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3-BROMOMETHYL-6,7-DIMETHOXY-1-METHYL-2(1*H*)-QUINOXALINONE AS A NEW FLUORESCENCE DERIVATIZATION REAGENT FOR CARBOXYLIC ACIDS IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

3-Bromomethyl-6,7-dimethoxy-1-methyl-2(1*H*)-quinoxalinone was found to be a selective and highly sensitive fluorescence derivatization reagent for carboxylic acids in high-performance liquid chromatography. Its reactivity was investigated for various linear C_3–C_{20} saturated fatty acids. The reagent reacts with the fatty acids in acetonitrile in the presence of 18-crown-6 and potassium carbonate to produce the corresponding fluorescent esters, which can be separated on a reversed-phase column, Radial-Pak C_{18} cartridge, with gradient elution using 57–100% (v/v) aqueous methanol; the detection limits for the acids were 0.3–1 fmol for an injection volume of 5 μl. The reagent also reacts with unsaturated fatty, dicarboxylic, aromatic carboxylic and hydroxycarboxylic acids and acidic nucleotides to form fluorescent derivatives. α-Keto acids and α-amino acids do not give fluorescent derivatives under these conditions.

INTRODUCTION

Many fluorescence derivatization agents have been developed for the determination of carboxylic acids by high-performance liquid chromatography (HPLC), *e.g.*, 4-hydroxymethyl-7-methoxy[1], 4-diazomethyl-7-methoxy[2], 4-bromomethyl-6,7-dimethoxy[3] and 4-bromomethyl-7-methoxycoumarins (Br-MMCs)[4–6], N,N′-dicyclohexyl-O-(7-methoxycoumarin-4-yl)methylisourea[7], 8-(chloromethyl)-[8], 9-(hydroxymethyl)-[9] and 9-aminophenanthrene[10], 9-anthryldiazomethane[11,12], 9,10-diaminophenanthrene[13], *p*-(9-anthroyl)phenacyl bromide[14], 1-aminoethyl-4-dimethylaminonaphthalene[15] and 1-bromoacetylpyrene[16]. HPLC methods with these reagents are fairly sensitive but, in general, do not permit the determination of carboxylic acids at sub-femtomol level. Of these reagents, Br-MMC may be most suit-

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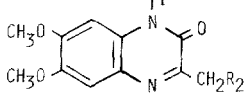
able, because it has the smallest molecular size. Thus, Br-MMC has been most widely used for the determination of carboxylic acids. However, the fluorescence intensities of the MMC derivatives of carboxylic acids are greatly influenced by the solvent. Furthermore, the fluorescence intensities of these derivatives depend on the kind of carboxylic acids[17]. 9,10-Diaminophenanthrene derivatives are also subject to solvent effects[13]. Thus, these disadvantages make it difficult to separate various carboxylic acids using gradient elution and determine them simultaneously at the same detector sensitivity.

Recently, a fluorimetric HPLC method for the determination of carboxylic acids using 4-bromomethyl-7-acetoxycoumarin (Br-MAC) has been developed[18]. It is based on the reaction of Br-MAC with carboxylic acids to give esters, which are then separated by HPLC. The derivatives in the eluates are hydrolyzed by an alkaline solution in a post-column system and the resulting fluorescence is detected. The method is very sensitive and does not suffer from the above disadvantages, but requires both pre- and post-column techniques.

We have reported that pyruvic acid reacts with 1,2-diamino-4,5-dimethoxybenzene (DDB) in acidic solution to form a highly fluorescent product, 6,7-dimethoxy-3-methyl-2(1*H*)-quinoxalinone (DQ)[19,20]. Recently, we found that 6,7-dimethoxy-1,3-dimethyl-2(1*H*)-quinoxalinone (DDQ), a methylation product of DQ, gives a more intense fluorescence. Thus 3-bromomethyl-6,7-dimethoxy-1-methyl-2(1*H*)-quinoxalinone (Br-DMEQ) was synthesized as a fluorescence derivatization reagent for carboxylic acids. In order to investigate its reactivity with carboxylic acids, linear saturated C_3–C_{20} fatty acids have been employed as model carboxyl compounds. Br-DMEQ reacts with the fatty acids in acetonitrile or acetone in the presence of 18-crown-6 and potassium carbonate to produce the corresponding fluorescent esters. The esters are separated on a reversed-phase column with gradient elution using aqueous methanol. The reactivity of Br-DMEQ with various carboxylic acids (unsaturated fatty, dicarboxylic, hydroxycarboxylic, aromatic carboxylic, α-keto and α-amino acids) and acidic nucleosides has also been studied.



R_1=R_2=H : DQ; R_1=CH_3, R_2=H : DDQ
R_1=CH_3, R_2=Br : Br-DMEQ

EXPERIMENTAL

Apparatus

Uncorrected fluorescence spectra and intensities were measured with a Hitachi MPF-2A spectrofluorimeter in 10 × 10 mm quartz cells; spectral bandwidths of 6 nm were used in both the excitation and emission monochromators.

Infrared (IR) spectra were recorded with a Shimadzu 430 spectrophotometer using potassium bromide pellets. ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were obtained with Hitachi R-22 and JEOL FX-100 spectrometers at 90 and

25.1 MHz, respectively, using a *ca.* 5% (w/v) solution of [$^2H_6$]dimethylsulphoxide (DMSO-d_6) containing tetramethylsilane as an internal standard. The splitting patterns were designated as follows: s, singlet; d, doublet; t, triplet; q, quartet. In ^{13}C NMR spectra, signals were assigned by both the complete decoupling and off-resonance decoupling techniques. Electron impact mass spectra were taken with a JEOL DX-300 spectrometer. The pH was measured with a Hitachi-Horiba M-7 pH meter at *ca.* 25°C. Uncorrected melting points were measured with a Yazawa melting point apparatus.

Materials and reagents

All chemicals were of analytical reagent grade, unless noted otherwise. Deionized and distilled water was used. Acetonitrile and acetone for the derivatization reaction were refluxed over calcium hydroxide for more than 10 h and distilled through a 120-cm column filled with helical coils. The solvents were used within 2 days, because they were contaminated with some fatty acids present in air. Propionic (C_3), butyric (C_4), valeric (C_5), caproic (C_6), caprylic (C_8), capric (C_{10}), lauric (C_{12}), myristic (C_{14}), palmitic (C_{16}), margaric (C_{17}), stearic (C_{18}) and arachidic (C_{20}) acids were purchased from Sigma (St. Louis, MO, U.S.A.).

Synthesis of Br-DMEQ

DQ was prepared by the reaction of pyruvic acid with DDB as described previously[20]. DQ (1 g, 4.5 mmol) in 50 ml of anhydrous methanol was treated with an ethereal diazomethane solution prepared by the established method[21]. The reaction mixture was evaporated to dryness *in vacuo*. The residue dissolved in 5 ml of ethyl acetate was purified by column chromatography (25 × 3.5 cm I.D.) on silica gel 60 (*ca.* 130 g, 70–230 mesh; Japan Merck, Tokyo, Japan) with *n*-hexane–ethyl acetate (1:3, v/v) as eluent, to give DDQ (380 mg, 1.6 mmol) as yellow needles, m.p. 170–171°C. IR $\nu_{max.}^{KBr}$ (cm^{-1}): 1640 (C=O); 1620 (aromatic C=N). ^1H NMR (DMSO-d_6): δ 2.39 (3H, s, C–CH_3); 3.61 (3H, s, N–CH_3); 3.83 and 3.94 (each 3H, each s, O–CH_3); 7.21 and 6.91 (each 1H, each s, aromatic H). ^{13}C NMR (DMSO-d_6): δ 20.7 (q), 29.1 (q), 55.9 (q), 56.2 (q), 97.5 (q), 110.6 (d), 126.1 (s), 128.0 (s), 145.6 (s), 150.9 (s), 153.8 (s), 154.3 (s). Analysis (%) calculated for $C_{12}H_{14}N_2O_3$: C, 61.53; H, 6.02; N, 11.96; found: C, 61.71; H, 6.11; N, 11.77. MS: *m/z* 234 (M^+); 219 ($M^+ - CH_3$); 163 ($M^+ - CH_3 - CH_3O$). DDQ was stable in the crystalline state for at least a year in daylight at room temperature.

To a solution of DDQ (350 mg, 1.5 mmol) in 3 ml of acetic acid placed in 20-ml screw-capped test-tube were added *ca.* 350 mg of anhydrous sodium acetate and 2 ml of 1.5 *M* bromine in acetic acid. The tube was tightly closed and heated at 100°C for *ca.* 15 min, then cooled. The precipitates formed on adding *ca.* 10 ml of diethyl ether were removed by filtration and washed two or three times with small portions of ether. The combined filtrates and washings were evaporated to dryness. The residue dissolved in 5 ml of ethyl acetate was chromatographed on silica gel 60 (*ca.* 130 g, 70–230 mesh, Japan Merck; column size, 25 × 3.5 cm I.D.) with ether. The main fraction was concentrated to dryness and the residue was recrystallized from *n*-hexane–ethyl acetate (1:1, v/v) to give Br-DMEQ (110 mg, 0.6 mmol) as yellow needles, m.p. 161–163°C. IR $\nu_{max.}^{KBr}$ (cm^{-1}): 1640 (C=O); 1620 (aromatic C=N). ^1H NMR (DMSO-d_6) :δ 3.71 (3H, s, N–CH_3); 3.93 and 4.01 (each 3H, each

s, O–CH_3); 4.62 (2H, s, CH_2–Br); 6.64 and 7.24 (each 1H, each s), aromatic H). ^{13}C NMR (DMSO-d_6) :δ 25.5 (t), 29.5 (q), 56.2 (q), 56.8 (q), 98.1 (q), 111.1 (d), 127.0 (s), 128.8 (s), 146.1 (s), 151.3 (s), 154.4 (s), 155.0 (s). Analysis (%) calculated for $C_{12}H_{13}BrN_2O_3$: C, 46.03; H, 4.18; N, 8.95; found: C, 46.40; H, 4.12; N, 8.63. MS: *m/z* 312 and 314 (M^+); 297 and 299 ($M^+ - CH_3$). Br-DMEQ was stable in the crystalline state for a year or longer when kept dry in the dark at room temperature. The reagent dissolved in acetonitrile could be used for more than a week when stored in a refrigerator at 5°C.

Derivatization procedure

To *ca.* 100 mg of finely powdered potassium carbonate placed in a PTFE screw-capped test-tube were added 0.5 ml of a test solution of fatty acids in acetonitrile, 0.25 ml each of 3.8 m*M* 18-crown-6 and 0.8 m*M* Br-DMEQ (both in acetonitrile). The tube was tightly closed and heated at 80°C for 20 min in the dark. After cooling, 5 μl of the reaction mixture were injected into the chromatograph. For the reagent blank, 0.5 ml of acetonitrile in place of 0.5 ml of a test solution were subjected to the same procedure.

HPLC apparatus and conditions

A Hitachi 655A high-performance liquid chromatograph equipped with a high-pressure sample injector and a Hitachi F1000 fluorescence spectrometer equipped with a 12-μl flow cell operating at the excitation wavelength of 370 nm and the emission wavelength of 450 nm were used. The column was a Radial-Pak C_{18} cartridge (100 × 4 mm I.D., particle size 10 μm; Waters Assoc., Milford, MA, U.S.A.). It can be used for more than 1000 injections with only a small decrease in the theoretical plate number. The column temperature was ambient (20–27°C). For the separation of the DMEQ derivatives of fatty acids, a gradient elution with 57–100% (v/v) aqueous methanol (Fig. 1) was carried out by using a Hitachi 833A solvent-

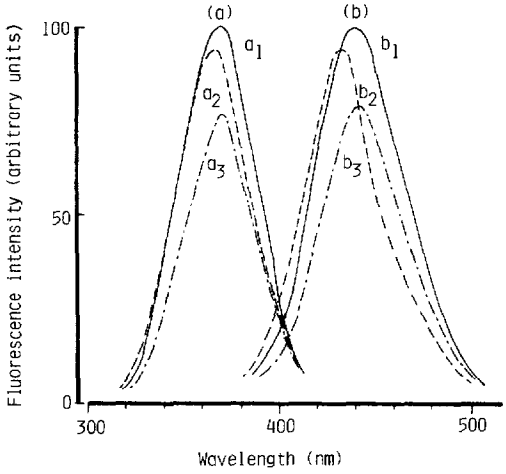


Fig. 1. Fluorescence excitation (a) and emission (b) spectra of DDQ (1.0 nmol/ml) in methanol (a_1, b_1), acetonitrile (a_2, b_2) and water (a_3, b_3).

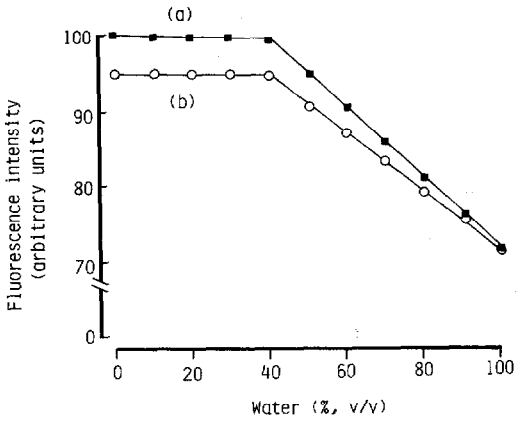


Fig. 2. Effect of water concentration in aqueous methanol (a) and aqueous acetonitrile (b) on the fluorescence intensity of DDQ (1.0 nmol/ml). The fluorescence intensity was measured at the excitation and emission maxima.

gradient device. The flow-rate was 2.0 ml/min. Peak areas were measured on a Waters QA-1 Data System.

RESULTS AND DISCUSSION

Fluorescence properties of DDQ and Br-DMEQ

The fluorescence properties of DDQ and Br-DMEQ in methanol, acetonitrile, water and their mixtures, which have widely been used as mobile phases in reversed-phase chromatography, were examined to find a suitable mobile phase for the HPLC separation of DMEQ derivatives of the fatty acids.

The fluorescence excitation (maximum, 370 nm) and emission (maximum, 450 nm) spectra of DDQ in methanol were practically identical with those in water (Fig. 1). The maxima in aqueous methanol were independent of the concentration of water. On the other hand, the fluorescence excitation and emission maxima (363 nm and 429 nm, respectively) in acetonitrile were slightly blue-shifted compared with those

TABLE I

FLUORESCENCE INTENSITY OF DDQ (1.0 nmol/ml) DISSOLVED IN THE BRITTON-ROBINSON BUFFER AT VARIOUS pH VALUES

The fluorescence intensity was measured at the excitation and emission maxima. The intensity at pH 7.0 was taken as 100.

pH	*Relative fluorescence intensity*	*pH*	*Relative fluorescence intensity*
2.0	52	6.0	96
3.0	84	7.0–12.0	100
4.0	92	7.0–12.0	
5.0	95	7.0–12.0	

in methanol and water (Fig. 1). The fluorescence intensity was almost maximum and constant at water concentrations of 0–40% (v/v) in both aqueous methanol and acetonitrile, but was slightly decreased in proportion to the water concentration at >40% (v/v) (Fig. 2). The most intense and constant fluorescence of DDQ occurred in neutral and alkaline solution (Table I). These results suggest that aqueous methanol is suitable as a mobile phase in reversed-phase chromatography of DMEQ derivatives of fatty acids with gradient elution.

The fluorescence excitation and emission spectra of Br-DMEQ in methanol and acetonitrile were almost identical to those of DDQ. However, the fluorescence intensity of Br-DMEQ in these solvents was much lower than that of DDQ, probably due to the heavy atom effect of bromine; the intensity of Br-DMEQ was *ca.* one tenth of that of DDQ.

Separation of DMEQ derivatives of fatty acids

The simultaneous separation of DMEQ derivatives of (C_3–C_{20}) fatty acids was studied on a reversed-phase column, Radial-Pak C_{18} cartridge, with aqueous methanol. Methanol–water (95:5, v/v) gave a complete separation of DMEQ derivatives of (C_{10}–C_{20}) long chain fatty acids, though the peaks for (C_3–C_8) short chain fatty acids were almost overlapped with that for Br-DMEQ. When methanol–water (57:43, v/v) was used, DMEQ derivatives of the short chain fatty acids were completely separated, but those of the long chain fatty acids were eluted late with peak broad-

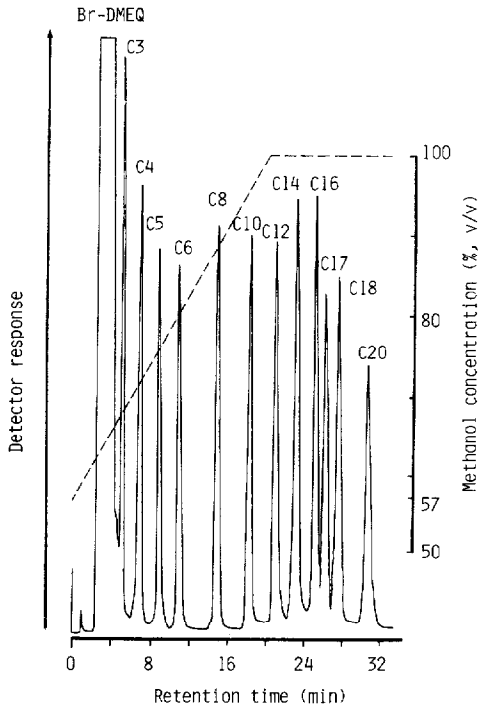


Fig. 3. Chromatogram of DMEQ derivatives of C_3–C_{20} acids. A portion (0.5 ml) of a standard mixture of the acids (each 1.0 nmol/ml) was treated according to the described procedure.

TABLE II

RELATIONSHIPS BETWEEN THE CARBON NUMBERS OF LINEAR SATURATED FATTY ACIDS AND THE PEAK AREAS IN THE CHROMATOGRAMS

A portion (0.5 ml) of a standard mixture of C_3–C_{20} acids (each 1.0 nmol/ml) was treated according to the described procedure. The peak area obtained with propionic acid was taken as 100.

Carbon number	*Peak area*	*Carbon number*	*Peak area*
3	100	12	84
4	92	14	82
5	80	16	75
6	83	17	77
8	85	18	75
10	82	20	73

ening. Gradient elution with aqueous methanol served to minimize the elution times and also sharpen the peaks. Fig. 3 shows a typical chromatogram obtained with a gradient of methanol between 57 and 100% (v/v) in the mobile phase. All the peaks were completely separated within 32 min. The change in methanol concentration actually had no effect on the fluorescence excitation and emission maximum wavelengths and intensities of the DMEQ derivatives of all the fatty acids; the spectra were virtually identical with those of DDQ.

The peak areas were slightly decreased for fatty acids with higher carbon numbers (Table II). This means that the individual fatty acids may be determined at almost the same sensitivity. On the other hand, the fluorescence intensity of MMC derivatives of fatty acids greatly varies depending on the individual fatty acids[17].

Derivatization conditions

The conditions were examined using a mixture of the fatty acids (each 1.0 nmol/ml). The most intense peaks were obtained at concentrations greater than *ca.* 3.0 m*M* of the reagent solution for all the fatty acids; 3.8 m*M* was used in subsequent work.

18-Crown-6 and potassium carbonate have been used to facilitate the derivatization of fatty acids with reagents having bromomethyl groups[1–3,6,14,16,18]. Maximum and constant peak areas could be attained at 18-crown-6 concentrations in the solution in the range 2.0–5.0 *M* for C_3–C_5 short chain fatty acids, and in the range 3.0–5.0 m*M* for C_{10}–C_{20} long chain fatty acids; 10 m*M* was selected as optimum for the simultaneous derivatization of all the fatty acids. The peak areas for the fatty acids were maximal and constant at amounts of potassium carbonate higher than 20 and 150 mg for the short and the long chain fatty acids, respectively; 100 mg were employed in subsequent work.

The derivatization reaction of butyric acid with Br-DMEQ apparently occurred even at moderately low temperatures; higher temperatures allowed the fluorescence to develop more rapidly (Fig. 4). However, at 100°C, the peak areas were decreased at heating times of 10 min or longer. At 80°C, the peak areas for all the fatty acids were almost maximal after heating for 20 min. Thus, heating for 20 min at 80°C was employed in subsequent work. The derivatization reaction proceeded

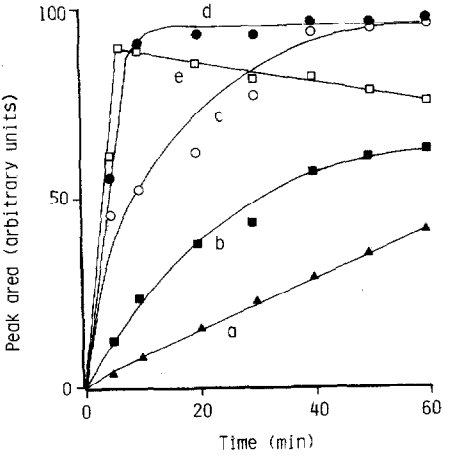


Fig. 4. Effect of reaction time and temperature on the peak area. Portions (0.5 ml) of butyric acid (1.0 nmol/ml) were treated according to the described procedure. Temperatures: a, 20; b, 37; c, 50; d, 80; e, 100°C.

effectively in acetonitrile or acetone; acetonitrile was utilized because of its easy purification. The DMEQ derivatives in the final mixture were stable for at least 72 h in daylight at room temperature.

Precision, calibration and detection limit

The precision was established by repeated determination using a standard mix-

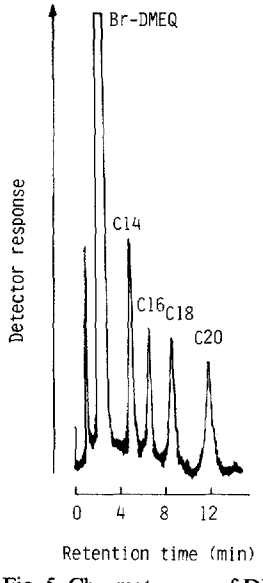


Fig. 5. Chromatogram of DMEQ derivatives of C_{14}–C_{20} acids. A portion (0.5 ml) of a standard mixture of the acids (each 1.0 nmol/ml) was treated according to the derivatization procedure. The reaction mixture was diluted 1000 times in acetonitrile and applied onto the chromatograph. HPLC conditions: mobile phase, methanol–water (95:5, v/v); for the other conditions, see Experimental.

TABLE III

COMPOUNDS WHICH REACT WITH Br-DMEQ TO PRODUCE FLUORESCENT ESTERS, AND THEIR RETENTION TIMES

Portions (0.5 ml) of 1.0 nmol/ml solutions of the compounds were treated according to the described procedure.

Compound	*Retention time (min)*	*Compound*	*Retention time (min)*
Unsaturated fatty acids		*Aromatic carboxylic acids*	
Myristoleic acid	20.7	Benzoic acid	9.4
Palmitoleic acid	22.2	Salicylic acid	7.1
Linolenic acid	22.2	*p*-Aminobenzoic acid*	17.0
Arachidonic acid	22.3	*Nucleosides*	
Linoleic acid	22.4	Uridine	13.7
Oleic acid	23.8	Deoxyuridine	14.1
Dicarboxylic acids		Thymidine	15.2
Oxalic acid	1.0	*Miscellaneous*	
Malonic acid	1.3	Imidazole-4-acetic acid	8.3
Succinic acid	1.5	1-Methyl-4-imidazoleacetic acid	7.9
Adipic acid	1.8	Glucuronic acid*	10.2
Hydroxycarboxylic acids			
Lactic acid	3.7		
Malic acid	1.2		

* Linear gradient elution with aqueous methanol during 30 min [methanol concentration (%, v/v): initial, 30; final, 70] was used for the HPLC separation of DMEQ derivatives of these compounds. When the mobile phase in the described procedure was used, DMEQ derivatives of these compounds were not separated from Br-DMEQ.

ture of fatty acids (each 2.0 pmol/ml). The coefficients of variation did not exceed 2.0% for all the fatty acids examined ($n = 10$ in each case).

The relationships between the peak areas and the amounts of the individual fatty acids were linear from 50 fmol to at least 100 pmol per injection volume (5 μl).

To ascertain the sensitivity, a standard mixture of C_{14}–C_{20} acids was treated according to the described procedure and the reaction mixture was successively diluted in acetonitrile and subjected to HPLC. Fig. 5 shows a chromatogram in which each peak corresponds to 5.0 fmol of fatty acid. It indicates that the detection limits for the fatty acids are 0.3–1 fmol at a signal-to-noise ratio of 2. The sensitivity is at least 100 times higher than that of the method with Br-MMC, and *ca.* 10 times higher than that with Br-MAC.

Reaction of Br-DMEQ with compounds other than linear saturated fatty acids

Many unsaturated fatty, dicarboxylic, hydroxycarboxylic and aromatic carboxylic acids and acidic nucleosides react with Br-DMEQ under the derivatization conditions described, to produce fluorescent derivatives which can be separated by HPLC. The retention times for the DMEQ derivatives of the compounds are shown in Table III. α-Keto acids (pyruvic, α-ketoglutaric, α-ketocaproic, α-ketovaleric and phenylpyruvic acids) and seventeen different α-amino acids did not fluoresce. Other substances such as alcohols, sugars, amines, aldehydes, ketones, phenols and sulphhydryl compounds gave no fluorescent derivatives under these conditions. These

observations suggest that the reagent is selective for carboxylic acids and compounds with active imino hydrogen. It is considered that the reactivity of Br-DMEQ is very similar to that of Br-MAC and Br-MMC.

Br-DMEQ as a fluorescence derivatization reagent should be useful for the detection of carboxylic acids of biologically importance at the femtomol level by HPLC using gradient elution.

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