[Chem. Pharm. Bull. 36(6)2263—2266(1988)]

3-Bromomethyl-6,7-methylenedioxy-1-methyl-2(1*H*)-quinoxalinone as a Highly Sensitive Fluorescence Derivatization Reagent for Carboxylic Acids in High-Performance Liquid Chromatography

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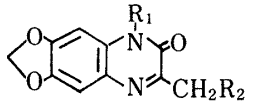
(Received November 27, 1987)

3-Bromomethyl-6,7-methylenedioxy-1-methyl-2(1*H*)-quinoxalinone was found to be a highly sensitive fluorescence derivatization reagent for carboxylic acids in high-performance liquid chromatography. Its reaction conditions were optimized 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 derivatives, which can be separated on a reversed-phase column, Radial-Pak C_{18} cartridge, by gradient elution with 35—100% (v/v) aqueous methanol. The detection limits for the acids were 0.2—0.8 fmol for an injection volume of 10 μl.

Keywords——3-bromomethyl-6,7-methylenedioxy-1-methyl-2-(1*H*)-quinoxalinone; carboxylic acid; fluorogenic reagent; high-performance liquid chromatography

Various fluorescence derivatization reagents have been proposed for the determination of carboxylic acids by high-performance liquid chromatography (HPLC), as cited in our previous paper.[1] Among the reagents, 3-bromomethyl-6,7-dimethoxy-1-methyl-2(1*H*)-quinoxalinone (Br-DMEQ) is one of the most sensitive and practically useful fluorescence derivatization reagents for carboxylic acids in HPLC. In the present paper, we show that 3-bromomethyl-6,7-methylenedioxy-1-methyl-2(1*H*)-quinoxalinone (Br-MMEQ, Chart 1) is an even more sensitive fluorescence derivatization reagent for carboxylic acids in HPLC.



$R_1 = R_2 = H$: MQ
$R_1 = CH_3$; $R_2 = H$: MMQ
$R_1 = CH_3$; $R_2 = Br$: Br-MMEQ

Chart 1

Experimental

Reagents and Solutions——All chemicals were of analytical reagent grade, unless otherwise noted. Deionized and distilled water was used. Acetonitrile and acetone for the derivatization reaction were purified as described previously.[1] Linear C_3–C_{20} saturated fatty acids were purchased from Sigma (St. Louis, Mo., U.S.A.).

Apparatus——Uncorrected fluorescence spectra and intensities were measured with a Hitachi 650—60 spectrofluorimeter in 10 × 10 mm quartz cells; spectral bandwidths of 10 nm were used in both the excitation and emission monochromators. Infrared (IR) spectra were recorded with a Shimadzu 430 IR spectrophotometer, in KBr pellets. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on a Hitachi R-90 spectrometer at 90 MHz in dimethyl sulfoxide-d_6 with tetramethylsilane as an internal standard. Electron impact mass spectra (MS) 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.

Synthesis of Br-MMEQ——6,7-Methylenedioxy-3-methyl-2(1*H*)-quinoxalinone (MQ 1 g, 4.9 mmol; Chart 1), prepared by the reaction of pyruvic acid with 1,2-diamino-4,5-methylenedioxybenzene as described previously,[2] was methylated with diazomethane according to the established method[3] to give 1,3-dimethyl-6,7-methylenedioxy-2(1*H*)-quinoxalinone (MMQ, Chart 1) (370 mg, 1.7 mmol) as pale yellow needles, mp 223—225°C. IR ν_{max}^{KBr} cm^{-1}: 1650 (lactam C=O), 1615 (aromatic C=N). ^{1}H-NMR δ: 2.37 (3H, s, C-CH_3), 3.58 (3H, s, N-CH_3), 6.15 (2H, s, OCH_2O), 7.17, 7.21 (1H each, s each, aromatic protons). MS *m/z*: 218 (M^+, base peak). *Anal.* Calcd for $C_{11}H_{10}N_2O_3$: C, 60.55; H, 4.62; N, 12.84. Found: C, 60.34; H, 4.84; N, 12.67.

MMQ (350 mg, 1.6 mmol) was treated in the same way as described for the synthesis of Br-DMEQ[1] to give Br-MMEQ (200 mg; yield, 14%) as yellow needles, mp 170 °C (dec.).IR ν_{max}^{KBr} cm^{-1}: 1645 (lactam C=O), 1615 (aromatic C=N). ^{1}H-NMR δ: 3.63 (3H, s, NCH_3), 4.61 (2H, s, CH_2Br), 6.21 (2H, s, OCH_2O), 7.25, 7.29 (1H, each, s each, aromatic protons). MS *m/z*: 296, 298 (M^+), 217 ($M^+ - Br$, base peak). *Anal.* Calcd for $C_{11}H_9BrN_2O_3$: C, 44.47; H, 3.05; N, 9.43. Found: C, 44.14; H, 3.10; N, 9.26.

Br-MMEQ 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——About 10 mg of finely powdered potassium carbonate was placed in a screw-capped test tube, and 200 μl of a test solution of fatty acids in acetonitrile and 100 μl each of 3.8 mM 18-crown-6 and 3 mM Br-MMEQ (both in acetonitrile) were added. The tube was tightly closed and heated at 80 °C for 15 min in the dark. After cooling, 10 μl of the reaction mixture was injected into the chromatograph. For the reagent blank, 200 μl of acetonitrile in place of 200 μl of test solution was subjected to the same procedure.

HPLC Apparatus and Conditions——A Hitachi 655A high-performance liquid chromatograph equipped with a sample injector (10-μl loop) was used. A Hitachi F1000 fluorescence spectromonitor equipped with a 12-μl flow cell was operated at an excitation wavelength of 363 nm and an emission wavelength of 437 nm. The column was a Radial Pak C_{18} cartridge (100 × 8 mm i.d., particle size 5μm; Waters Assoc., Milford, Mass., U.S.A.). A Hitachi 833A solvent gradient device was used for gradient elution with 35—100% (v/v) aqueous methanol at a flow rate of 2 ml/min (see Fig. 3). The column temperature was ambient (*ca.* 25 °C). Peak areas were measured on a Waters QA-1 Data System.

Results and Discussion

Fluorescence Properties of MMQ and Br-MMEQ

The fluorescence properties of MMQ and Br-MMEQ in methanol, acetonitrile, water

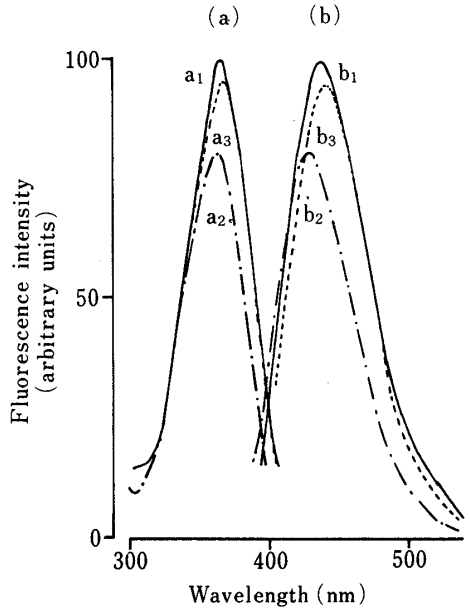


Fig. 1. Fluorescence Excitation and Emission Spectra of MMQ (1 nmol/ml) in Methanol, Acetonitrile and Water

a, excitation spectra; b, emission spectra.
a_1 and b_1, methanol; a_2 and b_2, acetonitrile; a_3 and b_3, water.

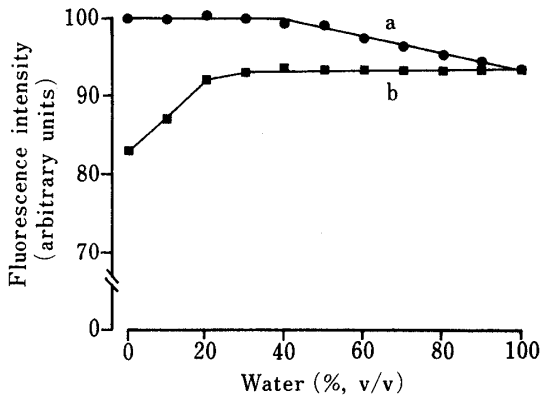


Fig. 2. Effect of Water Concentration in Aqueous Methanol and Aqueous Acetonitrile on the Fluorescence Intensity of MMQ (1 nmol/ml)

The fluorescence intensity was measured at the excitation and emission maxima.
a, aqueous methanol; b, aqueous acetonitrile.

and their mixtures, which have been widely used as mobile phases in reversed-phase chromatography, were examined to find a suitable mobile phase for the HPLC separation of MMEQ derivatives of the fatty acids.

The fluorescence excitation (maximum, 363 nm) and emission (maximum, 437 nm) spectra of MMQ in methanol were practically identical with those in water (Fig. 1). On the other hand, the fluorescence excitation and emission maxima (361 and 429 nm, respectively) in acetonitrile were slightly blue-shifted and the intensity in this solvent was slightly low, as compared with those in methanol and water (Fig. 1). The fluorescence intensity was slightly decreased in proportion to the water concentration in the range of 40—100% (v/v) in aqueous methanol, though that in aqueous acetonitrile remained constant in this water concentration range (Fig. 2). These results suggest that aqueous methanol is suitable as a mobile phase in reversed-phase chromatography of MMEQ derivatives of fatty acids with gradient elution.

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

Separation of MMEQ Derivatives of Fatty Acids

The separation of MMEQ derivatives of (C_3—C_{20}) fatty acids was studied on a reversed-phase column, Radial Pak C_{18} cartridge, with aqueous methanol. Simultaneous separation was attained by gradient elution with methanol between 35 and 100% (v/v) in the mobile phase. Figure 3 shows a typical chromatogram obtained with twelve fatty acids. All the peaks were completely separated within 60 min. The change in methanol concentration actually had no effect on the fluorescence excitation and emission maximum wavelengths or intensities of the MMEQ derivatives of all the fatty acids and their spectra were virtually identical with those of MMQ.

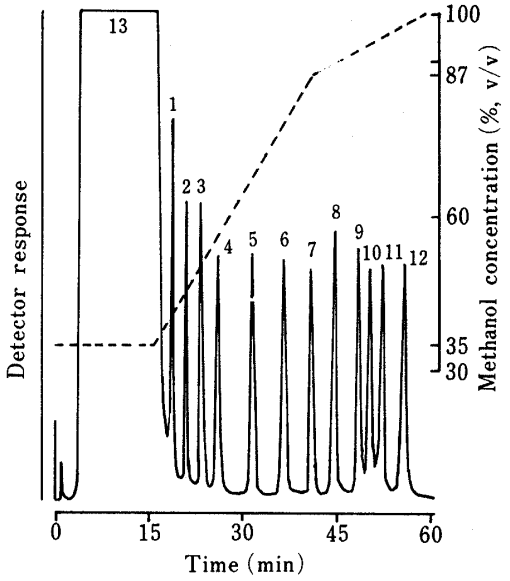


Fig. 3. Chromatogram of the MMEQ Derivatives of Fatty Acids

A portion (200 μl) of a mixture of twelve fatty acids (1 nmol/ml each) was treated according to the standard procedure.

Peaks: 1, propionic acid; 2, butyric acid; 3, valeric acid; 4, caproic acid; 5, caprylic acid; 6, capric acid; 7, lauric acid; 8, myristic acid; 9, palmitic acid; 10, margaric acid; 11, stearic acid; 12, arachidic acid; 13, reagent blank.

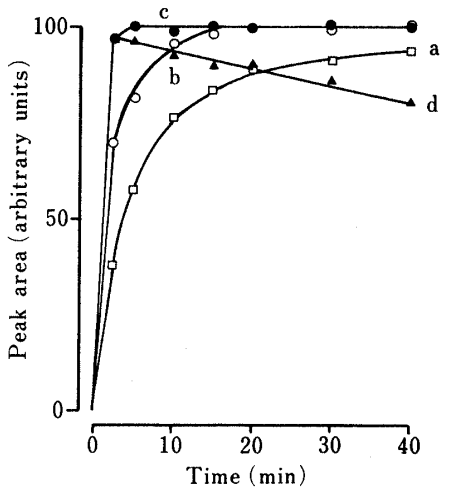


Fig. 4. Effect of Reaction Temperature and Time on the Peak Area in the Case of Arachidic Acid

Portions (200 μl) of arachidic acid (1 nmol/ml) were treated according to the standard procedure, except for temperature.

Temperature: a, 37 °C; b, 50 °C; c, 80 °C; d, 100 °C.

The peak areas were almost the same for all the fatty acids. Therefore, the individual fatty acids can be determined at almost the same sensitivity.

Derivatization Conditions

The derivatization conditions were examined using a mixture of the fatty acids (1.0 nmol each/ml). Br-MMEQ gave the most intense peaks at concentrations greater than *ca.* 2.0 mM in the solution (acetonitrile); 3.0 mM was used as a sufficient concentration. Maximum and constant peak areas were attained at 18-crown-6 concentrations in the solution (acetonitrile) in the range of 2.0—5.0 mM; 3.8 mM was selected for the procedure. The peak areas due to the fatty acids were maximal and constant at amounts of potassium carbonate higher than 5 mg; 10 mg was employed in the procedure.

The derivatization reaction of arachidic acid with Br-MMEQ apparently occurred even at 37 °C; higher temperatures allowed the fluorescence to develop more rapidly (Fig. 4). However, at 100 °C, the peak areas were decreased at heating times of 5 min or longer. At 80 °C, the peak areas for all the fatty acids were almost maximal after heating for 5 min. Thus, heating for 15 min at 80 °C was employed in the present procedure. The derivatization reaction proceeded effectively in acetonitrile or acetone; acetonitrile was utilized because of its easy purification. The MMEQ derivatives in the final mixture were stable for at least 2 d in daylight at room temperature.

Calibration, Detection Limit and Reaction of Br-MMEQ with Compounds Other than Linear Fatty Acids

The calibration curves for the individual fatty acids showed linear relationships between the peak areas and the concentrations (50 fmol—100 pmol/injection volume) of the acids. The limits of detection of the fatty acids were 0.2—0.8 fmol/injection volume (10 μl) at a signal-to-noise ratio of 2. The sensitivity is *ca.* 1.6 times higher than that of the method with Br-DMEQ.

Many other acidic compounds, dicarboxylic, hydroxycarboxylic, aromatic carboxylic and unsaturated fatty acids and acidic nucleosides, reacted with Br-MMEQ under the derivatization conditions described, to produce the corresponding fluorescent derivatives, which can be separated in HPLC by gradient elution with aqueous methanol. α-Keto acids, 17 different L-amino acids, alcohols, sugars, amines, aldehydes, ketones, phenols and sulfhydryl compounds gave no fluorescent derivatives under these conditions.

Br-MMEQ as a fluorescence derivatization reagent is more sensitive than Br-DMEQ and should be useful for the detection of carboxylic acids of biological importance at the subfemtomol level by HPLC.

References

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