Journal of Inclusion Phenomena and Molecular Recognition in Chemistry **18:** 69–79, 1994.

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Basic Studies of the Influence of β-Cyclodextrin and 2-Hydroxypropyl β-Cyclodextrin on the Photo-Oxidation Reaction of Phenothiazine in Inclusion Complexes, Using Fluorescence Detection

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(Received: 2 February 1994; in final form: 31 May 1994)

Abstract. The photo-oxidation reaction of phenothiazine has been studied in the presence of β-cyclodextrin (β-CD) and 2-hydroxypropyl β-cyclodextrin (HP β-CD). The influence of these organized media on the formation of the oxidation photoproduct upon UV irradiation has been investigated. Phenothiazine forms an inclusion complex with the cyclodextrins. The stoichiometry and formation constant of the complex formed with 2-hydroxypropyl β-CD have been calculated using the changes of the fluorescence emission signal and of the absorbance of the drug upon inclusion. An increase of the fluorescence intensity of the photogenerated product is attained when it becomes included inside the cyclodextrin cavity.

Key words: β-cyclodextrin, 2-hydroxypropyl β-CD, phenothiazine; photochemically-induced fluorescence spectroscopy, molecular absorption spectroscopy.

1. Introduction

Phenothiazine derivatives are usually employed in medicine as psychotropic drugs in pharmaceutical preparations. The dosage required for each patient depends on the particular phenothiazine chosen and the severity of the disease involved. A variety of methods based on the fluorescence of both unoxidized and oxidized compounds have been used for their determination. Chemical [1–3] and photochemical oxidation [4,5] of phenothiazine drugs have been utilized.

Cyclodextrins (CDs) are naturally occurring, torus-shaped, cyclic oligosacharides made of six, seven or eight glucose units (α-, β-, and γ-cyclodextrins) joined by $\alpha - (1,4)$ linkages. They can form stable inclusion complexes with a variety of compounds, depending on the dimensions of the molecules involved. The internal toroidal and relatively non-polar cavity can easily accommodate molecules, or parts

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of molecules, having the size of an aromatic ring. The secondary 2- or 3-hydroxyl groups of each glucopyranose ring in the CD are located axially at the mouth of the cavity. A cyclodextrin often accelerates or decelerates many types of reactions [6], which has interest for pharmaceutical applications.

The interactions of medically-important phenothiazines and cyclodextrin derivatives have been described. A 1 : 1 inclusion complex of β-CD with chlorpromazine has been reported [7]. Chlorpromazine is known to frequently cause cutaneous phototoxic and photoallergic responses in patients treated with prolonged and high doses, because of the toxic photoproducts of chlorpromazine [8]. Dimethyl-β-cyclodextrin significantly reduces the photosensitized skin irritation caused by chlorpromazine [9,10], which may be due to the alteration in the photochemical reactivity of chlorpromazine, rather than the direct interaction of the photoproducts with dimethyl-β-cyclodextrin. When chlorpromazine is photoirradiated in the presence of dimethyl-β-cyclodextrin, promazine, which is less toxic than chlorpromazine, is produced in a high yield [11]. The photochemical oxidation of phenothiazine derivatives is also interesting from an analytical point of view because the oxidation photoproducts formed show a relatively strong fluorescence emission suitable for analytical purposes [12–17].

The aim of this work is to investigate the influence of the presence of cyclodextrins on the photooxidation reaction of phenothiazine, in order to evaluate the potential application of these organized media for improving the room temperature photochemically-induced fluorescence analysis of this compound. We chose to study only β-CD and its derivative, 2-hydroxypropyl (HP) β-CD, because their intermediate-sized cavity allows the complexation of a great variety of analytes and HP β-CD generally yields the largest fluorescence enhancement factors [18].

2. Experimental

2.1. Reagents

Phenothiazine was purchased from Fluka AG (Bush, Switzerland). β-Cyclodextrin (β-CD) was obtained from Sigma and 2-hydroxypropyl β-cyclodextrin (HP β-CD) was purchased from Cyclolab Laboratory (Budapest, Hungary), and used as received. The buffer solutions (pH = 2) were prepared from KCl and HCl obtained from Panreac (Spain). $NaHCO_3$ (RPE, Italy) and NaOH (Merck) were also utilized. Solvents used were distilled and deionized water, and ethanol (Probus, Spain).

2.2. Apparatus

Fluorescence measurements were made on a SLM Aminco Bowman, Series 2 luminescence instrument, equipped with a 150 W continuous xenon lamp, interfaced by a GPIB card, and driven by a PC 386 microcomputer. Data acquisition and data analysis were performed using AB2 software version 1.40, running under OS/2 2.0. The scan rate of the monochromators was maintained at 4 nm s^{-1}. All mea-

surements were performed in a 10 mm quartz cell at 20°C, using a thermostatic cell holder and a Selecta Model 382 thermostatic bath. Absorption measurements were performed with a Beckman DU-64 spectrophotometer interfaced via a RS-232 to an Olivetti PC 286 microcomputer. The Beckman Data Leader Software, version 3.0, was used for spectral acquisition and analysis of the spectrophotometric data.

2.3. PROCEDURE

A 10^{-3} M stock solution of phenothiazine was prepared by dissolving the compounds in ethanol. Aqueous 10^{-5} M solutions were obtained by serial dilution. Various volumes of buffer solutions were used for obtaining the convenient pH value (pH = 2). The working solution of phenothiazine contained 1% of ethanol. An aliquot of phenothiazine aqueous solution was placed in a quartz cuvette and irradiated at 20°C with the xenon lamp of the instrument at the maximum of excitation ($\lambda_{ex} = 311$ nm) of the photoproduct. The kinetics of the photoreaction were monitored at the emission wavelength of the photoproduct ($\lambda_{em} = 385$ nm) for 10 min.

For the study of the influence of the HP β-CD (or β-CD) concentration on the intensity of fluorescence or on the absorption, several solutions were prepared, by maintaining a constant concentration of 10^{-5} M phenothiazine, and buffer solution, and varying the HP β-CD (or β-CD) concentration.

3. Results and Discussion

3.1. FLUORESCENCE CHARACTERISTICS OF PHENOTHIAZINE

The excitation and emission fluorescence spectra of phenothiazine in acid aqueous solution (pH = 2) are characterized by a wavelength of excitation at 301 nm, and emission maxima at 385 and 450 nm. After UV irradiation, the emission spectrum shows a large peak centered at about 385 nm, whereas the peak situated at 450 nm disappears.

3.2. KINETICS OF THE PHOTOREACTION. INFLUENCE OF THE PRESENCE OF CYCLODEXTRINS

In a previous study [16,17], the influence of pH on the photo-oxidation reaction of phenothiazine was investigated. The fluorescence intensity of the oxidation photoproducts changed considerably with the pH of the reaction medium. A pH 2 value, corresponding to the maximum photochemically induced fluorescence signal, was considered to be optimum.

In order to evaluate the influence of the presence of cyclodextrins in the medium on the kinetics of the photoreaction, the effect of β-cyclodextrin and of 2-hydroxypropyl β-cyclodextrin on the photochemically-induced fluorescence intensity was tested.

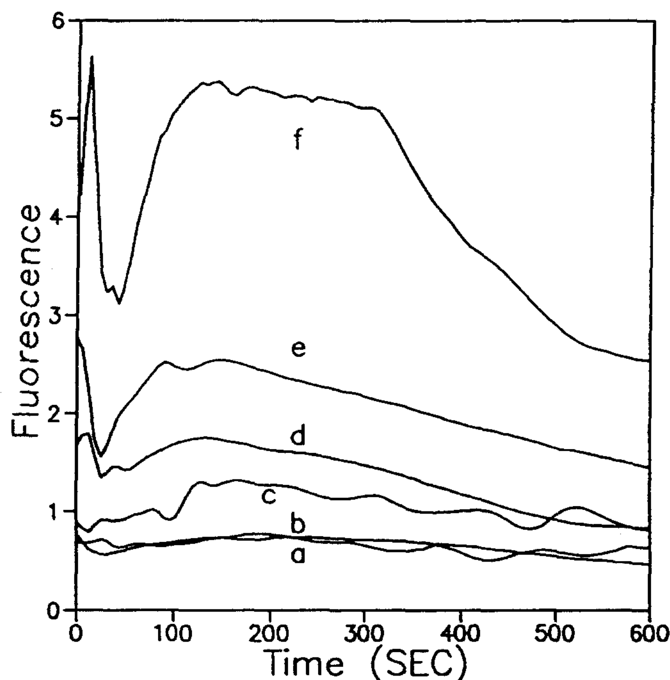

Fig. 1. Kinetics of the photoreaction of neutral (a,c,d) and acid (pH = 2) (b,e,f) aqueous solutions of phenothiazine, (a,b) in the absence of CDs, (c,e) in 10 mM β-CD, (d,f) in 85 mM HP β-CD, $\lambda_{ex} = 311$ nm, $\lambda_{em} = 385$ nm.

Fluorescence enhancements have been observed for a variety of analyte molecules in the presence of β-CD [18]. However, in many instances, it has been found that the magnitude of the fluorescence enhancement attainable with β-CD was restricted because of its limit of solubility in water (0.014–0.016 M). As a consequence, the fluorescence intensity of many analyte molecules increases with cyclodextrin concentration until the β-CD solubility in water is reached. Since the analyte-β-CD complexation process obeys dynamic equilibrium laws, the analyte fluorescence dependence upon the cyclodextrin concentration is due to the increased proportion of analyte molecule which becomes included in the protective β-CD cavity. The implication is that the fluorescence intensity of many analytes should be still further intensified if it were somehow possible to increase the β-CD concentration further above its water solubility limit. The reason for using the HP β-cyclodextrin derivative in addition to the parent compound results from its higher water solubility allowing the use of more concentrated HP β-CD solutions.

In Figure 1 the kinetics of the photooxidation of phenothiazine in pure water and in acid medium (pH = 2) are shown in the absence and in the presence of the cyclodextrins, at the excitation and emission wavelengths of the generated oxidation photoproduct. These phenothiazine oxidation photoproducts, formed on

exposure to UV irradiation, have been characterized spectrofluorimetrically as sulphoxide derivatives [19]. It can be observed that the photochemically-induced fluorescence signal is higher at pH = 2 than in pure water in all cases.

It can also be seen that the maximum fluorescence intensity is attained very quickly, in about 2 minutes in all instances, and that the photogenerated product, after reaching the maximum fluorescence, is unstable, and the fluorescence signal decreases with further irradiation of the sample. At pH = 2, the photo-oxidation rates of phenothiazine are significantly faster in the presence of HP β-CD than in β-CD.

The fluorescence intensity of the photoproduct is increased in the presence of β-CD or HP β-CD. This increment is higher when using HP β-CD instead of native β-CD. Also, in this case, a zone of certain stability is attained after 3 minutes of irradiation.

It is probably due to the formation of an inclusion complex between the phenothiazine oxidation photoproduct and the cyclodextrin, which enhances fluorescence emission. The kinetic curve was not smooth and the reproducibility was poor. This was the result of the fact that only a small portion of the solution in the conventional cell is being irradiated by the excitation radiation [12], and consequently, the photochemical product of the analyte diffused into the other regions of the cell after it was formed and concentrated in the small zone under UV irradiation.

3.3. Influence of the Presence of β-CD and HP β-CD on the Fluorescence Spectra

Figure 2 shows the emission spectra of phenothiazine after 10 minutes of irradiation, in the absence and in the presence of the cyclodextrin derivatives.

The emission maximum of phenothiazine, irradiated in acid medium, remains unshifted in the presence of β-CD or HP β-CD. But the excitation maximum is displaced from 301 nm to 311 nm when adding β-CD or HP β-CD.

3.4. Effect of the β-CD and HP β-CD Concentration

The effect of the β-CD and HP β-CD concentration on the fluorescence intensity of the analyte was investigated. As can be seen in Figure 2, the addition of β-CD and HP β-CD produces an enhancement of the photochemically induced fluorescence emission of phenothiazine. The range of concentration of β-CD between 10^{-3} M and 1.2×10^{-2} M, which is close to the aqueous solubility limit of β-CD, was studied. It was found that the maximum enhancement of fluorescence could not be reached at this β-CD concentration, and that the data obtained did not allow determination of the stoichiometry and formation constant of the complex formed between irradiated phenothiazine and β-CD.

Increasing amounts of HP β-CD in the range $10^{-2} - 8.5\ 10^{-2}$ M progressively enhanced the fluorescence intensity, when phenothiazine was irradiated for ten

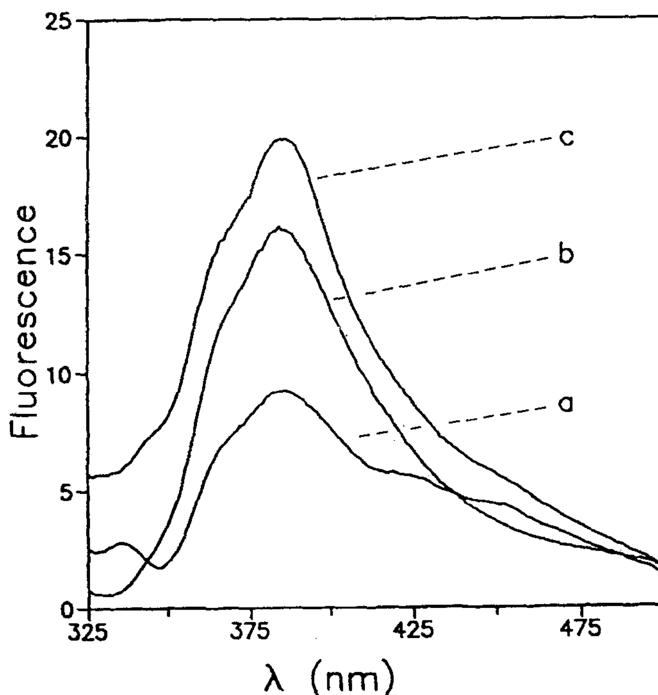

Fig. 2. Fluorescence emission spectra of phenothiazine irradiated at 20°C for 10 min in acid solution (pH = 2), (a) in water, (b) in a 10 mM solution of β-CD and (c) in the presence of 85 mM HP β-CD.

minutes with UV radiation in a pH = 2, 1% EtOH aqueous solution. Therefore, the relative fluorescence intensity of phenothiazine oxidation photoproduct increased in the presence of HP β-CD. This emission enhancement phenomenon can be attributed to the photo-oxidation of phenothiazine included in the complex with HP β-CD. It was used to determine the stoichiometry and the formation constant of the inclusion complex between the phenothiazine oxidation photoproduct (sulfoxide) and HP β-CD.

In Figure 3 we show the increase in relative fluorescence intensity of the phenothiazine photoproduct with increasing HP β-CD concentrations. The intensity was mesured at the maximum of the photochemically generated fluorescence peak at 385 nm. The phenothiazine oxidation photoproduct fluorescence intensity increases sharply for low concentrations of HP β-CD, and then reaches a plateau value for larger HP β-CD concentrations. This indicates that practically all the molecules have been included inside the HP β-CD cavity, forming an inclusion complex.

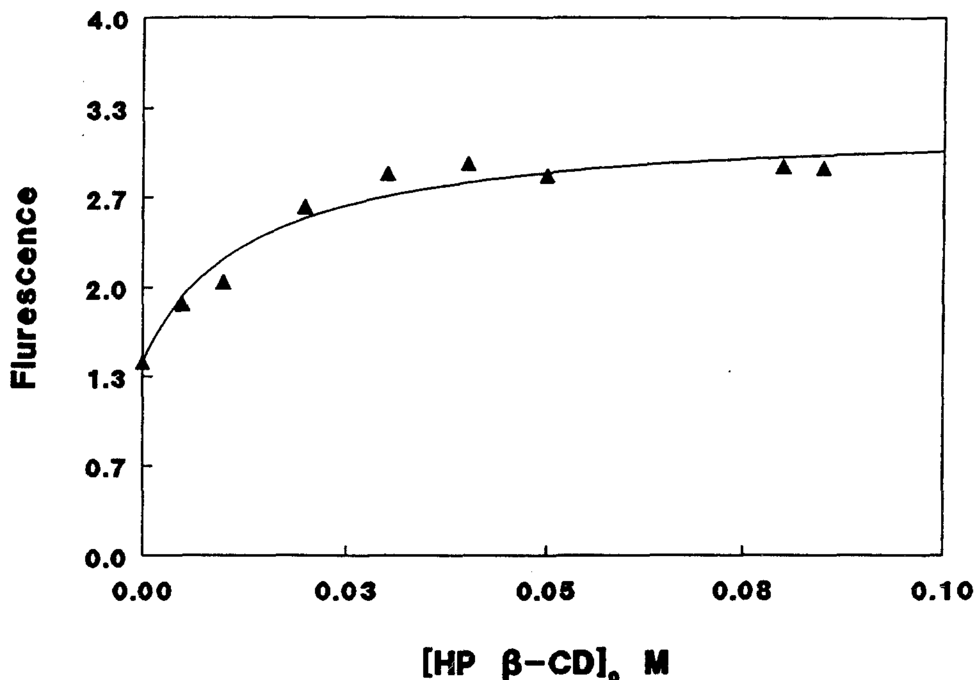

Fig. 3. Influence of HP β-CD concentration on the fluorescence intensity of 10^{-5} M phenothiazine (pH = 2, 1% EtOH) irradiated for 10 min. The solid line was calculated through the use of Equation (8), assuming a 1 : 1 stoichiometry for the complex and using the obtained value of K_1.

3.5. Characteristics of the HP β-CD Inclusion Complex

The stoichiometry and the formation constant of the phenothiazine oxidation photoproduct:2-hydroxypropyl β-cyclodextrin (PHE: HP β-CD) complex were calculated as follows. Assuming a 1 : 1 stoichiometry ratio, according to the following equilibrium:

$$[\text{HP }\beta\text{-CD}] + [\text{PHE}] \rightleftarrows [\text{HP }\beta\text{-CD:PHE}] \qquad (1)$$

the formation constant of the complex (K_1) is given Equation (2):

$$K_1 = \frac{[\text{HP }\beta\text{-CD:PHE}]}{[\text{HP }\beta\text{-CD}][\text{PHE}]} \qquad (2)$$

where [HP β-CD], [PHE] and [HP β-CD:PHE] are the corresponding equilibrium concentrations of these species, respectively. As the initial concentration of HP β-CD, ([HP β-CD]$_0$) is in a large excess over the complex concentration, we can approximate:

$$[\text{HP }\beta\text{-CD}] = [\text{HP }\beta\text{-CD}]_0 - [\text{HP }\beta\text{-CD:PHE}] \approx [\text{HP }\beta\text{-CD}]_0 \qquad (3)$$

and from the mass balance, we have

$$[\text{PHE}]_0 = [\text{PHE}] + [\text{HP } \beta\text{-CD:PHE}] \tag{4}$$

where $[\text{PHE}]_0$ is the initial concentration of phenothiazine. Consequently, Equation (2) can be simplified to:

$$K_1 = \frac{[\text{HP } \beta\text{-CD:PHE}]}{[\text{HP } \beta\text{-CD}]_0\ ([\text{PHE}]_0 - [\text{HP } \beta\text{-CD:PHE}])} \tag{5}$$

As can be observed in Figure 3, the fluorescence intensity of PHE increased on interacting with HP β-CD and forming the inclusion complex. The relation between this fluorescence increase and the HP β-CD concentration allowing the evaluation of the stoichiometry and the formation constant of the inclusion complex, can be represented by the following Equation [20]:

$$\frac{1}{F - F_0} = \frac{1}{(F_\infty - F_0)\, K_1\ [\text{HP } \beta\text{-CD}]_0} + \frac{1}{F_\infty - F_0} \tag{6}$$

where $[\text{HP } \beta\text{-CD}]_0$ is the initial HP β-CD concentration, F_0 denotes the fluorescence intensity of PHE in the absence of HP β-CD, F_∞ is the fluorescence intensity when all of the PHE molecules are essentially complexed with HP β-CD, F is the observed fluorescence at each HP β-CD concentration tested, and K_1 is the formation constant of the complex. The representation of $1/(F - F_0)$ vs $1/[\text{HP } \beta\text{-CD}]_0$, known as a double-reciprocal plot [21], allows the determination of the stoichiometry and of the formation constant. If the stoichiometry is 1 : 1, a linear plot should be obtained. In the case that a 2 : 1 stoichiometry is predominant, the application of Equation (7) should give a linear plot:

$$\frac{1}{F - F_0} = \frac{1}{(F_\infty - F_0)\, K_2\ [\text{HP } \beta\text{-CD}]_0^2} + \frac{1}{F_\infty - F_0} \tag{7}$$

where K_2 is the formation constant of the complex with a 2 : 1 stoichiometry, and the remaining symbols have the same meaning as in Equation (6). Typical double-reciprocal plots for the HP β-CD:PHE complex are shown in Figure 4. A linear relationship is obtained when $1/(F - F_0)$ is plotted against $1/[\text{HP } \beta\text{-CD}]_0$, ($r = 0.993$), indicating that the stoichiometry of the complex is 1 : 1 (Figure 4a). In contrast, a downward concave curvature is obtained when these data are fitted to a 2 : 1 complex, using Equation (7) (Figure 4b). The linear plot can be used to obtain K_1, by simply dividing the intercept by the slope, but Benesi–Hildebrand plots tend to place more emphasis on lower concentration values than on higher ones. As a result, the slope of the line is more sensitive to the ordinate value of the point having the smallest concentration. Therefore, a better estimation can be made, using nonlinear regression analysis [21]. Rearranging the data, we obtain the direct relationship between the observed fluorescence intensity, F and $[\text{HP } \beta\text{-CD}]_0$:

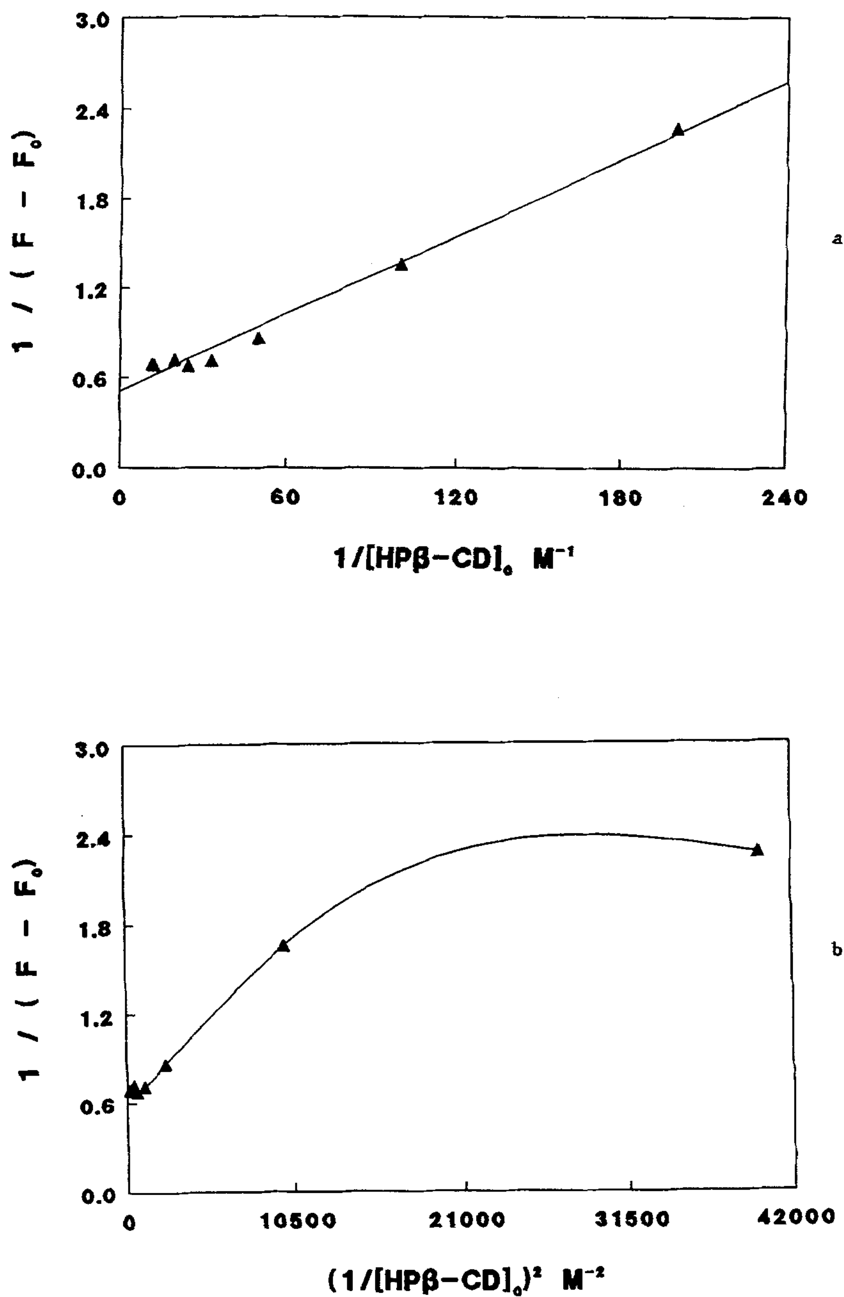

Fig. 4. Double-reciprocal plots. A linear relationship when the data are plotted assuming a 1 : 1 PHE : HP β-CD stoichiometry (4a) and a downward concave curvature when the data are plotted assuming a 1 : 2 PHE : HP β-CD stoichiometry (4b).

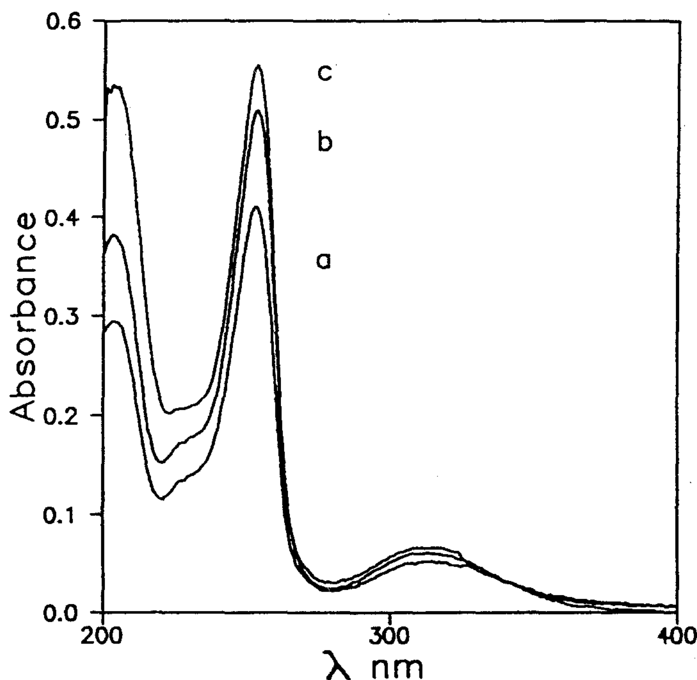

Fig. 5. Effect of HP β-CD concentration on the absorption spectrum of phenothiazine, (a) no HP β-CD, (b) 17 mM HP β-CD, and (c) 80 mM HP β-CD.

$$F = F_0 + \frac{(F_\infty - F_0)\, K_1\, [\text{HP } \beta\text{-CD}]_0}{1 + K_1\, [\text{HP } \beta\text{-CD}]_0} \tag{8}$$

Using Equation (8) allows a direct fit of the experimental data. The initial parameter estimates needed for the nonlinear regression method have been obtained from the linear plots. The calculated association constant is $80 \pm 45\ M^{-1}$.

3.6. UV ABSORPTION METHOD

The UV absorption spectra of phenothiazine in the absence and in the presence of HP β-CD are shown in Figure 5. The absorption spectrum of phenothiazine is characterized by peaks at 205, 253 nm and 320 nm. A small increase of the absorbance is observed upon addition of the HP β-CD and the wavelength of the maximum of absorption does not change significantly.

Similar calculations to those applied to the fluorescence emission data gave a 1 : 1 stoichiometry and an association constant of $100 \pm 50\ M^{-1}$ for the PHE : HP β-CD complex, by using the changes in the absorption spectrum. These results are in agreement with those found by the fluorescence approach (Section 3.5).

4. Conclusions

The stoichiometry and stability constant of the complex formed between phenothiazine photoproduct and HP β-CD in acid solution (pH = 2), have been determined by using the changes in the fluorescence emission intensity and in the absorbance upon inclusion.

We have found that the fluorescence intensity reaches a maximum with increasing HP β-CD concentration, suggesting that the complexation reaction between the phenothiazine oxidation photoproduct and HP β-CD has reached completion. We have determined the stoichiometry and the formation constant of the phenothiazine oxidation photoproduct inclusion complex. The results of these studies are currently being examined in our laboratories for developing specific analytical and pharmaceutical methodologies.

The effect of several physicochemical variables, such as ionic strength, polarity changes, foreign species, is also being considered for improving the performances of analytical methods based on the enhanced fluorescence of photo-oxidized phenothiazine-β-CD and HP β-CD inclusion complexes.

Acknowledgements

This work was supported by a Spanish–French integrated action (HF-41) of the Spanish and French governments.

References

1. J.B. Ragland and V.J. Kinross-Wright: *Anal. Chem.* **36**, 1356 (1964).
2. F.J. Mellinger and C.E. Keeler: *Anal. Chem.* **35**, 554 (1963).
3. F.J. Mellinger and C.E. Keeler: *Anal. Chem.* **36**, 1840 (1964).
4. V.R. White, C.S. Frings, J.E. Villafranca and J.M. Fitzgerald: *Anal. Chem.* **48**, 1314 (1976).
5. A.H.M.T. Scholten, V.A. Brinkman, and R.W. Frei: *Anal. Chim. Acta* **114**, 137 (1980).
6. M.L. Bender and M. Komiyama: *Cyclodextrin Chemistry*, Springer-Verlag, Berlin (1978).
7. M. Otagiri, K. Uekama, and K. Ikeda: *Chem. Pharm. Bull.* **23**, 188 (1975).
8. I.E. Kochevar and J. Hom: *Photochem. Photobiol.* **37**, 163 (1983).
9. T. Irie and K. Uekama: *Photomed. Photobiol.* **5**, 25 (1983).
10. T. Irie and K. Uekama: *J. Pharmacobiol.-Dyn.* **8**, 788 (1985).
11. K. Uekama, T. Irie, and F. Hirayama: *Chem. Lett.*, 1109 (1978).
12. J.J. Aaron: in S.G. Schulman (Ed.), *Molecular Luminescence Spectroscopy*, Part 3, Wiley, New York, p. 85 (1993).
13. D. Chen, A. Rios, M.D. Luque de Castro, and M. Valcarcel: *Analyst* **116**, 171 (1991).
14. D. Chen, A. Rios, M.D. Luque de Castro, and M. Valcarcel: *Talanta* **38**, 1227 (1991).
15. J. Martínez Calatayud and C. Gómez Bendito: *Anal. Chim. Acta* **256**, 105 (1992).
16. B. Laassis, J.J. Aaron, and M.C. Mahedero: *Anal. Chim. Acta* **290**, 27 (1994).
17. B. Laassis, J.J. Aaron, and M.C. Mahedero: *Talanta*, in press.
18. R.P. Frankewich, K.N. Thimmaiah, and W.L. Hinze: *Anal. Chem.* **63**, 2924 (1991).
19. E.O. Fadiran and A.G. Davidson: *J. Chromatogr.* **442**, 363 (1988).
20. V.K. Smith, T.T. Ndou, A. Muñoz de la Peña, and I.M. Warner: *J. Incl. Phenom.* **10**, 471 (1991).
21. K.A. Connors: *Binding Constants, The Measurement of Molecular Complex Stability*, John Wiley and Sons, New York (1987).