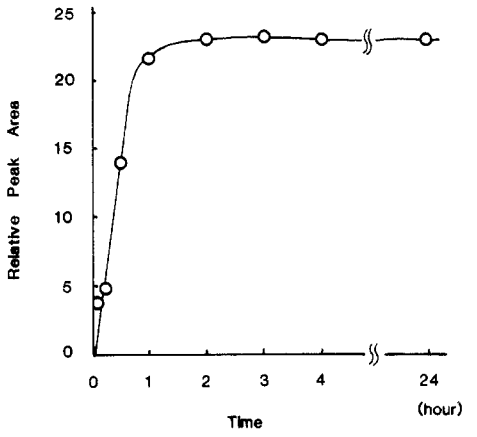

Figure 2. Yield of PDAM derivative of palmitic acid as a function of reaction time at room temperature.

(Japan Spectroscopic). The column was stainless steel (150 × 4-mm i.d.) packed with a octadecylsilyl silica gel, TSK-GEL-120A (particle size 5 μm, TOSOH Co., Ltd., Tokyo). Chromatographic conditions are described in the figure legends.

RESULTS AND DISCUSSION

The stability of aromatic diazoalkanes generally depends mainly on the aromaticity of the aromatic ring and electron-withdrawing activity of the ring substituents. Accordingly, a more stable diazoalkane reagent may be developed by converting the anthryl moiety of ADAM into a more aromatic one. However, the stability of the agent decreases its reactivity, and its reaction with carboxylic acids consequently requires heating. Taking into account these conflicting properties of diazoalkanes, we designed a new reagent having a pyrene ring, which seemed to have moderate aromaticity.

1-Pyrenyldiazomethane (PDAM) was synthesized by oxidation of pyrenecarboxaldehyde hydrazone obtained by condensation of pyrenecarboxaldehyde with hydrazine hydrate. Activated manganese dioxide was found to be a suitable oxidant to give PDAM in excellent yield. The oxidation was complete in 80 min at room temperature. The obtained crystalline PDAM could be used without recrystallization.

Figure 1 shows the schema of the reaction of PDAM with a carboxylic acid. PDAM was found to react readily with carboxylic acids at room temperature without catalysts in various protic and aprotic solvents such as methanol, ethyl acetate, diethyl ether, hexane, and so on. Among the solvents tested, ethyl acetate was found to be the best solvent not only for the esterification but for the storage of the reagent.

Figure 2 shows the fluorescence intensity of the reaction product of palmitic acid with PDAM periodically monitored by HPLC. The yield of the palmitate reached its maximum in 90 min while that of the palmitate ester of ADAM reached its maximum in 60 min (*9*). The difference in the reaction time may reflect the stability of PDAM. The palmitate ester was isolated, and its structure was confirmed by mass spectrometry as illustrated in Figure 3. The molecular ion peak of the pyrenylmethyl palmitate appeared at m/z 470. Furthermore, the fragment ions formed by ester cleavage also appeared at m/z 215 and 256. These results suggested that

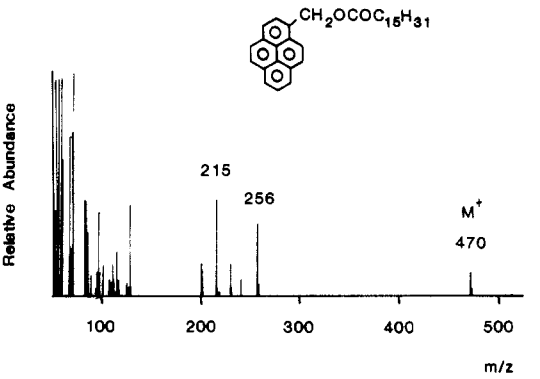

Figure 3. Mass spectra of the reaction product of palmitic acid with PDAM. The structural formula indicates the proposed structure of palmitic acid 1-pyrenylmethyl ester.

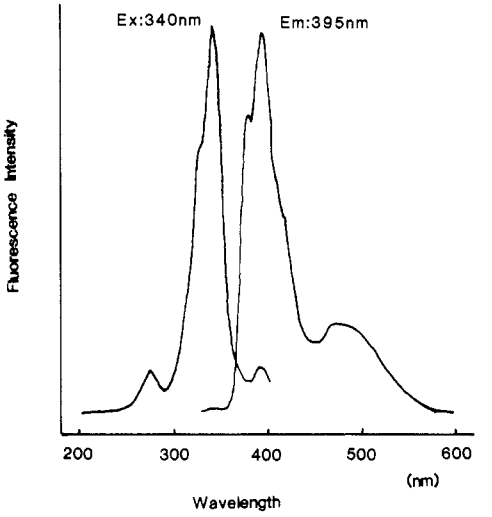

Figure 4. Fluorescence spectra of 1-pyrenylmethyl palmitate in acetonitrile–water (9:1).

the product from the reaction of PDAM with palmitic acid was palmitic acid 1-pyrenylmethyl ester, whose structural formula is illustrated in Figure 3.

Figure 4 shows the excitation and fluorescence spectra of the purified PDAM ester of palmitic acid in acetonitrile–water (9:1). The excitation maxima were at 270 and 340 nm, and the emission maximum at 395 nm. For the detection of PDAM esters in HPLC was employed the excitation wavelengths at 340 nm, which gives greater fluorescence intensity. The PDAM esters also can be spectrophotometrically detected by their absorption maxima near 241 or 340 nm. The fluorescence intensity of the ester derived from PDAM was approximately 5 times stronger than that from ADAM.

The stability of PDAM was examined by periodic monitoring of the ester formed by its reaction with palmitic acid because PDAM itself was difficult to quantitate directly. The residual PDAM stored in the solid state and in solutions of various solvents at various temperatures was measured. Figure 5 shows the stability of PDAM stored under three different conditions. The amount of PDAM was represented by the peak area of its palmitic acid ester. At room temperature, PDAM was stable for 20 days in the solid state and 48 h in solution, whereas 25% of ADAM decomposed in 24 h in ethyl acetate solution. The stability increased at lower temperatures. Solid PDAM proved stable for 5 years in a freezer at −20 °C. The reactivity of the reagent solution of PDAM was also preserved at −20 °C for at least 1 week after its preparation.

Figure 6A shows a chromatogram of PDAM esters of lactic acid, α-hydroxy acid, and short-chain fatty acids eluted with

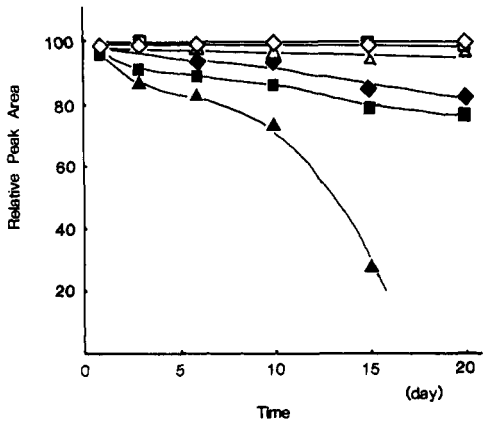

Figure 5. Stability of PDAM under various conditions: in the solid state at −20 °C (◇), 5 °C (□), and room temperature (△); in a solution of ethyl acetate at −20 °C (◆), 5 °C (■), and room temperature (▲).

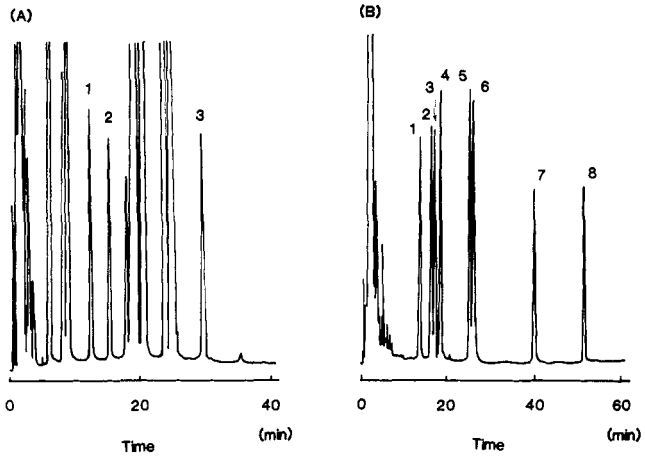

Figure 6. Chromatograms of PDAM derivatives of short-chain fatty acids (A) and long-chain fatty acids (B) with fluorometric detection. (A): peaks, (1) lactic acid, (2) formic acid, (3) propionic acid; mobile phase, acetonitrile:water (1:1); flow rate, 1.0 mL/min. Each peak corresponds to about 5 pmol of fatty acid. (B): peaks, (1) $C_{18:3}$, (2) $C_{16:1}$, (3) $C_{14:0}$, (4) $C_{16:2}$, (5) $C_{16:0}$, (6) $C_{18:1}$, (7) $C_{18:0}$, (8) $C_{20:0}$; mobile phase, gradient elution of water with acetonitrile; acetonitrile concentration, 0–30 min, 85%, 30–60 min, 85%–100%; flow rate, 1.0 mL/min. Each peak corresponds to about 5 pmol of fatty acid.

acetonitrile–water (1:1) as a mobile phase. Interfering peaks from the reagent were observed between PDAM derivaties of formic and propionic acid. These peaks overlapped with those of the PDAM derivative of acetic acid, which accordingly could not be determined under this condition. However, such contaminants did not interfere with the sensitive detection of the polar carboxylic acids, except for acetic acid, at the subpicomole level. Figure 6B shows a chromatogram of PDAM esters of saturated and unsaturated long-chain fatty acids separated by gradient elution. Excess reagent and its degradation products did not interfere with the determination, because these were eluted before the PDAM derivatives under this condition.

Figure 7 demonstrates the sensitive detection of the long-chain fatty acids. Each 100 fmol of the acids was clearly separated and detected, as shown on the chromatogram. The sample solution (100 μL) containing 4 pmol of each of the fatty acids was used for the analysis. This chromatogram implies that the detection limits of PDAM derivatives of fatty acids are 15–30 fmol (S/N = 3). The esters of unsaturated fatty acids were stable as well as those of saturated fatty acids. No remarkable change in the peak heights was observed until 24 h after the initiation of the reaction.

Figure 8 displays the standard curves for several fatty acids. Excellent linearities with the correlation coefficient over 0.999,

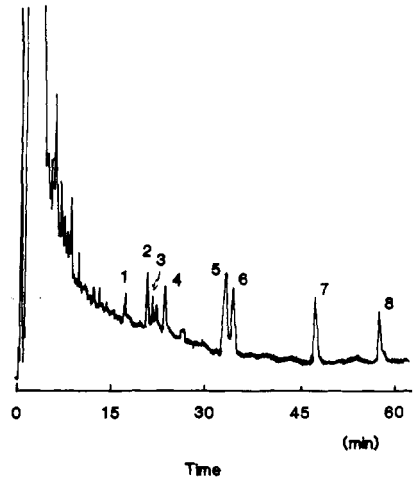

Figure 7. Chromatogram of PDAM derivatives of long-chain fatty acids with fluorometric sensitive detection. Identification of peaks and chromatographic conditions are same as in Figure 6B. Each peak corresponds to about 100 fmol of fatty acid.

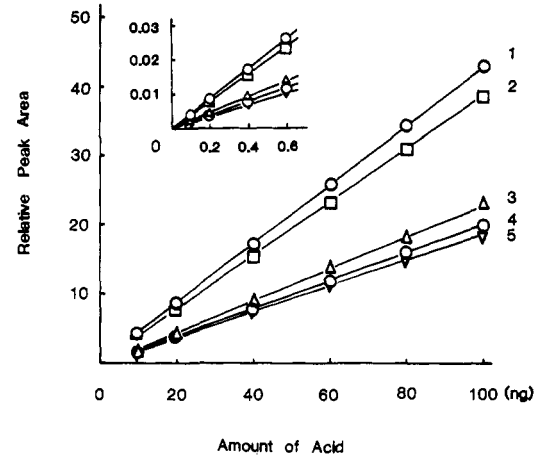

Figure 8. Standard curves for PDAM derivatives of various fatty acids: (1) $C_{18:1}$, (2) $C_{18:2}$, (3) $C_{14:0}$, (4) $C_{16:0}$, (5) $C_{18:0}$.

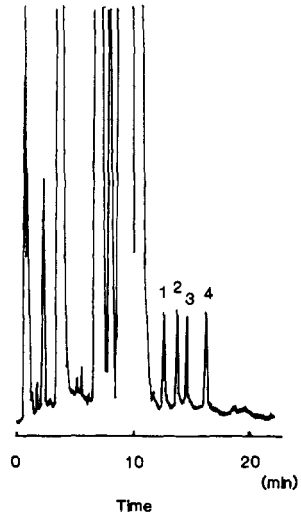

Figure 9. Chromatogram of PDAM derivatives of prostaglandins with fluorometric detection: peaks, (1) PG E_2, (2) PG $F_2\alpha$, (3) PG E_1, (4) PG $F_1\alpha$; mobile phase, acetonitrile–water (75:25); flow rate, 1.0 mL/min. Each peak corresponds to 100 fmol of prostaglandin.

ranging from 0.1 to 100 ng, were observed. The coefficient of variation for palmitic acid of 1 ng per injection (n = 7) was 1.1%. The later-eluted fatty acid derivatives showed lower fluorescence intensity compared with that of faster-eluted ones. This fact may be due to the difference in the polarity

of the mobile phase used in the gradient elution. Fluorescence intensity of PDAM derivatives was found to decrease with the increase in the content of organic solvents such as acetonitrile or methanol in the solution.

Figure 9 shows the chromatogram of each 100 fmol of prostaglandins (PGs) E_1, E_2, $F_1\alpha$, and $F_2\alpha$. All the PGs tested were excellently separated and sensitively detected. The detection limits for the PGs were about 15–20 fmol (S/N = 3). Since the degradation products of PDAM may interfere with the determination of hydrophilic PGs such as 6-keto $PGF_1\alpha$, they were removed by the pretreatment described previously (*16*, *21*).

The above results suggests that PDAM is suitable for the liquid chromatographic determination of biologically important carboxylic acids.

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Quantitative Trace Element Analysis of Microdroplet Residues by Secondary Ion Mass Spectrometry

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This paper reports the results of secondary ion mass spectrometry (SIMS) analyses of the elemental components contained in microvolume liquid residues deposited onto high-purity graphite substrates. These residues were formed by evaporating the solvent in 25-nL volumes of standard solutions containing the analyte element and a known mass of a yttrium internal standard. The capability of the SIMS technique to quantitatively measure the mass of the analyte was determined from these standard samples. The relative ion yields of Al, Ca, Mn, Fe, Co, Cu, Zn, Se, and Pb with respect to the Y internal standard were determined. The minimum detectable quantities (MDQ) of these elements were measured along with the precision of the SIMS analysis. Gram detectivities for this set of elements dissolved in the 25-nL volumes ranged between 85 pg and 2.0 fg, corresponding to molar detectivities ranging between 16 μM and 1.6 nM. Stable isotope dilution analysis of samples containing enriched ^{206}Pb demonstrated quantitative measurement accuracy within 3% of the true values for samples containing 4.0 mM Pb. SIMS analyses of the NBS bovine serum reference standard indicate that this technique can provide useful quantitative analysis of selected elements contained in a microvolume of biological fluids.

Contemporary inorganic elemental analysis of fluid samples can be subdivided into two volumetric regimes: microliter (μL) or larger aliquots analyzed by techniques such as inductively coupled plasma (ICP) atomic emission spectroscopy (AES) or mass spectrometry (MS) and picoliter (pL) volume samples analyzed by electron probe microanalysis (EPMA). Although the ICP-AES or MS techniques are capable of providing sub-part-per-million detection limits, these detection limits are necessarily degraded when the amount of available sample is less than approximately 1 mL (*1*). Part-per-million (ppm) detection limits are achievable with the electron probe technique at sample volumes in the 100-pL range, and these detection limits are not reduced as the sample volume increases (*2*). Thus, the need exists for a trace element analysis technique having sub-ppm detection limits for sample volumes in the 100 pL to 100 nL range. The development of a technique that could perform trace element analyses on these sample volumes would find extensive applications in such areas as microbiology, medical research, forensic science, and industrial quality control.

In the past 10 years, secondary ion mass spectrometry (SIMS) as performed on the Cameca IMS-3f or 4f ion microanalysers has emerged as a viable analytical tool for the quantitative trace element analysis of solid samples (*3*). The applications of this technique have been primarily in semiconductor (*4*) and metallurgical (*5*) characterizations, although a number of biomedical researchers have applied the SIMS technique to the microanalysis of a wide range of biological materials (*6*, *7*). The technique employs sputtering of the surface atoms from relatively small areas (typically, 250 μm

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