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. Among the reagents, 6,7-dimethoxy-1-methyl-2-oxo-1,2-dihydroquinolin-3-ylpropionohydrazide (DMEQ-hydrazide) is one of the most sensitive and practically useful fluorescence derivatization reagents for carboxylic acids in HPLC. In the present paper, we show that 6,7-methylenedioxy-1-methyl-2-oxo-1,2-dihydroquinolin-3-ylpropionohydrazide (MMEQ-hydrazide, Fig. 1) is an even more sensitive fluorescence derivatization reagent for carboxylic acids in HPLC.

**Experimental**

**Chemicals**

All chemicals were of analytical reagent grade, unless otherwise noted. Water was passed through an Elix 3 and a Milli-Q systems (Japan Millipore Ltd., Tokyo Japan). Linear C₆ - C₂₀ saturated fatty acids and valproic acid were purchased from Sigma (St. Louis, MO, USA). 1,2-Diamino-4,5-methylenedioxybenzene (DMB) was prepared as described previously; it is now commercially available from Dojindo Laboratories (Kumamoto, Japan).

**Apparatus**

Uncorrected fluorescence spectra and intensities were measured with a Hitachi 650-60 spectrofluorometer in 10×10 mm quartz cells; the spectral bandwidths of 10 nm were used in both the excitation and emission monochromators. Proton nuclear magnetic resonance (¹H-NMR) spectra were recorded on a JEOL JNM-GX 400 spectrometer at 400 MHz in dimethyl sulfoxide-d₆ with tetramethylsilane as an internal standard (abbreviations used: s, singlet; t, triplet; and m, multiplet). Fast atom bombardment (FAB) mass spectra (MS) were taken with a JEOL JMS-HX 110 spectrometer. The pH was measured with a Fisher Scientific accumet model 15 pH meter at ca. 25°C. Uncorrected melting points were measured with a Yawata melting point apparatus.

**Synthesis of MMEQ-hydrazide**

6,7-Methylenedioxy-1-methyl-2-oxo-1,2-dihydroquinolin-3-ylpropionic acid (MQ, Fig. 1) (1.3 g, 5 mmol), prepared by the reaction of α-ketoglutaric acid with DMB as described previously, was methylated with an ethereal diazomethane solution prepared by the established method to give methyl 6,7-methylenedioxy-1-methyl-2-oxoquinolin-3-ylpropionate (MMEQ-hydrazide, Fig. 1) (780 mg, 2.7 mmol) as pale yellow needles, mp 149 – 151°C. ¹H-NMR: δ 2.75 (t, J = 7.3 Hz, 2H), 3.02 (t, J = 7.3 Hz, 2H), 3.59 (s, 3H), 6.16 (s, 2H), 7.18 (s, 1H), 7.20 (s, 1H). MS m/z: 291(MH⁺, base peak), 259 (MH-OCH₃⁺).

Anal. Calcd for C₁₄H₁₄N₂O₅: C, 57.93; H, 4.86; N, 9.65. Found: C, 58.05; H, 4.91; N, 9.81%.

MMEQ (750 mg, 2.6 mmol) was treated in the same way as described for the synthesis of DMEQ-hydrazide to give MMEQ-hydrazide (560 mg; yield, 75%) as pale yellow needles, mp 237 – 239°C. ¹H-
NMR: δ 2.47 (t, J=7.3 Hz, 2H), 2.98 (t, J=7.3 Hz, 2H), 3.59 (s, 3H), 6.16 (s, 2H), 7.19 (s, 1H), 7.20 (s, 1H). MS m/z: 291(MH⁺, base peak), 259 (MH–OCH₃⁺).


MMEQ-hydrazide was stable in the crystalline state for a year or longer when kept dry in the dark at room temperature. The reagent dissolved in N,N-dimethylformamide and kept in the dark could be used for more than a month.

Reaction of valproic acid with MMEQ-hydrazide
MMEQ-hydrazide (200 mg), valproic acid (100 mg) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, 100 mg) were dissolved in 100 ml of 1% pyridine in methanol and the mixture was allowed to stand at room temperature for 2 h. The mixture was evaporated to dryness under reduced pressure. The residue, dissolved in a small amount of ethyl acetate, was chromatographed on a silica gel 60 column (25×2 cm i.d., 60 g, 70 – 230 mesh) with the same solvent. The main fraction was evaporated to dryness and the residue was recrystallized from ethanol to give product I (Fig. 1)(136 mg; yield, 47%) as white needles, mp 237–239˚C. δH-NMR: δ 0.84 (s, 3H·2), 1.22 – 1.45 (m, 8H), 2.58 (t, J=7.3 Hz, 2H), 3.00 (t, J=7.3 Hz, 2H), 3.60 (s, 3H), 6.16 (s, 2H), 7.20 (s, 1H), 7.24 (s, 1H), 9.62 (s, 1H), 9.72 (s, 1H). MS m/z: 417 (MH⁺), 300 (MH–C₇H₁₅⁺, base peak). Anal. Calcd for C₂₁H₂₈N₄O₅: C, 60.56; H, 6.78; N, 13.45. Found: C, 60.28; H, 6.82; N, 13.32%.

Derivatization procedure
To 100 μl of a test solution of fatty acids in water were added 50 μl each of 0.5 M EDC and 10% pyridine (both in water) and 100 μl of 10 mM MMEQ-hydrazide in N,N-dimethylformamide. The mixture was warmed at 37˚C for 60 min and then a portion (10 μl) was injected into the chromatograph. For the reagent blank, 100 μl of water in place of the test solution were subjected to the same procedure.

HPLC apparatus and conditions
A Shimadzu LC-10AT high-performance liquid chromatograph equipped with a sample injector (10-μl loop) and a Shimadzu RF-10Axl fluorescence spectrometer equipped with a 12-μl flow cell operated at an excitation wavelength of 365 nm and an emission wavelength of 440 nm were used. The column temperature was kept at 30˚C with a Shimadzu CTO-10A column oven. For the separation of the MMEQ derivatives of fatty acids, a gradient elution was carried out by using a Shimadzu LC-10AT solvent gradient device. A YMC-Pack ODS-A column (250×4.6 mm i.d., 5 μm; YMC Co., Ltd, Kyoto, Japan), TS Kel 180T column (250×4.6 mm i.d., 5 μm; Tosoh, Tokyo, Japan) and Biofine RPC-SC 18B column (250×4.6 mm i.d., 5 μm; JASCO, Tokyo, Japan) were used. Other chromatographic conditions are as given in the figure captions.

Results and Discussion

Fluorescence properties of product I (MMEQ-VPA)
The fluorescence properties of the product given by the reaction of valproic acid with MMEQ-hydrazide were examined to find a suitable mobile phase for the HPLC separation of MMEQ derivatives of the fatty acids. Product I was confirmed as MMEQ-VPA (Fig. 1) by the elemental analysis data and by the FAB-MS and δH-NMR spectral data.

The fluorescence excitation (maximum, 365 nm) and emission (maximum, 440 nm) spectra of product I in methanol were practically identical with those in water and acetonitrile (Fig. 2). On the other hand, the fluorescence intensity in acetonitrile was lower than those in methanol and water. Thus, the intensities in aqueous acetonitrile were dependent on the concentration of water, but almost maximum at water concentration >50% (Fig. 3). These results suggest that aqueous methanol is suitable as a mobile phase in reversed-phase chromatography of the MMEQ derivatives of the fatty acids with gradient elution.

Derivatization conditions
The conditions were examined using a mixture of the fatty acids (1.0 nmol/ml each). MMEQ-hydrazide gave the most intense peaks at concentrations greater than ca. 5.0 mM in the solution (N,N-dimethylformamide); 10 mM was used in the procedure.

EDC and pyridine were used to facilitate the derivatization of fatty acids with MMEQ-hydrazide. Maximum and constant peak heights were attained at...
pyridine concentrations in the solution in the range 7 – 15%; 10% was selected as optimum. The peak heights for the acids were maximum and constant at concentrations of EDC higher than 0.2 M; 0.5 M was employed.

The derivatization reaction of fatty acids with MMEQ-hydrazide apparently occurred even at 0°C; higher temperatures allowed the fluorescence to develop more rapidly. However, at 60°C the peak heights decreased. At 37°C, the peak heights for all the fatty acids were almost maximum after standing for 40 min. Hence, the solution was allowed to stand at 37°C for 60 min in the procedure. The MMEQ derivatives in the final mixture were stable for at least 24 h in daylight at room temperature. The yield of fluorescent derivative from valproic acid under the conditions employed was found to be 54.7% by comparing the value of the peak height for valproic acid with that of product I.

Separation of MMEQ derivatives of fatty acids

The simultaneous separation of MMEQ derivatives of C6 – C20 fatty acids was achieved on a reversed-phase column, YMC-Pack ODS-A, by gradient elution with mixture of methanol and 20 mM acetic acid-ammonium acetate buffer (pH 3.5). Figure 4 shows a typical chromatogram obtained with twelve fatty acids. All the peaks were completely separated within 38 min. The change in methanol concentration had no effect on the fluorescence excitation and emission maximum wavelengths of the MMEQ derivatives of all fatty acids; the spectra were virtually identical with those of product I.

It appears that different fatty acids have different peak responses (Fig. 4). This might be due to the differences in the yields of the fluorescent derivatives from fatty acids.

Precision, calibration curve, detection limit and reaction of MMEQ-hydrazide with compounds other than saturated fatty acids

The precision was established by repeated determinations using a standard mixture of fatty acids (1.0 nmol/ml each). The relative standard deviations did not exceed 2.0% for any of the fatty acids examined (n=10 in each instance).

The calibration curves for the individual fatty acids showed linear relationships between the peak heights and the concentrations (50 fmol – 50 pmol/injection volume) of the acids. The limits of detection of the fatty acids were 1 – 4 fmol at a signal-to-noise ratio of 3. The sensitivity is ca. 1.6 times higher than that of the method with DMEQ-hydrazide. The high sensitivity is because that MMEQ moiety, which has a planar structure of methylenedioxy group, has higher fluorescence intensity than DMEQ moiety.

Many other acidic compounds, dicarboxylic, hydroxy-carboxylic, aromatic carboxylic and unsaturated fatty acids reacted with MMEQ-hydrazide 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 α-amino acids, alcohols, sugars, amines, aldehydes, ketones, phenols and sulphydryl compounds did not fluoresce under the
derivatization conditions employed.

MMEQ-hydrazide permits the derivatization of carboxylic acids in aqueous solution and is more sensitive than DMEQ-hydrazide as a fluorescent reagent. Thus, the reagent should be useful for the determination of biologically important carboxylic acids, particularly thermally labile acids such as arachidonic cascades and short chain fatty acids with volatility such as valproic acid.

References


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