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SENSITIVE FLUORESCENCE LABELLING FOR ANALYSIS OF CARBOXYLIC ACIDS WITH 4-BROMOMETHYL-6,7-METHYLENEDIOXYCOUMARIN

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SUMMARY

A fluorescence labelling reagent, 4-bromomethyl-6,7-methylenedioxycoumarin (BrMDC), was synthesized from sesamol and citric acid by Pechman condensation followed by bromination. Nanomole amounts of saturated aliphatic fatty acids were converted into the corresponding fluorogenic esters in the presence of anhydrous potassium carbonate and a crown ether as a catalyst and were separated by reversed-phase high-performance liquid chromatography (HPLC). Quantitative studies revealed that *n*-caproic acid was esterified completely at low temperature and with sufficient reproducibility. The detection limit was just below 15 fmol per injection at a signal-to-noise ratio of 3. The fluorescence quenching of the BrMDC derivative was the lowest in conventional mixed solvent systems in comparison with those of two previously reported coumarin compounds. BrMDC was also applied to the simultaneous analysis of some acidic non-steroidal anti-inflammatry agents by reversed-phase HPLC.

INTRODUCTION

A large number of biologically interesting substances contain carboxylic moieties, both as intermediates and end-products, from endogenous metabolism of carbohydrates or lipids. Several xenobiotics represented by drugs also possess carboxylic groups. In therapeutic drug monitoring to design an individualized dosage regimen or in the investigation of pharmacokinetic–pharmacodynamic relationships, reliable and sensitive analytical methods for the drug itself and/or its active metabolites in biological specimens are required[1,2]. High-performance liquid chromatography (HPLC) is now widely use for the trace analysis of numerous organic substances because it provides rapid and sufficient resolution even when two or more closely related analogues exist homogeneously. In early work, some fatty acids or their methyl esters were also successfully separated directly by carbon number using reversed-phase bonded columns[3–5]. With such substances, however, which do not have any chromogenic substituents, detection has to depend on their refraction or weak

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absorption near the extreme ultraviolet region. This has hindered the application of HPLC to the trace analysis of carboxylic acids in biological systems[6].

Chemical derivatization of specific functional groups to give UV-sensitive or strong fluorogenic probes overcame this disadvantage and improved the detectability of the compounds of interest[7]. Durst *et al.*[8] first employed *p*-bromophenacyl bromide (*p*-BPB) as a labelling reagent for calboxylic acids to achieve sensitive UV detection in HPLC, and this was extended to the resolution diastereomers of some long-chain unsaturated fatty acids[9]. Dünges and co-workers introduced 4-bromomethyl-7-methoxycoumarin (BrMMC), a highly sensitive fluorescence labelling reagent, for both thin-layer chromatography[10,11] and HPLC[12,13], and since then various other fluorescence probes have been developed, *e.g.*, 1-bromoacetylpyrene (BAP)[14], 4-bromomethyl-6,7-dimethoxycoumarin (BrDMC)[15], 9-anthryldiazomethane (ADAM)[16], 4-bromomethyl-7-acetoxycoumarin (BrMAC)[17], 3-bromomethyl-6,7-dimethoxy-1-methyl-2(1H)-quinoxalinone (BrMQ)[18] and *p*-(9-anthroyloxy)phenacyl bromide[19]. Among these, BrMMC and ADAM have been commonly applied to the trace analysis of biologically important acid compounds such as prostaglandins[20,21] in enzymatic preparations.

In this paper, we report the preparation of 4-bromomethyl-6,7-methylenedioxycoumarin (BrMDC), a fluorescence labelling reagent for caboxylic acids, which possesses a more fluorogenic 6,7-methylenedioxycoumarin moiety than in previously reported coumarin compounds, and its applicability to the simultaneous analysis of a series of fatty acids and some acidic non-steroidal anti-inflammatry agents (NSAIDs) by reversed-phase HPLC.

EXPERIMENTAL

Materials

3,4-Methylenedioxyphenol (sesamol) and 4-bromomethyl-6,7-dimethoxycoumarin (BrDMC) were purchased from Aldrich (Milwaukee, WI, U.S.A.), 4-bromomethyl-7-methoxycoumarin (BrMMC), aspirin, ketoprofen and ibuprofen from Wako (Osaka, Japan) and all C_3–C_{19} saturated fatty acids, naproxen, indomethacin and the crown ethers 18-crown-6, dicyclohexano-18-crown-6, dibenzo-18-crown-6 and kryptofix 222 from Sigma (St. Louis, MO, U.S.A.). Loxoprofen was prepared and supplied by the Chemical Research Laboratories of Sankyo (Tokyo, Japan)[22]. Flurbiprofen was purchased as commercially available tablets (Furoben; Kaken Pharmaceutical, Tokyo, Japan) and extracted and purified in our laboratories. 4-Methyl-6,7-methylenedioxycoumarin for fluorescence spectral studies was prepared according to Fukui and Nakayama[23]. All other chemicals for synthesis were of guaranteed reagent grade and all organic solvents for chromatographic purpose were of special grade for HPLC, obtained from Wako.

Instruments

Proton nuclear magnetic resonance (^{1}H NMR) spectra were measured on a Model JNM-GX270 spectrometer (Jeol, Tokyo, Japan) at 270 MHz and chemical shift values (δ) were expressed in parts per million downfield from tetramethylsilane as internal standard. IR spectra were recorded on a Model 60-SX Fourier transform infrared spectrometer (Nicolet Japan, Tokyo, Japan). Mass spectra were measured

with a Model DX-300 mass spectrometer (Jeol, Tokyo, Japan). The HPLC system consisted of a Model 655 solvent delivery pump unit (Hitachi, Tokyo, Japan) and a Model F-1000 fluorescence spectrophotometer (Hitachi), which was linked to a Model C-R3A chromatographic integrator (Shimadzu, Kyoto, Japan). The sample was applied to a μBondapak C_{18} reversed-phase packed column (300 mm × 3.9 mm I.D.) (Millipore-Waters, Milford, MA, U.S.A.) by a WISP 710B automatic sample processor (Millipore-Waters).

Determination of fluorescence quantum yield

Fluorescence spectra of 6,7-substituted 4-methylcoumarins in various solvents were measured on a Model RF-540 fluorescence spectrophotometer (Shimadzu). The fluorescence intensity was evaluated as the integrated area under the spectrum and the relative fluorescence quantum yield was calculated according to Weber and Teale[24] using quinine sulphate as a reference.

Synthesis of 4-carboxymethyl-6,7-methylenedioxycoumarin (I)

4-Carboxymethyl-6,7-methylenedioxycoumarin (I) was prepared according to Baker *et al.*[25]. Finely powdered crystalline citric acid (250 mmol) was poured stepwise at 70°C into 67.5 ml of sulphuric acid. To the ice-cold solution was added an equal number of moles of finely ground sesamol, keeping the temperature below 5°C. A further 29 ml of sulphuric acid were poured in gradually and stirred gently overnight. The reaction mixture, diluted with 50 ml of chilled water, was filtered and the remaining brown material was washed sequently with 0.01 *M* sulphuric acid, 2 *M* sodium hydroxide solution and 2 *M* hydrochloric acid. After being kept stationary overnight, the ash-coloured precipitate was separated by centrifugation and recrystallized from acetonitrile to give I as faintly white prisms; yield 33–51%, m.p. 170°C. Analysis: calculated for $C_{12}H_8O_6$, C 58.07, H 3.25; found, C 57.96, H 3.08%. ^{1}H NMR (perdeuterated dimethyl sulphoxide: 3.96 (2H, s, $-CH_2COO-$), 6.23 (2H, s, $-OCH_2O-$), 6.39 (1H, s, $=C=CH-CO-$), 7.02 (1H, s, aromatic), 7.26 ppm (1H, s, aromatic). Mass spectrum: *m/z* 248 (M^+), 204 (base peak). IR (KBr pellet): 3050, 2920, 1720, 1410, 1280, 1040, 940 cm^{-1}.

Synthesis of 4-bromomethyl-6,7-methylenedioxycoumarin (BrMDC, II)

To 10 mmol of I suspended in 7.5 ml of acetic acid was gradually added an equimolar amount of acetic acid containing 10 mmol of bromine, the solution was refluxed for 1 h. After cooling, the resulting crude material was subjected to silica gel column chromatography and eluted with acetone. The main fraction was evaporated to dryness and the residue was recrystallized from methanol to give II as yellow prisms; yield 76%, m.p. 241°C. Analysis: calculated for $C_{11}H_7O_4Br$, C 46.46, H 2.47, Br, 28.23; found, C 46.93, H 2.34, Br 27.86%. ^{1}H NMR (perdeuterated acetone): 4.79 (2H, s $-CH_2Br$), 6.20 (2H, s, $-OCH_2O-$), 6.50 (1H, s, $=C=CH-CO-$), 6.94 (1H, s, aromatic), 7.32 ppm (1H, s, aromatic). Mass spectrum: *m/z* 283 (M^+), 175 (base peak). IR (KBr pellet): 3070, 2920, 1730, 1450, 1270, 1040, 630 cm^{-1}.

Preparation of n-caproic acid derivatives (III) as fluorescence reference

The authentic BrMDC derivative of *n*-caproic acid was synthesized on a semi-micro preparative scale in order to evaluate its reactivity. To a solution of

n-caproic acid (1.2 mmol) in 5 ml of acetonitrile were added BrMDC (0.6 mmol) and triethylamine (1.2 mmol). The resulting solution was allowed to stand at 40 C for 1 h, then, evaporated to dryness *in vacuo*. The residue was purified on a silica gel column with *n*-hexane–dichloromethane (50:50) as eluent. The main fraction was evaporated to dryness *in vacuo* and the residue was purified by repeated recrystallization from methanol to give III as white needles; yield 53%, m.p. 106°C. Analysis: calculated for $C_{17}H_{18}O_6$, C 64.14, H 5.70; found, C 64.33, H 5.58%. ^{1}H NMR (deuterochloroform): 0.91 (3H, t, J = 6.8 Hz, $-CH_3$), 1.31–1.72 [6H, m, $-(CH_2)_3-$], 2.45 (2H, t, $-OCOCH_2-$), 5.20 (2H, s, $-CH_2OCO-$), 6.09 (2H, s, $-OCH_2O-$), 6.36 (1H, s, $=C=CH-CO-$), 6.86–6.88 ppm (2H, m, aromatic). Mass spectrum: m/z 318 (M^+), 220 (base peak). IR (KBr pellet): 3080, 2840–2960, 1740, 1720, 1270, 1170, 1030 cm^{-1}.

Analytical derivatization of carboxylic acids

Fatty acids or NSAIDs for working standards were prepared as solutions in ethanol or acetone. An aliquot (0.1–100 nmol) was dispended into a 15-ml brown tube and evaporated to dryness *in vacuo*. To the residue were added a 500 μl solution of acetonitrile containing BrMDC (1.0 mM, corresponding to at least a 5-fold excess over the acids) and a crown ether (1.0 mM, saturated with an excess of finely powdered potassium carbonate and then sonicated briefly) as to become 1 ml of acetonitrile mixture. The reaction mixture was heated at 40°C for 1 h, then 100 μl of acetic acid–acetonitrile (1:9) was added in order to consume the remaining reagent and to stabilize the derivative formed. After cooling to ambient temperature, a 10-μl aliquot of the reaction mixture was applied to HPLC.

Chromatographic conditions

Optimum separation of C_3–C_{19} straight-chain fatty acid derivatives with BrMDC could be achieved by using μBondapak C_{18} as an analytical reversed-phase column and three different mobile phases, *i.e.*, acetonitrile–water (50:50, 70:30 and 90:10, v/v), containing 1.5% acetic acid. By using same column and acetonitrile–water (50:50, v/v) containing 1% acetic acid as the mobile phase was also separated six NSAID derivatives with BrMDC. All solvents were degassed by brief sonication just before use and then pumped isocratically at 2 ml/min under ambient temperature. The detection wavelengths were adjusted to 355 nm excitation and 435 nm emmision. The sensitivity range of the fluorescence detector was varied between 0.5 and 20.

RESULTS AND DISCUSSION

Synthesis of 4-substituted 6,7-methylenedioxycoumarin as a fluorescence probe

Many fluorescence labelling reagents are known for the sensitive chromatographic determination of carboxylic acids[10,14–19]. ADAM[21] and BrMMC[20] have been used extensively for the trace analysis of prostanoids in biological specimens. However, these reagents had the disadvantages that the former was unstable[8,21] and the fluorescence intensity of the latter derivatives was affected by solvents[26]. It is well recognized that electron-donating substituents at the 6- or 7-position of the coumarin moiety, such as alkylamino, hydroxy and alkyloxy, contribute to enhancing the fluorogenecities and show significant Stokes shifts[27,28].

In order to overcome the drawbacks of the previous fluorescence probes, we

therefore examined the effect of the solvent on the fluorescence properties of several 6- or 7-substituted 4-methylcoumarins, and found that a 6,7-methylenedioxy substituent possessed about a 2–10 times higher relative fluorescence quantum yield than the 7-monomethoxy analogue, as shown in Table I. The smaller difference in the fluorescence quantum yields among aqueous solvents such as ethanol, acetonitrile and water suggested less quenching in their mixed solvent systems, which might make them utilizable as mobile phases for reversed-phase HPLC.

We have already demonstrated that N-(6,7-methylenedioxy-4-methyl-3-coumarinyl)maleimide (MDCM), a labelling reagent for thiol groups, gave a higher sensitivity than DACM, because it showed less quenching in aqueous mixed solvents used as HPLC mobile phases[29]. These findings strongly suggested that BrMDC, with an active vinylogous halocarbonyl at the 4-position of the coumarin moiety, might be also be also applicable as a labelling reagent for carboxylic acids. BrMDC could be readily prepared from sesamol and citric acid by a two-step reaction, *i.e.*, condensation[25] of a phenolic alcohol with an α-keto acid followed by bromination under mildly acidic conditions, although the overall reaction yield was low (25–39%). The reagent proved to be stable at room temperature for at least 6 months with protection from light.

Fluorescence characteristics of BrMDC derivatives

A carboxylic acid is easily esterified with a β-halocarbonyl group under alkaline catalysis[30]. Durst *et al.*[8] demonstrated that inorganic alkaline crown ethers might be effective as solid–liquid phase transfer catalysts because of the small amount of reagent required, quantitative reactivity and low solvent effect. We first compared the derivatization efficiencies of BrMDC by combining seven aprotic solvents and four crown ethers, 18-crown-6, dicyclohexo-18-crown-6, dibenzo-18-crown-6 and kryptofix 222, as catalysts. The first two crown ethers adequately catalysed the formation of ester derivatives with *n*-caproic acid. The reaction velocity was the highest in acetonitrile, followed by acetone, tetrahydrofuran and dichloromethane, in that order; no fluorescent product could be obtained in methanol or water. Consequently, 18-crown-6 saturated with potassium carbonate in acetonitrile was chosen as the

TABLE I

COMPARISON OF MAXIMUM FLUORESCENCE EMISSION WAVELENGTHS (λ) AND RELATIVE FLUORESCENCE QUANTUM YIELDS (Φ_f) OF 6- AND 7-SUBSTITUTED COUMARINS IN VARIOUS SOLVENTS

Fluorescence properties of 4-methyl-7-methoxycoumarin are taken from ref. 28.

Solvent	*4-Methyl-6,7-methylenedioxycoumarin*		*4-Methyl-7-methoxycoumarin*	
	λ *(nm)*	Φ_f	λ *(nm)*	Φ_f
Diethyl ether	385	0.029	368	0.003
Dichloromethane	398	0.09	372	0.02
Ethanol	406	0.38	374	0.11
Acetonitrile	404	0.10	370	0.01
Acetic acid	405	0.51	375	0.18
Distilled water	410	0.88	381	0.58

catalyst for the subsequent study. The fluorescence characteristics of *n*-caproic acid ester derivatives with BrMDC, BrMMC and BrDMC were compared in acetonitrile water mixtures as shown in Fig. 1.

The BrMDC derivative gave a higher fluorescence than equimolar amounts of the other two fluorescent derivatives, and its quenching on increasing the concentration of acetonitrile was the least, as expected from a spectral study. The reaction kinetics of BrMDC with *n*-caproic acid are shown in Fig. 2.

The derivatization reaction was completed almost quantitatively within 1 h at 40°C. A constant amount of *n*-caproic acid (250 nmol) was titrated with various amounts of BrMDC, as shown in Fig. 3. The results confirmed that the derivatization occurred in a 1:1 molar stoichiometric manner as already reported for β-halocarbonyl[8].

We further intend to study application of BrMDC to the quantitative analysis of acidic compounds in biological specimens. During a preliminary study on some acidic NSAIDs, it was found that equimolar BrMDC could not achieve complete derivatization with solvent extracts from biological fluids, such as human plasma and urine. This might be due to the coexistence of undesirable reactive substances or a small amount of water remaining in the extracts. On the other hand, none of interference peaks derived from the reagent could be found in the eluate for most carboxylic acids of interest, even when a large excess of BrMDC (2 μmol or more per reaction cuvette) was present. Therefore, it seemed preferable that the derivatization should be performed with an adequate excess of reagent, which was therefore set at 0.5 μmol, corresponding to at least a 5-fold excess over the total amount of acids expected, in further chromatographic studies.

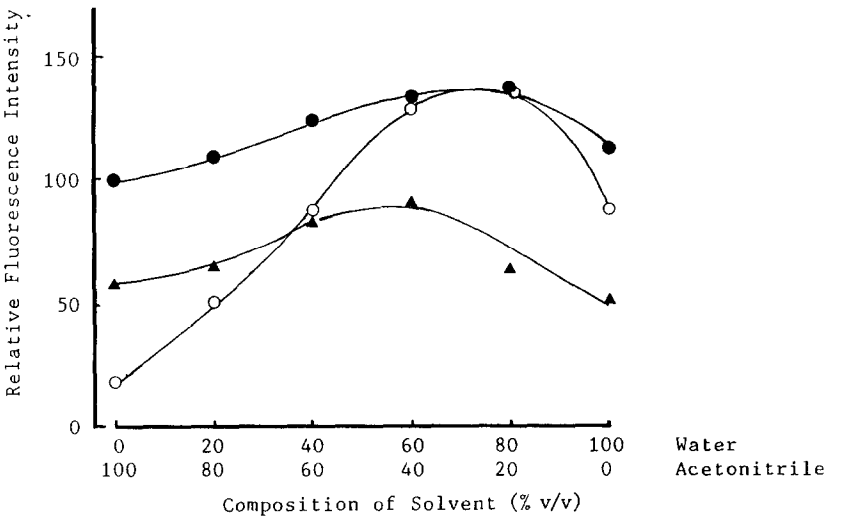

Fig. 1. Relative fluorescence intensity of derivatives of *n*-caproic acid with (●) BrMDC, (○) BrMMC and (▲) BrDMC in acetonitrile–water mixtures. A 40-nmol amount of *n*-caproic acid was derivatized with each fluorescence probe as described in the text and 5-μl aliquots of the reaction mixtures were applied to 20 × 20 cm silica gel thin-layer plates and developed with *n*-hexane–ethyl acetate (50:50). The corresponding reaction products were scraped off and reconstituted in aqueous acetonitrile to give a final concentration of 40 n*M*. The fluorescence intensity of the BrMDC derivative in distilled water is arbitrarily expressed as 100.

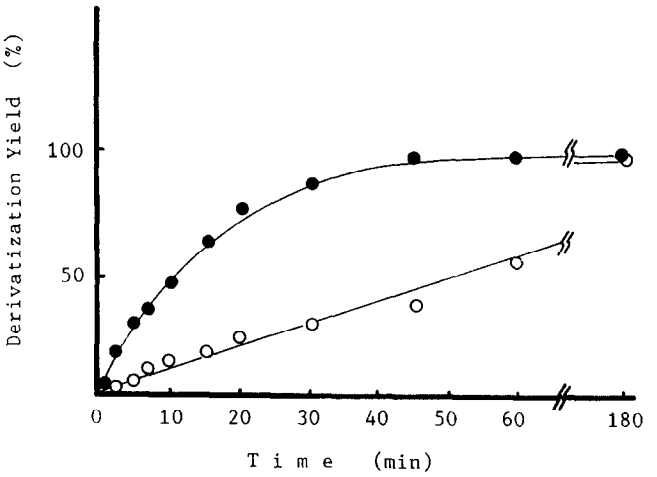

Fig. 2. Time course of the development of the fluorescence of *n*-caproic acid derivative with BrMDC. (●) 40°C; (○) room temperature. The derivatization yield was obtained from the fluorescence response against that of a synthetically prepared derivative (III).

Chromatographic application

A reversed-phase HPLC separation of BrMDC derivatives of thirteen aliphatic fatty acids could be achieved within 15 min per run by using three different mobile phases, as illustrated in Fig. 4.

The derivatives were almost completely resolved from each other, except the propionic acid derivative, which was eluted relatively faster than others and was overlapped by reagent peaks. The accuracy and the validity of the derivatization were

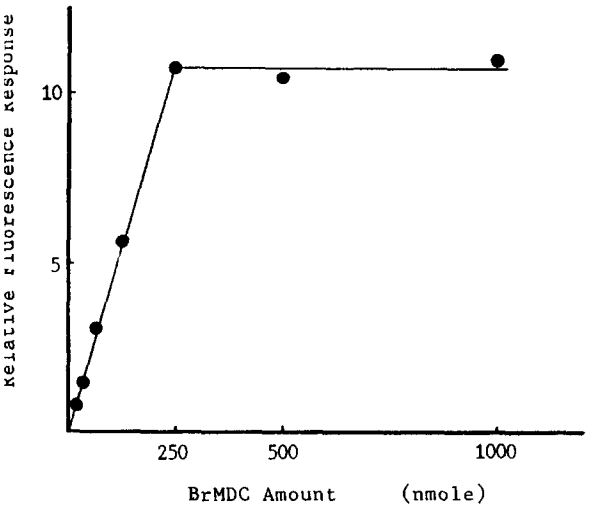

Fig. 3. Quantitative titration curve of *n*-caproic acid with BrMDC. A constant amount of *n*-caproic acid (250 nmol) was derivatized with different amounts of BrMDC (15–1000 nmol) and subjected to HPLC. The relative fluorescence response (ordinate) is represented in arbitrary units.

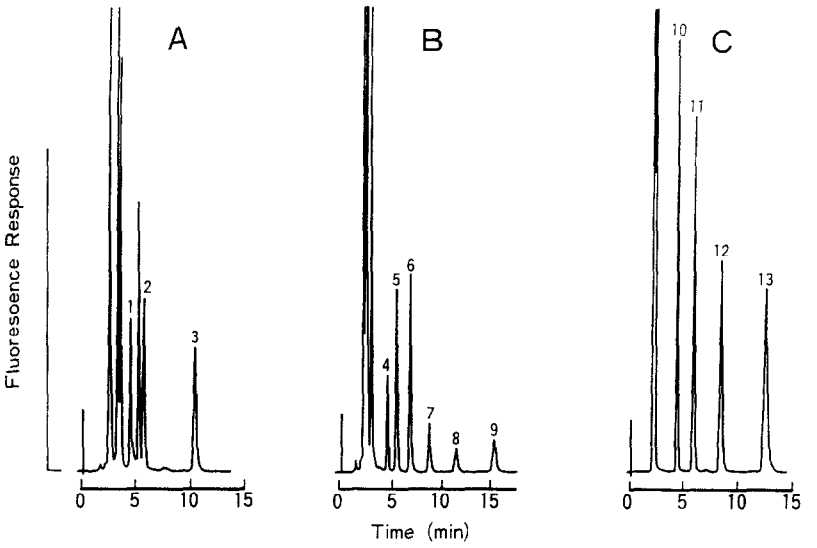

Fig. 4. Chromatographic separations of BrMDC derivatives of saturated fatty acids. Peaks: 1 = C_3; 2 = C_4; 3 = C_6; 4 = C_7; 5 = C_8; 6 = C_9; 7 = C_{10}; 8 = C_{11}; 9 = C_{12}; 10 = C_{13}; 11 = C_{15}; 12 = C_{17}; 13 = C_{19}. HPLC conditions: column, μBondapak C_{18} (300 mm × 3.9 I.D.); mobile phase, acetonitrile–water, (A) 50:50, (B) 70:30 and (C) 90:10, containing 1.5% acetic acid; flow-rate, 2.0 ml/min (isocratic); temperature, ambient; detection, excitation at 355 nm, emission at 435 nm; detector sensitivity, 0.5.

examined by five replicated analyses at different amounts of *n*-caproic acid, as shown in Table II.

The calibration graph of amount of acid *versus* integrated peak area of the fluorescent response was linear over the range 0.10–25 nmol and passed through the origin ($r = 0.999$). All the results for *n*-caproic acid were sufficiently accurate, with coefficients of variation of not more than 7%. The detection limit of the *n*-caproic acid derivative as a representive compound was as low as 15 fmol per injection when the signal-to-noise ratio was 3. This sensitivity is similar to those of previously reported fluorescence probes[11,12,17].

Some commercially available NSAIDs could also be derivatized with BrMDC. The derivatives of aspirin and five propionate anti-inflamatory agents in the

TABLE II

DERIVATIZATION REPRODUCIBILITY OF BrMDC WITH *n*-CAPROIC ACID

Each value is expressed as the mean ±S.D. of five replicate determinations.

Amount of n-caproic acid (nmol)		*Coefficient of variation (%)*
Added	*Found*	
25	25.2 ± 0.6	2.34
5	5.15 ± 0.12	2.33
1	0.94 ± 0.04	4.04
0.1	0.103 ± 0.007	6.98

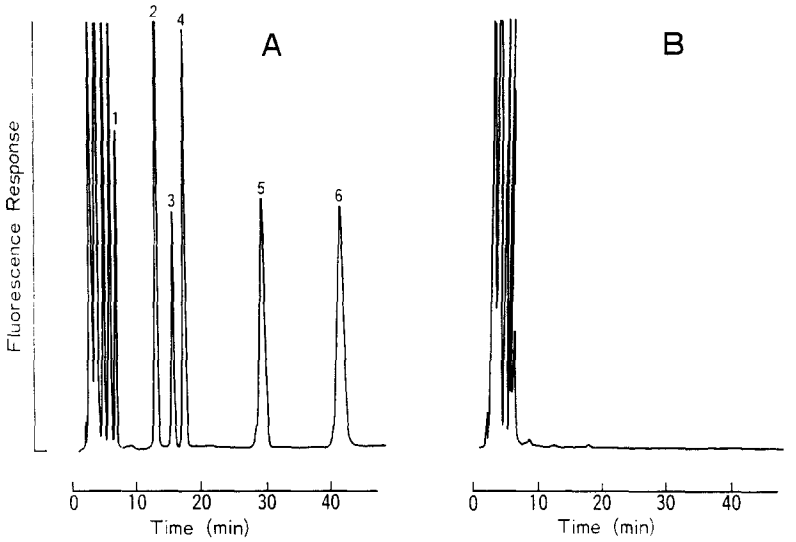

Fig. 5. Chromatographic separation of BrMDC derivatives of commercially available acidic NSAIDs. (A) Authentic NSAID mixture. Peaks: 1 = aspirin (25.2); 2 = loxoprofen (37); 3 = ketoprofen (72); 4 = naproxen (197); 5 = flurbiprofen (37); 6 = ibuprofen (44 pmol per injection). (B) Blank of reaction mixture containing only reagents. HPLC conditions: mobile phase, acetonitrile–water (50:50) containing 1% acetic acid; detector sensitivity, 2; other conditions in Fig. 4.

pharmacokinetically interesting concentration range (1–10 μg/ml) were adequately separated under the same isocratic conditions, as shown in Fig. 5.

Neither indomethacin nor mefenamic acid were completely esterified with BrMDC, even when a large amount of reagent was used, so the method seems inapplicable to their trace analysis, especially in biological specimens. The reason for this extremely low reactivity was suspected to be that each of the two compounds possessed a weakly basic nitrogen atom, which might prevent the formation of an anionic intermediate in aprotic solvents such as another carboxylic compound. We also ascertained that BrMDC could form corresponding ester derivatives with a variety of other substituted carboxylic acids, such as branched fatty acids, unsaturated acids, aryl acids, diacids and N-acylamino acids, but did not react with amino acids and peptides.

In conclusion, BrMDC might be applicable to the trace analysis of several series of acid compounds with satisfactory accuracy and reliability with slight modifications of the chromatographic condition. It is expected that its high sensitivity may provide a much more precise knowledge of biologically important organic acids, *e.g.*, as a diagnostic technique for inherited metabolic diseases. The reagent is now being applied to the determination of prostaglandins, which are produced enzymatically from arachidonic acid by bovine seminal microsome preparations, and to some NSAIDs and their metabolites in biological fluids for pharmacokinetic studies.

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