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3,4-DIHYDRO-6,7-DIMETHOXY-4-METHYL-3-OXO-QUINOXALINE-2-CARBONYL CHLORIDE AS A HIGHLY SENSITIVE FLUORESCENCE DERIVATIZATION REAGENT FOR ALCOHOLS IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

3,4-Dihydro-6,7-dimethoxy-4-methyl-3-oxo-quinoxaline-2-carbonyl chloride was found to be a highly sensitive fluorescence derivatization reagent for primary and secondary alcohols in high-performance liquid chromatography. Its reactivity was investigated for benzyl alcohol, *n*-hexanol and cyclohexanol. The reagent reacts with the alcohols in benzene to produce the corresponding fluorescent esters, which can be separated on a reversed-phase column, YMC Pack C_8, with aqueous 70% (v/v) methanol; the detection limits for the alcohols were 2–3 fmol for an injection volume of 10 μl. The reagent also reacts with hydroxysteroids with primary and/or secondary alcoholic group(s) to form fluorescent derivatives. Tertiary alcohols, hydroxycarboxylic acids and phenols do not give fluorescent derivatives under these conditions.

INTRODUCTION

Many fluorescence derivatization reagents have been developed for the determination of alcohols by high-performance liquid chromatography (HPLC), *e.g.* 4-dimethylamino-1-naphthoylnitrile[1], (+)- and (−)-2-methyl-1,1′-binaphthalene-2′-carbonyl nitriles[2], 2-dansylethyl chloroformate[3], 1- and 9-anthroylnitriles[4], 3- and 4-chloroformyl-7-methoxy-[5], 7-[(chlorocarbonyl)methoxy]-4-methyl-[6] and 4-diazomethyl-7-methoxycoumarins[7], 7-methoxycoumarin-3- and -4-carbonyl azides[8], 2-(4-isocyanatophenyl)-6-methyl-benzothiazole[9,10] and naphthyl isocyanate[11]. HPLC methods with these reagents are not always satisfactory with respect to sensitivity; the reagents do not permit the determination of alcohols at the femtomole level per injection volume except for 7-methoxycoumarin-3-carbonyl azide.

We have previously reported that 3,4-dihydro-6,7-dimethoxy-4-methyl-3-oxo-

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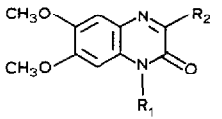
TABLE I

3,4-DIHYDRO-6,7-DIMETHOXY-3-OXO-QUINOXALINES AND THEIR ANALYTICAL DATA



Compound	R_1	R_2	*Yield (%)*	*Appearance**	*m.p. (°C)*	*Formula*	*Analysis (%) calc. (found)*		
							C	*H*	*N*
DQ-COOH	H	COOH	55.9	ON	268	$C_{11}H_{10}N_2O_5$	52.80 (52.71	4.00 4.02	11.20 11.13)
DMEQ-$COOCH_3$	CH_3	$COOCH_3$	14.3	YN	164	$C_{13}H_{14}N_2O_5$	56.12 (56.13	5.04 5.04	10.07 9.98)
DMEQ-COOH	CH_3	COOH	78.6	YN	222	$C_{12}H_{12}N_2O_5$	54.54 (54.51	4.54 4.52	10.60 10.58)
DMEQ-COCl	CH_3	COCl	96.0	ON	261	$C_{12}H_{11}N_2O_4Cl$	50.97 (50.94	3.89 3.77	9.91 9.81)
I	CH_3	$COOCH_2$–C$_6$H$_5$	11.0	ON	193	$C_{19}H_{18}N_2O_5$	64.41 (64.32	5.08 5.10	7.91 7.98)
II	CH_3	$COOC_6H_{13}$	6.10	PYN	198	$C_{18}H_{24}N_2O_5$	62.02 (61.80	6.90 7.08	8.05 8.17)
III	CH_3	COO–C$_6$H$_{11}$	17.0	PYN	190	$C_{18}H_{22}N_2O_5$	62.42 (62.46	6.36 6.51	8.09 8.17)

* O = orange; p = pale; y = yellow; N = needles.

quinoxaline (DMEQ) derivatives fluoresce highly intensely in aqueous methanol and acetonitrile[12], and thus we developed 2-bromomethyl-DMEQ as a fluorescence derivatization reagent for carboxylic acids in HPLC[12]. Recently, we found that DMEQ-2-carboxylic acid (DMEQ-COOH; Table I) also gives an intense fluorescence in aqueous methanol and acetonitrile. Thus, DMEQ-2-carbonyl chloride (DMEQ-COCl; Table I) was synthesized as a fluorescence derivatization reagent for alcohols. In order to investigate its reactivity with alcohols, we have used benzyl alcohol, *n*-hexanol and cyclohexanol as model primary and secondary alcohols. DMEQ-COCl reacts with these alcohols in benzene to produce the corresponding fluorescent esters. The esters are separated on a reversed-phase column with aqueous methanol. The reactivity of DMEQ-COCl with various other alcohols (tertiary alcohols, hydroxysteroids and hydroxycarboxylic acids) has also been examined.

EXPERIMENTAL

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. Quantum yields of fluorescence were obtained on the fluorimeter by the method of Parker and Rees[13].

Infrared (IR) spectra were recorded with a Shimadzu 430 spectrophotometer using potassium bromide pellets. ^1H nuclear magnetic resonance (NMR) spectra were obtained with a Hitachi R-90H spectrometer at 90 MHz using a *ca.* 5% (w/v) solution of [$^2H_6$]dimethyl sulphoxide containing tetramethylsilane as an internal standard.

Field desorption mass spectra (MS) were taken with a Jeol DX-300 spectrometer. Uncorrected melting points were measured with a Yazawa melting point apparatus.

Reagents and materials

All chemicals were of analytical reagent grade, unless noted otherwise. Deionized and distilled water was used. Organic solvents were distilled and dried in the usual manner. 1,2-Diamino-4,5-dimethoxybenzene monohydrochloride was prepared as described previously[14]; it is now available from Dojindo Labs. (Kumamoto, Japan).

Synthesis of DMEQ-COCl

1,2-Diamino-4,5-dimethoxybenzene monohydrochloride (8 g, 40 mmol) and α-ketomalonic acid (8 g, 44 mmol) were dissolved in 200 ml of 0.5 *M* hydrochloric acid. The mixture was heated for 2 h in a boiling water-bath. The precipitates that separated on cooling the mixture in ice-water were filtered off, washed with water and then recrystallized from aqueous 90% (v/v) 1,4-dioxane to give 3,4-dihydro-6,7-dimethoxy-3-oxo-quinoxaline-2-carboxylic acid (DQ-COOH;Table I).

DQ-COOH (5.5 g, 22 mmol) in 50 ml of anhydrous methanol was treated with ethereal diazomethane solution prepared by the established method[15]. The reaction mixture was evaporated to dryness *in vacuo*. The residue dissolved in 30 ml of chloroform was purified by column chromatography (25 × 5.7 cm I.D.) on silica gel 60

(*ca.* 130 g, 70–230 mesh; Japan Merck, Tokyo, Japan) with *n*-hexane–ethyl acetate (1:1, v/v) as eluent to give methyl DMEQ-2-carboxylate (DMEQ-$COOCH_3$; Table I).

DMEQ-$COOCH_3$ (2.5 g, 9.5 mmol) was dissolved in 200 ml of 1.0 *M* sodium hydroxide. The solution was allowed to stand at room temperature for *ca.* 70 min and then washed five times with 200 ml of ethyl acetate. The aqueous layer was neutralized with dilute hydrochloric acid and the resulting precipitates were collected by filtration and recrystallized from aqueous 80% (v/v) 1,4-dioxane to give DMEQ-COOH (Table I).

A solution of DMEQ-COOH (1 g, 3.6 mmol) in 20 ml of freshly distilled thionyl chloride was refluxed for 1 h and cooled. The precipitates formed on adding *ca.* 50 ml of light petroleum (b.p. 30–60°C) were collected by filtration and recrystallized from benzene–light petroleum (9:1, v/v) to give DMEQ-COCl (Table I).

DMEQ-COCl was stable in the crystalline state for three months or longer when kept dry in the dark at room temperature. The reagent dissolved in benzene could be used for a week when stored in a refrigerator at 5°C.

Preparation of the fluorescent compounds from benzyl alcohol, n-hexanol and cyclohexanol

DMEQ-COCl (100 mg , 0.35 mmol) and an alcohol (0.35 mmol) were dissolved in 10 ml of benzene. The solution was placed in a screw-capped test-tube (20 ml) and heated at 100°C for 1 h and cooled. The reaction mixture was evaporated to dryness *in vacuo*. The residue, dissolved in 5 ml of chloroform, was chromatographed on a silica gel-60 column (25 × 2.7 cm I.D.) with *n*-hexane–ethyl acetate (1:1, v/v). The main fraction was evaporated to dryness *in vacuo*, and the residue was recrystallized from methanol to give the corresponding product (I, II or III; Table I).

Derivatization procedure

To 0.5 ml of a test solution of alcohols in benzene (from 20 pmol to 25 nmol each per 0.5 ml) placed in a PTFE screw-capped reaction vial (2 ml) was added 0.5 ml of 3 m*M* DMEQ-COCl in benzene. The vial was tightly closed and heated at 100°C for 40 min in the dark. After cooling, 20 μl of the reaction mixture was diluted with 2 ml of methanol, and the resulting solution (10 μl) was injected into the chromatograph. For the reagent blank, 0.5 ml of benzene in place of a test solution was subjected to the same procedure.

HPLC apparatus and conditions

A Waters 510 high-performance liquid chromatograph equipped with a U6K universal injector (10-μl loop) and a Hitachi F1100 fluorescence spectrometer equipped with a 12-μl flow-cell operated at the excitation wavelength of 400 nm and the emission wavelength of 500 nm were used. The column was a YMC Pack C_8 (150 × 6 mm I.D.; particle size, 10 μm; Yamamura Chemical Labs., Kyoto, Japan). It can be used for more than 1000 injections with only a small decrease in the theoretical plate number, when washed with methanol every day after analyses. The mobile phase used for the separation of the DMEQ derivatives of the examined alcohols was aqueous 70% (v/v) methanol at a flow-rate of 2.0 ml/min (*ca.* 90 kg/cm^2). The column temperature was ambient (15–25°C).

RESULTS AND DISCUSSION

Fluorescent products of reaction between alcohols and DMEQ-COCl

The reaction products from benzyl alcohol, *n*-hexanol and cyclohexanol were confirmed as the corresponding DMEQ-2-carboxylates, compounds I, II and III, respectively, by IR, MS and NMR data (Table I).

Fluorescence properties of DMEQ derivatives

The fluorescence properties of the DMEQ derivatives in methanol, acetonitrile and water, which have widely been used as components of mobile phases in reversed-phase HPLC, were examined. The DMEQ esters of alcohols (DMEQ-$COOCH_3$ and compounds I–III) showed almost the same fluorescence excitation and emission maxima; the maxima are almost independent of the kind of the alcohol and of the solvent. On the other hand, the fluorescence intensities (or the quantum yields) in acetonitrile were smaller than those in methanol and water. The intensity of DMEQ-COOH in these solvents was low compared with that of the DMEQ esters (Table II).

HPLC conditions

The separation of DMEQ derivatives of benzyl alcohol, *n*-hexanol and cyclohexanol was studied on a reversed-phase column, YMC Pack C_8, with aqueous methanol. At methanol concentrations higher than 80% (v/v), the peak for the DMEQ derivative of benzyl alcohol partially overlapped an unknown peak (Fig. 1, peak 4), whereas methanol concentrations of 60–65% (v/v) caused a delay in elution with broadening of the peaks. Optimum separation was attained by using aqueous 70% (v/v) methanol. Fig. 1 shows a typical chromatogram obtained with the alcohols.

Derivatization conditions

Benzene as a solvent for the derivatization reaction provided the most intense peaks for the alcohols examined, and toluene gave less intense peaks (*ca.* 70% of those in benzene). On the other hand, the fluorescence reaction was very limited in chloroform, dimethyl sulphoxide, dimethylformamide, tetrahydrofuran, ethyl acetate, acetonitrile, acetone or 1,4-dioxane. Water interfered with the reaction owing to the decomposition of DMEQ-COCl. Thus, benzene was chosen for the procedure. Acceleration of the reaction was not observed in the presence of pyridine, trimethylamine, dimethylaminopyridine, N,N-diisopropylethylamine, potassium carbonate, potassium hydrogen carbonate or 18-crown-6.

The reaction of DMEQ-COCl with the alcohols occurred more rapidly with as the reaction temperature was increased. An example for cyclohexanol is shown in Fig. 2. The peak heights reached a maximum and constant after heating at 100–150°C for 30 min. Thus, heating for 40 min at 100°C was employed in the procedure. The most intense peaks were obtained at reagent concentrations greater than *ca.* 2 m*M* and 3 m*M* was employed in the procedure. The DMEQ derivatives in the final mixture were stable for at least 72 h in daylight at room temperature.

Calibration curve, precision and detection limit

The relationships between the peak heights and the amounts of the individual

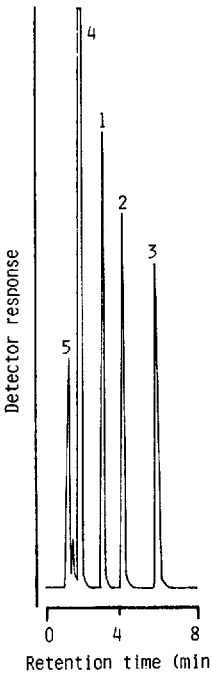
TABLE II

FLUORESCENCE PROPERTIES OF DMEQ DERIVATIVES IN METHANOL, ACETONITRILE AND WATER

Compound	*Excitation maximum (nm)*			*Emission maximum (nm)*			*Relative fluorescence intensity**			*Quantum yield*		
	Methanol	*Acetonitrile*	*Water*	*Methanol*	*Acetonitrile*	*Water*	*Methanol*	*Acetonitrile*	*Water*	*Methanol*	*Acetonitrile*	*Water*
DMEQ-$COOCH_3$	399	397	406	503	487	512	99	73	83	0.99	0.65	0.86
DMEQ-COOH	400	413	379	479	489	480	17	24	16	0.16	0.06	0.16
DMEQ-COCl**	–	401	–	–	473	–	–	76	–	–	0.76	–
I	400	399	408	501	489	503	100	74	84	0.99	0.67	0.85
II	399	394	406	501	488	509	97	61	84	0.95	0.56	0.87
III	399	396	408	502	486	513	78	52	85	0.89	0.56	0.84

* The fluorescence intensity was measured at the excitation and emission maxima. The intensity of compound I in methanol was taken as 100.

** DMEQ-COCl reacted partly with methanol to produce DMEQ-$COOCH_3$ even at room temperature, and decomposed with water to form DMEQ-COOH.



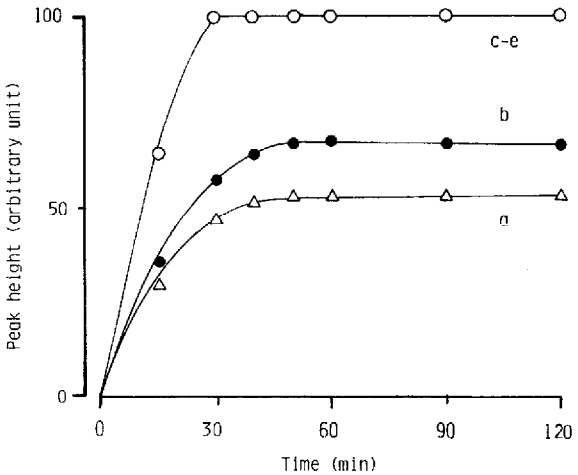


Fig. 1. Chromatogram of DMEQ derivatives of benzyl alcohol, *n*-hexanol and cyclohexanol. A portion (0.5 ml) of a mixture of the alcohols (1.0 nmol each per ml) was treated according to the described procedure. Peaks: 1 = benzyl alcohol; 2 = cyclohexanol; 3 = *n*-hexanol; 4 and 5 = unknown (probably due to decomposition products of DMEQ-COCl).

Fig. 2. Effect of reaction time and temperature on the peak height. Portions (0.5 ml) of cyclohexanol (1.0 nmol/ml) were treated as in the procedure at various temperatures. Temperatures: a = 60°C; b = 80°C; c = 100°C; d = 130°C; e = 150°C.

alcohols were linear from 2 fmol to at least 2.5 pmol per 10-μl injection volume (corresponding to 20 pmol to 25 nmol in 0.5 ml of a test solution). The precision was established by repeated determinations ($n = 10$) using a mixture of the three alcohols (25 nmol each per 0.5 ml). The coefficients of variation were 0.7, 1.2 and 0.9% for benzyl alcohol, *n*-hexanol and cyclohexanol, respectively. The detection limits for the alcohols were 2–3 fmol for an injection volume of 10 μl, at a signal-to-noise ratio of 2. The sensitivity is comparable with that of the method using 7-methoxycoumarin-3-carbonylazide[8], and much higher than those of the methods with the other fluorescence derivatization reagents[1–11].

Reaction of DMEQ-COCl with other substances

Many hydroxysteroids reacted with DMEQ-COCl under the derivatization conditions recommended for the formation of fluorescent derivatives. The retention times and detection limits for the DMEQ derivatives of the compounds are shown in Table III. Tertiary alcohols (2-methyl-2-propanol, 2-methyl-2-butanol and 4-androsten-17α-ethynyl-17β-ol-3-one) and hydroxycarboxylic acids (lactic and malic acids) did not fluoresce. Other substances, such as carboxylic acids, seventeen different L-α-amino acids, aldehydes, ketones, phenols and sulphydryl compounds gave

TABLE III

RETENTION TIMES AND DETECTION LIMITS FOR DMEQ DERIVATIVES OF HYDROXYSTEROIDS

Compound	*Retention time (min)*	*Detection limit (fmol/10 μl)*
4-Pregnen-21-ol-3,11,20-trione (1-dehydrocorticosterone)	3.4	12.6
4-Pregnen-21-ol-3,20-dione (deoxycorticosterone)	6.6	8.4
5-Pregnen-3β-ol-20-one (pregnenolone)	28.8	15.2
1,3,5(10)-Estratriene-3,17α-diol (17α-estradiol)	9.6	10.4
5-Androsten-3β-ol-17-one (dehydroisoandrosterone)	13.5	6.6
5-Cholesten-3β-ol (cholesterol)*	4.9	4.6
5α-Cholestan-3β-ol (cholestanol)	5.4	7.5

* Methanol was used as a mobile phase for the separation of the DMEQ derivatives of these compounds. When the mobile phase in the described procedure was used, the DMEQ derivatives were strongly retained on the column and not eluted.

no fluorescent derivatives. Therefore, DMEQ-COCl should be useful as a fluorescence derivatization reagent in HPLC of primary and secondary alcohols.

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