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Complexation Study and Spectrofluorimetric Determination of Pipemidic Acid with γ-Cyclodextrin

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Abstract

The fluorimetric characteristics of pipemidic acid (PIPE) have been investigated. It has been proven that the fluorescence emission band of pipemidic acid at 439 nm is significantly intensified in the presence of γ-cyclodextrin. The inclusional complexation between the antibacterial pipemidic acid and γ-cyclodextrin (γ-CD) has been studied. A 1:1 stoichiometry of the complex was established and its association constant was calculated by a nonlinear regression method, monitoring the changes in the fluorescence signal of pipemidic acid in the presence of γ-CD. According to the results obtained, a spectrofluorimetric method for the determination of PIPE has been proposed. The best limits of detection and quantification were obtained in presence of γ-CD, in acidic media. The dynamic range of the method was comprised between 0.18 and 1.40 μg/ml.

Introduction

It is known that cyclodextrins (CDs) have the property of forming inclusion complexes with guest molecules that have suitable characteristics of polarity and dimension [1–6]. The inclusion complex formation in the CD systems is favoured by substitution of the high-enthalpy water molecules located inside the CD cavity, with an appropriate guest molecule of low polarity. An overview of the non-chromatographic analytical uses of CDs has been presented by Szente and Szejtli [7]. Aspects covered in that review included: sustrate/analyte solubilization and stabilization by CD; sensitivity improvement by CD; CDs in enzimological analysis and development of CD-based sensors and detectors with particular emphasis on biopharmaceutical and clinical analysis. The non-radiative decay processes of the analyte are often significantly attenuated and the fluorescence emission increases. This fact can be used as a resource for improving the performance of fluorimetric analytical methods.

We have previously reported studies concerning the interactions of γ-CD with other quinolones [8–10], and the aim of this paper is the continuity of those studies. Pipemidic acid, 8-ethyl-5,8-dihydro-5-oxo-2-(1-piperazynil)pyrido[2,3-*d*]pyrimidine-6-carboxylic acid (Scheme 1), is a therapeutic agent for urinary tract infections owing to its antibacterial spectrum against gram-negative bacteria [11–13]. A number of different instrumental methods involving spectrophotometric [14–16], electroanalytical [17, 18], chromatographic [19–22] and electrophoretic [23–25] techniques for the determination of PIPE have been reported. Several spectrofluorimetric methods for the determination of PIPE in the presence of metal ions have been proposed and, in all cases, an enhancement of fluorescence signals has been observed. Egorova *et al*. [26] proposed a method based on the formation of a complex with europium. Durán *et al*. [27] studied several complexes of quinolones with Zn(II) and Al(III), and proposed a method for the determination of pipemidic acid in presence of Al(III). In the present work, the guest-host complex with γ-CD responsible for the analytical signal was investigated prior to the quantitative analysis. Based on the obtained results, the optimum working conditions were established and spectrofluorimetric methods for the determination of PIPE in both the presence and absence of γ-CD are discussed.

Experimental

Apparatus

Fluorescence measurements were made on a Perkin–Elmer Model LS50 luminescence spectrometer, equipped with a xenon discharge lamp equivalent to 20 kW for 8 μs duration. The instrument was connected *via* an RS-232 interface to a Pentium PC

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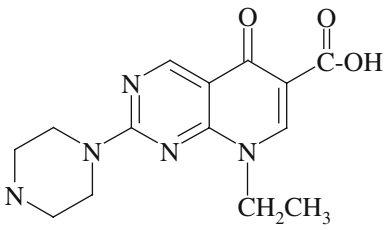

Scheme 1

microcomputer. Data acquisition and data analysis were performed by use of the Perkin–Elmer fluorescence data manager software, version 2.70. Fluorescence measurements were made with excitation and emission bandwidths of 4 and 8 nm, respectively. The scan rate of the monochromators was maintained at 10 nm/s. All measurements were made at 10 ± 0.1 °C, by use of a thermostatically controlled cell holder, and a Selecta Model 382 thermostatically controlled water-bath.

Reagents and sample preparation

All experiments were performed with analytical-reagent grade chemicals. Purified liquid chromatographic grade water (Milli-Q system) was used. Pipemidic acid and γ-CD were obtained from Sigma and used as received.

A 1.06×10^{-3} mol/l stock standard solution of PIPE was prepared in dymethylformamide (DMF). A 4×10^{-2} mol/l stock solution of γ-cyclodextrin was prepared in water. Solutions of lower concentrations were prepared by appropriate dilution of the stock solutions with Milli-Q water.

General procedure for fluorescence measurements

For the fluorimetric study of PIPE, the initial concentration was maintained at 5.8×10^{-6} M. The excitation spectrum was registered between 240 and 400 nm, and the emission spectrum between 350 and 550 nm.

For the fluorimetric study of the PIPE-γ-CD complex, the PIPE concentration was held constant at 5.84×10^{-6} M, while γ-CD concentrations were varied from 0 to 1.85×10^{-2} M.

The influence of pH in the fluorescence spectra of both pipemidic acid and PIPE-γ-CD systems was studied with constant concentrations of pipemidic acid and γ-CD of 5.84×10^{-6} M and 1.55×10^{-2} M, respectively. The pH of all solutions were adjusted, over the range 2.3–6.7, by the addition of trace amounts of hydrochloric acid or sodium hydroxide. The temperature was maintained at 20 °C. From the profiles of fluorescence signals at the wavelength of the maxima ($\lambda_{ex} = 276$ nm, $\lambda_{em} = 439$ nm) *versus* pH, the deprotonation constants of pipemidic acid, both in presence and in absence of γ-CD, were evaluated. These calculations were performed applying the methods of Stenström and Goldsmith [28], adapted to fluorescence measurements, and of Wilson and Lester [29].

Determination of the stoichiometry and association constant of the complex

The stoichiometry of the complex with γ-CD was established by the methods of Scathard [30] and Benesi–Hildebrand [31]. The following equilibrium can be considered:

$$\text{PIPE} + \gamma\text{-CD} \rightleftharpoons \text{PIPE-}\gamma\text{-CD}.$$

In the Scatchard's method, in the case of a 1:1 complex, the relationship between the observed fluorescence intensity enhancement (F–F_0) and the γ-CD concentration is given by:

$$\frac{(F - F_0)}{[\gamma\text{-CD}]_0} = (F_\infty - F_0)K_1 - (F - F_0)K_1.$$

In this expression, $[\gamma\text{-CD}]_0$ is the analytical concentration of γ-cyclodextrin, F_0 denotes the fluorescence intensity of PIPE in the absence of γ-CD, F_∞ denotes the fluorescence intensity when all of the PIPE molecules are essentially complexed with γ-CD, and F is the observed fluorescence at each γ-CD concentration tested.

If the assumption of a 1:1 stoichiometry for the complex is applied, plotting $(F-F_0)/[\gamma\text{-CD}]_0$ *versus* $(F-F_0)$ should give a straight line.

In the Benesi–Hildebrand's method, in the case of a 1:1 complex, the following equation is applicable:

$$\frac{1}{(F - F_0)} = \frac{1}{(F_\infty - F_0)K_1[\gamma\text{-CD}]_0} + \frac{1}{(F_\infty - F_0)}$$

If the stoichiometry is 1:1, the representation of $1/(F-F_0)$ versus $1/[\gamma\text{-CD}]_0$ should give a linear plot.

Association constant of the inclusion complex In the case of the Scatchard method, the association constant is given by the slope of the straight line, while in the Benesi–Hildebrand method, it is determined by dividing the intercept by the slope of the straight line. We used non-linear least-squares regression analysis (NLR) [31], an alternative and more appropriate approach than the graphical methods. The initial parameter estimates needed for this method were obtained from the linear plots, and the NLR analysis of the data was performed by an iterative Marquardt-type process, by using the following equation:

$$F = F_0 + \frac{(F_\infty - F_0)K_1[\gamma\text{-CD}]_0}{1 + K_1[\gamma\text{-CD}]_0}.$$

AM1 calculations

Ground-state geometry optimization of the proposed structures for the inclusion complexes was performed with the AM1 method contained in the Hyperchem package, version 6.00, on a Pentium PC microcomputer. Molecular mechanics method MM+ was used to obtain

the initial structures. Afterward, the energy was minimized by the AM1 method, using the conjugate gradient Fletcher–Reeves algorithm with a RMS gradient lower than 1 kcal/mol Å.

Results and discussion

Preliminary studies suggested an inclusion complex formation between pipemidic acid and γ-CD. Pipemidic acid is a carboxylic acid and therefore it is present in solution as an equilibrium between its protonated and deprotonated forms, according to the pH value. The carboxylic group, in turn, may or may not be included into the CD. Conclusions about the spatial distribution of this group could be obtained by comparison of the pK_a values calculated in the presence and in the absence of γ-CD.

Fluorimetric study of the inclusion complex of pipemidic acid with γ-CD

Pipemidic acid presents native fluorescence. The excitation spectrum shows two maxima located at 276 and 327 nm, and the emission spectrum shows only a maximum located at 439 nm, (Figure 1). The shortest excitation wavelength has been used for all the spectrofluorimetric studies, as the emission intensity obtained is higher when exciting at this wavelength. In consequence, the wavelengths chosen for the measurements were 276 and 439 nm, for excitation and emission, respectively.

Although α-, β-, and γ-CDs were investigated, only γ-CD produced changes on the fluorescence spectra of PIPE. The different inner cavities of the CDs enable them to discriminate among guest molecules on the basis of their sizes. It is apparent that γ-CD possesses the optimal conditions for the partial inclusion of PIPE. Figure 2 shows the emission spectra of aqueous PIPE solutions with different concentrations of γ-CD. As may

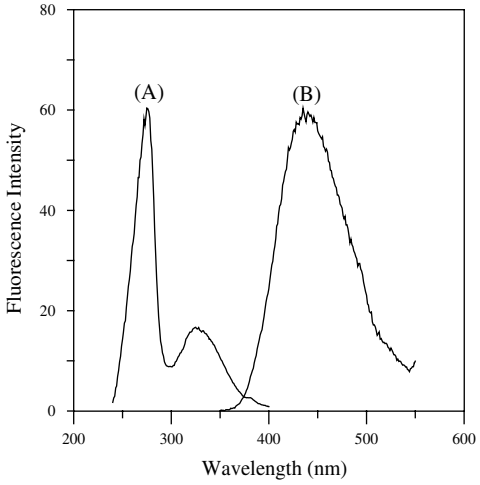

Figure 1. Excitation (A) and emission (B) spectra of pipemidic acid (PIPE) in aqueous media.

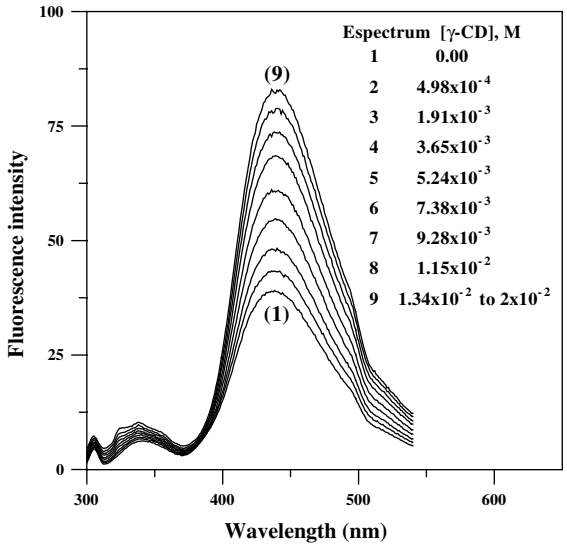

Figure 2. Influence of γ-CD concentration on the fluorescence emission spectra of PIPE. [PIPE] = 5.84×10^{-6} M; $\lambda_{ex} = 276$ nm; $\lambda_{em} = 439$ nm.

be appreciated, the changes in the fluorescence signals are significant, but the wavelengths do not change. Upon inclusion of a fluorophore, in the cyclodextrin cavity, generally the fluorescence of the guest molecule is enhanced by shielding the excited species from non-radiative processes occurring in the bulk solution. It can be observed that the intensity of fluorescence increases when increasing the concentration of γ-CD. A value of γ-CD concentration of 1.55×10^{-2} M was selected for the experiments.

Study of the influence of pH

A study of the influence of pH in the fluorescence spectra of both PIPE and PIPE-γ-CD systems has been carried out. The results obtained are presented in Figure 3. As can be appreciated, the fluorescence, in presence of CD and when CD is absent, is constant for pH values between 2.5 and 4.6. For values of pH higher than this, the fluorescence decreases. For pH > 4.5, a small hipochromic displacement of the maximum of emission can be observed. The fluorescence disappears at pH > 7.5, presumably due to the direct quenching by OH^-. Similar effects have been described previously for other systems [32, 33].

The variation of fluorescence with the pH allowed the quantification of the pK_a, corresponding to the dissociation of the 6-carboxylic acid group of the molecule of PIPE, and of the complex PIPE-γ-CD. The results obtained are summarized in Table 1. The fact that the values of the pK_a found, in the absence and in the presence of γ-CD, are not statistically different is an indication that, in the inclusion complex, the carboxylic functional group must be located outside the cavity. These values are in agreement with the value

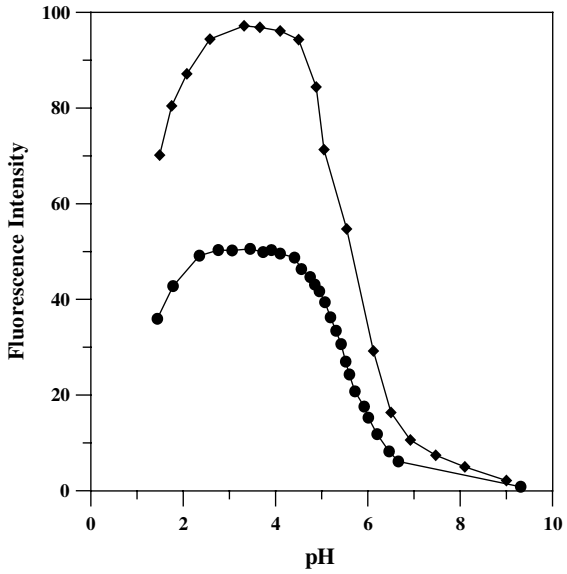

Figure 3. Influence of pH on the fluorescence intensity of PIPE (●) and PIPE-γ-CD complex (♦). [PIPE] = 5.84×10^{-6} M; [γ-CD] = 1.55×10^{-2} M; $\lambda_{ex} = 276$ nm; $\lambda_{em} = 439$ nm, for both PIPE and PIPE-γ-CD complex.

Table 1. Experimental acidity constants, pK_a, for PIPE and PIPE-γ-CD

	Stenström and Goldsmith method	Wilson and Lester method
PIPE	5.60 ± 0.03	5.59
PIPE-γ-CD	5.72 ± 0.06	5.84

reported previously by Barbosa *et al.* (5.42 ± 0.03) obtained by electrophoretic measurements [34, 35].

Influence of the buffer concentration, temperature, and order of addition of reagents

Several buffer solutions were studied with the aim of proposing methods for the determination of PIPE, in the absence and in the presence of γ-CD. An acetic acid/sodium acetate buffer (pH 3.5) was selected as the dependence of the fluorescence intensity with the buffer concentration is not appreciable. A 0.10 M concentration was chosen as the optimum.

The fluorescence emission decreases as the temperature increases. A constant temperature of 10 ± 0.1 °C is recommended for the method.

The order of addition of the reagents has no influence on the complexation, and the inclusion process is attained immediately.

Stoichiometry of the inclusion complex

The straight line obtained when, in accordance with Scatchards method, $(F-F_0)/[\gamma\text{-CD}]_0$ is plotted against $[F-F_0]$, supports the existence of a 1:1 complex (Figure 4A).

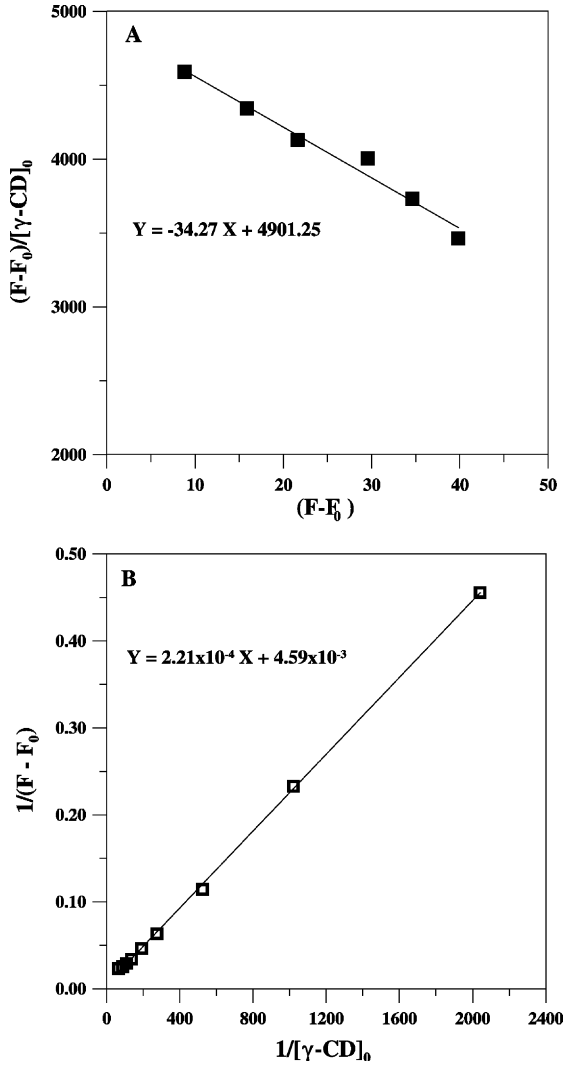

Figure 4. (A) Scatchard plot for the PIPE-γ-CD complex. (B) Benesi–Hildebrand plot for the complex.

Applying the Benesi–Hildebrand's method, the representation of $1/(F-F_0)$ *versus* $1/[\gamma\text{-CD}]_0$, is also giving a linear plot (Figure 4B), confirming the 1:1 stoichiometry.

Association constant of the inclusion complex

Once the stoichiometry of the system is known, the association constant can also be calculated by application of the methods previously described. In the case of the Scatchard method, the association constant is given by the slope of the straight line and the value obtained was 34 ± 3 M^{-1}.

By the Benesi–Hildebrand's method, the association constant is determined by dividing the intercept by the slope of the straight line obtained in the double-reciprocal plot. The value obtained was 21 ± 3 M^{-1}.

However, the linear transformations used in these graphical methods do not properly weigh the data [31]. The double-reciprocal plots tend to place more emphasis

on lower concentration values in comparison to higher ones. As a result, the value of the slope is very dependent upon the ordinate value corresponding to the point having the smaller cyclodextrin concentration. Therefore, a more adequate estimation can be made by using non-linear least-squares regression analysis (NLR). The formation constant calculated from the linear method may be used, however, as a parameter estimate in the NLR method. With the equation described in the experimental section, the experimental data can be directly fitted and a value of $28 \pm 4\ M^{-1}$ was obtained.

Characterizing the inclusion complex

With the purpose of further characterizing the inclusion complex, semiempirical MO calculations using the AM1 program were performed. This program is commonly used to study geometric and thermodynamic properties of organic molecules, especially when hydrogen bonding occurs [36]. Several initial modes of inclusion were probed and optimized by energy minimization. The complex structure leading to the minimum heat of formation shows the amino ring located inside of the γ-CD cavity. This fact is not surprising, since the most probable mode of binding in the CD inclusion complexes involves the insertion of the less polar part of the molecule into the cavity, while the more polar groups are exposed to the bulk solvent outside the opening of the cavity. The optimized structure of the complex, obtained by energy minimization, is displayed in Figure 5.

Analytical parameters

The spectrofluorimetric determination of PIPE, in both the absence and the presence of γ-CD, involves the construction of the corresponding calibration curves. In order to attain the pH-dependent fluorescence emission, the acidic medium was maintained constant with a pH 3.8 acetic acid/sodium acetate buffer. Analytical characteristics of the determination of PIPE with the proposed methods are summarized in Table 2. As can be appreciated, the quality of the calibration in presence of γ-CD significantly improves with respect to that without CD, while the limit of detection is significantly lower in presence of γ-CD.

Conclusions

On the basis of spectrofluorimetric measurements, the complex formation between PIPE and γ-CD was

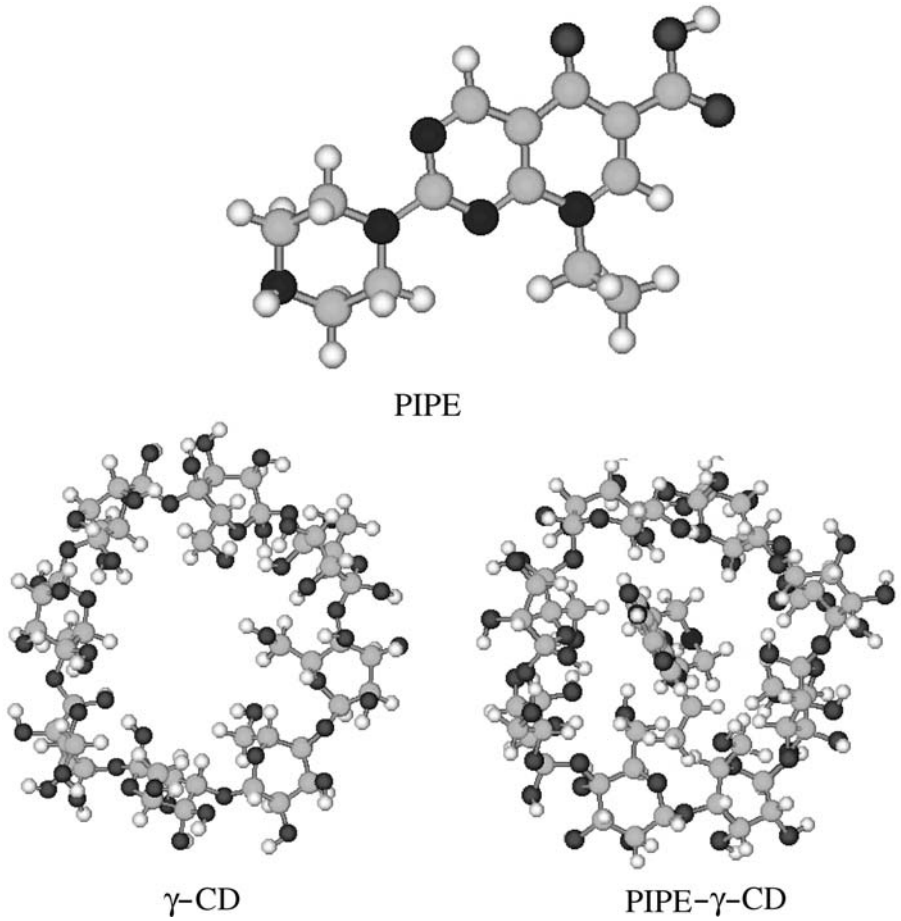

Figure 5. Optimized structures of PIPE, γ-CD and PIPE-γ-CD.

Table 2. Analytical and statistical parameters for the determination of PIPE

Parameter	In absence of γ-CD	In presence of γ-CD
Linear range (μg/ml)	0.40–2.15	0.18–1.40
Calibration curve	$Y = 32.52X + 1.43$	$Y = 43.13X + 4.57$
Correlation coefficient (r)	0.994	0.994
Reproducibility (%)	1.7 (1.2 μg/ml)	0.8 (0.80 μg/ml)
LOD[a] (μg/ml)	0.2	0.1
LOD[b] (μg/ml)	0.3	0.2

[a]Limit of detection according to Long and Winefordner [37].
[b]Limit of detection according to Clayton *et al.* [38].

studied. Both the stoichiometry and equilibrium constant for the inclusion complex were evaluated, and a structural model was proposed. The studies performed suggest that the 6-carboxylic group remains outside the γ-CD cavity, because the pK_a values obtained are similar for both systems, in the absence and in the presence of γ-CD. A method for the spectrofluorimetric determination of PIPE was developed.

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