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FATTY ACID ESTERS OF 4-HYDROXYMETHYL-7-METHOXYCOUMARIN: FLUORESCENCE CHARACTERISTICS AND THEIR VARIATION IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SOLVENTS

J. B. F. LLOYD

Home Office Forensic Science Laboratory, Priory House, Gooch Street North, Birmingham B5 6QQ (Great Britain)

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SUMMARY

UV absorbance and corrected fluorescence spectral data and fluorescence quantum yields from some representative fatty acid esters of 4-hydroxymethyl-7-methoxycoumarin are presented. The fluorescence quantum yields vary extensively between different solvents. In methanol values of less than 0.1 are typical. With the addition of water the yield from the lower molecular weight derivatives rises to values in the region of 0.4; but for derivatives above the *n*-octanoate a presumed micelle formation occurs, with a consequent fall in yield after the initial water-promoted increase. In non-hydrogen bonding solvents the yields, where examined, are less than 0.02. These results place considerable restrictions on the choice of high-performance liquid chromatographic (HPLC) conditions appropriate to the analysis of these derivatives. A published fluorescence excitation spectrum is subject to an intense inner filter effect, and, therefore, the HPLC-monitoring conditions selected on the basis of this spectrum are entirely inappropriate. An intrinsic fluorescence sensitivity parameter, calculated from spectral characteristics, is suggested as a basis of future comparisons between different fluorescent derivatives.

INTRODUCTION

The 7-methoxycoumarin nucleus was introduced as a fluorescent label for chromatographic use by Baker *et al.*[1]. It has since been used by Dünges[2] for the derivatisation, with 4-bromomethyl-7-methoxycoumarin, of fatty acids and other acidic compounds. Thin-layer chromatographic[2,3] and high-performance liquid chromatographic (HPLC)[4–6] applications have been described.

Although all of the derivatives examined are reported to give about the same molar fluorescence quantum yield[2], no actual measurements have been reported; and the variation that occurs between peak areas of equimolar amounts of different fatty acid derivatives on gradient-elution HPLC[4] suggests that the quantum yields are exceptionally solvent-sensitive or that extensive spectral shifts occur as the solvent composition is changed. The few spectral data that have been published are incon-

sistent with one another. Hence, it is neither possible to select HPLC conditions appropriate to the efficient use of these particular derivatives, nor to assess the value of these derivatives in terms of spectral selectivity and sensitivity relative to possible alternatives.

EXPERIMENTAL

Instrumental details

The fluorescence spectra were recorded with a Perkin-Elmer MPF-4 fluorescence spectrometer fitted with a corrected spectra accessory. All of the excitation and emission spectra shown and the tabulated data have been corrected. The UV absorbance spectra were recorded with a Pye-Unicam SP 8000 spectrometer. Both instruments were periodically checked and calibrated according to the manufacturers' manuals.

Materials

Methanol, ethanol (both Spectrosol grade, Hopkins & Williams, Chadwick Heath, Great Britain) and acetonitrile (spectroscopic grade, BDH, Poole, Great Britain) contained no significant fluorescent or absorbing impurities detectable at the highest instrumental sensitivities used. Cyclohexane was purified over activated charcoal and distilled under nitrogen.

9,10-Diphenylanthracene scintillation grade; m.p. 250–252°, ref. 7: 245–247° (Koch-Light, Colnbrook, Great Britain), after evacuation at 0.1 Torr and 100° for 30 min, contained by HPLC no UV-absorbing (254 nm) or fluorescent (266/425 nm) impurity at a level greater than 1 part per 10^4.

4-Bromomethyl-7-methoxycoumarin (Regis, Morton Grove, Ill., U.S.A.) and the fatty acids (none of specified purity less than 99%) were used as received.

4-Hydroxy-7-methoxycoumarin esters

The acetate, decanoate and stearate esters were prepared essentially according to Dünges[2] except that they were further purified by classical column chromatography on neutral alumina (E. Merck, Darmstadt, G.F.R.; deactivated with 3.7% water) with *n*-hexane and ethyl acetate solvent mixtures, and recrystallised from the same solvents. Each product was a colourless crystalline material: acetate, m.p. 166–167°, ref. 2: 164–167° dec; stearate, 92–93°, ref. 2: 90–93° dec; decanoate, 73–74°. None of the derivatives appeared to decompose on heating: the same melting point was obtained when the melted compound was cooled and reheated. The decanoate has not been reported before. Its identity was confirmed by mass spectrometry.

Quantum yields

These were determined with reference to 9,10-diphenylanthracene in deoxygenated methanol at ambient temperature, essentially according to Parker[8]. The required absorbances were measured using the fluorescence spectrometer with the sample interposed in the same light beam used, subsequently, for excitation of the emission spectra. The spectral band width at half peak height of the excitation, at 337 nm, was 1 nm. The transmitted light was monitored with a rhodamine B quantum counter. None of the absorbances at the 2.5-mm pathlength used for the fluorescence

measurements exceeded 0.01. A correction for the slight inner filter absorption of the excitation was applied.

The quantum yield of the diphenylanthracene under these conditions was taken to be 0.95. Recent work by Morris *et al.*[9] gives a result of 0.95 in deoxygenated ethanol. Relative to this value the yield in methanol was found to be either 0.92 or 0.96, depending on whether or not the usual refractive index correction was made. (These results are the means of 6 determinations distributed with a standard deviation of 0.04). In cyclohexane, relative to ethanol, the result was either 0.90 or 0.82. Morris *et al.*[9] report 0.86. In view of the doubtful validity of the refractive index correction[9], because of the small difference in refractive index between ethanol and methanol, and because of their similar solvent properties, the yield in methanol was taken to be not significantly different from that in ethanol, as the above result indicates, and no refractive index correction was made. None of the following conclusions would be affected in any way if the value used had been taken from anywhere within the range of values that have been reported in the literature.

RESULTS AND DISCUSSION

In Table I are given the UV absorbance and the corrected fluorescence spectral data and quantum yields from the acetyl, decanoyl and stearyl derivatives in methanol and in 90% (v/v) water in methanol. All of these determinations were made in duplicate, as the table shows. The quantum yields were measured both in aerated and in nitrogen-purged solutions. For the stearyl derivative are also given fluorescence spectral data and quantum yields in solutions in acetonitrile and in cyclohexane, each containing 0.5% methanol derived from the stock solutions of the derivative. These yields are limited in accuracy by the weakness of the fluorescences concerned. The corrected fluorescence excitation and emission spectra of the decanoate are shown in Fig. 1.

The effect of the variation in the concentration of water in methanol on the fluorescence intensity excited at 323 nm and monitored at 395 nm is shown in Fig. 2. The derivatives used are the same as in Table I. On the methanol-rich side of each curve, before its maximum, the intensities correspond to the indicated quantum yields (Q). Beyond this point the spectral characteristics of the derivatives tend to vary, for instance the emission is red-shifted and varyingly broadened; hence, these intensities are not directly correlated to quantum yields, although the general trends are still apparent.

In Fig. 3 is shown the effect of variation in the carbon number of the fatty acid residue in the derivatives on the fluorescence intensity in the 90% water solvent, relative to the maximum intensity attained at any solvent composition for each derivative. When the isolated derivative was not available, the results were obtained from reaction mixtures containing an excess of each acid over an amount of the reagent calculated to give the required concentration (1 μM) of derivative after dilution. For the present purpose, quantitative formation of the derivative is unnecessary because only ratios of intensities are being compared. Even so, the reaction is reported to go to completion[2,3,6].

TABLE I

UV ABSORPTION AND FLUORESCENCE DATA OF SOME FATTY ACID ESTERS OF 4-HYDROXYMETHYL-7-METHOXYCOUMARIN IN METHANOL, IN 90% (v/v) WATER IN METHANOL, AND IN TWO APROTIC SOLVENTS

The UV absorption measurements were made at concentrations of 20 μM, and the fluorescence measurements at 2 μM. Pairs of results are from independent duplicate determinations. The following abbreviations are used: A = molar absorptivity; 90% aq. = 90% (v/v) water in methanol, Ex. and Em. = wavelengths (nm) of corrected fluorescence excitation and emission maxima; Q = quantum yield. Further details are given in the text.

Fatty acid	*UV absorption max., nm (A)*		*Fluorescence*													
	Methanol	*90% aq.*	*Methanol*				*90% Water in methanol*				*Acetonitrile*			*Cyclohexane*		
			Ex. (nm)	*Em. (nm)*	*Q (N_2)*	*Q (air)*	*Ex. (nm)*	*Em. (nm)*	*Q (N_2)*	*Q (air)*	*Ex. (nm)*	*Em. (nm)*	*Q (N_2)*	*Ex. (nm)*	*Em. (nm)*	*Q (N_2)*
Acetic	322(14593)	323(14749)	323	395	0.080	0.078	325	402	0.44	0.44						
	323(14534)	323(14765)	323	395	0.095	0.093	325	401	0.41	0.41						
Decanoic	322(14792)	322(13154)	323	397	0.082	0.082	323	403	0.14	0.13						
	322(14522)	323(12576)	324	395	0.094	0.088	324	402	0.14	0.13						
Stearic	323(14633)	323	323	396	0.082	0.079	323	403	0.054	0.051	322	393	0.02	323	388	0.006
	323(14539)	325	324	395	0.086	0.084	325	402	0.054	0.050						

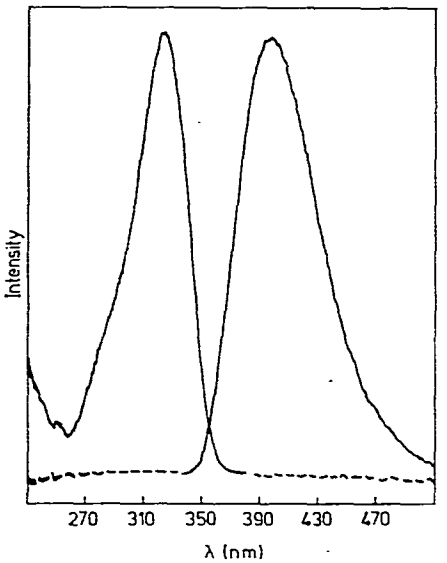


Fig. 1. Corrected fluorescence excitation and emission spectra of the decanoate ester of 4-hydroxymethyl-7-methoxycoumarin in methanol (deoxygenated) at a concentration of 1 μM. The solvent blanks are idicated by broken lines, each truncated to avoid its merging into the other spectra.

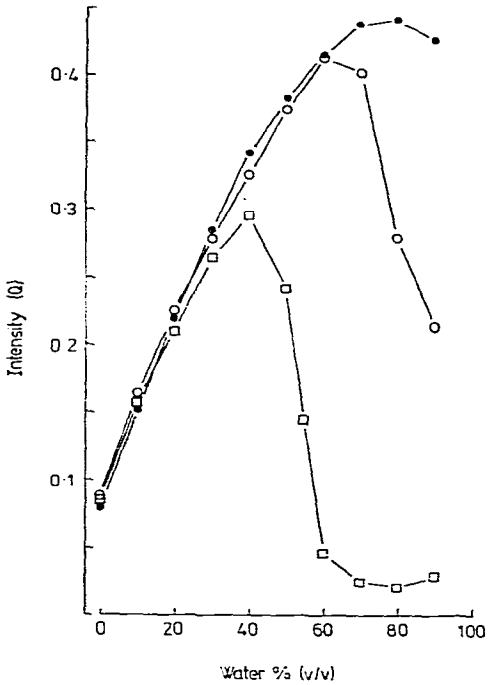
Spectral characteristics and solvent effects

The wavelength of the absorption maximum and of the corresponding fluorescence excitation maximum of each derivative in methanol and in 90% water are in close agreement with one another (Table I), and vary little between or within the two solvents. In methanol the mean of all of the absorbance and excitation maxima is 322.9 nm; and in 90% water, 323.7 nm. A similar result, for the stearyl derivative, is obtained from cyclohexane and acetonitrile solutions.

The wavelength of the fluorescence emission maximum in methanol or in the aqueous solvent does not vary significantly between the derivatives, but is red-shifted by 6.7 nm, on average, in the latter relative to the former solvent. The mean value in methanol is 395.5 nm; and in 90% water, 402.2 nm. In acetonitrile and in cyclohexane the maximum shifts back, from the methanol value, to 393 and 388 nm, respectively.

In general appearance all of the absorbance and excitation spectra, and all of the emission spectra, compare with the respective excitation and emission spectra of the decanoate shown in Fig. 1, but the stearate differs slightly. A weak shoulder occurs on the long-wave side of its absorbance and excitation spectra in the aqueous solvent, and the width at half peak height of the corresponding emission is 6200 cm^{-1}, in contrast to 4700 cm^{-1} for the aqueous decanoate, and to a mean of 4000 cm^{-1} for the derivatives in methanol-rich solvents.

The wavelength of the emission maximum of the aqueous stearate solution used for the absorbance measurements, where the concentration was 20 μM instead of the 2 μM used for the fluorimetry, appeared at 394 nm instead of the anticipated 402 nm, and shifted to 389 nm after 30 min (in the absence of light). Other spectral changes occurred in the absorption and excitation spectra of the solution, which



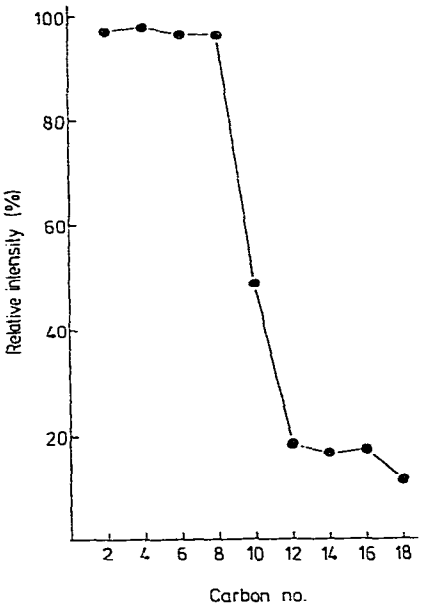


Fig. 2. Variation of fluorescence intensity, excited at 323 nm and monitored at 395 nm, with concentration of water in methanol. The compounds are the acetate (filled circles), the decanoate (open circles), and the stearate (squares) esters of 4-hydroxymethyl-7-methoxycoumarin at a concentration of 1 μM. On the methanol-rich side of the maxima the points are the quantum yields (Q) indicated on the vertical axis. Beyond the maxima spectral changes occur and the relationship between intensity and quantum yield tends to become indirect.

Fig. 3. Variation of the fluorescence intensity in 90% (v/v) water in methanol, relative to the maximum intensity reached at any solvent composition, with carbon number of some linear, saturated fattyacid esters of 4-hydroxymethyl-7-methoxycoumarin. Each is at an approximate concentration of 1 μM. See the text for further details.

sometimes became slightly opalescent and evidently represents the limit of solubility of the stearate. Because of this the molar absorptivity obtained (mean, 16220) has been excluded from Table I.

The decanoate absorptivity in the aqueous solvent (mean, 12865) also differs significantly from the remainder, probably because of the processes, discussed below, affecting the fluorescence yield of the aqueous solutions rather than any incipient precipitation (there were no features directly comparable to the stearate case). None of the other molar absorptivities differ significantly from one another. Their mean is 14641.

Fluorescence quantum yields and solvent effects

In methanol the quantum yields are in close agreement with each other, negligibly affected by oxygen quenching, and unaffected by the size of the fatty acid residue. The means are 0.087, in deoxygenated methanol, and 0.084, in the aerated solvent. These disappointingly low yields are even lower in the non-hydrogen bonding solvents acetonitrile and cyclohexane, where the observed yields (Table I) are probably

enhanced by the small amount of methanol present. Thus, an upper limit of 0.02 is indicated for xuch solvents.

As water is added to methanolic solutions of the derivatives an initial rapid increase in quantum yields occurs at approximately the same rate for each derivative. Then, at a point depending on the fatty acid residue, the yield decreases. Three examples are shown in Fig. 2. From the acetate the maximum yield is 0.44, when the water content of the solvent is 80%. Thereafter the yield slightly decreases. The maximum from the stearate is 0.30, at a water content of 40%. Above the 40% level, a particularly rapid fall in yield occurs. The behaviour of the decanoate is intermediate between the other two derivatives. These results have been obtained at a concentration of 1 μM of each derivative. At other concentrations the inflexion points differ slightly, but the effect is small relative to the differences between these derivatives. It is of course well known that the fluorescence of the solutions of many compounds depends on the water content of the solvent[10]. Examples and other references are given by Beddard *et al.*[11] of some coumarins, although most of these carry an unsubstituted 7-hydroxyl that results in a proliferation of the possible ground and excited state structures.

The manner in which the sharp reduction in fluorescence yield with the addition of water, shown for the stearate in Fig. 2, depends on the chain length of the fatty acid residue, is shown in Fig. 3. Up to octanoate there is little change as the water concentration is increased once the maximum yield has been reached. Beyond octanoate the fluorescence is strongly depressed in the more aqueous solvents. It is apparent, therefore, that as the water content is increased a relatively sharp change occurs in the environment of the higher molecular weight derivatives. A presumed cause is the formation of molecular aggregates such as micelles, which in this instance are weakly fluorescent.

In aqueous solvents the derivatives are noticeably photolabile. Although the effect on the spectra is negligible at the 1-nm half band width used for the excitation beam, when a value of 4 nm is used, corresponding to a 16-fold increase in light input, the effect is appreciable. For instance, the fluorescence intensity from an aqueous deoxygenated solution of the decanoate fell to 78% of its original value in 120 sec. Acetonitrile solutions are also strongly subject to the effect, and to a lesser extent the other solutions are too.

Comparison with other reported results

With allowance for the different conditions used, in particular for the use of uncorrected fluorescence spectrometers, the absorption and the fluorescence emission spectra of the palmitate derivative given by Dünges[2], and the emission spectrum of the laurate given by Lam and Grushka[5] are consistent with the results reported here. However, the excitation spectrum given for the laurate[5] is entirely inconsistent both with the present results and with Dünges' absorption spectrum[2]. Apparently, inner filter effects[12] in the relatively concentrated solutions used (350–440 μM) were not taken into account, so that the recorded excitation spectra are virtually inverted absorption spectra. The effect is reproduced in Fig. 4. Spectrum D is from a solution of the decanoate at a concentration of 500 μM in 20% water in methanol. The same spectrum at a 10-fold increased sensitivity is shown at E. Spectra C, B and A are from cumulative 10-fold dilutions of the original solution at correspondingly increased

sensitivities. In the absence of inner filter effects spectra D-A would be identical to one another.

The optical pathlength at which these spectra were obtained is 5 mm, with right angle illumination. Hence, the corresponding absorbance from the data in Table I is about 3.7 for the most concentrated solution. Generally, the value should not exceed 0.02 unless special precautions are taken[12]. The spectrum E (Fig. 4) is of the same form as Lam and Grushka's[5]. In view of the huge inner filter absorption, and of the different instruments involved, the "maxima" in spectrum E at 270 and 355 nm are consistent with the reported[5] values of 295 and 362 nm. The latter was directly transposed to the fluorescence monitoring system used by Lam and Grushka[5]. But the fluorescence intensity, from dilute solutions, obtained on excitation at this wavelength is a negligible amount (about 3%) of the intensity obtained at the excitation maximum, particularly when short pathlength flow-through cells are used.

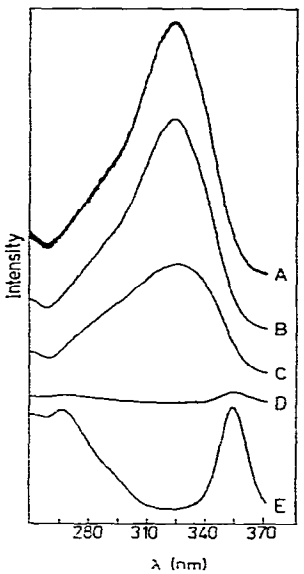


Fig. 4. Corrected fluorescence excitation spectra of the decanoate ester of 4-hydroxymethyl-7-methoxycoumarin, in 20% (v/v) water in methanol, at concentrations of 0.5 (A), 5 (B), 50 (C), and 500 (D, E) μM. The relative instrumental sensitivities are 1 (D), 10 (C, E), 100 (B), and 1000 (A).

Fluorescence characteristics with reference to HPLC

The considerable variation of the fluorescence quantum yield of these derivatives, both between different solvents and between different derivatives, and the low values of the yield in many chromatography solvents, considerably limits the derivatives' usefulness.

Because of the dependence of the fluorescence on the presence of water, separations in non-aqueous systems[6] will not give the high sensitivity typical of fluorescence detection, and UV absorption detection might equally well be applied[4].

For reversed-phase separations of the higher molecular weight derivatives[4,5] relatively low concentrations of water in methanol have been used, under which

conditions the quantum yields are still less than 0.1. Although the sensitivity could be improved by the use of less retentive columns, which would permit an increase to be made in the water content of the solvent, the extent to which this is possible is limited to the range within which the molecular aggregation does not occur.

The water sensitivity of the fluorescence is likely to cause inconvenience when comparisons are made between reversed phase isocratic separations differing in solvent composition, and in solvent programmed separations where, as Dünges and Seiler[4] point out, the molar fluorescence response varies throughout the chromatogram. Under their conditions the variation in quantum yield must be over the range of 0.4 to 0.09. At the start of the chromatogram the higher molecular weight derivatives will be in an aggregated form in the mobile phase, but at a low concentration, and, therefore, probably without effect on chromatographic performance.

Although the optimum corrected excitation and emission wavelengths for the detection of these derivatives in aqueous methanolic solvents are 323 and 396 nm respectively, the "apparent" (uncorrected) values will be determined by the optical characteristics of the fluorescence detector used for chromatography. Obviously, the wavelengths should be checked, if possible, in the relevant detector. The solutions used must be sufficiently dilute to eliminate inner filter effects, although provided the usual short pathlength cells are fitted in the detector, such effects are generally small.

Because of a chromatographic peak's short transit time in a detector, it is unlikely that the photolability of the derivatives could affect their peak heights. If spectra are plotted under stopped flow conditions, however, the intense excitation sources sometimes used could result in considerable photodegradation.

CONCLUSIONS

Intrinsic fluorescence sensitivity (IFS) values

When new fluorescence derivatives are developed it would be of value if details of their quantum yields and of their fluorescence and UV absorption spectra, obtained under conditions relevant to the derivatives' chromatographic use, could be made available. Without this information the value of a new derivative cannot be fully assessed.

The sensitivity with which a compound can be detected by fluorescence monitoring is determined not only by the fluorescence quantum yield (Q), and by extrinsic instrumental factors, but also by the molar absorptivity (A) at the excitation wavelength, and, inversely, by the width at half peak height ($W_{1/2}$) of the emission band. The latter controls the intensity distribution over the part of the emission seen by the detector. Hence, an IFS parameter may be defined: $AQ/W_{1/2}$.

From the data in Table I, and taking $W_{1/2}$ as 4000 cm^{-1}, for the present compounds under optimal conditions the IFS value is 1.6. Hopefully, future work will furnish data appropriate to the comparison of fluorescent derivatives either on this or a similar basis.

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