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Spectrofluorimetric Determination of Coumarin in Commercial Tablets

Mirian Marcolan · Priscila Alfonso Martins · Valber A. Pedrosa · Maira R. Rodrigues · Hueder P. M. de Oliveira · Lucia Codognoto

Abstract A simple, rapid and effective analytical method based on fluorescence spectroscopy for the determination of coumarin in pharmaceutical formulations without pre-treatment or pre-concentration step was development. Coumarin had maximum excitation and emission at 310 nm and 390 nm, respectively. Optimum conditions for the detection of coumarin were investigated. Under optimized conditions, we observed a linear behavior for the sign of coumarin in the concentration range of 2.5×10^{-6} to 1.0×10^{-4} mol L^{-1}, with linearity of 0.998 and sensitivity of 2.9×10^{10} u.a/mol L^{-1}. The proposed method was validated in terms of accuracy, precision and specificity of coumarin using the standard addition and external calibration. It was noted that the results support ($P < 0.05$), indicating that the matrices were not an interference in the determination of coumarin by fluorescence spectroscopy. The results were favorable compared with those obtained by reference chromatographic methods.

Keywords Coumarin · Fluorescence spectroscopy · Pharmaceutical preparations

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Introduction

Coumarin (Fig. 1) is an active principle of various natural plants like Guaco, Emburana Watercress, Cumaru, Chicory, and in fruits such as strawberry, cherry, raspberry and damask. Also, it is used as a fixative in perfumes, additives for paints and spray, and food flavoring [1]. Most coumarins have pharmacological properties, and are used in various areas of medicine [2, 3]. More than 1300 types of coumarins have been identified from natural sources, especially green plants [4]. Coumarin and derivatives have great applicability in anticoagulant drugs, which alter the kinetics of blood coagulation. The mechanism of action is due to the chemical similarity to vitamin K1, which it is in final stage the synthesis of factors II, VII, IX and X and protein C and S, act causing the appearance of carboxyl forms of these factors, unable to act adequately in kinetics of coagulation [5]. In the topical application of products containing coumarins, the absorption (about 60%) is rapid and extensive by the human skin (and rodents). The coumarins remain metabolically unchanged during absorp-

Fig. 1 Molecular structure of coumarin
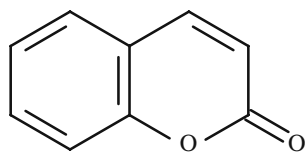
tion. In many studies in humans, coumarin is rapidly absorbed in the gastrointestinal tract (oral ingestion) distributed by the organism and extensively metabolized by hepatic CYP2A6 to 7-hydroxicoumarin, which is excreted in urine in the form of sulfate and glucuronide conjugates [6].

The intensive use of this compound in recent years has required the development of methods of analysis, not only for the quality control in pharmaceutical preparations, but also for biological fluids and cosmetics [7]. In the literature, several methods, including gas chromatography-mass spectrometry [8] high-performance liquid chromatography with UV [9], conductometric [10], amperometric [11], are proposed for determination of pharmacy and drugs [12]. However, in practice, these techniques exhibit some major limitations, such as: requiring complex and expensive instrumentation, highly trained operators, production of a large amount of organic solvents and lengthy measurement processes. The great diversity of coumarins structures and their wide range of polarities present special problems for their simultaneous determination. Also, spectroscopic method has been used to determination of 6-methylcoumarin and 7-methylcoumarin in cosmetics, but they had used pretreatments for analysis [7]. Recently, Fery-Forgues [13] reported a fluorescent method to determinate coumarin in dye. Also, the absorption properties were studied on the suspensions and compared with those of the dissolved dye. The determination of coumarin in different matrices can be carried out by direct fluorescence [14], and the notorious advantages of the proposed methodology are the reduction of analytical costs and a very interesting alternative to those labs, which do not have such sophisticated equipments as required to carry out chromatography techniques.

In this paper, we present an easy and suitable method to detect coumarin by fluorescence spectroscopy with a simple dissolution of sample that can be a viable alternative for the quantification of these compounds in different matrices. Moreover, the figures of merit involving sensitivity, selectivity and limit of detection were investigated, and the accuracy of the proposed method was also estimated by using the external calibration method. Also, the developed method was applied to the analysis of commercial pharmaceutical tablets obtaining good results compared to those acquired through chromatograph method.

Experimental

Reagents

All reagents and chemicals used were of analytical reagent grade. Coumarin (Adrich 99%) was obtained from Sigma-Aldrich (St. Lois, USA). All the solvents (acetonitrile, dioxane, and ethanol) used were purchased from Aldrich (St. Louis, USA). Stock solutions of coumarin (1.0×10^{-3} mol L^{-1}) were freshly prepared by dissolving the compound in acetonitrile. Serial dilutions were performed to obtain working standard solutions using Milli-Q water as solvent. All solutions were protected against light with aluminum foil and stored in a refrigerator.

Apparatus

The measurements were performed using spectrofluorimetric Jobin-Yvon Spex fluoromax-2 with scanning from 200 to 800 nm using a 1 cm path length quartz cell. The spectra were obtained with slits of 2 in the excitation and 3 in the emission.

Measurements of pH were made with a DM-20 pH-meter from Digimed (Brazil), using combined glass electrode.

The HPLC experiments were performed using a binary gradient chromatographic system from Waters, model 1525, coupled to a Waters photodiode array detector (PDA) model 2996 and a Rheodyne injector, model 7725 with a sample loop of 20 μL. Data acquisitions were performed by the Millenium 4.0 software. The chromatographic column was LiChrospher 100 C-18 reversed-phase (250 mm× 4,0 mm, 5 μm) from Varian. The column was kept at room temperature. The mobile phase was acetonitrile/water containing 0.75% of acetic acid (60:40, v/v) at a flow-rate of 1.0 mL min^{-1} and the detection wavelength was 340 nm. The retention time for coumarin was found at 6 min.

Drugs Samples Analysis

Pharmaceutical samples were purchased in a Brazilian drugstore located in São José dos Campos city. The nominal composition of the pharmaceutical formulation (tablets) used consisted of 15.0 mg of coumarin and 90.0 mg troxerutin per tablet. The five tablets were maceration and 0.0102 g dissolving in a volumetric flask of 5.0 mL with acetonitrile (working standard solution). Extract solutions of the coumarin were prepared by transferring 80 μL aliquots of the acetonitrile working standard solutions into a 2.0 mL volumetric flask and adjusting to marker with the Milli-Q water required volume. The solutions were then shaken vigorously before analytical measurements. The standard addition procedure was used for the recovery experiments and the results obtained were compared to the chromatographic technique.
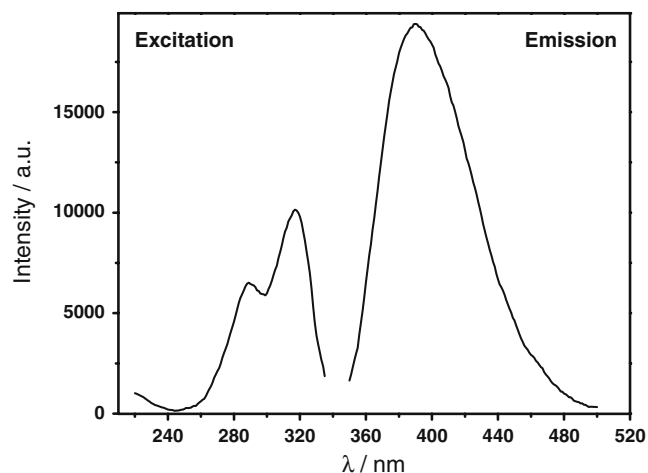
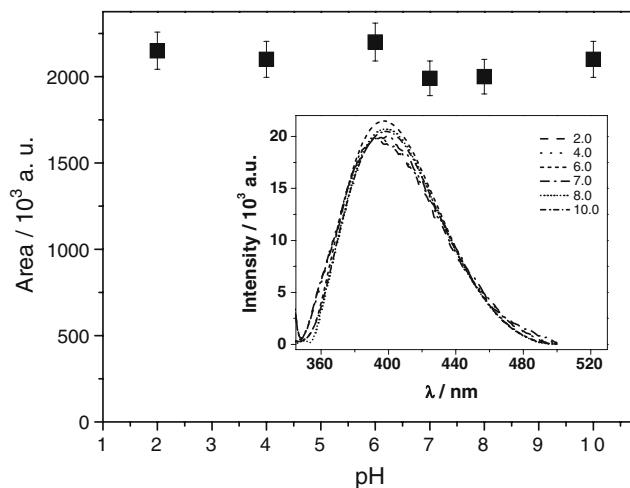**Fig. 2** Excitation and emission spectra of coumarin (4.0×10^{-5} mol L^{-1}) in pure water

Fig. 4 Integrated emission spectra for coumarin in different pH values ($\lambda_{\text{emission}} = 390$ nm). Insert: Emission spectra of coumarin in different pH values (concentration 5.0×10^{-5} mol L^{-1})
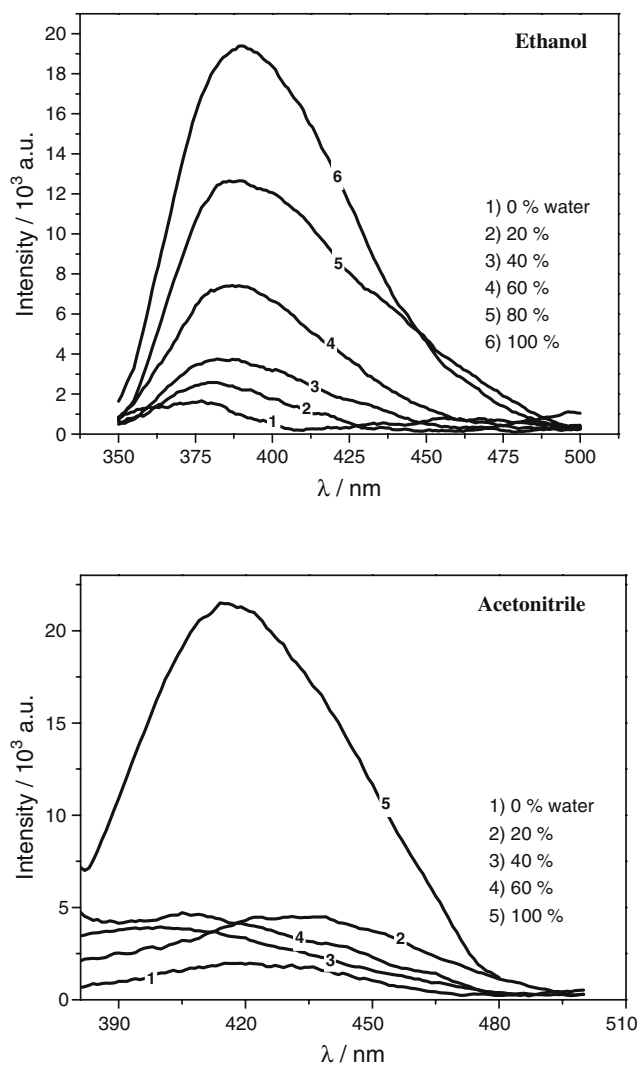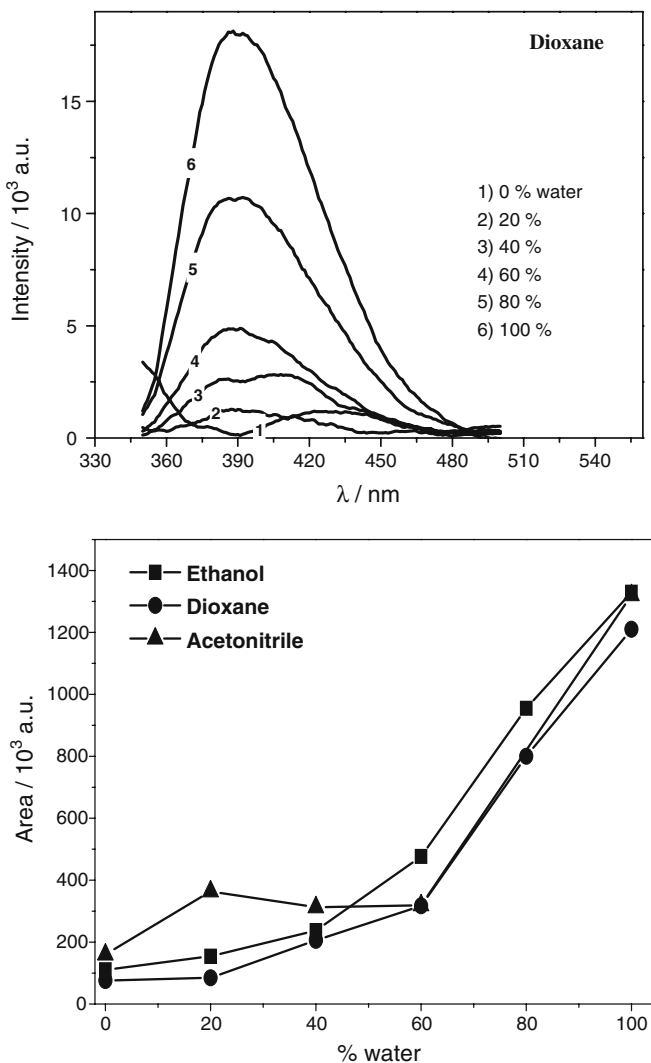

Fig. 3 Emission spectra and variation of emission intensity (by integrated area of spectrum) ($\lambda_{\text{emission}} = 390$ nm) of coumarin in different solvents (coumarin concentration 4.0×10^{-5} mol L^{-1})
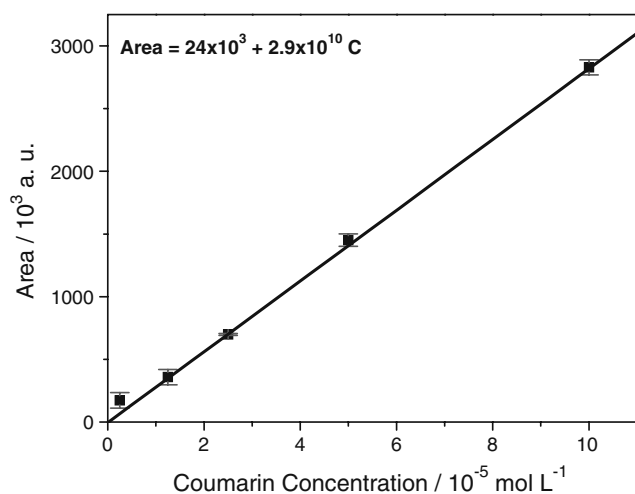
Fig. 5 Analytical curve for coumarin in Milli-Q water ($\lambda_{excitation}$ de 310 nm e $\lambda_{emission}$ de 390 nm)

Results and Discussion

Optimization of Parameters

Figure 2 shows the fluorescence spectra (excitation and emission) obtained for coumarin, which notes that the maximum excitation and emission wavelengths shows a band peak at 310 nm and 390 nm, respectively. Thus the value of 310 nm was the wavelength of excitation used for obtaining the emission spectra.

The solvent and the pH solution influence on the relative intensity fluorescence of coumarin were evaluated. Solvent is an important parameter for evaluating the development of an analytical method using fluorescence as the signal may undergo modifications according to the solvent used. Changing solvent polarity can causes an alteration of the absorption and emission spectra of the coumarin. Coumarins are sensitive to solvent polarity; the excited singlet state of these compounds appears to form a twisted internal charge-transfer state only in very polar solvents such as water [15–17]. Solvents evaluated were water, acetonitrile, dioxane and ethanol. As can be seen in Fig. 3, a significant enhancement of the signal was observed for coumarin in aqueous solution, which appears to be the convenient

medium for analytical purposes. This suggests that the interaction between the water molecules with coumarin increase with time due to salvation affect the shift of the absorption and fluorescence spectra maxima. The compound analyzed contains carbonyl group which can form hydrogen bonds with certain solvents both in the ground state and the excited state. Hydrogen-bond formation leads to solvation of the molecules as a result of dipole-dipole interaction. The degree of solvation is governed by the electron-density distribution in the molecules, on which the value of the permanent (for polar molecules) or induced (for nonpolar molecules) dipole moment depends [18].

The influence of pH of the medium in the intensity of the fluorescence signal of the coumarin was also evaluated (see Fig. 4). No influence of pH on the energy of the absorption transitions has been observed. This indicates that for the case of coumarin, the ionic strength of the medium has no influence on the excited states of the same [18]. Thus, to obtain the analytical curve, Milli-Q water was used as the only solvent. Additionally, the use of saline solutions as solvent also did not influence the spectral profile of coumarin.

Analytical Applications

The linearity, linear range and sensitivity were obtained from calibration graphs using an external standard at five concentration levels, in triplicate, between 2.5×10^{-6} to 1.0×10^{-4} mol L^{-1} coumarin in Milli-Q water (Fig. 5). The linearity was tested using a pure error lack of fit test with simple regression, which was not significant at the 5% level. The sensitivity (slope of the calibration graph) and linearity (correlation coefficient) are calculated as 2.9×10^{10} a. u./mol L^{-1} and 0.998, respectively. The corresponding linear equation was determined as Area $= 24 \times 10^3 + 2.9 \times 10^{10}$ C, where C is coumarin concentration. The inter-assay precision, expressed as the estimate relative standard deviation, established through the analyses of a 1.0×10^{-5} mol L^{-1} coumarin solution ($n=10$) was 1.1%.

The limit of detection (LOD) established as 1.0×10^{-6} mol L^{-1} coumarin represents the lowest concentration of an analyte in sample solutions that can be detected in the

Table 1 Determination of coumarin in tablets (coumarin nominal value 15 mg/tablet)

Sample	Standard addition method Average ± s[a] (coumarin mg)	External calibration method Average ± s[a] (coumarin mg)	Chromatographic method Average ± s[a] (coumarin mg)
1	14.2 ± 1.1	14.1 ± 1.0	13.8 ± 2.5
2	13.7 ± 0.6	13.9 ± 0.5	13.4 ± 4.2
3	15.3 ± 1.0	14.8 ± 0.9	14.5 ± 3.2
4	15.1 ± 0.9	15.2 ± 1.0	14.7 ± 3.7
5	15.7 ± 1.0	15.2 ± 1.2	14.8 ± 3.0
6	13.5 ± 1.0	13.7 ± 1.2	14.1 ± 3.5

[a] Confidence interval ($P=0.05$); s: estimate of the absolute standard deviation ($n=10$)

fluorimetric cell and was calculated by the following expression LOD=3 $s_{y/x}b^{-1}$, where $s_{y/x}$ is the residual standard deviation of the regression line and "b" is the slope of the calibration graph. The quantitation limit of the method are not presented, due to the fact that the active compound is the major constituent of the formulation and this parameter is not required for method validation for the quality control of pharmaceutical products. Furthermore, these limits would depend on sample dilution before analysis [19, 20]. These analytical parameters compare favorably with those reported for coumarin by more sophisticated methods such as HPLC-UV in extracts of guaco [21], capillary electrophoresis in extracts from roots [22], and by capillary electrochromatography in extracts of angelica dahurica [23].

Spectrofluorimetric method was *in-house* validated for the determination of the coumarin in pharmaceutical formulations (tablets) by evaluation of the following parameters: linear range, linearity, sensitivity, limit of detection, intra-assay precision and recovery tests [19]. The recovery tests were evaluated by comparing the results obtained from the analysis of tablets by addition standard method with the external calibration method. It was verified that the slope of both regression lines did not differ significantly, indicating that no matrices effect is present ($P<0.05$). These results are in the Table 1.

The accuracy was evaluated by comparing the results obtained from the analysis of pharmaceutical formulations by the proposed spectrofluorimetric method with a previous validated HPLC method (Table 1). The intra-assay precision was determined by using one sample containing coumarin in the concentration of 5.0×10^{-5} mol L^{-1}, $n=10$, where the relative standard deviation (RSD) it was of 2.0%. Also, the obtained results did not exhibit significant differences compared to the data obtained by the chromatographic method.

Recovery studies were performed using the standard addition method. In this study, known amounts of coumarin (analytical standard) were added to two samples (coumarin concentration in cell was 5.0×10^{-5} mol L^{-1} pre-analyzed. According to the results obtained the average percentage of recovery of coumarin are among 99.0–102.7%, indicating the good accuracy of the proposed method. The applied method shows goods results in order to quantify coumarin in drugs contained in pharmaceutical formulations (tablets). And also, the fluorescence technique shows faster responses than chromatographic methods, and with the advantage of not consuming a large volume of solvents.

Conclusions

In summary, a simple and direct fluorescent probe has been successfully developed for the determination of coumarin.

The proposed quantitative method proved to be a capable of permoing determination of coumarin and thee analytical curve showed linearity in the concentration range of 2.5×10^{-6} to 1.0×10^{-4} mol L^{-1}. Evaluating the parameters for validation it was concluded that the methodology developed can be applied in the analysis of pharmaceutical formulations, presenting the advantages of no need of a laborious preparation of samples, be quick and use small quantities of organic solvents. In the analysis of pharmaceutical formulation (tablets) no interference in the matrix method. Thus, the method has shown to be suitable for the quantification of coumarin in pharmaceutical tablets and might possess great potential to be further modified as a general and promising alternative for practical applications, and is reasonably in good agreement with the chromatographic method for coumarin determination.

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