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# Solubility, spectroscopic properties and photostability of Rhein/cyclodextrin inclusion complex

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ABSTRACT

The host–guest interaction between Rhein (Rh) – an anthraquinonic drug characterized by low water solubility and recently considered for its potential antidiabetic and antitumoral activities other than for the well-established anti-inflammatory properties – with cyclodextrins (CDs) was investigated using phase-solubility diagrams. The typical $A_L$ phase-solubility profiles suggest the formation of the 1:1 inclusion complexes between Rh and the two CDs investigated, namely β-cyclodextrin and 2-hydroxypropyl-β-cyclodextrin and the resulting constant values of complex formation, $K_c$, were estimated. Due to the higher $K_c$ value, complex of Rhein with 2-hydroxypropyl-β-cyclodextrin was chosen for further investigation. Characterization in solution of 2-hydroxypropyl-β-cyclodextrin/Rhein complex was achieved both by fluorescence and visible spectroscopic techniques. These results confirm the formation of inclusion complexes in solution and the 1:1 stoichiometry of the binary system. With respect to Rhein aqueous solution behavior, the inclusion complex appears to be able: (i) to enhance Rhein solubility; (ii) to control its neutral/anionic equilibrium; (iii) to affect both its electronic absorption and fluorescence spectra. Finally, the photostability of Rhein in the presence of cyclodextrins was evaluated.



## 1. Introduction

Rhein (Rh), 4,5-dihydroxy-9,10-dioxoanthracene-2-carboxylate, occurs in the free form as well as glucoside in many plants of Polygonaceae and Leguminosae [1,2]. According to the pH values, the aglycone Rh lead to three different structural formulas (Scheme 1), with $pK_{a1}$ and $pK_{a2}$ of $4.53 \pm 0.310$ and $8.52 \pm 0.050$, respectively [3].

Apart from its well-established effects on the gastrointestinal tract [4], Rh is used in osteoarthritis [5–7] and it has been proved as a potential antitumoral agent [6,8] as well as an effective substance in experimental treatment of diabetic nephropathy [9]. Based on these peculiar and promising properties an original procedure for the total chemical synthesis of Rh, instead of the high-cost semisynthetic route, was recently proposed [10].

With regard to the chemico-physical properties, Rh is affected by low solubility in water, poor absorption properties and photosensitivity. Cyclodextrins (CDs) are well known to form inclusion complex with a wide variety of hydrophobic molecules. The formation of such complexes improves chemical and physical properties of the guest and, consequently, it has lead to an extensive application of these cyclic carbohydrate derivatives in the pharmaceutical field [11–14], especially in terms of solubility increase. An in-depth screening of the literature provided only an article written in Chinese in which the inclusion complexes of Rh with unspecified CDs were studied by oscilloscope polarography [15]. In its abstract, the indication that the inclusion procedure spontaneously occurs, at normal temperature, with a 1:1 molar ratio of inclusion complexes has been reported.

Aim of the present work was to study the influence of CDs on Rh properties, in solutions. Specifically, Rh solubility in natural β-CD and in HP-β-CD buffered solutions has been examined. Since a significant increase in Rh solubility was mainly shown in HP-β-CD solution, Rhein/HP-β-CD interaction has been deeply investigated by Visible and Fluorescence spectroscopy. The formation of the inclusion complex as well as its stability evaluation have been studied. Finally, in relation to the well-known anthraquinone phototoxicity [16], the Rh photostability under visible light and aerobic conditions both in the absence and in the presence of CDs were also investigated.

## 2. Materials and methods

### 2.1. Materials

β-CD (Kleptose®, MW = 1135, batch number 813448) and HP-β-CD (Kleptose® HP, MW = 1400, degree of molar substitution

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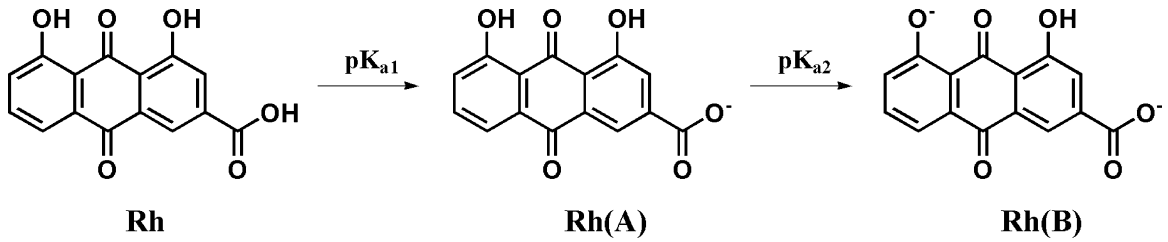


**Scheme 1.**

0.75–0.95, batch number 813447) were the generous gift from Roquette (France). Rh (batch number 1377) was provided by TRB Chemedica (Switzerland). All chemicals used were of reagent analytical grade. Freshly distilled water was used throughout the experiments.

### 2.2. Instruments

A PerkinElmer Lambda 25 UV/vis spectrophotometer with matched 10-mm quartz cells and a PerkinElmer LS50 B luminescence spectrofluorophotometer (PerkinElmer, USA) were used to measure the absorbance and fluorescence, respectively. All the spectra were recorded using PerkinElmer FL WinLab and UV WinLab version 2.85.04 software packages for fluorescence and visible spectroscopy, respectively. A thermostatic bath was used to control experiment temperatures. A Mettler pH-meter was use to control the pH value of the buffered solutions.

### 2.3. Phase-solubility study

Phase-solubility studies were performed according to the method reported by Higuchi and Connors [17]. Rh, in constant amounts exceeding its solubility, was transferred to vials containing 10 mL of either β-CD ranging from 0 to 16 mM or HP-β-CD ranging from 0 to 100 mM pH 7.2 buffered solutions. The contents were stirred (400 rpm) on electromagnetic stirrer (VELP Scientifica, Italy) at 25 ± 0.5 °C, unless otherwise stated, for a suitable time (14 days) well-above the time required for reaching the equilibrium. After reaching equilibrium, samples were filtered through a 0.45 μm nylon membrane filter (Millipore, USA). The filtered samples suitably diluted, were assayed for Rh by measuring absorbance at the wavelength of 435 nm. Solubility studies were performed in triplicate. The stability constant of the inclusion complex ($K_c$) were calculated from the phase-solubility diagrams using Eq. (1):

$$K_c = \frac{\text{slope}}{S_0(1 - \text{slope})} \quad (1)$$

where $S_0$ (intercept value) is Rh solubility in the absence of CD [18].

### 2.4. Fluorescence and absorption studies

A stock solution of Rh ($10^{-3}$ M) was prepared by dissolving Rh into the minimum amount of NaOH 1 M and diluting with phosphate buffer of different pHs. Concentrations ranging from $10^{-6}$ to $10^{-4}$ M both in the absence and in the presence of different amount of HP-β-CD were investigated.

Fluorescence emission spectra were acquired with an excitation wavelength at 435 nm and the emission intensities were monitored between 450 and 600 nm. Excitation and emission bandwidths were set at 5 and 10 nm, respectively.

Visible spectra were measured against a corresponding reagent blank, in the 360–600 nm spectral range.

All the experiments were carried out at 25 ± 0.5 °C. The pH values, ranging from 7.2 to 11 unless otherwise stated, have been controlled by a pH-meter.

### 2.5. Analysis of spectrofluorimetric and spectrophotometric data: determination of ionization constant ($pK_{a2}$) and complex formation constant ($K_c$)

The fluorescence enhancement due to the inclusion in HP-β-CD allows to calculate the stability constants of the complexes by using the Benesi–Hildebrand linear regression analysis [19]. The fluorescence increasing was therefore detected at different Rh concentrations ($10^{-6}$ to $10^{-4}$ M) in the various buffered solutions with and without HP-β-CD (5–25 mM).

For 1:1 stoichiometry Eq. (2) can be considered

$$\frac{1}{F - F_0} = \frac{1}{F_c - F_0} + \frac{1}{K_c(F_c - F_0)} \frac{1}{[\text{CD}]_0^n} \quad (2)$$

where $F$ is the observed fluorescence intensity of guest and guest–host mixture, $F_0$ the initial fluorescence intensity of free guest, $F_c$ the intensity of guest–host complex, and $K_c$ is the association constant for the 1:1 complex formation.

The p$K_a$ value of Rh was also evaluated according to Eq. (3) as an arrangement of both the absorbance and pH values in the mass balances [20]

$$\frac{1}{A - A_0} = \frac{1}{(\varepsilon_c - \varepsilon_0)[\text{Rh}]_0} + \frac{1}{(\varepsilon_c - \varepsilon_0)[\text{Rh}]_0 K_a} \frac{1}{[\text{CD}]_0^n} \quad (3)$$

where $A$ and $A_0$ are Rh solution absorbances at the corresponding maxima, evaluated at pH values comprised between 7.6 and 11 where both the ionized Rhein species are concurrent. The extinction molar coefficients, of HP-β-CD/Rh and free Rh have been indicated as $\varepsilon_c$ and $\varepsilon_0$, respectively. $[\text{Rh}]_0$ represents initial Rh concentration. Moreover, in the presence of equilibria that involve the ionization of the complexes formed in one of the other processes can also be written according to Eq. (4):

$$\frac{1}{A - A_0} = \frac{1}{(\varepsilon_c - \varepsilon_0)[\text{Rh}]_0} + \frac{1}{(\varepsilon_c - \varepsilon_0)[\text{Rh}]_0 K_a^*} \frac{1}{[\text{CD}]_0^n} \quad (4)$$

where $K_a^*$ stands for the apparent acidity constant in the presence of HP-β-CD. Starting from these values, $K_c$ can also be evaluated according to Eq. (5) [21]

$$K_c = \frac{10^{(pK_a^* - pK_a)} - 1}{[\text{CD}]} \quad (5)$$

### 2.6. Photostability investigations

Buffered Rh solutions ($10^{-4}$ M), both in the absence and in the presence of HP-β-CD (1, 5 and 25 mM) were exposed at a distance of 10 cm under aerobic conditions and constant stirring to the visible light of a Osram® lamp (800 lx) at room temperature (25 ± 0.5 °C). Samples withdrawn at fixed time intervals (0, 1, 2, 3, 4, 7 and 9 days) were spectroscopically analyzed as previously reported, to monitor the cyclodextrin-induced effects in/on the photodegradation process. Control experiments were carried out in the darkness. The results are expressed as the ratio among spectroscopic variations.



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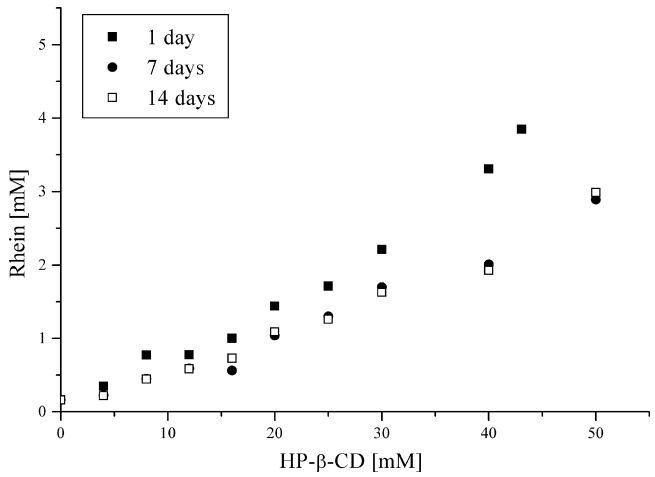


**Fig. 1.** Phase-solubility diagram of the Rh/HP-β-CD system at different contact times. Phosphate buffer, pH 7.2; 25 °C.

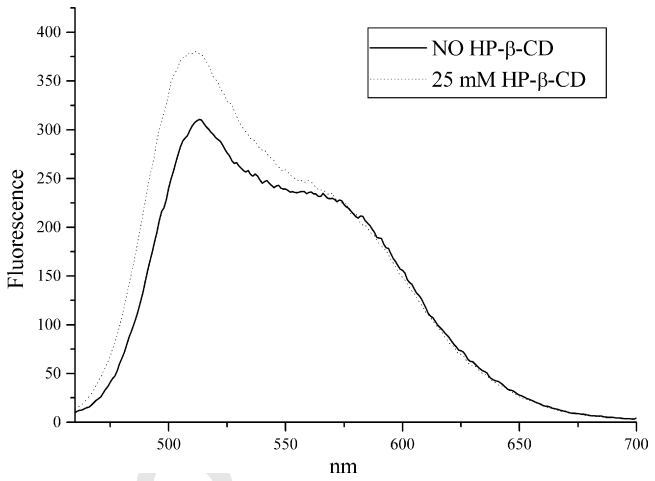


**Fig. 3.** Fluorescence spectra of Rh $10^{-5}$ M (phosphate buffer, pH 7.2) in the absence and in the presence of HP-β-CD (25 mM).

### 2.7. Statistical analysis

Results are expressed as the mean of at least three independent measurements (CV% < 2). ANOVA one-way performing the Bonferroni post-test (Instat software, version 3.0 GraphPAD Software Inc., San Diego, CA) were used for the statistical analysis of the results. Significance was defined as a $p$ value less than 0.05.

## 3. Results and discussion

### 3.1. Phase-solubility studies

Fig. 1 shows the experimental values of Rh apparent solubility in aqueous HP-β-CD solutions ranging in concentration from 0 to 50 mM, obtained with procedures described in method section after different contact times with excess amounts of solid Rh. As reported in the figure, Rh concentrations in the HP-β-CD solutions at 24 h seem higher than those obtained after 1 and 2 weeks ($p < 0.001$). Such a behavior, already described for other molecules [11], could be explained admitting a time-dependent over-saturation phase. This phenomenon stops when new equilibrium conditions take place and leads to interactions Rh/CD, not only of a soluble inclusion complex type. Anyway, a stable value has been obtained starting from 7 days and the solution appeared unaltered for prolonged storage (1 month). The values obtained after 14 days have been selected for the phase-solubility studies. Up to [CD] 50 mM the phase-solubility plot shows an $A_L$ type solubility curve [17] which indicates formation of 1:1 HP-β-CD/Rh inclusion complex. According to this hypothesis, a $K_c$ value of 253 $M^{-1}$ has been estimated from Eq. (1). At high CD concentration (60–100 mM) a solubility plateau has been observed, suggesting the precipitation of HP-β-CD/Rh complex (data not reported).

HP-β-CD has been much more effective in increasing the Rh solubility than β-CD: in fact, at same CD concentrations, the increase of Rh solubility obtained with HP-β-CD at the temperature of 37 °C was about three times higher than that obtained with β-CD; the HP-β-CD/Rh complex $K_c$ value is also higher than that $K_c$ 1:1 of β-CD/Rh binary system, 350 and 110 $M^{-1}$, respectively.

Due to the higher increase in water solubility of Rhein/HP-β-CD complex respect to β-CD complex, only interaction of Rh with HP-β-CD has been deeply investigated.

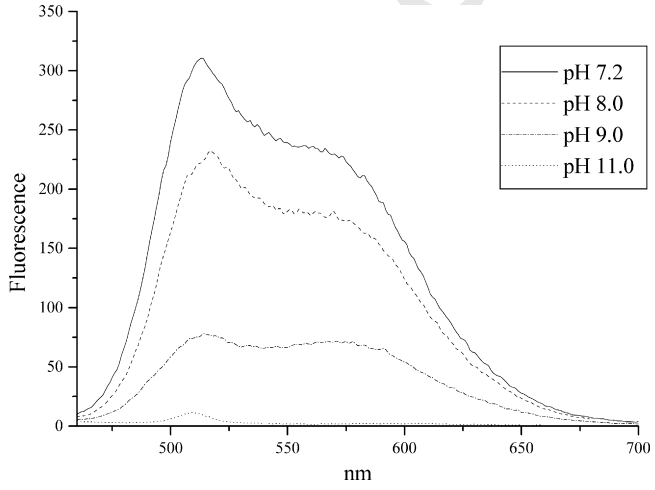


**Fig. 2.** Fluorescence spectra of Rh $10^{-5}$ M at various pHs.

### 3.2. Fluorescence and UV–vis spectral studies

Interaction between Rhein and HP-β-CD was deeply investigated by fluorescence and visible spectroscopy both to substantiate the Rh/HP-β-CD inclusion complex formation in solution and to evaluate the stability complex constant.

All spectroscopic investigations have been done at two times ($t = 0$ and at $t = 1$ week) to evaluate influences on Rh/HP-β-CD inclusion complex formation in solution. No remarkable spectral differences were achieved, so the spectra immediately obtained after dilution have been reported.

Fluorescence spectra of Rh ($10^{-5}$ M) at various pHs are reported in Fig. 2. At pH 7.2 all the Rh is present in the A form and the spectra shows two emission bands, at 513 and 580 nm with $F_{513/580} = 1.35$. The fluorescence intensity and the value of $F_{513/580}$ decrease with increasing pH and very low fluorescence is detected at pH 9. Fluorescence is a linear function of Rh concentration until ($2.5 \times 10^{-5}$ M), whereas at higher Rh concentration (range $2.5 \times 10^{-5}$ to $10^{-4}$ M) a non-linear trend of fluorescence vs. concentration is observed, due to an inner filter effect phenomenon [22].

Fig. 3 shows the fluorescence spectra of Rh ($10^{-5}$ M), both in the absence and in the presence of HP-β-CD, 25 mM at pH 7.2.

With the addition of HP-β-CD, the fluorescence intensity of the emission band at 513 nm was markedly enhanced due to an interaction between CD and Rh, which implied the inclusion of the Rh in CD cavity and the formation of CD–Rh complexes. Such a variation may be due to either an increase of the quantum yields, that could



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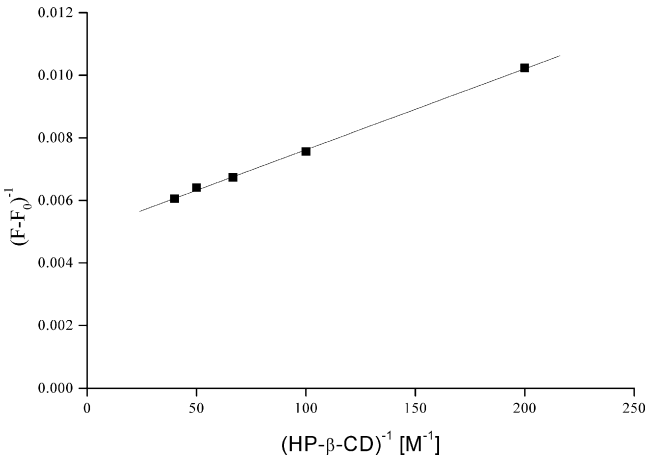



**Fig. 4.** Benesi–Hildebrand plot for complex formation.

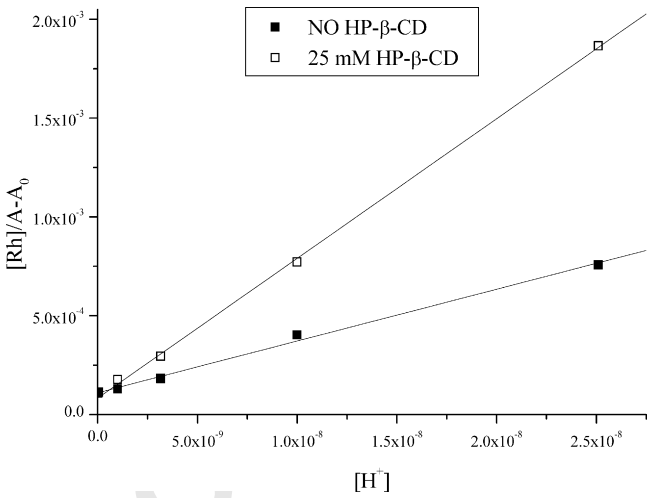



**Fig. 6.** $[Rh]/A - A_0$ plots as function of pHs, at fixed Rh concentration both in the absence and in the presence of HP-β-CD (25 mM).

be referred to a stiffening of the molecular structure of Rh inside the host molecule, or a decrease of hydrophobic interactions.

When the guest molecules were entrapped in the CDs cavity, the microenvironment with a smaller polarity and stronger rigidity would limit the freedom of guest molecules increasing the fluorescence quantum yield and consequently its emission fluorescence spectra [21]. Similar behaviors are observed for all the range of testing Rh concentrations ($10^{-6}$ to $10^{-4}$ M).

To estimate the complex stoichiometry and the corresponding complex formation constant, experimental data have been reported in a double reciprocal plot of fluorescence enhancement vs. HP-β-CD concentration according to Eq. (2). A linear trend is observed ($r^2 = 0.998$, Fig. 4) confirming the 1:1 stoichiometry ratio. Moreover, the value of $K_c = 240\ M^{-1}$ obtained is of the same order of magnitude of that derived by solubility phase value, bearing out the assumption that the increase in solubility is related to the Rh inclusion in HP-β-CD cavity.

Absorption spectra of Rh at different pH values have been reported in Fig. 5. As can be observed, the intensity of the two absorption bands at 435 nm and 505 nm detectable in the spectra, appeared to be clearly dependent from pH, and the $A_{435nm}/A_{505nm}$ ratio decreased in an inverse manner with respect to pH. At pH values lower than 7.2 or higher than 10, only one peak, either near 435 nm or 505 nm was detected, respectively.

The presence of a sharp isosbestic point in the spectra indicates that, in the range of the pH investigated, two species were present,

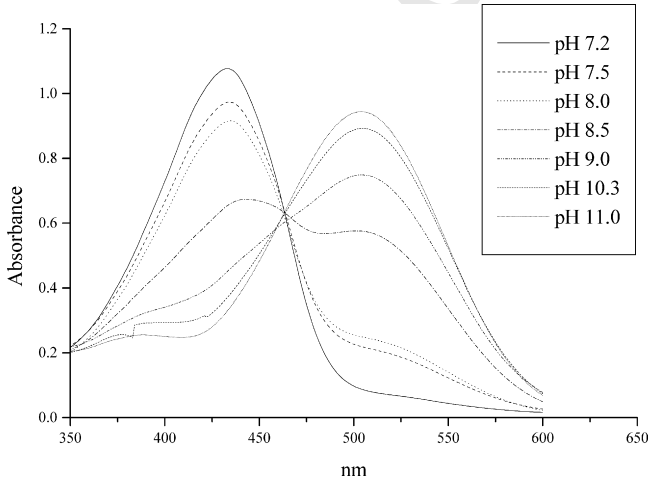



**Fig. 5.** Visible absorption spectra of Rh $10^{-5}$ M at all the pHs investigated.

in equilibrium each other under the experimental conditions. These results are in agreement with the acid–base equilibrium in solution, already reported by Wang et al. [3]: the two peaks representing the two different chromophores that are present in solution, i.e. the acidic and the alkaline resonant forms of Scheme 1. Experimental absorbance data are plotted according to Eq. (3) that allows to calculate Rh acidity constant. As shown in Fig. 6, at fixed Rh concentration, a linear trend of $[Rh]/A - A_0$ vs. $[H^+]$ is obtained, confirming that the absorption bands at 435 and 505 nm correspond to the Rh(A) and Rh(B) form of the molecule under investigation. A $pK_{a2}$ value of $8.38 \pm 0.12$ ($r^2 = 0.9997$) has been obtained, in agreement with the one reported in literature [3].

As for the presence of HP-β-CD, no effect on the absorption spectra (except than a slight bathochromic shift, specifically $\lambda_{max} = 434.7$ nm to $\lambda_{max} = 436.8$ nm and $\lambda_{max} = 505$ nm to $\lambda_{max} = 511$ nm at pH 7.2 and 11, respectively) was observed with [HP-β-CD] ≤ 25 mM at the pH values where only one Rhein species is predominant, namely pH 7.2 and pH 11 for the form A and B of Fig. 1, respectively. Such an assertion has been based on the practical overlapping of the tarature curves obtained both in the absence and in the presence of HP-β-CD, $y = 10{,}731x + 0.011$ and $y = 10{,}791x + 0.0051$, as well as $y = 9373x - 0.0006$ and $y = 9244x - 0.0003$, $r^2 \geq 0.9995$, for pH 7.2 and pH 11, respectively) while a slight Lambert–Beer's law deviation has been observed at [HP-β-CD] ≥ 30 mM.

Moreover, since both solubility phase studies and fluorescence data suggest the formation of Rh/HP-β-CD, it can be argued that inclusion in HP-β-CD cavity did not affect the spectrophotometric absorption behavior. On the other hand, Fig. 7 shows that at basic pHs, the presence of CD modified the Rh absorption spectra. This behavior can be explained considering that HP-β-CD preferably complexes the less polar Rh(A) form – as depicted in Scheme 2 which represents the host–guest interaction of Rh with CDs – leading to a shift of acidic equilibrium and, consequently, of molecular absorption of the Rh. According to this assumption, the apparent stability constant $pK^*_{a2}$ can be obtained from the absorption spectra at different pHs and at fixed HP-β-CD concentration, according Eq. (4). In a similar manner, the stability constant can be obtained from the analysis of $pK^*_{a2}$ values at different HP-β-CD by Eq. (5). Both the agreement of the experimental data with the linear trend predicted by Eqs. (4) and (5), namely $pK^*_{a2} = 9.23 \pm 0.14$, $r^2 = 0.9983$ and $K_c = 243\ M^{-1}$, as well as the significant increase of the $pK^*_{a2}$ value with respect to the $pK_{a2}$ one [23], support the hypothesis (Fig. 6).

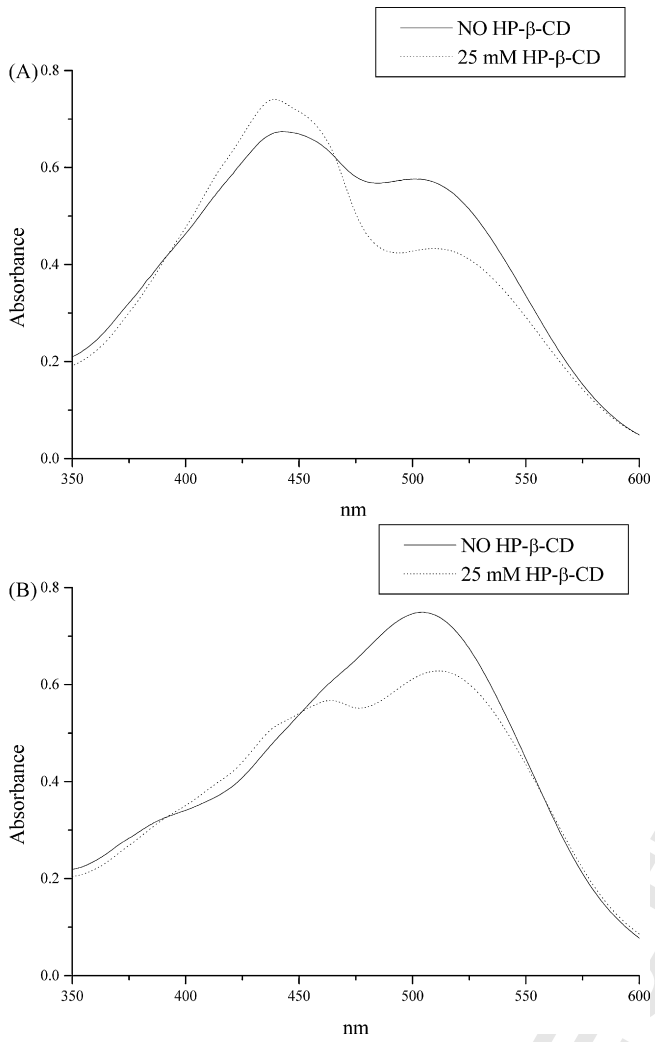
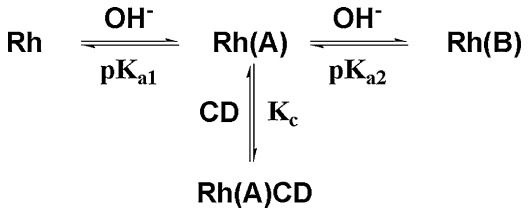



**Fig. 7.** Visible absorption spectra of Rh in the absence and in the presence of HP-β-CD (25 mM). (Panel A) pH 8.5. (Panel B) pH 9.0.

### 3.3. *Rh photostability and HP-β-CD effects*

In relation to the capacity of some anthraquinones to produce free radicals when they are irradiated with visible light [24], photostability of phosphate buffer solution of Rh and HP-β-CD at pH 7.2 has been also investigated. Fig. 8 shows absorbance and fluorescence data as a function of time obtained by exposing to the light Rh buffered solutions in the absence and in the presence of different amounts of HP-β-CD. It is worth noting that Rh solutions both without and with CD protected from light are stable for at least 1 month with change in absorbance and fluorescence minor than 5%. Light sensibility of Rh ethanolic solutions under aerobic conditions by irradiation with visible light has been previously described. The photodegradation was evidenced both by a gradual decrease and transformation of the native UV–vis spectra and




**Scheme 2.**

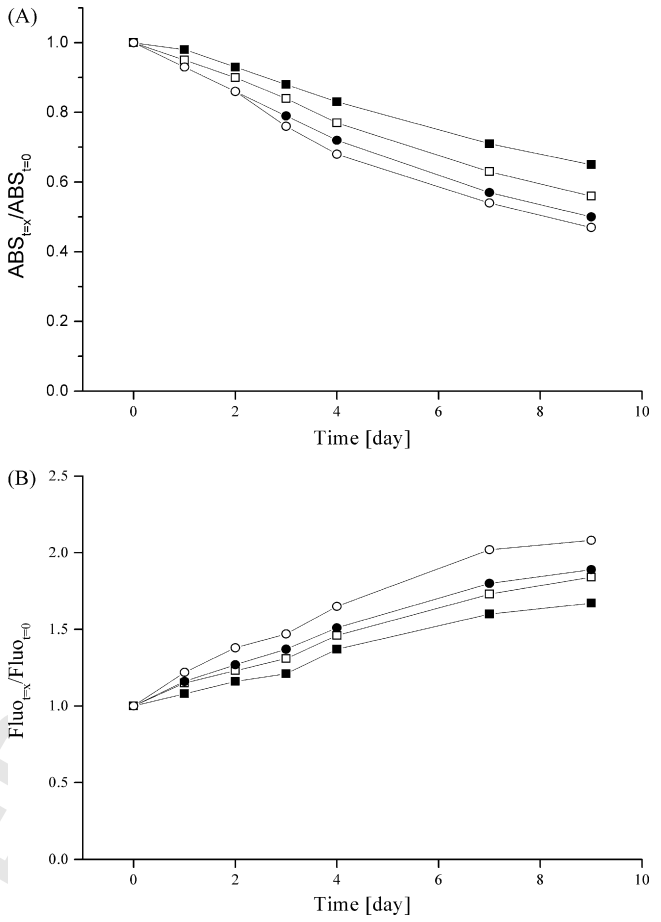



**Fig. 8.** Rh photostability in the absence and in the presence of different amounts of HP-β-CD, as evaluated by absorbance (panel A) and fluorescence (panel B) studies. Rh (■); Rh + 1 mM HP-β-CD (□); Rh + 5 mM HP-β-CD (●); Rh + 25 mM HP-β-CD (○).

by a higher emission band in the fluorescence studies [16]. Results in the present study confirm Rh instability to light and evidence that the inclusion of Rh into HP-β-CD decrease its photostability in a CD concentration-dependent manner. Such a behavior suggests a role of the macrocycle as a partner in the reaction involving radicals and that the radical species involved can more easily react when Rh is constrained within the HP-β-CD cages [25]. Preliminary study on the solid-state characterization of host–guest complex [26] seems to support this hypothesis.

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