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Effects of hydroxypropyl-β-cyclodextrin on the chemical stability of a naphthoquinone in aqueous solutions

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2-Hydroxy-*N*-(3,4-dimethyl-5-isoxazolyl)-1,4-naphthoquinone-4-imine (**1**), an antibacterial agent, was shown to form inclusion complexes with HP-β-CD in aqueous solution. In the present work the kinetics of **1** degradation in aqueous buffer solution was investigated as a function of pH (2.34–3.95), HP-β-CD concentration (0%–28% (w/v)) and temperature (60–90 °C). A second-order derivative spectroscopic methodology was developed for the kinetic investigations. The degradation showed to follow pseudo-first-order kinetics. Also, an specific acid catalysis was found and the introduction of up to 28% (w/v) HP-β-CD to the reaction medium did not change this kinetic behaviour. The obtained results indicated that HP-β-CD stabilises **1** against degradation in aqueous solutions.

1. Introduction

Cyclodextrins form a whole family of pharmaceutical excipients. They are cyclic oligosaccharides with a hydrophilic outer surface and a lipophilic cavity in the center [1]. Cyclodextrins have the remarkable property of forming molecular inclusion complexes with many drugs. These complexes have properties different from free drugs. This is used in the field of pharmaceutics to improve aqueous solubility, chemical stability, and bioavailability of drug molecules [2]. Drug-cyclodextrin complex formation can be regarded as encapsulation of drugs at the molecular level. The cyclodextrin molecules shield, at least partly, the drug molecule against attack by various reactive molecules. In this way, cyclodextrins can prevent or reduce drug hydrolysis, oxidation, steric rearrangement, racemisation, isomerization, polymerization and even enzymatic descomposition of drugs [3].

2-Hydroxy-*N*-(3,4-dimethyl-5-isoxazolyl)-1,4-naphthoquinone-4-imine (**1**) is an experimental drug for the treatment of Chagas disease which also exhibits antibacterial activity against *Staphylococcus aureus* [4, 5]. Unfortunately, **1** exhibits low water solubility, making it difficult to prepare the solutions for biological tests. In our previous work, we have shown that **1** forms inclusion complexes in either its neutral or its anionic form with HP-β-CD in aqueous solution. Phase solubility diagrams were used to study the complexation of **1** with hydroxypropyl-β-cyclodextrin (HP-β-CD). A 300-fold increase in **1** solubility was obtained and a stability constant ($K_{1:1}$) of $1 \times 10^3\ M^{-1}$ [6] was calculated from the straight line of the isotherms. Previously, it has been demonstrated that **1** attains its optimal chemical stability at pH values higher than its pKa value (5.40 ± 0.16), because no degradation was detected when **1** was in its ionized form [7]. The aim of this work was to study the influence of HP-β-CD on the acidic degradation of **1**. Since spectrophotometric techniques are faster, easier to use and less expensive than HPLC methods [8, 9], the possibility of applying such techniques in a complex chemical system like this was examined. For these studies we have developed a second-order derivative spectrophometric method to determine **1** and its hydrolysis products in the presence of HP-β-CD, which can be used as a stability indicating method as well.

2. Investigations, results and discussion

2.1. Second-order derivative spectrophotometry

We wanted to check the applicability of spectrophotometric techniques to these kinetic measurements. However, as can be seen in Fig. 1, zero-order spectra of **1** and **2** overlap in the range 250–550 nm. Consequently, the simultaneous determination of **1** and **2** in a mixture seems impossible. In the second derivative spectra traced with $\Delta\lambda = 2$ nm (Fig. 2), exits a zero-crossing point at 322.5 nm for **1** giving opportunity for its determinations by reading $d^2A/d\lambda^2$ values at this wavelength without interference from **2**. In the method, the mean recoveries found for synthetic mixtures prepared in our laboratory at the selected wavelength (322.5 nm) are listed in Table 1.

Calibration graphs were established at 322.5 nm and straight lines were observed in the concentration range 1.0×10^{-5}–9.5×10^{-5} M for **1**. Intercept, slope values and correlation coefficients are shown in Table 2. From these satisfactory results, it can be concluded that the selected derivative method is suitable to study the stability of **1** in HP-β-CD solutions. Detection limit (DL) and quantitation limit (QL) are defined as the concentration which gives a signal equal to $3S_a/b$ and $10S_a/b$, respectively, where S_a is the standard deviation of the intercept and b is the slope of the calibration curve [10]. The values obtained are given in Table 3.

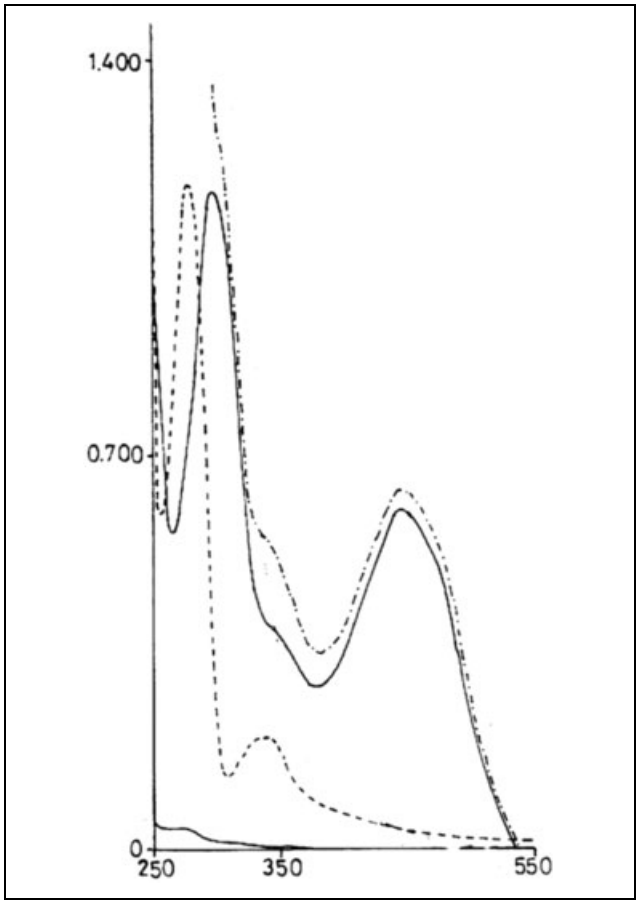


Fig. 1: Zero-order absorption spectra.
Key: —— 1; – – – 2; ·–·–· 1 + 2, —— (down) HP-β-CD

Table 1: Recovery of 1 in the presence of it acid degradation product 2 and HP-β-CD

pH	Concentration of 1 (M)[e]	Recovery (%)
2.34[a]	2.81×10^{-5}	100.3
	5.68×10^{-5}	101.4
	8.38×10^{-5}	99.9
2.34[b]	2.78×10^{-5}	99.3
	5.61×10^{-5}	100.2
	8.27×10^{-5}	98.6
3.95[c]	4.64×10^{-5}	99.6
	7.48×10^{-5}	100.3
	9.54×10^{-5}	1002.4
3.95[d]	4.51×10^{-5}	98.6
	7.48×10^{-5}	100.3
	9.37×10^{-5}	100.5

[a] 8% (w/v) HP-β-CD; [b] 20% (w/v) HP-β-CD; [c] 4% (w/v) HP-β-CD; [d] 14% (w/v) HP-β-CD; [e] amounts of **2** added are 6.92×10^{-5} M

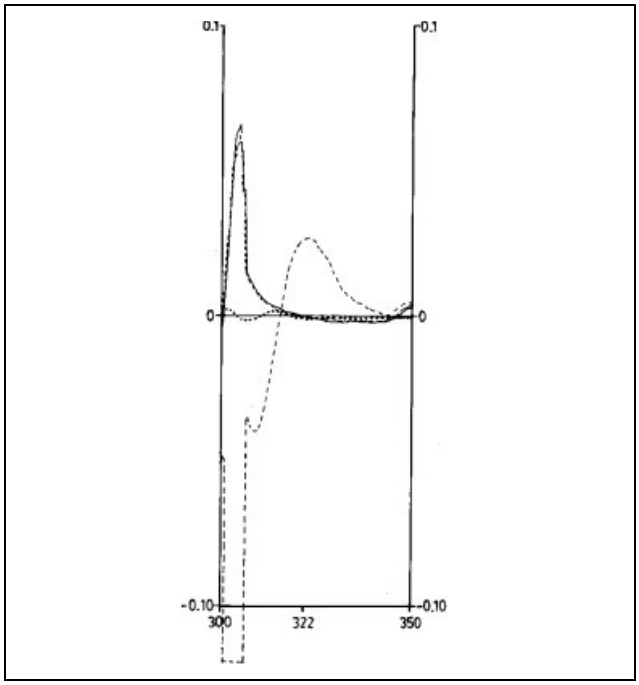


Fig. 2: Second-order derivative spectra
Key: – – – 1; —— 2, · · · · · HP-β-CD

2.2. Effects of cyclodextrin

To explore the influence of complexation with CDs on the stability of **1**, the observed degradation rate constants (k_{obs}) were determined in the presence of HP-β-CD and compared with those obtained in the absence of HP-β-CD. These constants (k_{obs}) were determined from the disappearance of compound by least-square linear regression of natural logarithmic of derivative spectra heights versus time (t) as represented by the following equation: $\ln h_t = \ln h_0 - k_{obs}t$.

A linear relationship was observed between the logarithmic percent of remaining concentration and time, from

Table 3: Detection limits and quantitation limits

pH	DL (M)	QL (M)
2.34[a]	7.12×10^{-6}	1.35×10^{-5}
2.34[b]	8.54×10^{-6}	0.81×10^{-5}
2.93[c]	1.70×10^{-6}	5.61×10^{-6}
2.93[b]	8.02×10^{-7}	2.64×10^{-6}
3.95[c]	3.50×10^{-7}	1.15×10^{-6}
3.95[d]	7.55×10^{-7}	2.49×10^{-6}

[a] 8% (w/v) HP-β-CD; [b] 20% (w/v) HP-β-CD; [c] 4% (w/v) HP-β-CD; [d] 14% (w/v) HP-β-CD

Table 2: Analytical parameters of the calibration curves of 1

pH	Concentration range of 1 (M)	Regression equation		
		Intercept (a ± S)	Slope (b ± S)	r^2 (n) [e]
2.34[a]	$2.0–9.0 \times 10^{-5}$	0.0022 ± 0.0005	30,818 ± 806	0.9990 (5)
2.34[b]	$1.0–9.0 \times 10^{-5}$	0.0016 ± 0.0004	32,426 ± 476	0.9987 (5)
2.93[c]	$2.0–8.5 \times 10^{-5}$	0.0012 ± 0.0001	24,144 ± 102	0.9997 (6)
2.93[b]	$2.0–8.5 \times 10^{-5}$	0.0009 ± 0.0001	26,336 ± 153	0.9991 (6)
3.95[c]	$2.0–9.5 \times 10^{-5}$	0.0023 ± 0.0003	23,430 ± 466	0.9998 (6)
3.95[d]	$2.0–9.5 \times 10^{-5}$	0.0020 ± 0.0006	24,690 ± 669	0.9997 (6)

[a] 8% (w/v) HP-β-CD; [b] 20% (w/v) HP-β-CD; [c] 4% (w/v) HP-β-CD; [d] 14% (w/v) HP-β-CD; [e] n, number of points in each calibration curve, each point is the mean of two or three experimental measurements

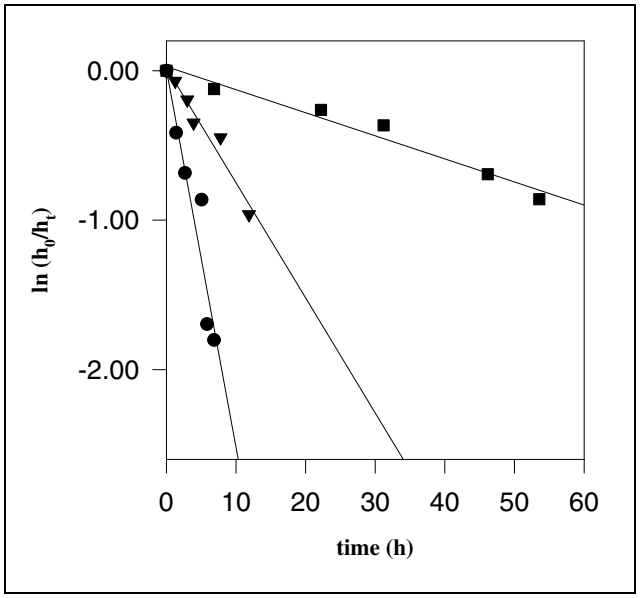


Fig. 3: Plots of the observed pseudo-first-order kinetic degradation of 1 at different pHs, in the presence of 20% HP-β-CD at 80 °C. h_o = height at t = 0 min, h_t = height at t = t min.
Key: (■) pH 3.95; (▼) pH 2.93; (●) pH 2.34.

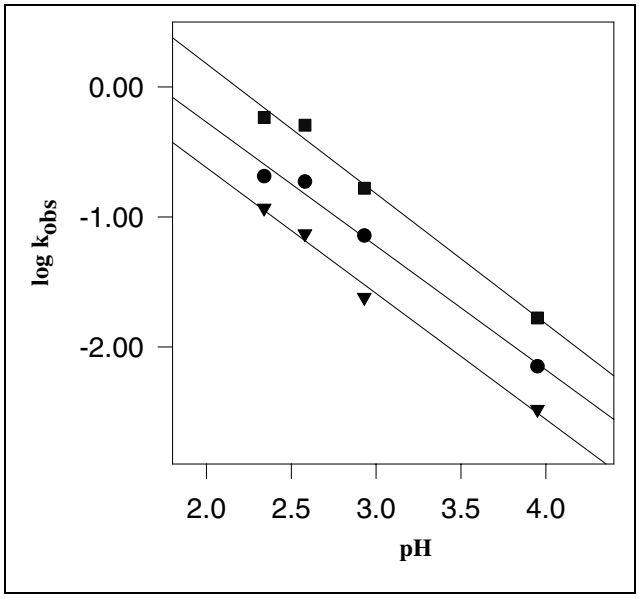


Fig. 4: pH-Rate profiles for the observed first-order degradation of 1 in aqueous buffer solutions in the presence or absence of HP-β-CD at 70 °C.
Key: (■) 0% HP-β-CD; (●) 8% (w/v) HP-β-CD; (▼) 20% (w/v) HP-β-CD.

Table 4: Observed pseudo-first-order rate constants (k_{obs}) and half-lives ($t_{1/2}$) for the degradation of 1 in aqueous solutions in the presence or absence of HP-β-CD at 70 °C

pH	% HP-β-CD (w/v)	$k_{obs} \times 10^2$ (h^{-1})	$t_{½}$ (h)	Relative rate[a]
2.34	0.00	58.2	1.19	1.00
	4.00	42.1	1.65	0.72
	8.00	20.6	3.36	0.35
	14.00	13.4	5.17	0.23
	20.00	11.7	5.93	0.20
	28.00	8.51	8.13	0.15
2.93	0.00	16.6	4.18	1.00
	4.00	13.6	5.09	0.82
	8.00	7.20	9.63	0.43
	14.00	4.14	16.74	0.25
	20.00	2.41	28.76	0.14
	28.00	1.49	46.52	0.09
3.95	0.00	1.67	41.50	1.00
	4.00	1.39	49.87	0.83
	8.00	0.71	97.63	0.42
	14.00	0.51	135.91	0.31
	20.00	0.33	210.04	0.20
	28.00	0.20	346.57	0.12

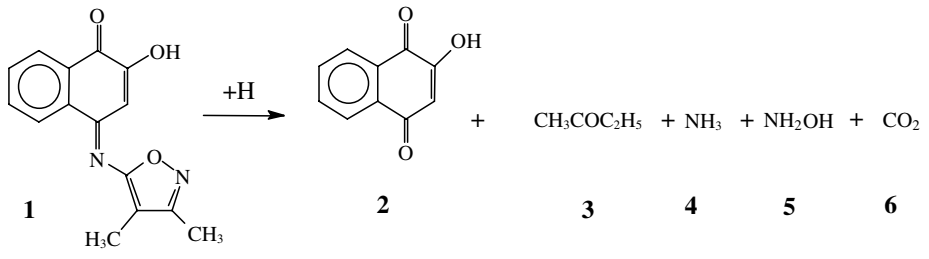
[a] k_{obs} (in the presence of HP-β-CD)/k_{obs} (in the absence of HP-β-CD)

which the observed degradation rate constant appeared to be pseudo-first order (Fig. 3).
The most striking result from this study was the significant difference between the stability of **1** in HP-β-CD solutions and in water under the same conditions.

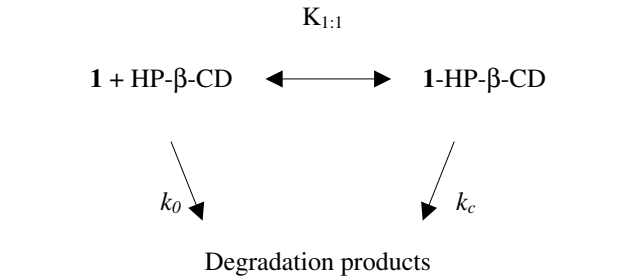
2.3. Influence of pH and stabilization with cyclodextrins

The influence of pH on the degradation of **1** in aqueous buffer solutions with or without HP-β-CD was studied over the pH-range of 2.34–3.95. The ionic strength of the buffer solutions was constant ($\mu = 0.5$). Table 4 summarises the effects of solution pH and HP-β-CD concentration on the degradation of **1**.
As it was observed for similar structures [7, 11, 12], our data give evidence of a specific acid catalysis (Scheme 1). The presence of HP-β-CD in the reaction medium did not appear to affect this mechanism; however, the increments of the HP-β-CD concentration decreased the hydrolysis rate of **1**.
The pH-rate profiles for the observed first-order degradation of **1** in aqueous solutions containing 0, 8 and 20% (w/v) of HP-β-CD at 70 °C are shown in Fig. 4.
The three parallel straight lines indicated a linear relationship between log k_{obs} and pH. Decreasing the $[H^+]$ concentration, the observed pseudo-first order rate constants

Scheme 1



Scheme 2



increased and the system became more stable. The degradation of **1** is pH dependent and showed a general catalytic effect at acid pH. The parallel course of the straight lines indicated that the stabilizing effect of the HP-β-CD is independent from pH.

2.4. Influence of cyclodextrin concentration

The stability of **1** and the effects of HP-β-CD on it were studied. The degradation of **1** in aqueous solution can follow the pathways indicated in Scheme 2.

The influence of HP-β-CD on the stability of **1** was determined by increasing the concentrations of HP-β-CD between 0 and 28%, in the reaction medium at various pH values (2.34–3.95), and determining the observed first-order rate constants for the overall loss of **1** (k_{obs}). The results are presented in Table 4. A nonlinear relationship between k_{obs} and HP-β-CD concentrations was obtained (Fig. 5).

The rate decreased fast when the HP-β-CD concentration was increased from 0 to 8%; then, it leveled off at higher HP-β-CD concentrations. The results are in agreement with kinetic systems where a compound degrades at a higher rate outside than inside the complex. The complexation equilibrium and outside/inside-the-complex degradations are illustrated in Scheme 2.

The relationship between the total HP-β-CD concentration and the observed rate constant for the degradation (k_{obs})

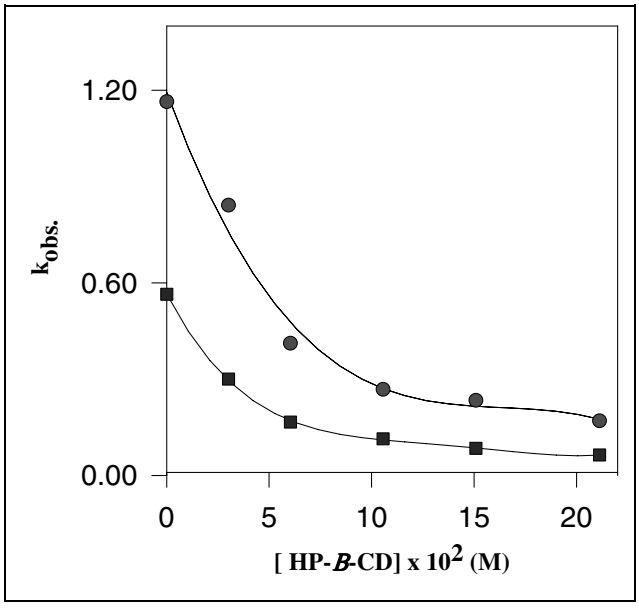


Fig. 5: Influence of HP-β-CD on the observed first-order constants for degradation of 1 at pH 2.34 at (●) 60 °C and (■) 70 °C.

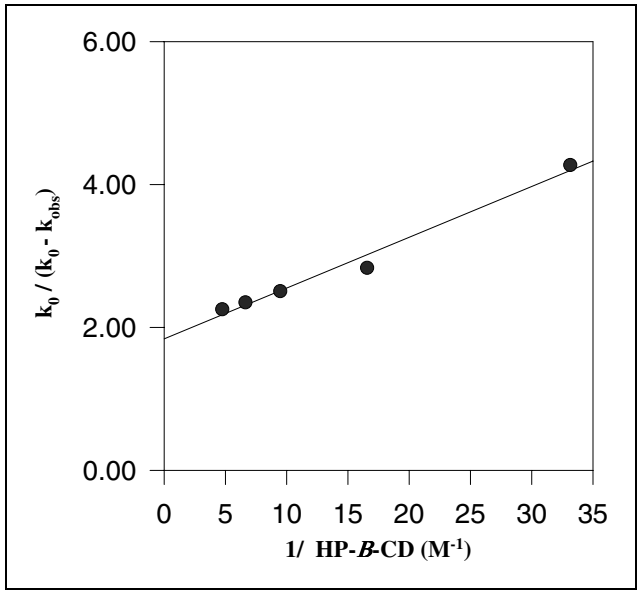


Fig. 6: Representative Lineweaver-Burk plot for degradation of 1 in pH 2.34 aqueous buffer solutions at 70 °C.

can be treated quantitatively by Lineweaver-Burk plots (Eq. 1):

$$\frac{k_0}{k_0 - k_{kobs}} = \frac{k_0}{K_{1:1}(k_0 - k_c)\,[HP - \beta - CD]} + \frac{k_0}{k_0 - k_c} \quad (1)$$

where k_0 represents the pseudo-first-order rate constant for the degradation of the free compound (when no HP-β-CD is present), k_c is the pseudo-first-order rate constant for the degradation of the drug in the complex, and $K_{1:1}$ is the stability constant of the inclusion complex, assuming 1 : 1 complexation [13]. The values of k_c and $K_{1:1}$ for **1**-HP-β-CD inclusion complex were calculated from the intercept and the slope of a linear plot when $k_0/(k_0-k_{obs})$ was plotted against the total reciprocal HP-β-CD concentration. The correlation coefficient of the linear plots obtained were in all cases higher than 0.996. A representative plot is shown in Fig. 6.

The values of k_c and $K_{1:1}$ are indicated in Table 5. As a consequence, it is obvious that HP-β-CD showed an stabilising effect over **1**.

As it can be seen, **1** degraded 11.5 times faster out in the solution than inside the HP-β-CD complex, at pH 2.34 and 60 °C.

2.5. Effect of temperature

The effect of temperature on the hydrolysis rate of **1** was investigated to gain further insight into the descomposition mechanism. Figure 7 shows the typical Arrhenius plots for the degradation rates of **1** at pH 2.93 in the absence and presence of HP-β-CD over the temperature range from 60 to 90 °C.

Table 5: The pseudo-first-order rate constants for the degradation and the complex stability constants of 1 in aqueous HP-β-CD buffer solutions at 60 °C

pH	$k_0 \times 10^2$ (h^{-1})	$k_c \times 10^3$ (h^{-1})	$K_{1:1}$ (M^{-1})
2.34	28.2	24.4	1590
2.93	6.52	1.40	1536
3.95	0.59	0.34	1614

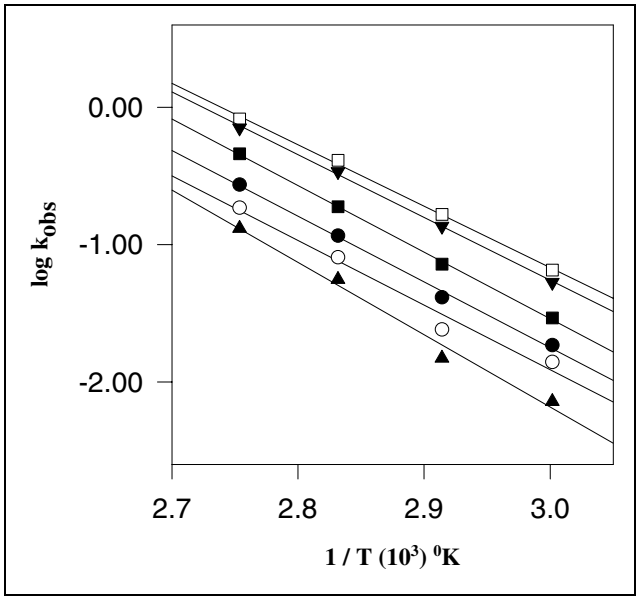


Fig. 7: Arrhenius plots of the hydrolysis of 1 at pH 2.93 in the presence or absence of HP-β-CD.
Key: (□) 0% HP-β-CD; (▼) 4% (w/v) HP-β-CD; (■) 8% (w/v) HP-β-CD; (●) 14% HP-β-CD; (○) 20% (w/v) HP-β-CD; (▲) 28% (w/v) HP-β-CD.

The half-lives ($t_{1/2}$, the time in which 50% of the drug is degraded) and shelf-lives ($t_{90\%}$, the time in which 90% of the drug remains) of **1** at 25 °C were calculated using the Arrhenius equation (Table 6). By decreasing the temperature of the solutions, the stabilizing effects of HP-β-CD increased. For example, at pH 2.93, HP-β-CD (28% (w/v)) increased the shelf-life of **1** 46.5 folds and 21.4 folds at 70 °C and 80 °C, respectively.
Linear plots of ln (k/T), where k is k_c, k_0 or $K_{1:1}$, versus 1/T, based on the Eyring equation (Eq. 2):

$$\ln (k/T) = \ln (k_B/h) + (\Delta S^{\ddagger}/R) - (\Delta H^{\ddagger}/R)\, 1/T \quad (2)$$

where k_B is the Boltzmann constant, T is the absolute temperature and R is the gas constant, were used to determine the enthalpy of activation ($\Delta H^{\ddagger}$) and the entropy of activation ($\Delta S^{\ddagger}$) at pH 2.93 (Table 7). The enthalpy change for the complex formation was negative, resulting in a decrease in the free energy through the complexation process. The enthalpy obtained for the stability constant of an inclusion complex formation is always negative and the complex dissociates when the temperature is increased [14] and the entropy has a large negative value associated with formation of highly ordered state. Conversely, the activation parameters for the degradation resulted in an increase in the free energy. Thus, when the temperature of the HP-β-CD containing reaction medium was lowered, both the decrease in the degradation rate constant and the increase in the stability constant for the inclusion complex would result in stabilization of **1**.
In summary, the aqueous stability of **1** can be increased by forming an inclusion complex with HP-β-CD since the drug degrades at much slower rate within the HP-β-CD cavity than outside in the aqueous solution. The aqueous stability of **1** increased at pH 3.95 and 70 °C, about 8-fold, when 28.0% (w/v) HP-β-CD was added to the reaction medium. The results also indicate that the stabilizing effects of HP-β-CD increase with decreasing temperature.
The increased solubility and stability of **1** in the complex, and the low toxicity [2] of HP-β-CD may lead to a practical formulation modality which could be an alternative to vehicles already studied [15–17] for parenteral administration of that compound. Therefore, HP-β-CD may be a useful additive in formulating this naphthoquinone derivative and potentially other related drugs.

Table 6: Observed half-lives ($t_{1/2}$) and shelf-lives ($t_{90\%}$) for overall hydrolysis of 1 in aqueous solutions at various temperatures in the presence or absence of HP-β-CD

% HP-β-CD (w/v)	$t_{1/2}$ (h), pH = 2.93				
	25 °C	60 °C	70 °C	80 °C	90 °C
0.00	16.1	10.6	4.2	1.7	0.8
4.00	21.4	13.0	5.1	2.0	1.0
8.00	54.1	23.8	9.6	3.7	1.5
14.00	122.9	37.4	16.7	5.9	2.5
20.00	329.2	49.6	28.8	8.6	3.7
28.00	759.3	96.3	46.5	12.4	5.3
	t_{90} (h), pH = 3.95				
	25 °C	60 °C	70 °C	80 °C	90 °C
0.00	22.5	17.7	6.2	2.7	1.4
4.00	31.7	21.9	7.5	3.1	1.6
8.00	84.7	39.0	14.6	5.1	2.6
14.00	117.8	59.2	20.5	9.3	3.9
20.00	227.7	72.9	31.5	10.9	4.8
28.00	534.8	95.0	51.3	18.6	6.9

Table 7: Pseudo-first-order rate constants for the degradation and the complex stability constants of 1 in aqueous HP-β-CD pH 2.93 McIlvaine Buffer solutions

T (°C)	$k_0 \times 10^2$ (h^{-1})	$k_c \times 10^3$ (h^{-1})	$K_{1:1}$ (M^{-1})
60.0	6.52	1.40	1536
70.0	16.6	3.68	1399
80.0	41.0	8.79	1245
90.0	82.0	20.0	745
ΔH (KJ/mol)[a]	82.9	86.3	−68.3
ΔS (J/mol/deg)[b]	−19.9	−41.7	−386.6

[a] Enthalpy of activation
[b] Entropy of activation

3. Experimental

3.1. Materials

The synthesis and identification procedures for **1** have previously been described [18]. HP-β-CD (mw 1326–1400, degree of molar substitution, 7.0) was a gift from CERESTAR USA, INC. (Hammond, IN). All other materials and solvents were of analytical reagent grade.

3.2. Buffers

McIlvaine buffers (pH 2.34–3.95) were prepared according to Elving et al. [19]. The ionic strength of the buffer solutions was adjusted to 0.5 by the addition of NaCl. The water used for buffer preparation was generated by a Millipore Milli-Q Water purification system.

3.3. Degradation products

Under acidic conditions, **1** undergoes hydrolysis to generate 2-hydroxy-1,4-naphthoquinone (**2**) as the major product. The IR spectrum and the melting points are in agreement with those of authentic samples. Compounds 2-butanone (**3**), ammonia (**4**), hydroxylamine (**5**), and carbon dioxide (**6**) were found to be the hydrolysis products of the 5-amine-3,4-dimethylisoxazole (Scheme 1) and were characterised as in a previous work [20].

3.4. UV Analysis

A Shimadzu UV 260 UV/visible spectrophotometer with 1 cm quartz cells was used, and the second derivative spectra were determined be-

tween 350 and 300 nm against a blank solution containing all components except from **1** and **2**. Suitable settings were a slit width of 1 nm and a fast scan speed. The absolute values of the derivatives were obtained by a zero-crossing technique with measurements at 322.5 nm for the analysis of **1**.

3.5. Validation parameters

Linearity, sensitivity, accuracy, precision and specificity were determined according to reported procedures [10] and ICH Guidelines for Validation of Analytical Procedures [21].

3.6. Kinetic study

Kinetics studies were carried out by adding stock solution of **1** in ethanol to 5.0 ml of an aqueous buffer HP-β-CD solution. The initial **1** concentration in the final solution was 5.20×10^{-5} M, and the HP-β-CD concentration ranged from 0 to 28%, w/v. The ethanol concentration in the final reaction mixture was 4.0%.

The sample solutions prepared of **1** were incubated at 25 °C (Haake F/3 thermostat with a $\pm$ 0.1 °C) for 72 h and remained stable until the solubility equilibrium was reached. Then, these flasks, as well as solutions without HP-β-CD, were stored at 60, 70, 80 and 90 °C. Samples were withdrawn at suitable time intervals and immediately cooled in an ice bath. Upon removal of the last samples, the stored solutions were allowed to warm up to room temperature and then, all samples were analysed by second-order derivative spectroscopy. These kinetics studies were done in duplicate. The pH of the final reaction mixture was determined at the end of each experiment with a pH-meter (Orion Model SA 520) standardised at appropriate temperature.

The pseudo-first-order rate constants were determined from the disappearance of the drug by least-square linear regression of natural logarithmic of the derivative spectra heights vs. time plots. The half-life and the correlation coefficient were calculated for each run.

Data from stability studies, as well as from method validation runs, were analyzed using Sigmaplot 3.0 (Jandel Scientific, San Rafael, CA).

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