Inclusion complexes and photo-stability studies of pyrazinamide with cyclodextrins: Spectrophotometric and kinetic determination of stability constant

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ABSTRACT

The present study offers valuable information in comparing two methods for determination of stability constants of inclusion complexes of two hosts; β-cyclodextrin (β-CD) and 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) as complexation reagents with Pyrazinamide (PZA) acting as the same "guest" molecule. The stoichiometry of the complex formation was examined by applying the continuous variation (Job plot) method and a 1:1 molar ratio has been identified for both complexes, at the examined concentrations. The formation of the complexes was investigated and confirmed by 1H NMR spectrometry. Stability constants Kst of the inclusion complexes were determined spectrophotometrically according to the spectral changes of PZA absorbance due to complexation and kinetically as a function of the changes in the rate constant of PZA photodegradation. The photodegradation rate constants for the free and complexed forms were also calculated. © 2011 Trade Science Inc. - INDIA

INTRODUCTION

Pyrazinamide (Figure 1) is a highly specific agent and is active only against Mycobacterium tuberculosis; it may be bacteriostatic or bactericidal depending on the concentration of the drug attained at the site of infection. In vitro and in vivo, the drug is active only at a slightly acidic pH[1]. Pyrazinamide (PZA) has been found to induce photosensitization in humans and its adverse effects have been previously reported[2]. Several clinical cases of photosensitivity have been reported in the literature, where PZA was found to induce cutaneous side effects such as flushing, photosensitivity, urticaria, etc[3-5]. In fact, a study conducted by Vargas et al.[6] has clearly shown that PZA had a phototoxic potential and its ability to participate in several types of photochemical reactions is relevant to the observed clinical phototoxicity and photoallergy caused by its administration in TB therapy.
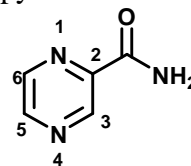

Figure 1 : Structure of PZA

Cyclodextrins (CDs) are doughnut-shaped molecules formed by six, seven or eight glucose units (α, β andγ, respectively) and are able to form inclusion complexes with a great variety of compounds.

Frequently, these supramolecular species enhance the luminescence properties of some compounds and this has been reported to have analytical implications[7].

In aqueous solutions, cyclodextrins are able to form inclusion complexes with many drugs by taking up a drug molecule or more frequently some lipophilic moiety of the molecule, into the central cavity. No covalent bonds are formed or broken during the complex formation and drug molecules in the complex are in rapid equilibrium with free molecules in the solution. The main driving forces between CD and a guest molecule were reported to be Van der Waals–London dispersion forces and hydrophobic interaction[8]. The physicochemical properties of free drug molecules and of free cyclodextrin molecules are different from those in the complex. In theory, any methodology that can be used to observe these changes in additive physicochemical properties may be utilized to determine the stoichiometry of the complexes formed and the numerical values of their stability constants[9-11].

CDs have extensively proven their potential as media for controlling chemical and photochemical reactions[12-18]. They have been used widely as solubilizing systems[19,20], enzyme models, catalysts, stationary and mobile phase additives for chiral and isomeric separations, etc. Because of the increased interest in CDs and their inherent usefulness, different studies have been carried out to evaluate the complexation procedure, the binding constant and the stoichiometries of the complexes formed. Different methods have been described for the determination of binding constants based on techniques[21-23] such as potentiometric titrations, spectrophotometric methods, solubility and competitive indicator binding.

The main goal of this study was to compare two methods for determination of stability constants of inclusion complexes of two hosts; β-cyclodextrin (β-CD) and 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) as complexation reagents with Pyrazinamide (PZA) acting as the same "guest" molecule. The stability constants of the formed complexes were determined spectrophotometrically using the Benesi–Hildebrand linear

approach[24]. On the other hand, monitoring the degradation rate constant of PZA in absence and presence of cyclodextrins revealed their role in increasing the photodegradation of the drug under UV light and allowed the determination of PZA-CD inclusion complex stability constant by using kinetic analysis.

MATERIALS AND METHODS

Chemicals and reagents

1 High purity β-CD and HP-β-CD were purchased from Aldrich and were used as received.
2 PZA was kindly supplied by Amoun Pharmaceutical Co.S.A.E.El-Obour City, Cairo, Egypt, and was used as received. Under license from: LUPIN CHEMICALS (THAILAND) LTD. (Assay: 99.91% according to the official USP 23 method[25].
3 De-ionized water was used as solvent.

All chemical and reagents used through this work are of spectroscopic analytical grade. De-ionized water is used throughout the whole work and is indicated by the word 'water'.

Instruments

1 Absorption spectra were recorded on SHIMADZU Dual-beam (Kyoto/Japan) UV-Visible spectrophotometer model UV-1601 PC with 1-cm quartz cuvettes connected to IBM compatible computer fitted with UV-PC personal spectroscopy software version 3.7 (SHIMADZU).
2 ^{1}H NMR spectra were recorded on a 300MHz Varian Mercury VX-300NMR spectrometer.
3 The photodegradation experiments were performed with DESAGA UV lamp (Sarstedt-Gruppe, Germany) short wavelength 254 nm (30 watt, 50/60 Hz) at a distance of about 10 cm.

Standard solutions

1 Solution of 10^{-3} M of PZA (solution A) was prepared by weighing and dissolving the appropriate amount in water.
2 Solutions of 10^{-2} M and 10^{-3} M of β-CD and HP-βCD were prepared by weighing and dissolving the appropriate amounts in water.

Procedures

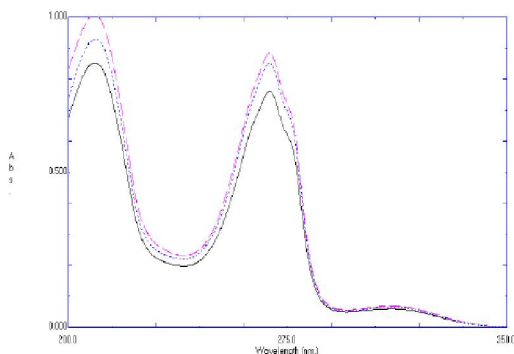Examination of the changes in PZA ultraviolet

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Figure 2 : UV spectra of 1×10^{-4} M of aqueous solution of PZA in the absence (-) and presence of 2.5×10^{-3} M β-CD (…) and 2.5×10^{-3} M HP-β-CD (- - -)

absorption spectra in the absence and in presence of CDs:

The examination of the changes in 1×10^{-4} M PZA spectra, in the absence and in presence of increasing CD concentrations (2.5 to 30 times that of PZA), was achieved by scanning the wavelengths between 200 and 350 nm, using water and an equimolar aqueous solution of CD as reference, respectively.

Determination of the time needed for the formation of the inclusion complex

The absorption spectrum of 1×10^{-4} M PZA solution was primarily scanned at initial time (t = 0), against water as blank. The same experiment was repeated, where aliquots of 1 ml of solution A (10^{-3} M PZA) were transferred into 10 ml calibrated flasks containing 1 ml of aqueous solution of CD (10^{-2} M) where the molar ratio was 1:10 ([PZA]: [CD] = 1:10). The contents were mixed, diluted to volume with water, stirred, and the spectrum was scanned at times: 5, 10, 15 and 25 min against a blank reference cell, containing CD at the same concentration.

Determination of stoichiometry by continuous variation method

The inclusion complexes of PZA with the two types of CDs were prepared by mixing different volumes of equimolar aqueous solutions(10^{-3} M) of PZA and CD resulting in certain molar ratios such that the total concentration remained constant ([PZA] + [CD] = M 1.2 X 10^{-4} M). Subsequently, ΔA[PZA] was plotted against r which varied between 0 and 1 {r = [PZA]/ ([PZA] + [CD])}. ΔA values were calculated by measuring the absorbance of PZA in the absence (A_0) and presence
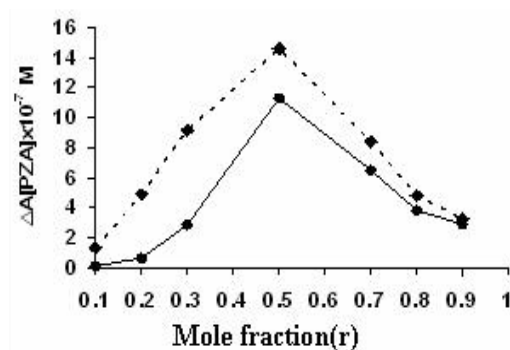

Figure 3 : Continuous variation plot (Job plot) of PZA: β-CD (-) and PZA: HP-β-CD (- - - -) inclusion complexes at 269 nm

(A) of the corresponding concentration of the CD. The quantities ΔA [PZA] are proportional to the concentrations of the complexes. The ratio (r) corresponding to the maximum absolute complex concentration does not depend on M or the binding constant[26].

Before measuring the absorbance of the prepared standard solutions, they were stirred continuously for 15 min and 10 min. time periods for the PZA-βCD complex and PZA-HP-β-CD complex, respectively. The method was repeated, using water instead of aqueous solutions of CD at the same volume proportion and the solutions were analyzed against a blank reference cell containing water.

Characterization of the complex in solution

In aqueous solution, the structures of the complexes were investigated by ^{1}H NMR spectroscopy using 1.625×10^{-2} M PZA solution and 5×10^{-3} M CD solution .The spectra were run at 300MHz in 99.9% D_2O (Sigma). Chemical shifts were quoted in δ and were related to that of the solvent (4.82 ppm at 303.1K).Typical conditions were: pulse 74.1 degrees, acquisition Time 4.004 sec, width 8000.0Hz and 32 repetitions.

Photostability studies

Monitoring of the photodegradation process of an aqueous solution of pyrazinamide in the absence and in presence of CDs was achieved by the examination of the changes in the spectra of the irradiated 1×10^{-4} M PZA solutions in the absence and presence of CD, by scanning the wavelengths between 200 and 350 nm at zero time and at 15 minutes intervals of irradiation for two hours, using water and an equimolar aqueous solution of CD as reference, respectively.
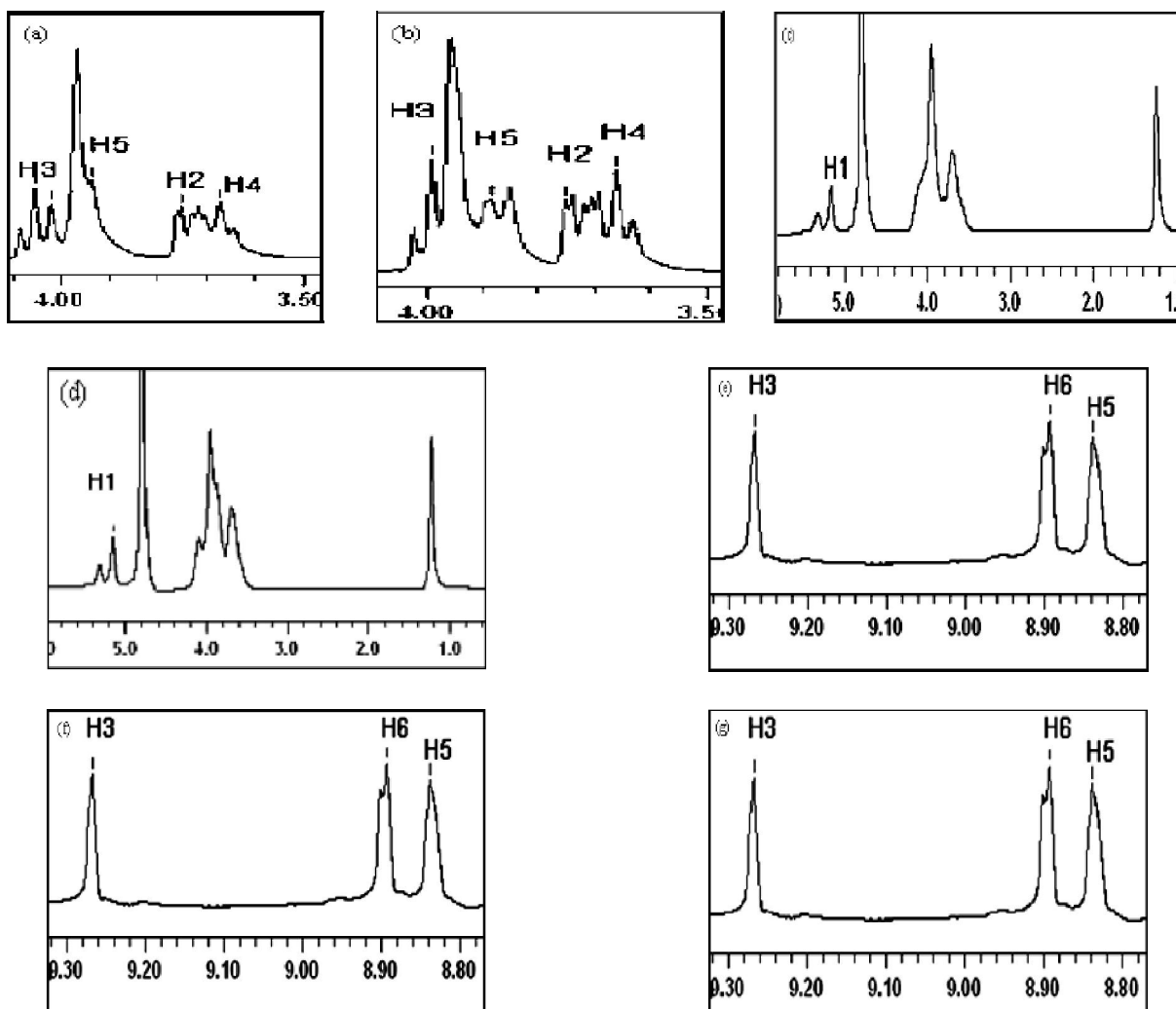
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Figure 4 : Partial 300 MHz ¹H NMR spectra of: (a) Pure β-CD, (b) β-CD in PZA-β-CD complex, (c) Pure HP-β-CD, (d) HP-β-CD in PZA-HP-β-CD complex, (e) Pure PZA , (f) PZA in PZA-β-CD complex, and (g) PZA in PZA-HP-β-CD complex

Determination of stability constant

Spectrophotometric method

Aliquots of 1 ml of solution A (10^{-3} M 1PZA) were transferred into 10 ml calibrated flasks containing increasing amounts of CD standard solutions (10^{-2} M) and diluted to volume with water (where the final concentrations of β-CD ranged from 10 to 20 times that of PZA while those of HP-β-CD were 5 to 25 times that of PZA). The prepared solutions were stirred continuously for 15 min and 10 min. time periods for the PZA-βCD complex and PZA-HP-β-CD complex, respectively and then the examination of the changes in PZA spectra was achieved by scanning the wavelengths between 200 and 350 nm, using equimolar aqueous solution of CD as reference.

Kinetic method

An aliquot of 1 ml of solution A (10^{-3} M PZA) was accurately transferred into a 10 ml calibrated flask and diluted to volume with water. Four ml of the prepared solution were accurately transferred into a quartz cuvette which was tightly closed and irradiated at a distance of 10 cm using a short wavelength 254 nm UV lamp at room temperature for 120 minutes. The examination of the changes in the spectra of the irradiated solution was achieved by scanning the wavelengths between 200 and 350 nm at zero time and then at 15 minutes intervals of irradiation for two hours, using a blank reference cell containing water. The previous procedure was followed using the same PZA concentration and increasing amounts of CD standard solutions (10^{-2} M) (where the final concentrations of CDs ranged
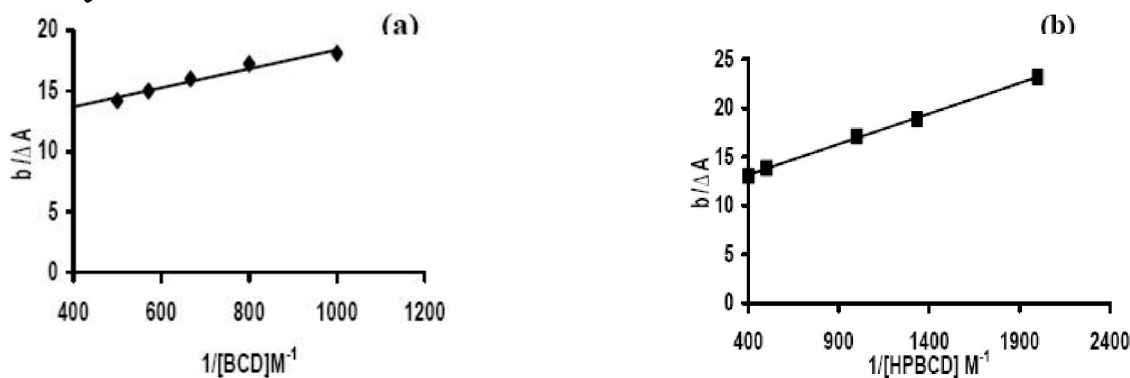
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Figure 5 : Benesi-Hildebrand plot for the effect of (a) β-CD and (b) HP-β-CD on PZA absorbance at 269 nm

from 10 to 25 times that of PZA). After dilution to volume with water, the prepared solutions were stirred continuously for 15 min and 10 min. time periods for the PZA-β-CD complex and PZA-HP-β-CD complex, respectively, and this was followed by irradiation as previously mentioned.

Aliquots of 1 ml of Pyrazinamide stock solution (4 x 10^{-3} M) were accurately transferred into 25-ml volumetric flasks containing increasing amounts of standard $FeCl_3$ solutions (5 x10^{-3} M) and diluted to volume with water (where the final concentrations of $FeCl_3$ ranged from 1.25 to 7.5 times that of PZA). The absorbance of each solution was recorded at 269 nm, using equimolar aqueous solutions of $FeCl_3$ as reference.

RESULTS AND DISCUSSION

The ultraviolet absorption spectrum of PZA showed three absorption maximum wavelengths at 209, 269 and 310 nm, which were slightly changed due to CD presence. These changes were intensified as the concentrations of CDs increased. The hyperchromic effects at the absorption maximum wavelengths of PZA were intense in the presence of both CDs (Figure 2). These results suggested that PZA formed stable complexes with CDs with expected large stability constants.

Determination of the time needed for the formation of the inclusion complex

The maximum difference in absorbance (ΔA) at 269 nm, for aqueous solution of PZA in the absence and presence of β-CD and HP-β-CD which corresponds to the formation of the PZA-β-CD complex, was reached after 15 min., and that corresponding to the PZA-HP-β-CD complex, was reached after 10 min.

However, this difference was constant for the rest of the time intervals.

Determination of complex formation stoichiometries

Job[27] described the continuous variation technique that can provide a reliable determination of the complex stoichiometries, based on the difference in a physical parameter, for example, the difference in absorbance ΔA (ΔA = A-A$_o$) of PZA in the presence of CD. On applying this technique, the resulting plots (Figure 3) demonstrate that since the ΔA [PZA] maximum has an r value of 0.5, therefore both PZA-β-CD and HP-β-CD complexes have 1:1 stoichiometry with respect to both PZA-β-CD and HP-β -CD complexes.

¹H NMR study

NMR spectroscopy is found to be the most effective tool for the investigation of the nature of inclusion complexes formation between cyclodextrins and guest molecules in solution[28].

The formation of inclusion complexes of PZA with CDs in aqueous solution (D_2O) is evidenced by significant modifications in the NMR spectra of the host molecules and slight modifications in the NMR spectra of the guest molecules. The partial 300 MHz ¹H NMR spectra of the pure compounds and of the complexes are displayed in (Figure 4).

Only shifts of the signals were observed and no new peaks that could be assigned to the pure complex appeared. This observation implies that the complexation is a dynamic process, the included guest being in fast exchange (relative to the NMR time scale) between the free and bonded states[29].
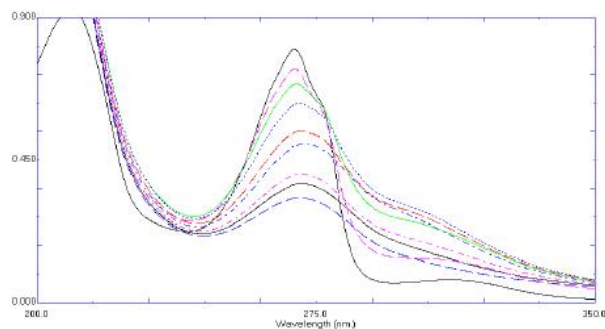
In general, the resonances of β-CD and HP- β-

Figure 6 : UV monitoring at 269 nm of the photolysis of aqueous solution of pyrazinamide (1×10^{-4}) in presence of HP-β-CD (1.5×10^{-3}) at zero time and at 15 minutes intervals of irradiation for two hours

Figure 7 : Effect of increasing CD concentrations on the pseudo-first-order rate constant for the photodegradation of PZA

CD protons located in the interior of the cavity (H-3 and H-5) show remarkable chemical shifts upon inclusion of the guest molecule , whereas those residing outside the cavity (H-1, H-2 and H-4) should undergo no or minimal changes. The H-6 protons are located at the smaller rim of the cavity and in most cases show a chemical shift similar to or slightly larger than those of the exterior protons (H-1, H-2 and H-4).

The ^{1}HNMRspectrum of β-CD and HP-βCD in D$_2$O showed remarkable up field shifts of H-3 and H-5 (TABLE 1 and 2) whereas the H-1, H-2 and H-4 showed minimal alterations. The effect of β-CD and HP-βCD on the ^{1}H NMR spectrum of PZA is not very significant showing slight chemical shifts of H-3 and H-5. The chemical structure of PZA and the chemical shifts described above suggest that the aromatic ring is the part of the drug that is inside the CD cavity and the aromatic π cloud is responsible for the shielding of the CD protons.

Furthermore, the hydrogen-bonding effect is superior to the PZA–HPβCD complex, due to the additional flexible hydroxyalkyl chains at the periphery of the cavity, explaining the enhanced stability of this complex, as compared with the PZA–β-CD 1:1 complex. It is presumed that hydrogen bonding occurs between the –NH$_2$, C=O and –N= of PZA and hydroxyl groups of HP-β-CD.

Determination of stability constant

Spectrophotometric method

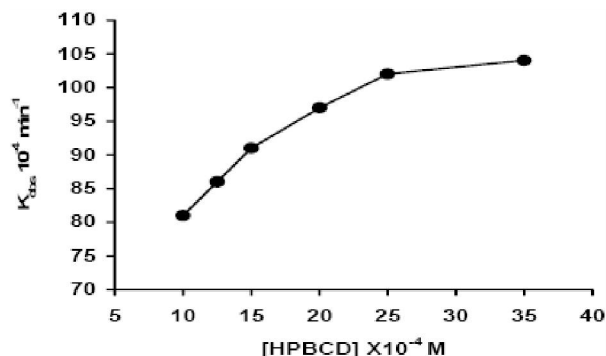The interactions with CDs may increase or decrease the absorbance and generally increase the fluorescence intensity of the included organic moiety[30,31].The molar absorptivity (ε) of any 'guest' molecule (drug to be complexed) changes depending on binding to cyclodextrins[29]. This may happen due to changes in the environmental polarity of the guest's chromophore on moving from the polar aqueous media to the apolar cyclodextrin cavity. The difference in absorbance ΔA at 269 nm as a function of CD concentration [CD] follows the typical binding isotherm of 1:1 complexes:

$$\frac{\Delta A}{b} = \frac{[PZA]Kst\,\Delta\varepsilon[CD]}{1 + Kst[CD]} \qquad (1)$$

When a high excess of [CD]t is being used compared to[PZA], the assumption that [CD] = [CD]t is employed and through this, (Eq. 1) is transformed, according to Benesi and Hildebrand, into the more widely utilized double reciprocal linear equation (Eq. 2)

$$\frac{1}{\Delta A} = \frac{1}{[PZA]Kst\,\Delta\varepsilon[CD]} + \frac{1}{[PZA]\Delta\varepsilon} \qquad (2)$$

The stability constant of each complex was determined by UV spectrophotometry according to the spectral shift method, which is based on the increase in absorbance (ΔA), due to the complexation. The measurement took place at 269 nm, the main of the three absorption maximum wavelengths, in order to achieve more reliable values of ΔA. The complex stability constant Kst was calculated from the ratio of intercept to slope using the Benesi–Hildebrand linear method which is solved graphically, using the linear least-squares regression analysis applied to known mathematical models such as Benesi-Hildebrand, Scatchard etc. Most of linear models suffer from theoretical and practical drawbacks, including assumed concentrations of the interacting moieties and products, poor solubility of certain

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TABLE 1 : Chemical shifts (ppm) of the protons of β-CD and PZA in the free and complexed

Proton*	δ_o(free)	δ_c(complex)	$\Delta\delta(\delta_c\text{-}\delta_o)$
β-CD			
H-3	4.054	3.991	0.063
H-5	3.938	3.883	0.055
PZA			
H-3	9.268	9.263	0.005
H-5	8.83	8.827	0.003

*Only the protons that show chemical shifts

TABLE 2 : Chemical shifts (ppm) of the protons of HP-β-CD and PZA in the free and complexed states

Proton*	δ_o(free)	δ_c(complex)	$\Delta\delta(\delta_c\text{-}\delta_o)$
Hp-β-CD			
H-3	3.96	3.897	0.063
H-5	3.704	3.667	0.037
PZA			
H-3	9.268	9.262	0.006
H-5	8.83	8.824	0.006

*Only the protons that show chemical shifts

TABLE 3 : A comparison of the kinetically determined values of Kst with those obtained by using the Benesi–Hildebrand graphical method

	Benesi-Hildebrand graphical method	Kinetic-photodegradation method
Kst of PZA-β-CD complex	1334±262	1335±74.94
Kst of PZA-HP- β-CD complex	1725±154	1778±102.98

compounds and the occasional formation of dimmers. But as the studies presented by Y.Dotsikas and Y.L.Loukas[32] and Y.Dotsikas et al.[33] confirm, in case of a complex with 1:1 stoichiometry, a series of linear procedures for the calculation of the binding constant could be applied without serious hesitations. The stability constants were calculated from the linear plots (TABLE 3), resulting from the regression analysis of (Eq. 2) (Benesi-Hildebrand, (Figure 5a and 5b). The results suggested that PZA formed stable complexes with CDs with large stability constants where a value of Kst of ($>$1000 M^{-1}) indicates high stability for the inclusion complex[32].

Apparently PZA-HP-β-CD complex showed much higher stability than PZA-β-CD complex since the presence of additional hydrogen groups of HP-β-CD stabilizes more effectively the complex via hydrogen bonds.

Kinetic method

It is well known that the presence of a CD in a solution of a sensitive compound plays the role of a catalyst and could result in an increase or decrease in its degradation rate[34]. There are numerous examples of compounds sensitive to external factors and the determination of the binding constant is based on simplified kinetic linear models[35].

Pyrazinamide was in fact found to be photolabile as revealed by the study conducted by Vargas et al.[6]. Comparative photo-stability studies of PZA in free and complexed forms were conducted by the UV irradiation of an aqueous PZA solution with or without the addition of CDs and the course of the reaction was followed by UV-Vis spectrophotometry. The absorption spectra of PZA in water showed three bands centered at 209, 269 and 310 nm, respectively, with a tail extending to 340 nm. The photolysis of PZA in free and complexed forms was followed by monitoring the disappearance of the band at 269 nm .The results are shown in (Figure 6). These studies revealed that the concentration of PZA in aqueous solutions decreased with increasing irradiation time, and the photodegradation rate of the drug in aqueous solutions with CD was evidently faster than that in aqueous solutions without CD. The rate constant of the photodegradation of PZA increased from 6×10^{-4} min^{-1} to 96×10^{-4} min^{-1} and 102×10^{-4} min^{-1} in the presence of 25×10^{-4} M, β-CD and HP-β-CD respectively, which indicates that CDs can enhance the photodegradation of PZA because of its inclusion inside the CD cavity and thus the stability constant could be kinetically determined.

A plot of the relationship between CDs concentration and the observed pseudo-first-order rate constant for the photodegradation of PZA (Figure 7) reveals that K_{obs} is not a linear function of the concentration of the added CD but it approached a maximum constant value with increasing CDs concentration. This so called "saturation behavior" is characteristic of the reaction through complex formation, occurring prior to the rate determining step and may be explained by the reaction

mechanism illustrated in the following scheme[35]:
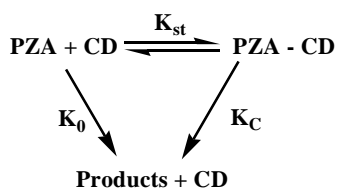
where K_0 is the rate constant for the decomposition of free PZA, K_c is the rate constant for the decomposition of totally complexed PZA and Kst is the apparent stability constant for complex formation. Usually, a ten-fold excess of CD should be present to ensure the conditions of a first-order reaction.

From the previously illustrated scheme, the following rate expression can be derived:

$$\frac{-d[PZA]}{d_t} = K_o + [PZA] + K_c [PZA - CD]$$

The observed reaction rate for PZA degradation in the presence of CDs is a weighted average of the rate of reaction of free PZA and the rate of reaction of PZA included in the CD cavity and therefore, Kst can be determined from the dependence of the observed rate constant on the concentration of added CD[29]. The actual measurable rate constant is:

$$K_{obs} = \frac{Ko + (K_c - K_o)[CD]}{\dfrac{1}{Kst + [CD]}} \quad (3)$$

This equation can be solved graphically according to the Lineweaver-Burke transformation and on rearrangement gives:

$$\frac{1}{(K_{obs} - K_o)} = \frac{1}{Kst(K_c - K_o)} \frac{1}{[CD]} + \frac{1}{(K_c - K_o)} \quad (4)$$

The plot of $1/(K_{obs} - K_0)$ versus $1/[CD]$ gives $1/K_{st}(K_c - K_0)$ as the slope and $1/(K_c - K_0)$ as the intercept. From the slope and intercept of the plot, the values of K_{st} were calculated and the results are shown in (TABLE 3) which shows the agreement of results obtained by the spectral shift method and the kinetic method. K_c was also calculated and found to be $119.41 \times 10^{-4} \pm 0.0004$ min^{-1} and $130.75 \times 10^{-4} \pm 0.0008$ min^{-1} for PZA- β-CD complex and PZA-HP-β-CD complex, respectively.

^{1}H NMR spectra revealed that PZA molecules can enter partly into the cavity of β-CD and HP-β-CD.

The marked enhancement effect of CDs on the photodegradation of PZA could depend on this moderate inclusion depth[37,38] allowing sufficient proximity of the reaction centre of PZA to catalytically active secondary hydroxyl groups of the CD cavity, which can enhance the degradation of excited PZA. On the other hand, this type of inclusion interaction between CDs and PZA could result in variation in the structural parameters (e.g. bond length) of PZA molecules because of interactions between non-bonded atoms[39]. The enhancement of photodegradation of PZA may result mainly from the lowering of bond energy between some atoms in the PZA molecule due to inclusion interaction with CDs.

CONCLUSION

The study of the electronic absorption spectral features of PZA in CD aqueous solutions which show significant increase of the absorbance intensity values with CD concentration clearly demonstrate the formation of inclusion complexes with CDs. 1H NMR studies show clear evidence for the inclusion of PZA inside the CD cavity as confirmed by the considerable chemical shifts of the interior protons of CD. Also, applying the continuous variation method described by Job, together with using the Benesi-Hildebrand treatments, we found a 1: 1 stoichiometry for the inclusion complexes. The kinetic study revealed the role of CDs in catalyzing the photodegradation of PZA which allowed the kinetic determination of the stability constants of the formed inclusion complexes. These Kst values were found to be in good accordance with those obtained by using the Benesi–Hildebrand graphical method. Evidently the graphical spectrophotometric method is suitable for determination of stability constants of inclusion complexes of CDs with compounds which show significant changes in absorption spectra upon complexation while the kinetic method has the advantage of being suitable for spectrophotometric determination of the stability constants when no changes in absorption spectra are observed upon complexation or even for non-absorbing compounds by kinetic monitoring of the degradation rate using other techniques such as HPLC.

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