Spectrofluorimetric determination of piroxicam in the presence and absence of β-cyclodextrin

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The complexation between β-cyclodextrin (β-CD) and piroxicam (PX) was investigated by both fluorescence and absorption spectrometry. A 1 : 2 guest : host stoichiometry for the complex was established, and its association constant was calculated by applying a non-linear regression method to the changes brought about by the presence of β-CD in both the fluorescence and absorbance spectra of PX. During the study of the influence of the pH on the fluorescence emission of the complex, an efficient enhancement of the signals at acidic pH was observed. This suggests that the protonated form of PX is included more effectively than the ionized form in the β-CD cavity. Based on the results obtained, spectrofluorimetric methods for the determination of PX were developed. The best limits of detection and quantification were obtained using β-CD at an acidic pH. The dynamic range in this latter case was 0.02–1 μg ml^{-1}. The method was applied satisfactorily to the determination of piroxicam in a pharmaceutical preparation.

Introduction

Piroxicam (PX), 4-hydroxy-2-methyl-*N*-2-pyridinyl-2*H*-1,2-benzothiazine-3-carboxamide 1,1-dioxide (Fig. 1), is a non-steroidal anti-inflammatory agent which is widely used in the treatment of rheumatic diseases.[1] Its commercial availability for medical use is relatively recent. The properties and therapeutic efficacy of PX have been extensively described.[2] The employment of several analytical methods (voltammetry, spectrophotometry, chromatography) for the determination of PX in pharmaceutical samples and biological fluids has been proposed.[3–6] Recently, the use of spectrofluorimetry for the quantification of PX was reported.[7] In this case, PX is dissolved in an alkaline medium and the solutions are strongly acidified before the spectrofluorimetric determination. Since spectrofluorimetry has the advantages of both sensitivity and simplicity, it has found extensive use in the determination of inorganic, organic and bioactive materials.[8] However, the technique could be improved by using the ability of cyclodextrins (CDs) to form inclusion complexes with certain analytes which may show different fluorescent properties than those of the free compounds.[9–13] The inclusion into the CDs depends on both the size and polarity of the guest,[14] hence the specificity of the method can be extended in addition to the sensitivity. This property of CDs has been used previously by analysts, and different techniques based on the fluorescence of inclusion complexes with CDs have been proposed for the determination of several pharmaceutical drugs and pesticides.[15–18] It is desirable to characterize the complex species responsible for the analytical signal prior to the quantitative analysis, and also to determine the optimum working conditions. Since the CDs may in principle modify both the photochemical and photophysical properties of their guest molecules, both fluorescence and absorbance signals can be used to gain insight into the characteristics of the complexes formed.[19] In this work, the inclusion complex of PX with β-CD was investigated through spectroscopic techniques. PX was dissolved in dioxane–water and the solutions were diluted with water in order to obtain samples which were almost entirely aqueous. Spectrofluorimetric methods for the determination of PX in both the presence and absence of β-CD are discussed.

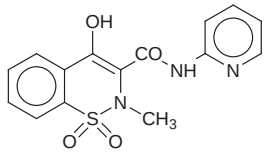

Fig. 1 Piroxicam.

Experimental

Apparatus

Fluorescence measurements were performed using a Shimadzu (Kyoto, Japan) RF-5301 PC spectrofluorimeter equipped with a 150 W xenon lamp, using 1.00 cm quartz cells, slit widths of 5 nm, excitation at 320 nm and fluorescence emission obtained at 440 and 450 nm for PX in the presence of β-CD at neutral and acidic pH, respectively, and at 460 nm for PX in the absence of β-CD. Absorbance data were obtained with a Beckman (Fullerton, CA, USA) Model DU 640 spectrophotometer. The pH of solutions was measured with a Metrohm (Herisau, Switzerland) 713 pHmeter equipped with a combined glass electrode.

Reagents

Analytical-reagent grade chemicals were used in all experiments. Piroxicam was obtained from Pfizer (Sandwich, Kent, UK). β-CD (Aldrich, Milwaukee, WI, USA) was recrystallized twice from water. 1,4-Dioxane was obtained from Merck (Darmstadt, Germany). Doubly distilled water was used throughout.

Influence of pH

The changes in the fluorescence of PX and the PX–β-CD complex as a function of pH were studied by the following procedure: to a stirred nitric acid solution of PX or PX–β-CD, NaOH solution was added in small increments (0.01–0.2 ml of *ca.* 0.05 mol l^{-1} solution). For each pH point a known aliquot of solution was extracted and the fluorescence spectrum was measured. The initial concentrations of PX and β-CD in the reaction vessel were 3.20×10^{-6} and 9.0×10^{-3} mol l^{-1}, respectively. The percentage of dioxane present in the experimental solutions (0.04%) was considered to be too low to have any significant influence on the acid–base behaviour of PX. The temperature and ionic strength (μ) were maintained at 20 °C and 0.10 mol l^{-1}, respectively. The small background fluorescence from β-CD was not affected by the pH. The measurements were performed in duplicate. The profiles of fluorescence at the wavelength of the emission maxima *vs.* pH were used to calculate the deprotonation constant of PX in the excited state, both in the presence and in the absence of β-CD. These calculations were performed with the aid of the program EPSILON, adapted to fluorescence measurements.[20] Standard deviations for the reported constants refer only to random errors.[21]

Influence of β-CD concentration

In both the absorbance and fluorescence measurements concerning the PX–β-CD complex, the PX concentration was held constant while the CD concentration was varied from 0 to 9.80×10^{-3} mol l^{-1}. A 4.00×10^{-3} mol l^{-1} stock standard solution of PX was prepared in 50% v/v dioxane–water. For fluorescence studies, 1.00 ml of this solution was diluted with water to 10.00 ml and a 40 μl aliquot of the latter PX solution was transferred into a 5.00 ml flask. An exact amount of 0.01 mol l^{-1} β-CD solution and 30 μl of 0.1 mol l^{-1} HNO_3 were then added and the flask was filled to the mark with water. In this way, the concentration of piroxicam was held constant at 3.20×10^{-6} mol l^{-1} in each flask and the pH was adjusted at 3.5. The dioxane concentration in each flask (0.04%) had a negligible effect on the complex formation. The solutions were read within 1 h. After this time, the fluorescence intensity decreases, possibly owing to the instability of the free and complexed PX in aqueous solution. For absorbance measurements the final concentration of piroxicam was 8.81×10^{-6} mol l^{-1}, with the other experimental conditions being similar to those for the fluorescence experiments. Corrected absorption and fluorescence spectra were obtained by subtracting the spectrum of a blank solution at the corresponding β-CD concentration from each spectrum. The absorbance spectra were read against a blank of water–nitric acid. All measurements were made in duplicate.

AM1 calculations

Ground-state geometry optimization was performed with the AM1 program contained in the Hyperchem package, version 5.02, on a Pentium 150 microcomputer.

Spectrofluorimetric calibration curves

Piroxicam in 1% v/v dioxane–water. Since the PX concentration range in this experiment is higher than that used in the presence of β-CD, a correspondingly higher percentage of dioxane was used with the purpose of maintaining the compound in solution. Although several ratios were checked, a minimum of 1% v/v dioxane–water was found to be adequate. A stock standard solution of PX (100 μg ml^{-1} in 5% v/v dioxane–water) was prepared by dissolving 10.0 mg of PX in 5 ml of dioxane and adding water to 100.0 ml. Working standard solutions for calibration were obtained by convenient dilutions of this stock standard solution with dioxane and water in order to obtain concentrations in the range 0–23 μg ml^{-1}. In all cases the final percentage of dioxane was 1%. The calibration curve was also measured in acidic medium (pH 1.5) by addition of 1mol l^{-1} HNO_3 to each experimental solution.

Piroxicam in β-CD solution. In order to construct the calibration curve, two stock standard solutions were prepared: a 9.0×10^{-3} mol l^{-1} solution of β-CD at pH 3.5 and a solution containing 63.9 μg ml^{-1} of PX and 9.0×10^{-3} mol l^{-1} of β-CD. An aliquot (3.00 ml) of the former solution was transferred into a spectrofluorimetric cell and small volumes of the latter solution were added, measuring the fluorescence spectrum after each addition. Thus, the concentration of PX was varied from 0 to 3 μg ml^{-1} while the β-CD concentration was kept constant. This procedure was replicated in order to obtain three or more fluorescence values for each of the PX concentrations studied.

Pharmaceutical preparation. Ten capsules were weighed in order to find the average mass of each capsule. Then the capsules were triturated and mixed. A sample of 10.0 mg taken from the latter mixture was accurately weighed and dissolved in 1 ml of dioxane and 3–4 ml of water. The mixture was stirred and filtered into a 25.00 ml calibrated flask, the residue was washed several times with water and the solution was diluted to the mark. For the analysis, a 50.0 μl aliquot of this solution and 30 μl of 1 mol l^{-1} HNO_3 were diluted to 3.00 ml with β-CD in order to obtain a 9.0×10^{-3} mol l^{-1} β-CD solution. The fluorescence was measured at 450 nm, with excitation at 320 nm.

Results and discussion

With the purpose of investigating the possible complex formation between PX and β-CD, preliminary studies were carried out. Both the absorption and fluorescence spectra of PX suffer modifications in the presence of β-CD, which are indicative of inclusion complex formation. Since the spectroscopic signals change with the acidity of the solutions, studies of the influence of pH were previously performed.

Influence of pH

PX is an enolic acid with a pK_a of 6.3 in dioxane–water (2 + 1).[22] Therefore, when PX is dissolved in this solvent medium, an equilibrium is present between the dissociated and undissociated PX forms. However, since the purpose of this work was to determine piroxicam by using spectrofluorimetric techniques, it is necessary to know the value of the deprotonation constant in the excited state (pK_a^*). From the changes in the fluorescence intensity at the emission maximum of PX with pH (Fig. 2), the pK_a^* value can be determined. The calculated pK_a^* value for PX was 2.70(6), which indicates that the enolic group is more acidic in the excited state than in the ground state. The pK_a^* value of PX in the presence of β-CD was also calculated from the fluorescence profile as a function of pH (Fig. 2). The value obtained [5.58(4)] is significantly larger than that obtained in the absence of β-CD. This suggests that the enolic group is located inside the β-CD cavity in the inclusion complex.

On the basis of these results, spectroscopic measurements were performed at low pH values, where PX is in its acid form

and the signals are more intense. Because of the instability of CD at very low pH, the use of strongly acidic solutions containing β-CD was avoided.[23]

Inclusion complex of PX with β-CD

Fig. 3 and 4 display absorption and fluorescence spectra, respectively, of PX at different concentrations of β-CD. As the β-CD concentration is increased, the absorption maximum of PX at 360 nm is slightly blue-shifted, with a concomitant decrease in the absorption intensity. An isosbestic point is observed around 336 nm. All these facts are rationalized as being indicative of complex formation. On the other hand, the fluorescence signals are intensified with increasing concentration of β-CD. Apparently, this is due to the protective environment offered by the CD from non-radiative decay processes occurring in the bulk solution. This enhancement of the fluorescence produced through the formation of the complex may be very useful from an analytical point of view. From the changes in either the absorption or the fluorescence spectra, a constant value for the inclusion complex can be determined. Fig. 5(A) shows the fluorescence emission at 450 nm of PX as a function of the β-CD concentration. These experimental data could not be fitted by considering the formation of a 1 : 1 PX–CD complex, but by an 1 : 2 guest–host complex according to the following reaction:

$$\mathrm{PX} + 2\ \beta\text{-CD} \overset{K}{\rightleftharpoons} \mathrm{PX}(\beta\text{-CD})_2 \qquad (1)$$

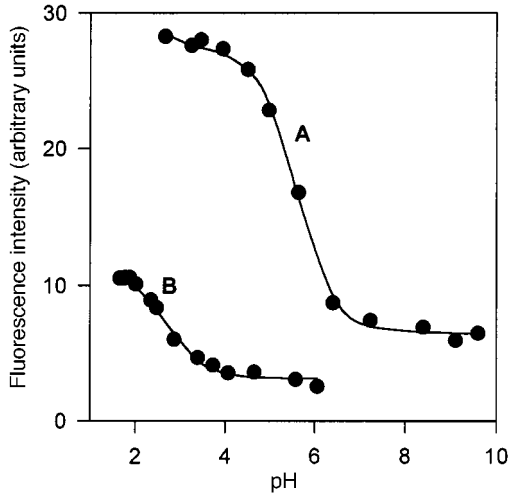

Fig. 2 Experimental (●) and calculated (——) fluorescence values *versus* pH for (A) PX–β-CD and (B) PX. Initial $C_{PX} = 3.20 \times 10^{-6}$ mol l^{-1}; initial $C_{\beta\text{-CD}} = 9.0 \times 10^{-3}$ mol l^{-1}; $\mu = 0.10$ M; $t = 20$ °C; $\lambda_{ex} = 320$ nm; $\lambda_{em} = 440$ nm.

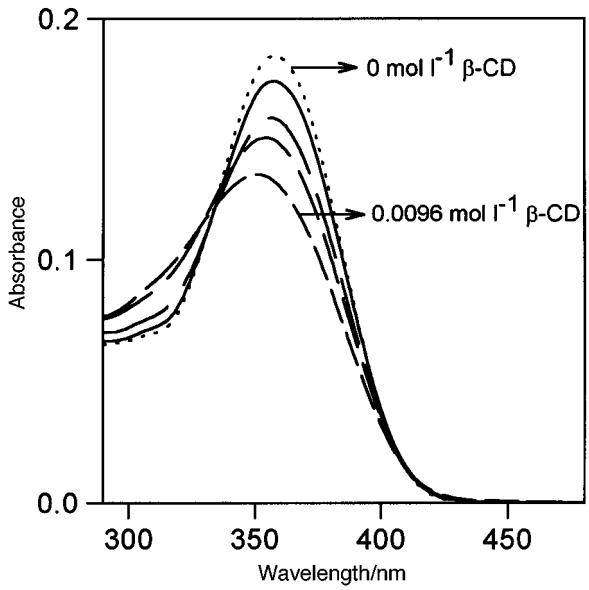

Fig. 3 Fig. 3 Absorption spectra of PX and PX with various concentrations of β-CD: from top to bottom, 0, 0.002, 0.004, 0.006 and 0.0096 mol l^{-1}. $C_{PX} = 8.81 \times 10^{-6}$ mol l^{-1}; pH = 3.5.

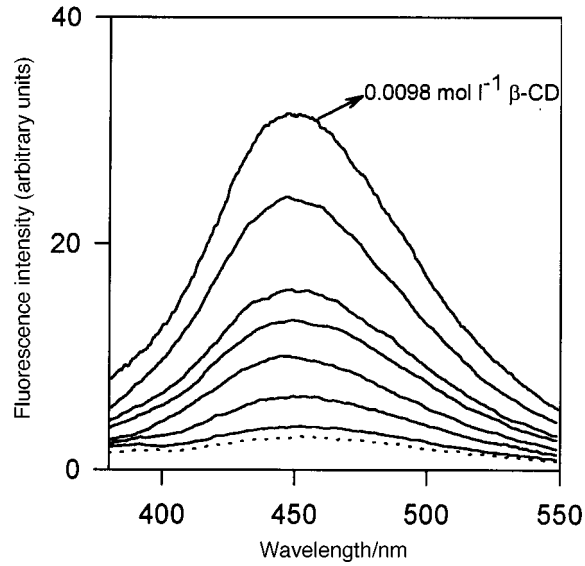

Fig. 4 Fluorescence spectra of PX (bottom, dashed curve) and PX with different concentrations of β-CD: from top to bottom, 0.001, 0.002, 0.003, 0.004, 0.005, 0.007 and 0.0098 mol l^{-1}. $C_{PX} = 2.93 \times 10^{-6}$ mol l^{-1}; pH = 3.5; $\lambda_{ex} = 320$ nm.

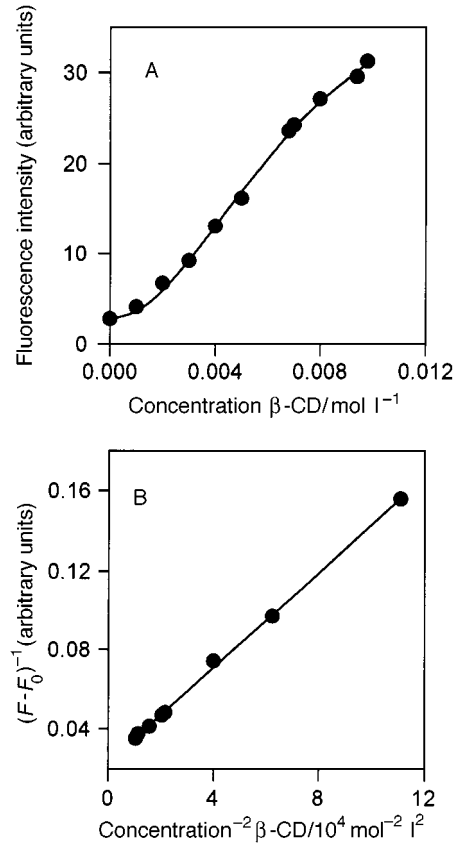

Fig. 5 (A) Influence of β-CD concentration on the fluorescence signal of PX. The solid line is the non-linear fit of the data assuming the formation of a 1 : 2 PX–β-CD complex. (B) plot of $1/(F - F_0)$ *versus* $1/C_{CD}^2$. A linear relationship is obtained when the data are plotted assuming a 1 : 2 stoichiometry of the PX(β-CD)$_2$ complex.

The K value was calculated taking into account that (1) the β-CD is in a large excess with respect to PX and therefore its free and analytical concentrations (C_{CD}) are similar, (2) the variations in either the fluorescence or absorbance signals are proportional to the complex concentration and (3) at high β-CD concentration essentially all of the PX molecules are complexed. Thus, the following equation is obtained:

$$W - W_0 = \frac{(W_\infty - W_0)KC_{CD}^2}{1 + KC_{CD}^2} \quad (2)$$

where W is the observed spectroscopic signal (fluorescence or absorbance) of the PX solution at each β-CD concentration tested and W_0 and W_∞ are the signals in the absence of β-CD and when all PX molecules are complexed, respectively. The best fitted curves provide evidence for the existence of a PX(β-CD)$_2$ complex. Since the β-CD cavity is too small to accommodate a single molecule of PX, it is conceivable that an additional β-CD molecule is involved in the inclusion complex. The straight line obtained when $1/(W - W_0)$ is plotted against $1/C_{CD}^2$ supports the existence of a 1:2 complex. Fig. 5(B) displays the plot corresponding to the fluorescence measurements. The constant values obtained from the changes in the fluorescence emission [$K = 2.0(1) \times 10^4$ mol^{-2} l^2] and from the absorption spectra [$K = 2.2(3) \times 10^4$ mol^{-2} l^2] are in good mutual agreement. Standard deviations refer only to random errors.[21]

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.[24] According to the molecular size, it is apparent that PX (*ca*. 6.0 Å wide and 13.7 Å long) is too large to fit entirely in the β-CD cavity (*ca*. 7.8 Å wide). It is reasonable to consider the complex formation with two molecules of β-CD, where PX appears to be totally included in the relatively hydrophobic β-CD cavities. Similar conclusions have been reported for a pyrene–β-CD complex.[25] Furthermore, PX is a molecule with electron donor groups able to form hydrogen bonds within the CD cavity, which may provide additional stabilization for the 1:2 complex. The optimized structure of the complex, obtained by energy minimization, is displayed in Fig. 6.

Analytical parameters and application

The spectrofluorimetric determination of PX in both the absence and the presence of β-CD involves the construction of the corresponding calibration curves. In order to attain a high and pH-independent fluorescence emission, the acidic media were selected in each case according to the pK_a* values of PX in the absence and in the presence of β-CD, *i.e.*, pH << 2.70 and pH << 5.58, respectively. Together with the calibration curves at acidic pH, additional curves without the addition of nitric acid were obtained with the purpose of comparing the results. The equation for the calibration graph is in all cases $F = h + mC$, where F is the fluorescence intensity (in arbitrary units) and C is the concentration of PX. The results obtained are given in Table 1. In the absence of β-CD, the calibration parameters significantly improve on going to an acidic pH. PX is more fluorescent in its neutral form and therefore the sensitivity of the technique is notably enhanced when PX is determined in this form. The addition of β-CD to the calibration solutions at neutral pH produces similar results to those obtained in the absence of CD at acidic pH. This suggests that the ability of CD to form an inclusion complex which is more fluorescent than the free compound enhances the sensitivity of the method even without pH adjustment. When the calibration in the presence of CD is carried out at acidic pH, the optimum conditions for the complex formation, significant decreases in both the limit of detection (LOD) and the limit of quantification (LOQ) are achieved with respect to the solutions without CD. It is also evident that the analytical sensitivity (γ) has significantly increased (see the last column in Table 1). This parameter allows one to compare analytical methods regardless of the specific technique, equipment and scale employed. It also establishes the minimum concentration difference (γ^{-1}) which is statistically discernible at any point on the calibration curve. Table 2 summarizes the results of determinations carried out on test samples under the different conditions studied.

Piroxicam was satisfactorily determined in the pharmaceutical preparation Solocalm (Microsules-Bernabo, Buenos Aires, Argentina) following the spectrofluorimetric method in the presence of β-CD at acidic pH. The indicated composition is 20 mg of PX and excipients (lactose, starch, povidone, magnesium stearate, cellulose acetophthalate, diethyl phthalate, tartrazine and croscarmellose). The result (average of five replicate

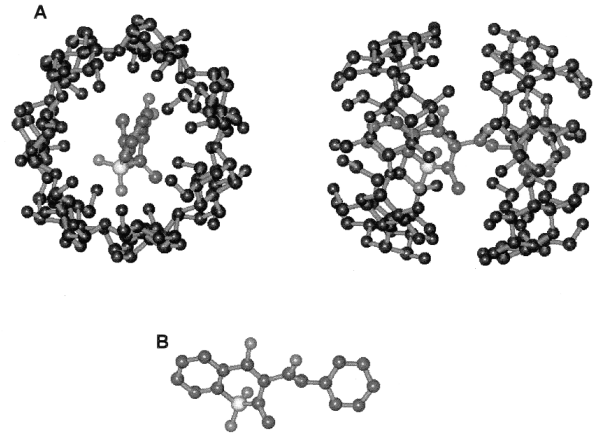

Fig. 6 (A) Two views of the model for the PX(β-CD)$_2$ complex. (B) PX optimized structure (as obtained within the CD cavities). For clarity the hydrogen atoms are not shown.

Table 1 Calibration results for PX[a]

Parameter	1% v/v Dioxane–water	1% v/v Dioxane–water, pH 1.5	9.0×10^{-3} mol l^{-1} β-CD, aqueous solution	9.0×10^{-3} mol l^{-1} β-CD, pH 3.5
Linear range/μg ml^{-1}	0.5–16	0.04–2	0.07–2	0.02–1
Correlation coefficient (r)	0.997	0.997	0.998	0.999
h	1.0 ± 0.1	1.2 ± 0.2	4.0 ± 0.2	2.9 ± 0.2
m[b]	0.61 ± 0.01	8.3 ± 0.1	12.6 ± 0.1	28.6 ± 0.3
γ[c]/μg^{-1}ml	3	22	29	61
LOD[d]/μg ml^{-1}	0.5	0.04	0.07	0.02
LOQ[e]/μg ml^{-1}	1.6	0.1	0.2	0.08

[a] The number of data for each calibration curve correspond to seven different concentration levels, with three replicates for each level (n = 21). [b] The slope of the calibration curve (m) is the calibration sensitivity according to IUPAC.[26] [c] Analytical sensitivity: $\gamma = m/S_s$ where S_s is the standard deviation of the regression residuals.[27,28] [d] Limit of detection: calculated as $3\sigma/m$, where σ is the standard deviation of the blank.[29] [e] Limit of quantification: calculated as $10\sigma/m$.[29]

Table 2 Determination of PX under different experimental conditions[a]

Parameter	1% v/v Dioxane–water	1% v/v Dioxane–water, pH 1.5	9.0×10^{-3} mol l^{-1} β-CD, aqueous solution	9.0×10^{-3} mol l^{-1} β-CD, pH 3.5
Actual PX/μg ml^{-1}	8.1	1.12	0.98	0.52
Found PX ± *s*/μg ml^{-1}	8.4 ± 0.2	1.11 ± 0.02	0.98 ± 0.02	0.52 ± 0.01
Recovery ± *s* (%)	103 ± 2	99 ± 2	100 ± 2	100 ± 2
RSD (%)	2.4	1.8	1.7	1.6
RE[b] (%)	2.1	1.6	1.5	1.4

[a] In all cases five replicates were measured. [b] Relative error (95% confidence level).

analyses) was 19.1 ± 0.3 mg with a recovery of 96 ± 2%. It should be noted that the recommended limits are within ±5% of the indicated amount.

Conclusions

On the basis of spectroscopic measurements, the complex formation between PX and β-CD was studied. Both the stoichiometry and equilibrium constant for the inclusion complex were evaluated and a structural model was proposed. Methods for the spectrofluorimetric determination of PX under different working conditions were developed. The results indicate that the inclusion of PX in its protonated form in the β-CD cavity produces fluorescence signals which are larger than those obtained in the absence of CD, leading to a corresponding improvement in the analytical method.

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