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Room temperature phosphorescence quenching study of coumarins. Indirect determination of warfarin in pharmaceuticals

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Room temperature phosphorescence of the 1-bromonaphthalene/β-cyclodextrin/cyclohexane inclusion complex was studied in the presence of coumarins: warfarin, coumarin, dicumarol and umbelliferone. The differences encountered when using right-angle or front-face illumination geometry are shown in order to mark the importance of a correct measurement. Phosphorescence static quenching was observed with the coumarins, as the phosphorescence lifetimes could reveal. Warfarin determination was performed using a commercial anticoagulant making use of the interaction between coumarins and the studied inclusion complex.

Introduction

Coumarins are natural compounds that exhibit a varied biological activity. Warfarin, (*RS*)-4-hydroxy-3-(3-oxo-1-phenylbutyl)-2*H*-chromen-2-one, is used as an anticoagulant and a rodenticide; coumarin, 2*H*-chromen-2-one, is used as an aroma enhancer and as a precursor molecule in the pharmaceutical industry and it is found in natural foodstuffs; dicumarol, 3,3′-methylenebis(4-hydroxy-2*H*-chromen-2-one), is a naturally occurring anticoagulant derived from coumarin due to the action of species of fungi; umbelliferone, 7-hydroxychromen-2-one, has antioxidant properties and is used in sunscreens. These compounds exhibit fluorescence whereas phosphorescence was only reported in the solid state in the case of warfarin, coumarin and dicumarol.[1] Fig. 1 shows the molecular structures of these compounds.

Cyclodextrin induced room temperature phosphorescence was widely applied in many fields. 1-Bromonaphthalene (1-BrN) forms an inclusion complex with β-cyclodextrin (β-CD) that exhibits weak room temperature phosphorescence (RTP) in the liquid state. This phenomenon was initially reported by Turro and co-workers for bromosubstituted naphthalene derivatives.[2] The inclusion of a third component enhances RTP without the need for deoxygenation.[3] In a previous study, we found the optimal experimental conditions when cyclohexane (CH) is used as the third component for the system 1-BrN/β-CD.[4] The room temperature phosphorescence quenching of the 1-BrN/β-CD/CH inclusion complex by a selected group of coumarins (warfarin – war-, coumarin – cou-, dicumarol – dic-, umbelliferone – umb-) is now studied when they are used as fourth components.

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Dissolutions of 1-BrN, at a concentration of 1.00×10^{-5} M, show considerable absorption at the excitation wavelength of 281 nm ($\varepsilon = 6466\ \mathrm{cm^{-1}\ M^{-1}}$). The high value of absorbance at this concentration ($A = 0.065$) makes the correction for inner filter effects necessary.

The apparent luminescence intensity and spectral distribution can be dependent upon the absorbance of the sample and the precise geometry of sample illumination. Instruments that use right-angle geometry generally suffer from inner filter effects (IFE) as the intensity of the exciting light at the point of observation (centre of the cuvette) and/or the observed luminescence are diminished due to the absorption of the sample at the excitation and/or emission wavelengths, respectively. Front-face illumination geometry, when the illuminated surface is orientated about 30° from the incident beam, overcomes the inconvenience presented by right-angle illumination geometry: (a) less reflected light enters into the emission monochromator and (b) the incident light is distributed over a larger surface area, decreasing the sensitivity of the measurement to the precise placement of the cuvette within its holder.[5]

Phosphorescence intensities, as well as fluorescence intensities, are influenced by the absorption of the sample and thus

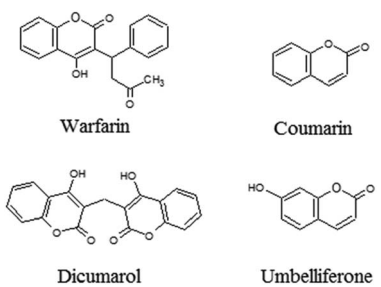

Fig. 1 Molecular structures of coumarins.

IFE may be present. Since usually the sample has no absorption at long emission wavelengths, the classical equation used to correct IFE ($I_{corr} = I_{obs}$ antilog$[(A_{exc} + A_{em})/2]$) is not efficient in phosphorescence studies as it cannot take into account all the effects. The use of front-face illumination geometry assures a good correction for IFE.

Experimental

Reagents and solutions

All chemicals were of analytical-reagent grade and used as received. β-Cyclodextrin (β-CD) was acquired from Cyclolab (Budapest, Hungary). 1-Bromonaphthalene (1-BrN), warfarin (war) and umbelliferone (umb) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Coumarin (cou) and dicumarol (dic) were provided by a local supplier. OmniSolv methanol (MeOH), spectroquality grade cyclohexane (CH) and OmniSolv acetonitrile were obtained from Merck (Darmstadt, Germany). Double-distilled water was used.

β-Cyclodextrin, coumarin and umbelliferone aqueous stock solutions and 1-bromonaphthalene, cyclohexane and warfarin methanolic stock solutions were prepared daily. A dicumarol standard solution was prepared daily in 0.1 M NaOH. From these solutions, more diluted water : methanol (80 : 20) working solutions were prepared.

Apparatus

Room temperature phosphorescence spectra were recorded on a Perkin-Elmer LS-50B luminescence spectrometer equipped with a pulsed xenon lamp (10 μs half-width, 60 Hz), an R928 photomultiplier tube and a computer working with FL Winlab software. All right-angle phosphorescence measurements were performed in a standard 1.0 cm pathlength quartz cuvette and complementary front-face phosphorescence measurements were performed using the front-face accessory and 1.0 cm pathlength cylindrical quartz cuvette of the same diameter of the powder holder. Excitation and emission bandwidths were set at 5.0 and 2.5 nm, respectively. A gate time of 5.00 ms and a delay time of 0.10 ms were used for the RTP measurements. 1-BrN excitation wavelength was set at 281 nm and phosphorescence intensities were measured at 519 nm.

Absorption spectra used to calculate molar extinction coefficients (ε) were recorded on a Shimadzu UV-240 recording spectrophotometer, equipped with a 1.0 cm pathlength quartz cuvette.

Results and discussion

The use of coumarins as fourth components for the system 1-bromonaphthalene/β-cyclodextrin/cyclohexane (1-BrN/β-CD/CH) led to room temperature phosphorescence quenching as shown in Fig. 2–5. Right-angle and front-face illumination geometries are compared in each figure. Data points are duplicates at the same concentration level obtained from independent solutions, except from the extremes and middle concentrations where data points are triplicates.

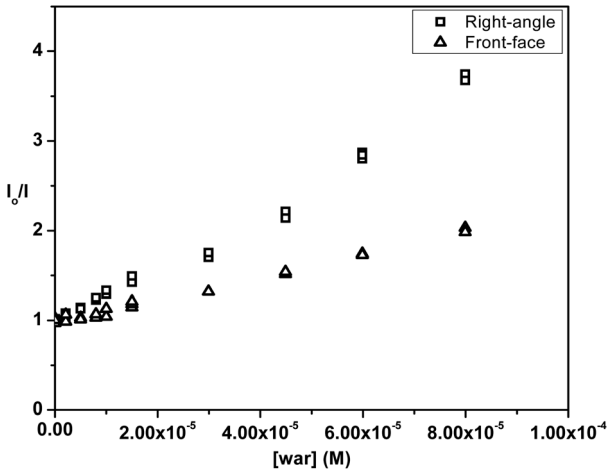

Fig. 2 Stern–Volmer plot for the room temperature phosphorescence quenching of 1-BrN at different concentrations of warfarin measured at right-angle and front-face illumination geometries. [β-CD] = 5.00 × 10^{-3} M; [1-BrN] = 1.00 × 10^{-5} M; [CH] = 1.85 × 10^{-3} M; 20% v/v methanol.

As can be appreciated, there are noticeable differences between RTP measurements at right-angle and front-face illumination geometries related to IFE as a result of the absorption characteristics of 1-BrN and coumarins. In the case of umbelliferone, the differences are less pronounced due to its low value of molar extinction coefficient at 281 nm (Table 1). The upward curvature seen in the figures is explained in terms of their high absorbance values (Table 1); as the concentration increases, the IFE becomes more important and even at front-face illumination geometry a loss of linearity can be observed.

An important feature also seen in the plots is the first region, at a low concentration of the fourth component. In all cases, front-face illumination geometry reveals a constant region

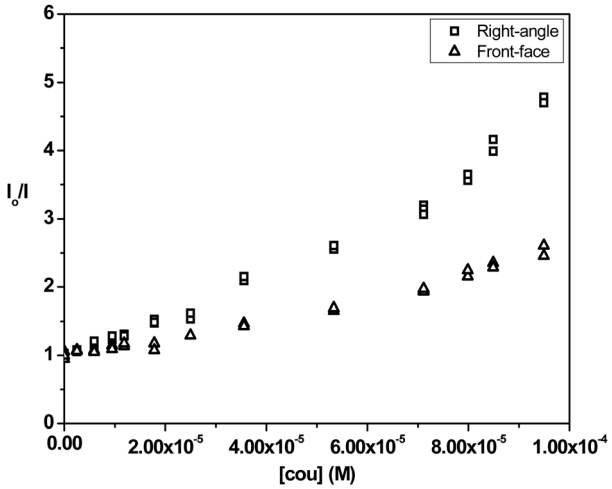

Fig. 3 Stern–Volmer plot for the room temperature phosphorescence quenching of 1-BrN at different concentrations of coumarin measured at right-angle and front-face illumination geometries. [β-CD] = 5.00 × 10^{-3} M; [1-BrN] = 1.00 × 10^{-5} M; [CH] = 1.85 × 10^{-3} M; 20% v/v methanol.

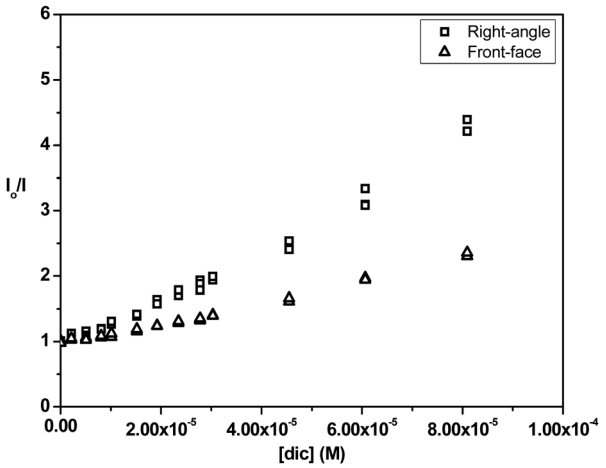

Fig. 4 Stern–Volmer plot for the room temperature phosphorescence quenching of 1-BrN at different concentrations of dicumarol measured at right-angle and front-face illumination geometries. [β-CD] = 5.00×10^{-3} M; [1-BrN] = 1.00×10^{-5} M; [CH] = 1.85×10^{-3} M; 20% v/v methanol.

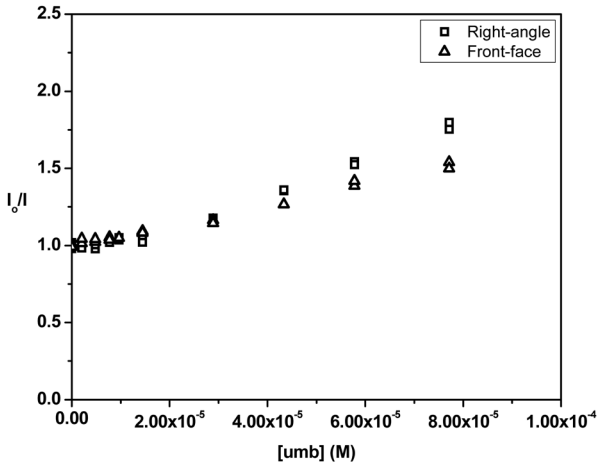

Fig. 5 Stern–Volmer plot for the room temperature phosphorescence quenching of 1-BrN at different concentrations of umbelliferone measured at right-angle and front-face illumination geometries. [β-CD] = 5.00×10^{-3} M; [1-BrN] = 1.00×10^{-5} M; [CH] = 1.85×10^{-3} M; 20% v/v methanol.

Table 1 Molar extinction coefficients of the studied coumarins at 281 nm

Analyte	ε (M^{-1} cm^{-1}) λ = 281 nm
Warfarin	10814 ± 240
Coumarin	10941 ± 168
Dicumarol	13115 ± 191
Umbelliferone	4422 ± 198

where the ratio I_o/I is almost unity, which is not observed in right-angle illumination geometry. This fact demonstrates that at low concentrations, the observed RTP quenching of 1-BrN at right-angle illumination geometry is caused by IFE. Care must be taken when analyzing coumarins (or any absorbing compound) because apparent quenching may be observed due to IFE. Front-face illumination geometry is a useful tool when analyzing absorbing systems which is an experimental procedure that assures reliable results.

Table 2 summarizes the values of K_{sv} for each compound obtained from the Stern–Volmer plots in the linear region (front-face illumination geometry).

In order to confirm the involved quenching mechanism, phosphorescence lifetimes were measured at front-face illumination geometry. A gate time of 1.00 ms and a varied delay time were used, setting the excitation and emission wavelengths at 281 and 519 nm, respectively.

Fig. 6 shows the Stern–Volmer plots in terms of phosphorescence lifetimes for the RTP quenching of 1-BrN. It can be noted that there is a negligible variation in the ratio τ_o/τ with quencher concentration indicating that the quenching mechanism is static. Static quenching removes a fraction of the phosphorophores from observation. The complexed phosphorophores are non-phosphorescent, and the only observed phosphorescence is from the uncomplexed phosphorophores. The uncomplexed fraction is unperturbed, and hence the lifetime is τ_o. Therefore, for static quenching $\tau_o/\tau = 1$. In contrast, for dynamic quenching $I_o/I = \tau_o/\tau$; the decrease in lifetime occurs because dynamic quenching is an additional rate process that depopulates the excited state.[5] A possible explanation for static quenching is the displacement of 1-BrN from

Table 2 Stern–Volmer constant values

Analyte	K_{sv} (M^{-1})
Warfarin	12540 ± 275
Coumarin	13345 ± 394
Dicumarol	12815 ± 470
Umbelliferone	6911 ± 227

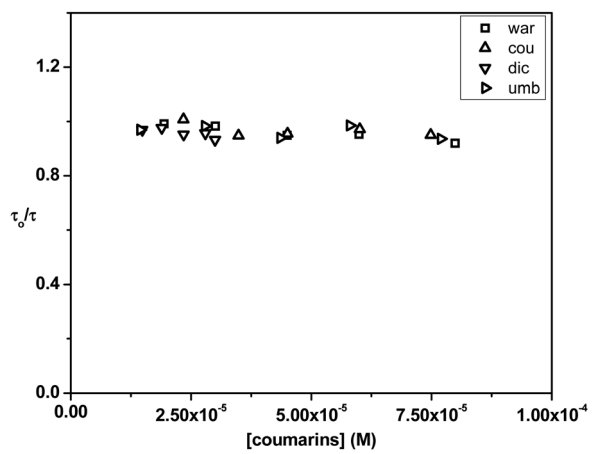

Fig. 6 Stern–Volmer plot for the room temperature phosphorescence quenching of 1-BrN at different concentrations of warfarin (□), coumarin (△), dicumarol (▽) and umbelliferone (▷) measured at front-face illumination geometry. [β-CD] = 5.00×10^{-3} M; [1-BrN] = 1.00×10^{-5} M; [CH] = 1.85×10^{-3} M; 20% v/v methanol.

Table 3 Figures of merit for the determination of warfarin by RTP quenching of 1-BrN[a]

$I_o/I = a + b$[war]			
a	0.98	s_a	0.01
b	12540 M^{-1}	s_b	275 M^{-1}
$s_{y/x}$	0.03		
r	0.99638		
SEN	12540 M^{-1}		
γ	4.34×10^5 M^{-1}		
LOD	7.15×10^{-6} M		
LOQ	1.65×10^{-5} M		
Dynamic range	7.15×10^{-6} M to 7.99×10^{-5} M		
Linear range	1.65×10^{-5} M to 7.99×10^{-5} M		

[a] a (Intercept), s_a (standard error of the intercept), b (slope), s_b (standard error of the slope), $s_{y/x}$ (standard error of the regression), r (correlation coefficient). Figures of merit: SEN (calibration sensitivity), γ (analytical sensitivity), LOD (limit of detection), and LOQ (limit of quantification).

the β-CD cavity by the coumarins, forming a new inclusion complex coumarins/β-CD and hence a decrease in the RTP intensity is observed.

Warfarin determination

A warfarin determination was performed in Circuvit 2 mg (Ariston S.A. Laboratory), a commercially sold anticoagulant. The pills were pre-treated following the procedure provided by Ariston S.A. Laboratory. This sample preparation method is analogous to the one reported in the USP36-NF31.[6] Twenty five pills were pulverized in an agate mortar with a pestle; 2000 mg of the resulting powder were weighed in a 50.00 mL volumetric flask and 30.0 mL of dilution solvent (phosphate buffer pH = 7.4 : acetonitrile, 85 : 15 v/v) were added. The solution was magnetically stirred for 60 minutes. The volume was completed with the dilution solvent. The resultant solution was filtered through a filter paper (Whatman #41). A 1 : 5 water dilution was made in order to use it as the stock solution in the calibration curve.

The sample was analyzed in triplicate, at three levels of concentration. The warfarin concentration found was 2.12 ± 0.09 mg per pill; it was in good agreement with the concentration reported by the laboratory and fits the USP36-NF31 requirements (warfarin sodium tablets contain not less than 95.0 percent and not more than 105.0 percent of the labeled amount of warfarin sodium).[6] No interference due to the excipients was observed. Table 3 summarizes the figures of merit of the proposed method.

Conclusions

The 1-bromonaphthalene/β-cyclodextrin/cyclohexane inclusion complex was studied using a set of coumarins as fourth components. Room temperature phosphorescence quenching affected by inner filter effects was observed. The absorption characteristics of 1-bromonaphthalene and coumarins make necessary the use of front-face illumination geometry in order to overcome the undesirable inner filter effects. This instrumental configuration assures that no apparent quenching occurs, especially at low concentrations of the coumarins. Stern–Volmer constants were calculated in the linear region. Phosphorescence lifetimes were measured and a static quenching mechanism was found for all coumarins. An alternative method for the determination of warfarin in commercial products was performed, leading to good figures of merit and in agreement with the United States Pharmacopeia. The experimental procedure followed in this work marks the importance of a required good analytical criterion when any compound is analyzed by phosphorescence methodologies in the liquid state. Generally, phosphorescence measurements reported in the literature are not corrected for inner filter effects despite their obvious contribution. This work provides guidance for future investigations in *Analytical Chemistry*.

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