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Precolumn fluorescence tagging reagent for carboxylic acids in high-performance liquid chromatography: 4-substituted-7-aminoalkylamino-2,1,3-benzoxadiazoles

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ABSTRACT

Four new 2,1,3-benzoxadiazole amine reagents having different functional groups at the 4- and 7-positions, [4-nitro-7-N-piperazino-2,1,3-benzoxadiazole (NBD-PZ), 4-(N,N-dimethylaminosulphonyl)-7-N-piperazino-2,1,3-benzoxadiazole (DBD-PZ), 4-(N,N-dimethylaminosulphonyl)-7-N-cadaverino-2,1,3-benzoxadiazole (DBD-CD) and ammonium 7-N-piperazino-2,1,3-benzoxadiazole-4-sulphonate (SBD-PZ)] were synthesized as fluorogenic tagging reagents for carboxylic acids in high-performance liquid chromatography. The reagents, except SBD-PZ, reacted with carboxylic acid at room temperature in the presence of activation agents to produce fluorescent adducts. The maximum wavelengths of arachidic acid tagged with DBD-PZ, DBD-CD and NBD-PZ were 569 nm (excitation, 440 nm), 561 nm (excitation, 437 nm) and 541 nm (excitation, 470 nm), respectively. Among various activation agents tested [diethyl phosphorocyanidate (DEPC), diphenyl phosphoroyl azide (DPPA), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)–pyridine, 2,2'-dipyridyl disulphide–triphenylphosphine (Mukaiyama A) and 2-chloro-1-methylpyridinium iodide–triethylamine (Mukaiyama B)], DEPC and Mukaiyama A were more effective than the others. When the piperazino reagents (DBD-PZ and NBD-PZ) were used as the tagging reagents, the derivatization reaction in the presence of Mukaiyama A was faster than that in the presence of DEPC. Although the reaction in the presence of Mukaiyama A was completed after 30 min, an unknown peak derived from the activation agent appeared on the chromatograms. The fluorescence peak intensities were compared in the presence of DEPC. The order of the fluorescence peak areas obtained after reaction for 6 h in the presence of DEPC was DBD-PZ > DBD-CD > NBD-PZ. Thirteen saturated free fatty acids (FFAs) derivatized with DBD-PZ (or DBD-CD) and DEPC (or Mukaiyama A) in acetonitrile were separated completely by linear gradient elution on a reversed-phase ODS column. Eight drugs (ibuprofen, indomethacin, dinoprost, prostaglandin E_1, dehydrocholic acid, ursodesoxycholic acid, hydrocartisone succinate and prednisolone succinate) were also tagged with DBD-PZ in the presence of DEPC and separated by isocratic elution. The detection limits (signal-to-noise ratio = 3) of FFAs tagged with DBD-PZ were in the range 3.2–4.7 fmol, whereas those of drugs were in the range 3.9–14 fmol.

INTRODUCTION

The sensitive detection of biologically active compounds with carboxylic acid groups such as fatty acids, prostaglandins and bile acid is difficult absorptiometrically owing to the weak UV and visible absorption. For the trace analysis of these compounds, fluorescence tagging followed by fluorimetric detection of the adducts is one of the plausible methods [1,2]. Various fluorescence tagging re-

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agents have been developed for the determination of carboxylic acids [3–17], but many of these have some disadvantages. Coumarin-type derivatives, such as 4-bromomethyl-7-methoxycoumarin (Br-Mmc) [3–5] and 4-bromomethyl-7-acetoxycoumarin (Br-Mac) [6,7], which are popular as fluorescence tagging reagents for carboxylic acids, are unstable to moisture, and aqueous reaction media should therefore be avoided in the derivatization step. Moreover, the reagents require a base catalyst such as K_2CO_3 for accelerating the reaction. Therefore, the derivatization reaction is usually carried out in aprotic solvents such as acetone at 50–60°C in the presence of a crown ether as a phase-transfer catalyst, which also increases the solubility of the base catalyst K_2CO_3 or $KHCO_3$. This derivatization procedure is complicated and the reaction is sometimes unsuccessful. Diazomethane-type reagents such as 9-anthryldiazomethane (ADAM) [12,13] and 1-pyrenyldiazomethane (PDAM) [14], on the other hand, are unstable in reaction solvents such as acetonitrile and methanol. In contrast, dansyl semipiperazide (DNS-PZ) [15] and monodansyl cadaverine (MDC) [16], aliphatic amine-type reagents, are suitable for carboxylic acid analysis owing to their excellent stability and selectivity toward the carboxylic acid moiety and the sensitivity of the adducts.

In a previous study [18], 4-(aminosulphonyl)-7-N-piperazino-2,1,3-benzoxadiazole (ABD-PZ), 4-(aminosulphonyl)-7-N-cadaverino-2,1,3-benzoxadiazole (ABD-CD) and 4-(aminosulphonyl-7-N-ethylenediamino-2,1,3-benzoxadiazole (ABD-ED) were synthesized and applied as precolumn tagging reagents to fatty acids in high-performance liquid chromatography (HPLC). The adducts of fatty acids with these reagents exhibited longer excitation (λ_{ex} 430–440 nm) and emission maxima (λ_{em} 570–580 nm) than those with other reagents (*e.g.*, Br-Mmc, λ_{ex} 360 nm and λ_{em} 410 nm; Br-Mac λ_{ex} 365 nm and λ_{em} 460 nm; MDC, λ_{ex} 340 nm and λ_{em} 518 nm; DNS-PZ, λ_{ex} 350 nm and λ_{em} 530 nm). The fluorescence characteristics of the adducts might be advantageous for the determination of carboxylic acids, as the other compounds having intrinsic fluorescence would not interfere.

This paper described further syntheses of similar new fluorogenic tagging reagents having a benzofurazan (2,1,3-benzoxadiazole) structure. Their reactivity toward fatty acids in the presence of various activation agents, the fluorescence properties and HPLC separation of the adducts with fatty acids and some drugs containing a COOH moiety in their structure were investigated. The effect of functional groups at the 4- and 7-positions in 2,1,3-benzoxadiazole reagents on the derivatization reaction and fluorescence properties of the adducts are also discussed.

EXPERIMENTAL

Materials and reagents

ABD-PZ and ABD-CD were synthesized as described previously [18]. Ammonium 7-fluoro-2,1,3-benzoxazole-4-sulphonate (SBD-F) [19], 4-(aminosulphonyl)-7-fluoro-2,1,3-benzoxadiazole (ABD-F) [20] and 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) [21] were purchased from Wako (Osaka, Japan). 4-(N,N-Dimethylaminosulphonyl)-7-fluoro-2,1,3-benzoxadiazole (DBD-F) [22,23] was obtained from Tokyo Kasei (Tokyo, Japan). Four saturated free fatty acids [myristic acid ($C_{14:0}$), palmitic acid ($C_{16:0}$), stearic acid ($C_{18:0}$) and arachidic acid ($C_{20:0}$)] were obtained from Wako, lauric acid ($C_{12:0}$), margaric acid ($C_{17:0}$), behenic acid ($C_{22:0}$) and lignoceric acid ($C_{24:0}$) from Tokyo Kasei and tridecanoic acid ($C_{13:0}$), *n*-pentadecanoic acid ($C_{15:0}$), nonadecanoic acid ($C_{19:0}$), heneicosanoic acid ($C_{21:0}$) and tricosanoic acid ($C_{23:0}$) from Nacalai Tesque (Kyoto, Japan). Dinoprost (prostaglandin $F_{2\alpha}$), ibuprofen, indomethacin and ursodesoxycholic acid were donated by Mochida Pharmaceutical (Tokyo, Japan), Kyowa Hakko Kogyo (Tokyo, Japan), Sumitomo Pharmaceuticals (Tokyo, Japan) and Tokyo Tanabe Seiyaku (Tokyo, Japan), respectively. Prostaglandin E_1 (Sigma, St. Louis, MO, USA), cadaverine (Sigma), piperazine (Wako), triphenylphosphine (Wako), dehydrocholic acid (Wako), diethyl phosphorocyanidate (DEPC) (Wako), diphenyl phosphoroyl azide (DPPA) (Tokyo Kasei), 2,2′-dipyridyl disulphide (Tokyo Kasei), 2-chloro-1-methylpyridinium iodide (Tokyo Kasei) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) for peptide synthesis (Nacalai Tesque) were used as received. Hydrocortisone succinate and prednisolone succinate were of biochemical-reagent grade (Wako). Acetonitrile and water were of HPLC

grade (Wako). All other chemicals were of analytical-reagent grade and were used as received.

Apparatus

Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a Varian (Palo Alto, CA, USA) Jemini-300 spectrometer at 300 MHz using tetramethylsilane as internal standard. For describing NMR characteristics, the following abbreviations are used: s = singlet, d = doublet, q = quartet and m = multiplet. For measurement of excitation and emission spectra, a Hitachi (Tokyo, Japan) Model 650-60 fluorescence spectrometer with a 1-cm quartz cell was employed without spectral correction. Melting points were measured with a Yanagimoto (Tokyo, Japan) micro melting point apparatus.

The high-performance liquid chromatograph consisted of two Model LC-9A pumps (Shimadzu, Kyoto, Japan) and an SCL-6B system controller (Shimadzu). All samples were injected using an SIL-6B autoinjector (Shimadzu). The analytical column was 5-μm Inertsil ODS-2 (150 × 4.6 mm I.D.) (GL Sciences, Tokyo, Japan). The column was maintained at 40°C with a Model 655A-52 column oven (Hitachi). A Shimadzu RF-550 fluorescence monitor equipped with a 12-μl flow cell was employed for the detection of the eluate from the column. The peak areas obtained from the fluorescence monitor were calculated with a C-R4A chromatopac (Shimadzu). All mobile phases were degassed with an on-line degasser (DGU-3A, Shimadzu). The flow-rate of the eluent was 1.0 ml/min.

Syntheses of DBD-PZ, DBD-CD, NBD-PZ and SBD-PZ

DBD-F (123 mg, 0.5 mmol) in 20 ml of acetonitrile was added dropwise to a stirred solution of piperazine (129 mg, 1.5 mmol) or cadaverine (153 mg, 1.5 mmol) in 20 ml of acetonitrile at room temperature (20–30°C). After stirring for 30 min at room temperature, acetonitrile in the reaction mixture was evaporated under reduced pressure. The residue was dissolved in 5% hydrochloric acid (50 ml) and extracted with 3 × 50 ml of ethyl acetate. The ethyl acetate solution was discarded and the pH of the aqueous solution including DBD-amine derivative (DBD-PZ or DBD-CD) was adjusted to 13–14 with 5% NaOH solution. Then 50 ml of ethyl acetate were added to the alkaline solution to extract DBD-amine derivative. The same extraction procedure was repeated five times. The combined ethyl acetate extracts were washed with 20 ml of water, dried over anhydrous sodium sulphate and evaporated *in vacuo*.

DBD-PZ: orange crystals; m.p. 121–122°C; yield 60%; NMR (ppm) in C^2HCl_3, 7.89 (1H, d, *J*ab = 8.15 Hz, a), 6.33 (1H, d, *J*ab = 8.15 Hz, b), 3.89 (4H, m, c), 3.15 (4H, m, d), 2.89 (6H, s, e); Analysis, calculated for $C_{12}H_{17}N_5O_3S$, C 46.29, H 5.50, N 22.49; found, C 46.34, H 5.49, N 22,17%.

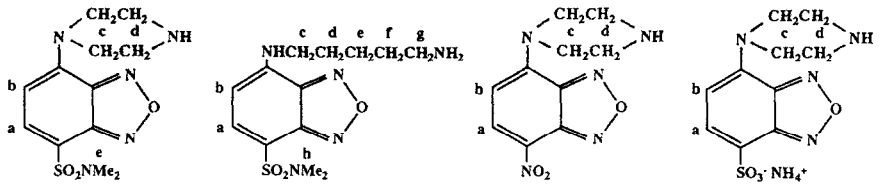
DBD-CD: orange crystals; m.p. 100–101°C; yield 30%; NMR (ppm) in C^2HCl_3, 7.90 (1H, d, *J*ab = 8.01, a), 6.12 (1H, d, *J*ab = 8.01, b), 3.41 (2H, q, c), 2.87 (6H, s, h), 2.79 (2H, m, g), 1.81 (2H, m, d), 1.56 (4H, m, e + f); analysis, calculated for $C_{13}N_{21}N_5O_3S$, C 47.69, H 6.46, N 21.39; found, C 47.24, H 6.39, N 21.51%

NBD-F (92 mg, 0.5 mmol) in 20 ml of acetonitrile and piperazine (129 mg, 1.5 mmol) in 20 ml of acetonitrile were reacted and treated as in the same manner for DBD-F and piperazine.

NBD-PZ: dark red crystals; m.p. 230–231°C; yield 62% NMR (ppm) in [$^2H_6$] dimethyl sulphoxide ([$^2H_6$]DMSO) 8.46 (1H, d, *J*ab = 9.23Hz, a), 6.65 (1H, d, *J*ab = 9.23Hz, b), 4.09 (4H, m, c), 2.93 (4H, m, d); analysis, calculated for $C_{10}H_{11}N_5O_3$, C 48.19, H 4.45, N 28.10; found, C 48.13, H 4.42, N 27.84%.

SBD-F (118 mg, 0.5 mmol) in 40 ml of ethanol and piperazine (215 mg, 2.5 mmol) in 20 ml of ethanol were reacted for 24 h at room temperature. Ethanol in the reaction mixture was evaporated under reduced pressure. The residue was dissolved in 20 ml of water and some impurities were extracted with 50 ml of ethyl acetate. The same extraction procedure was repeated twice. The ethyl acetate extract was discarded and the aqueous solution was evaporated *in vacuo* with heating at 50–60°C. The residue including crude SBD-PZ was chromatographed on a Bio-Gel P-2 (200–400 mesh) column (60 cm × 1.5 cm I.D.) (Bio-Rad Labs, Richmond, CA, USA) with water as eluent. The fluorescent fractions corresponding to SBD-PZ were collected, evaporated and crystallized.

SBD-PZ: orange crystals; m.p. >290°C (unknown); yield 20%; NMR (ppm) in [$^2H_6$] DMSO, 7.61 (1H, d, *J*ab = 7.65Hz, a), 6.65 (1H, d,



*J*ab = 7.65Hz, b), 3.75 (4H, m,c), 3.26 (4H, m, d); Analysis calculated for $C_{10}H_{15}N_5O_4S$, C 39.86, H 5.02, N 23.24; found, C 39.85, H 4.68, N 22.87%.

Spectral measurements of NBD-PZ, DBD-PZ, DBD-CD and SBD-PZ and their adducts

Excitation and emission spectra of 1 μM of each reagent (NBD-PZ, DBD-PZ, DBD-CD or SBD-PZ) in acetonitrile–water (9:1) were measured by a manual method. The fluorescence intensities of the reagents were determined at maximum excitation and emission wavelengths.

DEPC (70 mM) was added to 0.4 ml of dimethylformamide (DMF) solution containing the reagent (5 mM each) and arachidic acid (0.5 mM). After reaction at room temperature for 2 h, 5 μl of the solution were injected into the HPLC column. The peak corresponding to the adduct separated from those of the reagent and DEPC was collected in 2 ml for spectral measurement.

Time course for the derivatization reaction in the presence of DEPC

DEPC (70 mM) was mixed with 0.4 ml of DMF or acetonitrile containing the reagent (5 mM each of NBD-PZ, DBD-PZ, DBD-CD) and arachidic acid (50 μM) in a brown-glass vial. The vials were capped tightly and allowed to stand for 585 min at room temperature in the dark. At the fixed time intervals, an aliquot of the solution was automatically injected onto the column for HPLC and the fluorescence peak area corresponding to the adduct was calculated with an integrator.

Comparison of activation agents for the derivatization reaction

DBD-PZ (5 mM) was reacted with arachidic acid (50 μM) in 0.4 ml of DMF or acetonitrile at room temperature in the presence of each activation agent [DEPC (70 mM), DPPA (70 mM), EDC (35 mM) and pyridine (20 μl), 2,2'-dipyridyl disulphide (35 mM) and triphenylphosphine (35 mM) (designated Mukaiyama A) or 2-chloro-1-methylpyridinium iodide (35 mM) and triethylamine (20 μl) (designated Mukaiyama B)] in the same solvent (acetonitrile or DMF). After reaction for 6 h, an aliquot of the solution was injected onto the column and the fluorescent peaks corresponding to the adduct were compared with each other.

Effect of reaction solvent and concentration of activation agent on derivatization

Arachidic acid (50 μM) activated with DEPC (70 mM) or Mukaiyama A (10 mM) was reacted with DBD-PZ (5 mM) in acetonitrile, DMF or ethanol. Arachidic acid (50 μM) in acetontrile was also reacted with DBD-PZ (5 mM) in the presence of DEPC or Mukaiyama A at three different concentrations. The reaction solutions were allowed to stand for 585 min at room temperature in the dark. At fixed time interval, an aliquot of the solution was injected onto the HPLC column.

HPLC separation of fatty acids or drugs tagged with DBD-amines (DBD-PZ or DBD-CD)

To a 1-ml brown-glass vial was added 0.2 ml of DBD-amine (10 mM each of DBD-PZ of DBD-CD) in DMF or acetonitrile and 0.2 ml of mixed fatty acids (10 μM of each) in DMF containing DEPC (140 mM) or Mukaiyama A (70 mM). The reaction solution in the vial was allowed to stand for 6 h at room temperature. After reaction for 6 h at room temperature, 1 μl of the reaction solution was injected into the column. Linear gradient elution from 70% to 98% acetonitrile over 60 min and then isocratic elution with 98% acetonitrile for 20 min were adopted for the separation of fatty acids labelled with DBD-amines. The eluate was monitored at 569 nm (excitation at 440 nm) for the adducts with DBD-PZ and at 561 nm (excitation at 437 nm) for the adducts with DBD-CD.

Eight drugs (5 μM each of ibuprofen, indometha-

cin, dehydrocholic acid, ursodesoxycholic acid, hydrocortisone succinate, prednisolone succinate, dinoprost and prostaglandin E_1) and DBD-PZ (5 m*M*) in 0.4 ml of acetonitrile were reacted in the presence of DEPC (70 m*M*). After reaction for 6 h at room temperature, 1 μl of the solution was injected onto the HPLC column. The isocratic elution conditions were 65% acetonitrile for the adducts of ibuprofen and indomethacin, 50% acetonitrile for dehydrocholic acid and ursodesoxycholic acid and 45% acetonitrile for prednisolone succinate, hydrocortisone succinate, dinoprost and prostaglandin E_1.

RESULTS AND DISCUSSION

Fluoresence characteristics of 2,1,3-benzoxadiazole amine reagents and their adducts

To obtain the effective detection wavelengths of the adducts with carboxylic acid compounds, the fluorescence excitation and emission spectra of the reagents and their adducts with arachidic acid (Ar) were measured in acetonitrile–water (9:1) by a manual method. As shown in Table I, the excitation maxima of the adducts (DBD-PZ–Ar and DBD-CD–Ar) with arachidic acid were shifted towards the red compared with the reagents themselves, the shifts being 19 and 7 nm, respectively. Red shifts of the emission maxima were also observed in the solution of DBD-PZ–Ar (14 nm). Similar shifts were observed in the adducts of arachidic acid with ABD-PZ and ABD-CD [18]. In contrast, no shifts of the excitation and emission maxima were observed in the solution of NBD-PZ–Ar (Table I). On the other hand, no fluorescent product was obtained from the derivatization of arachidic acid with SBD-PZ.

The relative fluorescence intensities of the reagents themselves were also determined in the same medium. As indicated in Table I, the intensities of cadaverino reagents were higher than those of the corresponding piperazino reagents judging from the comparison between DBD-CD and DBD-PZ. Further, a dimethylaminosulphonyl group at the 4-position exhibits about a threefold higher fluorescence intensity than a nitro group [DBD-PZ (59.6) *versus* NBD-PZ (19.0)] and an aminosulphonyl group [DBD-CD (100) *versus* ABD-CD (32.6) and DBD-PZ (59.6) *versus* ABD-PZ (17.0)]. The fluorescence intensity of SBD-PZ was the weakest among all the reagents. Therefore, its is obvious that both the fluorescence wavelengths and the fluorescence intensity depend on the functional groups at the 4- and 7-positions in the 2,1,3-benzoxadiazole structure.

The shift of the fluorescence wavelengths between the reagent (DBD amine) and its adduct might be preferable for the determination of carboxylic acids, because the effect of the reagent would be less in the detection of the adducts.

Derivatization reaction with 2,1,3-benzoxadiazole amine reagents in the presence of activation agents

The 2,1,3-benzoxadiazole amine reagents essentially require the activation of carboxylic acids before the derivatization with the reagents. Various activation agents have been developed for the purpose of peptide syntheses. Some of them, *e.g.*, DEPC [24] and EDC [25], are miscible with solvents such acetonitrile, ethanol and DMF. As water-miscible solvents are suitable as a reaction medium for the determination of carboxylic acids in biological samples, the following five activation agents were selected in this work: DEPC [24], DPPA [26], EDC [25], Mukaiyama A (2,2'-dipyridyl disulphide and

TABLE I

MAXIMAL WAVELENGTHS OF SYNTHESIZED REAGENTS AND THEIR ADDUCTS WITH ARACHIDIC ACID

Reagent	λ_{max}		Relative fluorescence intensity	Adduct	λ_{max}	
	ex.	em.			ex.	em.
NBD-PZ	470	541	19.0	NBD-PZ-Ar	470	541
DBD-PZ	421	555	59.6	DBD-PZ-Ar	440	569
DBD-CD	430	560	100[a]	DBD-CD-Ar	437	561
SBD-PZ	399	576	5.6			

[a] The fluorescence intensity of DBD-CD was arbitrarily taken as 100%.

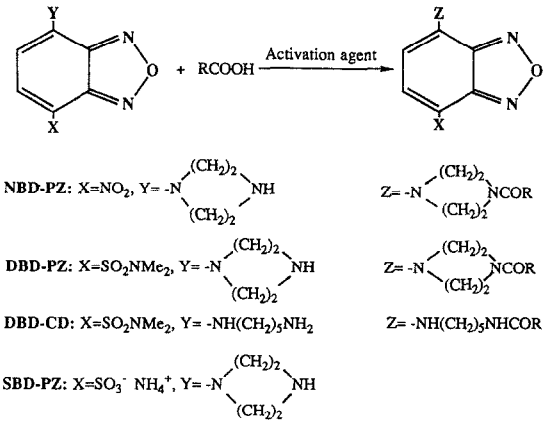


Fig. 1. Structures of the synthesized fluorescence reagents and their reaction with carboxylic acids in the presence of an activation agent.

triphenylphosphine) [27] and Mukaiyama B (2-chloro-1-methylpyridinium iodide and triethylamine) [28]. The scheme for the reaction of the 2,1,3-benzoxadiazole amine reagents with activated carboxylic acid is shown in Fig. 1.

The reactivity toward arachidic acid, which was selected as a representative carboxylic acid, was examined with three 2,1,3-benzoxadiazole amine reagents in the presence of DEPC, an activation agent. As depicted in Fig. 2, the production of the adducts with cadaverino reagents (DBD-CD) at room temperature reached a plateau after 15 min over the 585-min period tested. The derivatization reaction patterns with cadaverino reagents were almost identical in both acetonitrile and DMF (Fig. 2A and B), whereas the production of the adducts with piperazino reagents (NBD-PZ and DBD-PZ) gradually increased with increasing reaction time, and was constant after 5 h. Therefore, the rate of reaction with piperazino reagents seems to be slower than that with cadaverino reagents. With piperazino reagents, the reaction in acetonitrile gave higher fluorescent peaks than in DMF at all points (Fig. 2A and B). Among all the reagents tested, the fluorescence intensity of the adduct obtained from DBD-PZ in acetonitrile was the highest after reaction for 2 h (Fig. 2B). Therefore, the derivatization reaction with the reagents depends on the solvent in the reaction medium. This phenomenon was especially notable in the reaction with piperazino reagents (DBD-PZ and NBD-PZ). Although similar

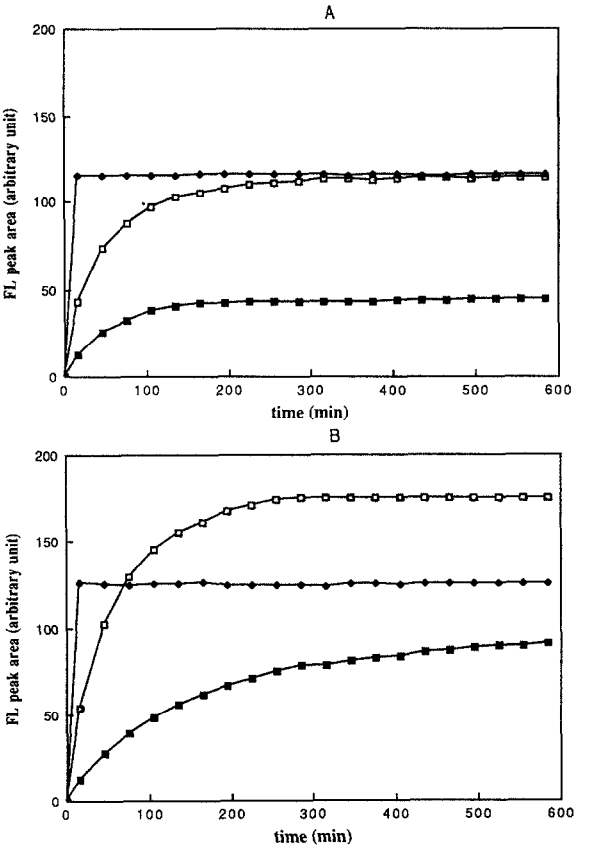


Fig. 2. Time course of the reaction of arachidic acid with various fluorescence reagents in the presence of DEPC. (A) in DMF; (B) in acetonitrile. □ = DBD-PZ; ◆ = DBD-CD; ■ = NBD-PZ. Arachidic acid (50 μM) and fluorescence tagging reagents (5 mM) in 0.4 ml of DMF (or acetonitrile) were reacted at room temperature in the presence of DEPC (70 mM). At fixed time intervals, an aliquot of the reaction solution was injected onto the HPLC column. Column, Inertsil ODS-2 (150 × 4.6 mm I.D.; 5 μm) at 40°C; flow-rate, 1.0 ml/min; isocratic elution with acetonitrile–water (9:1); fluorescence detection, λ_{ex} 470 nm and λ_{em} 541 nm for NBD-PZ–Ar, λ_{ex} 440 nm and λ_{em} 569 nm for DBD-PZ–Ar, λ_{ex} 437 nm and λ_{em} 561 nm for DBD-CD–Ar.

curves were also obtained with the reactions of ABD-PZ and ABD-CD [18], the maximum peak areas were 30–40% of those with DBD-PZ and DBD-CD. The reaction pattern with DBD-CD in the presence of DEPC (or with DBD-PZ in the presence of Mukaiyama A) seems to be comparable to that with MDC and DEPC reported by Lee *et al.* [16].

The effect of activation agents on the derivatization reaction was investigated with DBD-PZ in both acetonitrile and DMF. The relative activation

TABLE II

EFFECT OF ACTIVATION AGENTS ON THE DERIVATIZATION OF ARACHIDIC ACID WITH DBD-PZ

Activation agent	Relative peak area (%)	
	In acetonitrile	In DMF
DEPC	100[a]	63.2
DPPA	50.2	21.7
EDC–pyridine	81.6	71.2
Mukaiyama A	90.2	78.6
Mukaiyama B	24.4	25.9

[a] The peak area with DEPC in acetonitrile was arbitrarily taken as 100%.

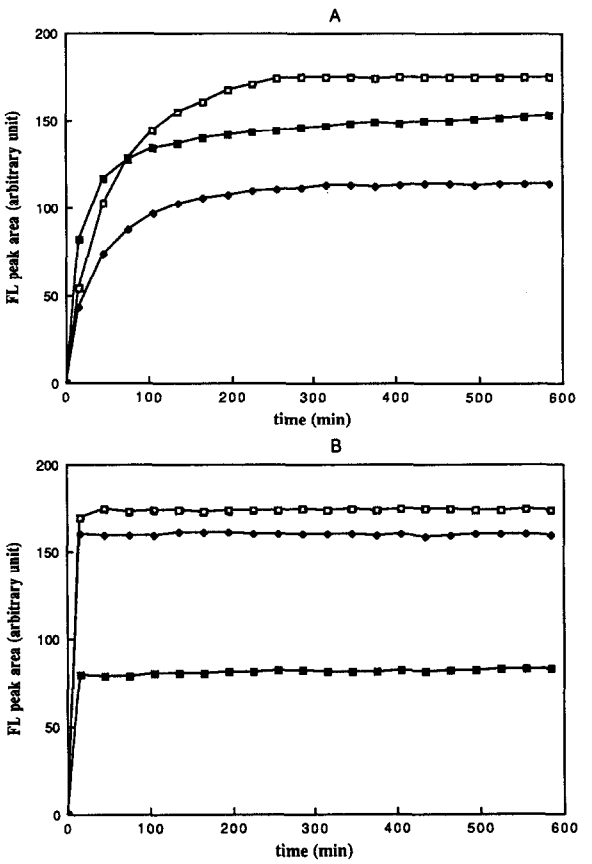


Fig. 3. Time course of the reaction of arachidic acid with DBD-PZ in various solvents. (A) DEPC (70 m*M*); (B) Mukaiyama A (10 m*M*). □ = acetonitrile; ◆ = DMF; ■ = ethanol. Arachidic acid (50 μM) and DBD-PZ (5 m*M*) in three solvents were reacted at room temperature in the presence of activation agents. Fluorescence detection; λ_{ex} 440 nm and λ_{em} 569 nm. Other HPLC conditions in Fig. 2.

capability was evaluated in terms of the fluorescent peak area of the adducts as shown in Table II. Among all the activation agents, DEPC and Mukaiyama A exhibited the highest activation in acetonitrile and DMF, respectively. The activation with Mukaiyama B was similar in both solvents. The order of activation in acetonitrile was DEPC > Mukaiyama A > EDC–pyridine > DPPA > Mukaiyama B. In DMF, the order was Mukaiyama A > EDC–pyridine > DEPC > Mukaiyama B > DPPA. Judging from the results in Table II both DEPC and Mukaiyama A were selected hereafter as the activation agents for carboxylic acids.

To decide a suitable reaction solvent, the derivatization reaction of arachidic acid with DBD-PZ was compared in three solvents (acetonitrile, ethanol and DMF). All three solvents dissolved DBD-PZ at relatively high concentration (>1000 ppm). As illustrated in Fig. 3, acetonitrile was the most effective solvent in the derivatization reaction with both DEPC and Mukaiyama A. The relative peak areas of the adduct with Mukaiyama A in DMF were about 90% of those in acetonitrile. The low fluorescent peaks with Mukaiyama A in ethanol seem to be due to side-reactions. The low production of the adduct might also depend on the amount of water in the solvent used as the medium. With DEPC, the order of the fluorescent peaks after reaction for 2 h was acetonitrile > ethanol > DMF. From these results, acetonitrile is the most effective solvent for the tagging reaction with DBD-PZ. However, the reaction solvent must also be selected by considering the solubility of the target carboxylic acid in real samples.

The derivatization reaction was compared at three different concentrations of DEPC and Mukaiyama A in acetonitrile. As shown in Fig. 4A, the adducts at 42 and 70 m*M* DEPC at room temperature gradually increased with increasing reaction time and reached a plateau after 5 h. With 14 m*M* DEPC, the reaction was slower than those at the other two DEPC concentrations and a constant peak area was not obtained even after 585 min. Consequently, a concentration of DEPC higher than 42 m*M* is necessary in order to obtain a constant peak area. On the other hand, the curves derived from Mukaiyama A were similar at three different concentrations (2, 10 and 35 m*M*) (Fig. 4B). The reaction of arachidic acid with DBD-CD in the

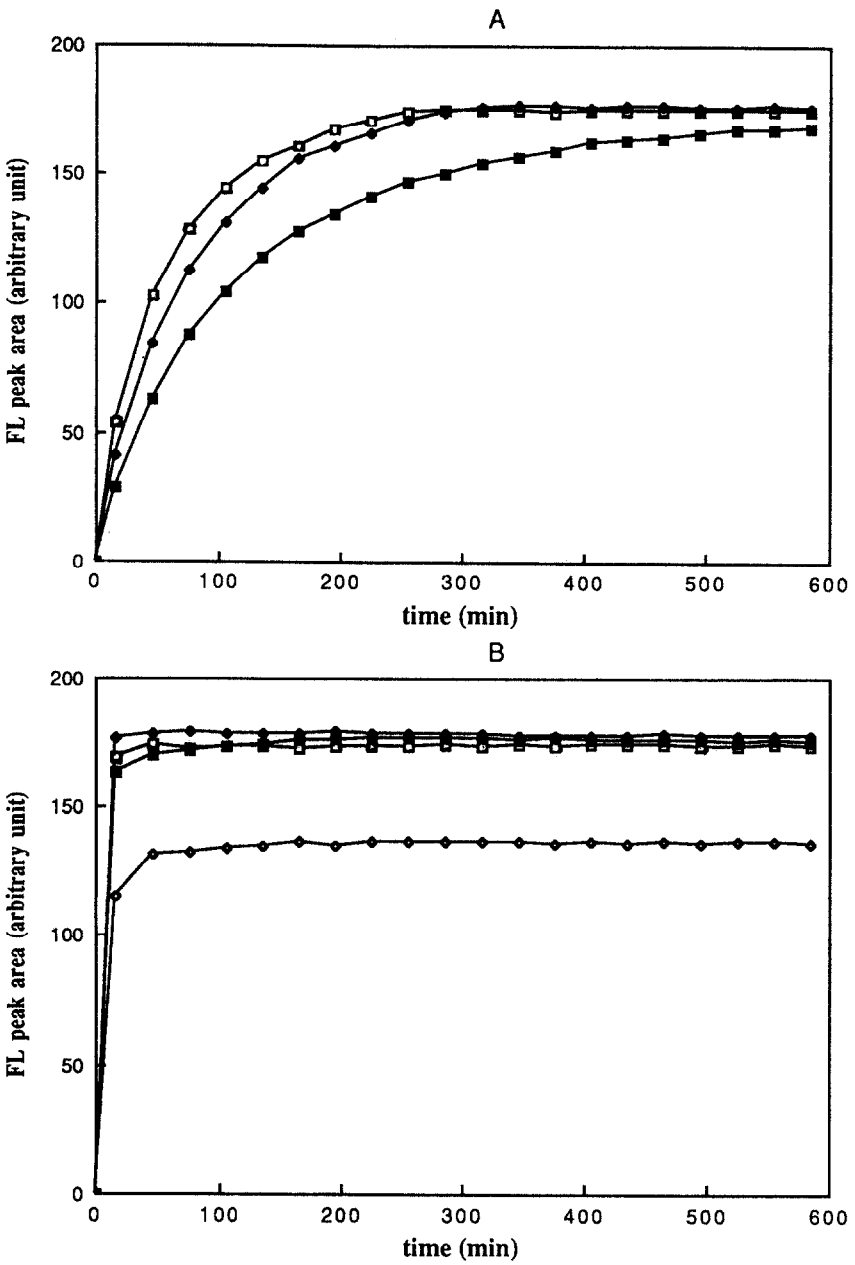


Fig. 4. Time course of the reaction of arachidic acid with various concentrations of activation agents in acetonitrile. (A) □ = with DBD-PZ and DEPC (70 m*M*); ◆ = with DBD-PZ and DEPC (42 m*M*); ■ = with DBD-PZ and DEPC (14 m*M*). (B) □ = with DBD-PZ and Mukaiyama A (35 m*M*); ◆ = with DBD-PZ and Mukaiyama A (10 m*M*); ■ = with DBD-PZ and Mukaiyama A (2 m*M*); ◇ = with DBD-CD and Mukaiyama A (35 m*M*). Arachidic acid (50 μM) and DBD-amine reagents (5 m*M*) in acetonitrile were reacted at room temperature in the presence of various concentration of activation agents. Fluorescence detection, λ_{ex} 440 nm and λ_{em} 569 nm for DBD-PZ–Ar, λ_{ex} 437 nm and λ_{em} 561 nm for DBD-CD–Ar. Other HPLC conditions in Fig. 2.

presence of Mukaiyama A was completed after 45 min, the same as those with DBD-PZ. However, the fluorescent peak areas of the adduct were about 25% lower than that with DBD-PZ at all points (Fig. 4B).

According to the time-course study described above, the adducts derived from DBD-PZ and DBD-CD in the presence of DEPC were stable for at least 6 h in both acetonitrile and DMF (Figs. 2, 3A and 4A). Therefore, reaction for 6 h at room temperature was selected for the derivatization of carboxylic acids with DBD-PZ or DBD-CD (10 m*M*) in the presence of DEPC (70 m*M*). As good stability of the adducts and faster reactions were observed in the reaction of carboxylic acids with DBD-PZ and DBD-CD in the presence of Mukai-

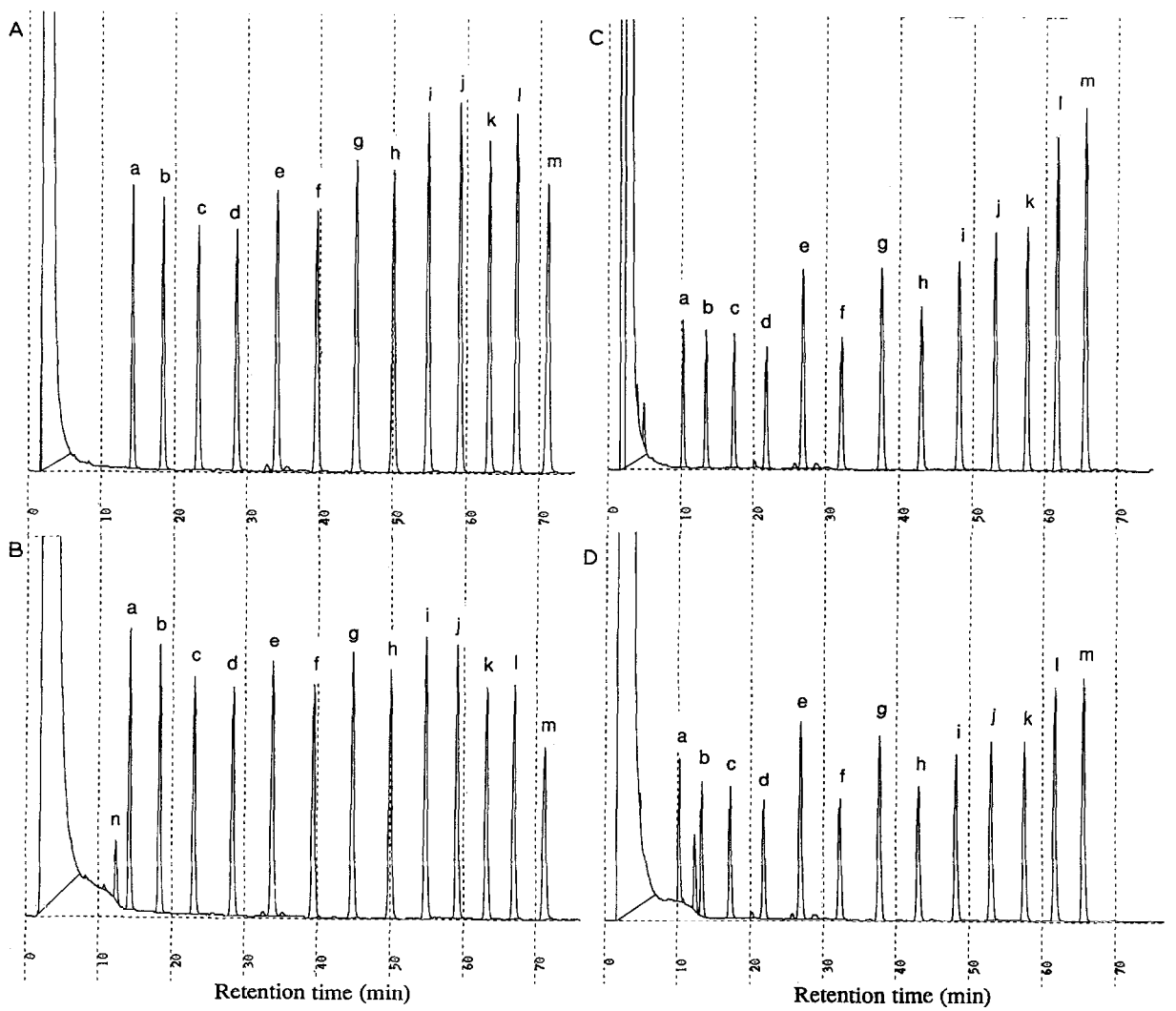


Fig. 5. Chromatograms of fatty acids labelled with DBD-amines in acetonitrile. (A) With DBD-PZ and DEPC (70 m*M*); (B) with DBD-PZ and Mukaiyama A (35 m*M*); C with DBD-CD and DEPC (70 m*M*); (D) with DBD-CD and Mukaiyama A (35 m*M*). Each peak corresponds to 5 pmol of fatty acid. (a) $C_{12:0}$ (b) $C_{13:0}$; (c) $C_{14:0}$; (d) $C_{15:0}$; (e) $C_{16:0}$; (f) $C_{17:0}$; (g) $C_{18:0}$; (h) $C_{19:0}$; (i) $C_{20:0}$; (j) $C_{21:0}$; (k) $C_{22:0}$; (l) $C_{23:0}$; (m) $C_{24:0}$; (n) unknown. Arachidic acid (5 μM) and DBD-amine reagents (5 m*M*) in acetonitrile were reacted at room temperature in the presence of activation agents. Linear gradient elution from acetonitrile–water (7:3) to acetonitrile–water (98:2) over 60 min and then isocractic elution with acetonitrile–water (98:2) for 20 min. Other HPLC conditions as in Fig. 4.

yama A (Figs. 2, 3B and 4B), reaction for 45 min was adopted for the derivatization in the presence of Mukaiyama A (35 m*M*). Judging from the reaction period affording the maximum fluorescence yield, Mukaiyama A might be more suitable than DEPC as an activation agent.

HPLC analyses of fatty acids and drugs tagged with DBD-amines

The HPLC separation of thirteen saturated free fatty acids (FFAs) and of eight drugs tagged with DBD-amine reagents (DBD-PZ and DBD-CD) in the presence of DEPC or Mukaiyama A were carried out on a reversed-phase column (Inertsil ODS-2). Fig. 5A and C show the separation of 5 pmol of each FFA, tagged with DBD-PZ or DBD-CD in the presence of DEPC, with mixtures of acetonitrile and water as the eluents. Peak separations of the adducts with each fluorescence reagent were completed within 75 min by a simple linear gradient. The FFAs tagged with DBD-PZ showed higher peaks compared than those of DBD-CD. Similar peak heights were observed among the DBD-PZ–FFAs in spite of the change in the solvent composition (Fig. 5A). For the adducts with DBD-CD in the presence of DEPC, the differences in the peak heights were larger than those with DBD-PZ (Fig. 5A *versus* C). Similar results were also obtained with the use of Mukaiyama A as an activating agent for carboxylic acids. However, an unknown peak (peak *n* in Fig. 5B and D) derived from the activating agent appeared on the chromatograms. With respect to the activation agents, DEPC and Mukaiyama A gave similar peak heights in the reaction with DBD-PZ (or DBD-CD) (Fig. 5A *versus* B and Fig. 5C *versus* D). Consequently, both activating agents are applicable to the determina-

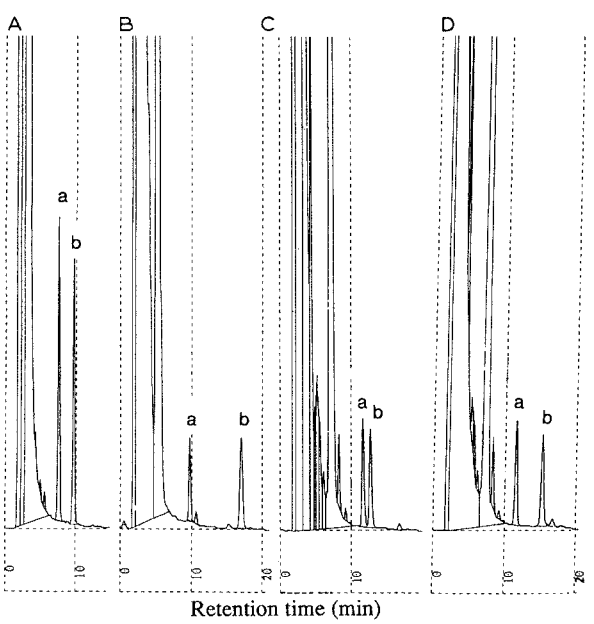


Fig. 6. Chromatograms of drugs derivatized with DBD-PZ in the presence of DEPC in acetonitrile. (A) (a) indomethacin and (b) ibuprofen; (B) (a) dehydrocholic acid and (b) ursodesoxycholic acid; (C) (a) prednisolone succinate and (b) hydrocortisone succinate; (D) (a) dinoprost (prostaglandin $F_{2\alpha}$) and (b) prostaglandin E_1. Each peak corresponds to 5 pmol of drug. Isocratic elution; (A) acetonitrile–water (65:35); (B) acetonitrile–water (5:5); (C) and (D) acetonitrile–water (45:55). Other HPLC conditions as those in Fig. 5.

TABLE III

DETECTION LIMITS OF FREE FATTY ACIDS AND DRUGS LABELLED WITH DBD-PZ AND DEPC IN ACETONITRILE

Carboxylic acid	Eluent[a]	Detection limit (signal-to-noise ratio = 3) (fmol)
Indomethacin	1	3.9
Ibuprofen	1	4.3
Dehydrocholic acid	2	13
Ursodesoxycholic acid	2	13
Prednisolone succinate	3	12
Hydrocortisone succinate	3	14
Dinoprost	3	12
Prostaglandin E_1	3	14
Lauric acid ($C_{12:0}$)	4	3.9
Tridecanoic acid ($C_{13:0}$)	4	4.1
Myristic acid ($C_{14:0}$)	4	4.5
n-Pentadecanoic acid ($C_{15:0}$)	4	4.7
Palmitic acid ($C_{16:0}$)	4	4.1
Margaric acid ($C_{17:0}$)	4	4.3
Stearic acid ($C_{18:0}$)	4	3.8
Nonadecanoic acid ($C_{19:0}$)	4	3.9
Arachidic acid ($C_{20:0}$)	4	3.3
Heneicosanoic acid ($C_{21:0}$)	4	3.2
Behenic acid ($C_{22:0}$)	4	3.6
Tricosanoic acid ($C_{23:0}$)	4	3.2
Lignoceric acid ($C_{24:0}$)	4	3.8

[a] Eluent 1, acetonitrile–water (65:35), isocratic elution; eluent 2, acetonitrile–water (1:1), isocratic elution; eluent 3, acetonitrile–water (45:55), isocratic elution; eluent 4, gradient elution from acetonitrile–water (7:3) to acetonitrile–water (98:2) over 60 min and isocratic elution with acetonitrile–water (98:2) for 20 min.

tion of carboxylic acids if the unknown peak derived from Mukaiyama A does not interfere with the separation of the objective acids.

HPLC separations of drugs containing a COOH moiety in the structure were also performed after tagging with DBD-PZ in the presence of DEPC. Each chromatogram in Fig. 6 shows the separation of 5 pmol of each drug divided into four groups, antiinflammatory, bile acids, hormones and prostaglandins.

As listed in Table III, the detection limits (signal-to-noise ratio = 3) of FFAs and drugs tagged with DBD-PZ in the presence of DEPC were 3–14 fmol, and were lower than those with ABD-PZ and ABD-CD (10–50 fmol) [18]. The detection limits were low compared with those obtained by other methods (*e.g.*, 10 fmol by Br-Mac [6], 20–30 fmol by PDAM [14], 100 fmol by MDC [16], 9 pmol by Br-Mmc [3]). Moreover, the long maximum wavelengths of the adducts might be an advantage for the detection of carboxylic acids in biological samples. Among the proposed reagents. DBD-amine reagents (DBD-PZ and DBD-CD) are recommended for the detection of carboxylic acids which are activated with DEPC or Mukaiyama A in acetonitrile or DMF. The DBD moiety was also preferable for more sensitive detection by peroxyoxalate chemiluminescence [29], so that attomole detection limits could be achieved for carboxylic acids. As the proposed method including the activation step under mild conditions is useful for the determination not only of FFAs but also of drugs such as prostaglandins, it might be applicable to the determination of various carboxylic acids in biological specimens. Further studies are in progress.

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