J Incl Phenom Macrocycl Chem (2011) 71:137–147
DOI 10.1007/s10847-010-9917-y

ORIGINAL ARTICLE

Comparison between 2-hydroxypropyl-β-cyclodextrin and 2-hydroxypropyl-γ-cyclodextrin for inclusion complex formation with danazol

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Received: 14 October 2010 / Accepted: 13 December 2010 / Published online: 25 January 2011

Abstract Complexation in solution between danazol and two different cyclodextrins [2-hydroxypropyl-β-cyclodextrin (HP-β-CD) and 2-hydroxypropyl-γ-cyclodextrin (HP-γ-CD)] was studied using phase solubility analysis, and one- and two-dimensional ^{1}H-NMR. The increase of danazol solubility in the aqueous cyclodextrin solutions showed a linear relationship (A_L profile). The apparent stability constant, $K_{1:1}$, of each complex was calculated and found to be 51.7×10^3 and 7.3×10^3 M^{-1} for danazol–HP-β-CD and danazol–HP-γ-CD, respectively. ^{1}H-NMR spectroscopic analysis of varying ratios of danazol and the different cyclodextrins in a mixture of EtOD–D_2O confirmed the 1:1 stoichiometry. Cross-peaks, from 2D ROESY ^{1}H-NMR spectra, between protons of danazol and H3′ and H5′of cyclodextrins, which stay inside the cyclodextrin cavity, proved the formation of an inclusion complex between danazol and the cyclodextrins. For HP-β-CD, the inclusion complex is formed by entrance of the isooxazole and the A rings of danazol in the cyclodextrin cavity. For HP-γ-CD, two different inclusion structures may exist simultaneously in solution: one with the isooxazole and A ring in the cavity and the other with the C and D ring inside the cavity. DLS showed that self-aggregation of the CD's was absent in the danazol HP-β-CD system up to a CD concentration of 10% and in the danazol HP-γ-CD system up to a CD concentration of 5%.

Keywords Danazol, cyclodextrins · Inclusion complexes · ^{1}H-NMR · ROESY, self-aggregation

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Introduction

Danazol (Fig. 1) is an oral androgenic agent that induces amenorrhoea through suppression of the hypothalamic-pituitary-ovarian axis, accompanied by increased serum androgen concentrations and low serum estrogen levels [1]. Danazol is used in the endometriosis and/or fertility treatment, gynaecomastia, and hereditary angioedema [1–4]. It has a very high potential for the treatment of various autoimmune diseases including human T-lymphotropic virus 1-associated myelopathy/tropical spastic paraparesis and idiopathic thrombocytopenic purpura [5, 6]. However, danazol is a very poorly water soluble compound and exhibits dissolution rate-limited absorption [7]. It is usually administered in a relatively high dose, ranging from 200 to 400 mg, in order to reach effective blood concentration [8]. Because the drug suppresses ovarian steroidogenesis causing low estrogen and high androgen conditions, and the fact that it is administered in high doses, many side effects such as weight gain, fluid retention, breast atrophy,

acne, oily skin, hot flushes and hirsutism have been reported [1].

Cyclodextrins (CDs) are cyclic oligosaccharides derived from starch containing six (α), seven (β), eight (γ) or more (α-1,4)-linked α-D-glucopyranose units; they are characterized by a truncated cone shape [9]. As all the polar groups are located at the exterior and the cavity is relatively hydrophobic, CDs allow the formation of an inclusion complex by admitting one or more molecules inside the cavity without establishing a covalent bond [10]. Inclusion complexation with CDs enables an increase in solubility, reduced bitterness, enhanced stability and a decrease in tissue irritation upon dosing and an increase in bioavailability [11]. Therefore, CDs have been extensively used in pharmaceutical research and development [12].

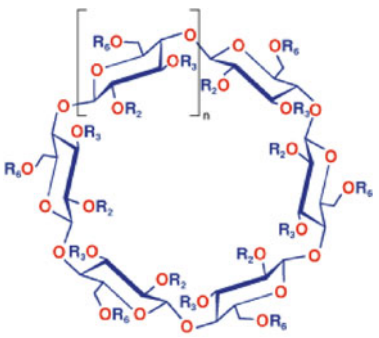
Danazol has been shown to form an inclusion complex with β-cyclodextrin (β-CD), leading to a better in vivo performance due to enhancement of danazol solubility in water [13]. However, the proposed structure of danazol–β-CD complex did not show any parts of danazol residing inside the CD cavity. The authors proposed that the methyl groups present at the junctions of A/B and C/D rings showed favorable interactions with β-cyclodextrin. The isooxazole ring, part of the A ring and the acetylene groups were exposed to the solvent. In order to contribute to the understanding of the inclusion phenomenon of danazol, we studied the complex formation between danazol and two different cyclodextrins: on the one hand a derivative of β-cyclodextrin, 2-hydroxypropyl-β-cyclodextrin (HP-β-CD), in order to compare with the underivatized product, and on the other hand 2-hydroxypropyl-γ-cyclodextrin (HP-γ-CD) to investigate the influence of the derivatization on the complexation behavior. The structures of the cyclodextrins are given in Fig. 1.

There are various methods to study inclusion complexation, such as X-ray diffraction, differential scanning calorimetry, UV–Vis spectroscopy and ^{1}H-NMR spectroscopy. In this study, both one and two dimensional (D) ^{1}H-NMR were used. While the 1D ^{1}H-chemical shift provides unambiguous evidence on the formation of a complex, NOESY or ROESY experiments provide information on the dynamics and the average relative inter- and intramolecular proton distances. In this study, to gain further information on the inclusion complexation mode and additional insights into the dynamic structure, 2D ROESY ^{1}H spectra were acquired. NMR techniques do provide a very clear picture of the inclusion complex formation by CDs in the solution phase, including the stoichiometry, the definition of the geometry of the new complex, and especially the orientation of the guest molecule in the CD cavity [14, 15].

Materials and methods

Materials

Danazol was purchased from Indis (Aartselaar, Belgium). 2-hydroxypropyl-β-cyclodextrin was kindly donated by Janssen Pharmaceutica (Beerse, Belgium). 2-hydroxypropyl-γ-cyclodextrin was purchased from Sigma-Aldrich (Bornem, Belgium). Deuterated ethanol and deuterium oxide were purchased from Cambridge Isotope laboratories, Inc. (MA, USA).



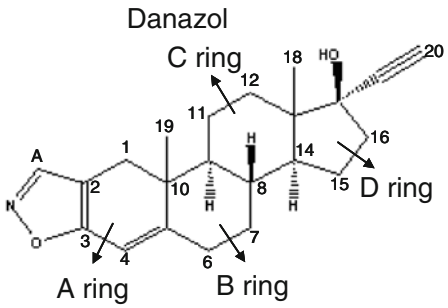


Fig. 1 Structural representation of cyclodextrins and danazol

Cyclodextrin	n	R
2-Hydroxypropyl-β-cyclodextrin (HP-β-CD)	2	–CH2CHOHCH3
2-Hydroxypropyl-γ-cyclodextrin (HP-γ-CD)	3	–CH2CHOHCH3

Methods

Phase solubility analysis

Phase solubility studies were performed based on the phase solubility technique established by Higuchi and Connors [16]. An excess amount of danazol was added in vials containing the cyclodextrin. For HP-β-CD, the concentration was varied from 0 to 0.072 M, and for HP-γ-CD from 0 to 0.064 M. Vials were shaken at 37 °C for 48 h (equilibrium). After equilibrium was attained, the solution was filtered through a 0.45 μm pore size membrane filter, diluted to fit within the linear range of the HPLC calibration line. The HPLC analysis was performed using a Merck Hitachi pump L-7100, an autosampler L-7200 and a UV–Vis detector L-7420. The column was a Lichrospher 100 RP8 5 μm 125 × 4.6 mm; the mobile phase consisted of acetonitrile:water (60:40, v/v) and the detection wavelength was set at 288 nm. The mobile phase was used at a flow rate of 1.0 mL/min. 20 μL sample volume was injected into the system.

The apparent stability constant ($K_{1:1}$) was calculated from the phase solubility diagram using the following equation:

$$K_{1:1} = \text{slope}/S_o(1 - \text{slope})$$

where S_o is the solubility of danazol in the absence of CDs, and 'slope' is the slope on the experimental phase solubility diagram.

^{1}H-NMR studies

^{1}H-NMR spectra were recorded on a Bruker Arian II 500 MHz spectrometer. The stock solutions of danazol (17 mM) and CDs (17 mM) were prepared in a mixture of deuterated water (D_2O) and deuterated ethanol (EtOD) in a ratio 1:2, and mixed in NMR tubes. The probe temperature was set at 298 K.

The ^{1}H-NMR spectra were recorded using a simple pulse-acquire sequence. Typical acquisition parameters consisted of 65 K points covering a sweep width of 7500 Hz and a pulse width of 6.5 μs, giving a resolution of 0.23 Hz/point.

The resonance at 1.20 ppm due to residual solvents present as impurities (EtOH and EtOD), was used as internal reference.

The 2 D DQF-COSY (DQF = double-quantum filter) and ROESY spectra were recorded with a sweep width of 7,000 in both dimensions. The DQF-COSY spectrum consisted of 4,096 data points in t2 and 512 increments in t1. The data were apodized with a shifted sine-bell square function in both dimensions and processed to a 4 K × 1 K matrix.

The total ROESY mixing time was set to 150 ms. The spectrum was acquired with 32 scans, 2,048 data points in t2 and 512 free induced decays (FIDs) in t1. The data were apodized with a shifted sine-bell square function in both dimensions and processed to a 2 × 1 K matrix.

1D spectra were analyzed using Topspin 2.1. For assignment and quantification of the ROESY cross peaks, the CARA (Computer assisted resonance assignment) program was used [17]. Distances were derived from the cross peak volume according to the formula $V = C/r^6$, where V represents the peak volume, r is the distance between the two 1-H nuclei and C is a constant, calibrated by using known distances within the molecule. The distance between H4 and H19 of danazol was used as reference (4.6 Å).

The continuous variation method was adopted to determine the stoichiometry of the complex. ^{1}H-NMR spectra were obtained for a series of danazol:CD mixtures in which the total initial concentration of the two species was kept constant but the mole fraction of each component was varied from 0 to 1. In case of NMR, under the fast exchange conditions, the observed signal (δ_{obs}) can be presented as:

$$\delta_{obs} = f_{\text{free drug}} \times \delta_{\text{free drug}} + f_{\text{com drug}} \times \delta_{\text{com drug}}$$

with $f_{\text{free drug}}$ = fraction of free drug; $\delta_{\text{free drug}}$ = NMR signal when the solution only contains the free drug; $f_{\text{com drug}}$ = fraction of drug in complex; $\delta_{\text{com drug}}$ = NMR signal when the solution only contains the complexed drug.

The observed chemical shift change of drug is not directly proportional with the amount of the compound in the complexes; however, the calculated quantity of the observed signal of the compound and the compound mole fraction are proportional to the complex concentration.

$$\Delta\delta_{obs} \times r_{drug} = \text{Constant} \times [\text{complex drug}]$$

with $\Delta\delta_{obs} = \delta_{obs} - \delta_{\text{free drug}}$; r_{drug} = mole fraction of drug; [complex drug] = concentration of drug in complex.

Determination of aggregation by dynamic light scattering (DLS)

The possibility of self-aggregation in CD solutions was investigated by photon correlation spectroscopy using a CGS-3 spectrometer (Malvern Instruments, Worcestershire, UK) equipped with a goniometry, auniphase 22 mV He–Ne laser operating at 632.8 nm, an avalanche photodiode and detector and an ALV-5000/EPP multi-angle tau correlator. Light scattering was monitored at 150°.

Results and discussion

Phase solubility analysis

Figure 2 shows the phase solubility diagram obtained for danazol in the presence of HP-β-CD and HP-γ-CD in water.

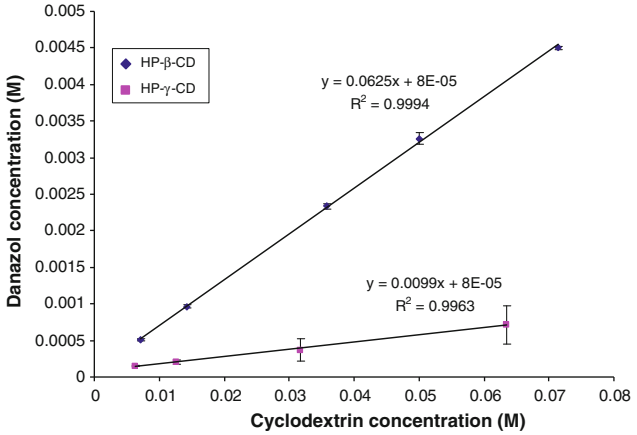


Fig. 2 Phase solubility diagrams of danazol in water in the presence of HP-β-CD (*filled diamond*) or HP-γ-CD (*filled square*) ($n = 3$). *Error bars* indicate standard deviation

The solubility of danazol increased from $1.2 \times 10^{-3} \pm 0.03 \times 10^{-3}$ mM (0.64 ± 0.09 μg/mL) to 4.5 ± 0.002 mM (1.52 ± 0.08 mg/mL) when the concentration of HP-β-CD was increased to 0.07 M (10%, w/v). The solubility of danazol increased from $1.2 \times 10^{-3} \pm 0.03 \times 10^{-3}$ mM (0.64 ± 0.09 μg/mL) to 0.7 ± 0.002 mM (0.24 ± 0.09 mg/mL) when the concentration of HP-γ-CD was elevated to 0.06 M (10%, w/v). The solubility increase of danazol in the presence of HP-β-CD and HP-γ-CD is linear within the range of the investigated concentrations and the resulting curve can be classified as an A_L type (linear positive slope isotherm), indicating the formation of water-soluble complexes. HP-β-CD and HP-γ-CD consist of a mixture of numerous structurally related isomers, thus HP-β-CD and HP-γ-CD and their complexes do generally not form crystalline precipitates [18].

The corresponding apparent stability constant values $K_{1:1}$ were estimated and found to be 51.7×10^3 and 7.3×10^3 M^{-1} for danazol–HP-β-CD and danazol–HP-γ-CD, respectively. The results show that HP-β-CD gives a higher $K_{1:1}$ value compared to that of HP-γ-CD. A similar observation was seen in an earlier study of Uekama et al. [19], where the structurally related steroid hydrocortisone showed a looser complex with γ-CD compared to β-CD and in a more recent study of Larsen et al. [20], where prednisolone also had a higher $K_{1:1}$ with β-CD than with γ-CD. Compared to β-CD, where a $K_{1:1}$ of 972.03 M^{-1} was reported for complexation with danazol [13], derivatization with hydroxypropyl groups clearly leads to a significant increase in apparent stability constant.

Study of the complexation by ^{1}H-NMR spectroscopy

^{1}H-NMR spectroscopy is a very effective method for studying inclusion complex formation between CDs and a variety of guest molecules, and has been used to confirm the conformation of inclusion complexes.

Stoichiometry determination

The continuous variation method, Job's method [21] based on ^{1}H-NMR chemical shifts, was used to determine the stoichiometry of the complexes. If a physical parameter directly related to the concentration of the complex is plotted as a function of the mole fraction of the host and guest, the maximum will be at the mole fraction ($r_{drug} = m/(m + n)$ or $r_{CD} = n/(m + n)$, where m and n are the molar ratios of drug and CD, respectively), in which they occur in the complex.

In the present conditions, only changes in chemical shift were observed, but no new peaks, which could be assigned to the pure danazol in inclusion complexes, were observed. Hence, complexation of danazol with HP-β-CD and HP-γ-CD appears to be a dynamic process with danazol being in a state of fast exchange (relative to the NMR timescale) [22]. In case of NMR, under the fast exchange conditions,

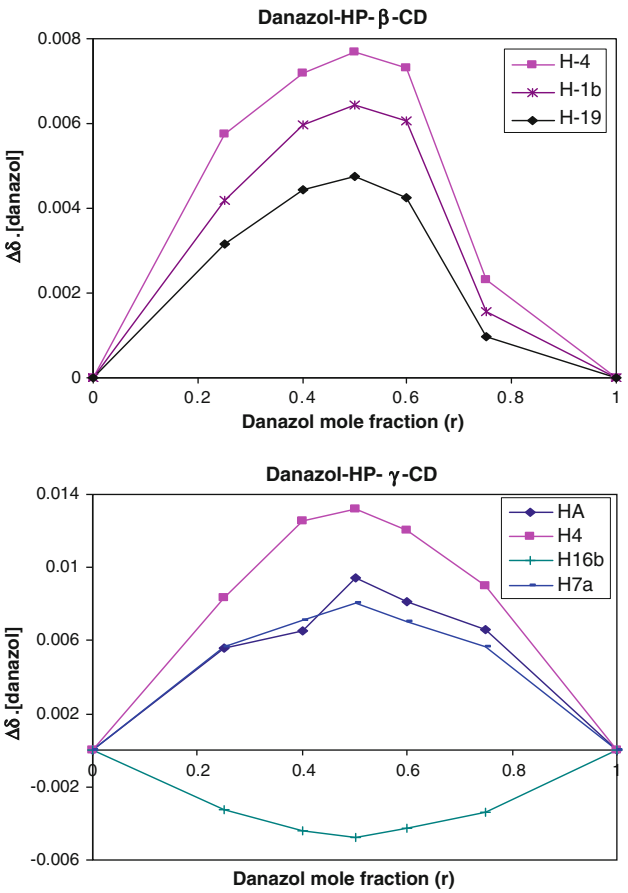


Fig. 3 Job's plots corresponding to the chemical shift displacement of H-A (*filled circle*), H-1b (*asterisk*), H-4 (*filled square*), H-7 (*minus*), H-16b (*plus*) and H-19 (*filled diamond*) protons of danazol for the danazol:HP-β-CD and the danazol:HP-γ-CD systems

the calculated quantity $\Delta\delta \cdot r_{drug}$ will be proportional to the complex concentration and thus can be plotted against the mole fraction r_{drug} [14, 22, 23].

The continuous variation method was applied for all protons of danazol and they all yielded the same results. For the sake of conciseness, only the danazol protons that showed the largest chemical shift change are reported in Fig. 3. In both cases, Job's plots show a maximum at $r = 0.5$, indicating the existence of complexes with a 1:1 stoichiometry. These results are in agreement with the A_L profile found in the phase solubility study.

One dimensional ^{1}H-NMR studies

Chemical shift variations of a specific host or guest nucleus can provide evidence for the formation of inclusion complexes in solution, since significant changes in microenvironment are known to occur between the free and bound states.

Danazol:HP-β-CD It was not possible to obtain definite information on the inclusion complex of danazol and HP-β-CD as changes in chemical shift of the HP-β-CD protons were not clear. Indeed, the CD derivatives consist of a mixture of a number of closely related derivatives with different degrees of substitution and isomeric forms, which produce very broad NMR peaks [24]. We therefore only examined the shifts of drug protons in the presence of HP-β-CD, which give information about the existence of an interaction in solution.

Danazol proton signals were assigned by analysis of one dimensional ^{1}H-NMR spectra and two dimensional ^{1}H–^{1}H chemical shift correlation spectra (COSY) (data not shown). The ^{1}H-NMR spectrum of danazol in the absence and presence of HP-β-CD is shown in Fig. 4.

The induced chemical shift changes of danazol protons in the presence of HP-β-CD are summarized in Table 1. The ^{1}H NMR shifts obtained are in agreement with what is often observed in inclusion complexes with CD, namely downfield shifts for drug protons. In the presence of HP-β-CD, most of the protons of danazol are deshielded, the largest downfield shifts were observed on H-1b, H-4, H-11(a,b), H-15a, H-16b and H19. The changes in chemical shift on H-1b, H-4, H-11(a,b), H19, which are in the A and B rings, indicate that a perturbation occurs at the A and B ring of the steroidal structure. This is not surprising as several of studies on complexes between CDs and steroid molecules also showed that the A and B ring of the molecule was inside the cavity of the CD. The observed downfield shift in guest protons has been attributed to variation of local polarity when these protons are inside the cavity, a deshielding effect due to Van der Waals forces between the drug and carbohydrate chains [14, 22].

Danazol:HP-γ-CD Similar to HP-β-CD, it was also not possible to obtain definite information about the inclusion

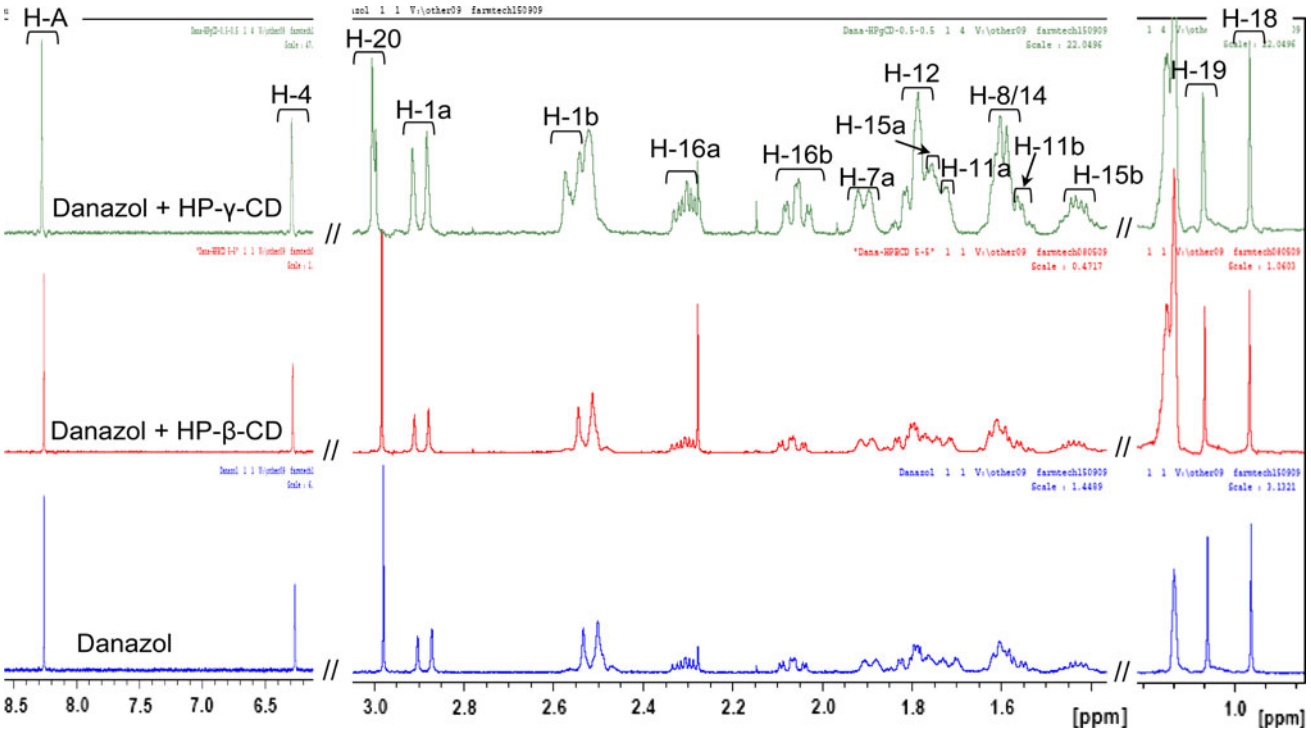


Fig. 4 Expanded part of the ^{1}H-NMR spectra of danazol in the presence, as well in the absence, of CDs

Table 1 The induced changes in the ^{1}H chemical shifts for danazol in the presence of HP-β-CD and HP-γ-CD

Danazol protons	$\Delta\delta^a$ (Hz) (HP-β-CD)	$\Delta\delta^a$ (Hz) (HP-γ-CD)
H-A	−1.8	9.4
H-1a	2.9	5.6
H-1b	6.5	10.3
H-4	7.7	13.2
H-7a	4.3	8.1
H-8/14	−0.8	3.9
H-11a	6.7	11.4
H-11b	4.7	3.9
H-12	4.2	−0.2
H-15a	6.9	11.9
H-15b	2.0	0.2
H-16a	0.7	−1.4
H-16b	5.4	−4.8
H-18	2.9	2.4
H-19	4.8	7.5
H-20	−0.8	8.3

[a] $\Delta\delta = \delta_{(complex)} - \delta_{(free)}$

complex of danazol and HP-γ-CD and only changes in chemical shift of danazol protons were observed in the presence of HP-γ-CD. The induced changes in the ^{1}H chemical shifts for danazol in the presence of HP-γ-CD are summarized in Table 1.

The significant chemical shift changes of danazol protons in the presence of HP- γ-CD are also observed with H-1b, H-4, H-11(a, b), H-15a, H-16b and H19, though the magnitude of these changes is different or even changes from downfield to upfield in the case of H-16b. Additionally, considerable changes in chemical shift are observed with H-A, H-20. The chemical shift changes on H-15, H-16 and H-20 point toward a new binding site at the D ring of the danazol molecule. These results strongly suggest that the complexation between danazol and HP-γ-CD can take place by the entering of the drug molecule into the cavity of CD in either direction.

Two dimensional ROESY ^{1}H-NMR studies

While the 1D ^{1}H-chemical shift provides unambiguous evidence on the formation of a complex, NOESY or ROESY experiments provide information on the dynamics and the average relative inter- and intramolecular proton distances. In this study, to gain further information on the inclusion complexation mode and additional insights into the dynamic structure, 2D ROESY ^{1}H spectra were acquired.

Danazol:HP-β-CD system An expansion of the 2D ROESY ^{1}H spectrum of danazol:HP-β-CD complex is reported in Fig. 5. The inspection of this ROESY map allows us to establish a spatial proximity between the guest protons and the inner protons of HP-β-CD. The bidimensional spectrum shows several intermolecular cross-peaks between H-3′ and H-5′ protons of HP-β-CD and protons of danazol. The most intensive cross-peaks are peaks with

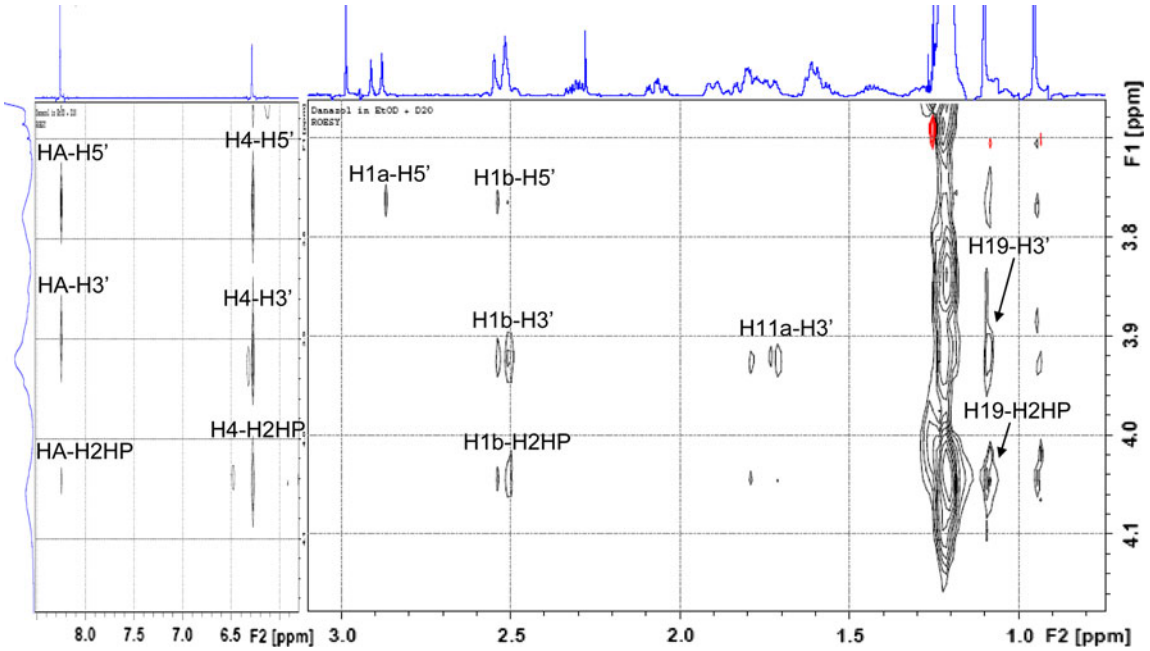


Fig. 5 Expanded region of the 2D ^{1}H-NMR spectrum of danazol:HP-β-CD mixture showing cross-peaks of danazol protons and HP-β-CD cavity protons

H-A and H-4, followed by cross-peaks with H-1 and H-19. This is clear evidence of the inclusion of danazol in the hydrophobic cavity of CD. Based on the intensity of the correlation band in the cross peaks, the distances between protons, which have interactions with each other, were estimated and if the estimated distances were >5 Å, they were eliminated. The results are shown in Table 2. The distance from H-A to H-5′ is shorter compared to that to H-3′, while distances from H-4, H-1b to H-5′ are longer compared to those to H-3′. The observed cross-peaks and estimated distances between protons of danazol molecule and H-3′ and H-5′, which are situated inside the cavity of HP-β-CD, indicate that danazol enters the cavity of HP-β-CD through the wider rim by the isooxazole and A-ring. The structure of the 1:1 danazol–HP-β-CD inclusion complex can therefore schematically be presented as shown in Fig. 7a.

This structure differs from the proposed structure of the danazol–β-CD complex in a study of Jadhav and Vavia [13]. Complex structures of a compound with native cyclodextrin and its derivatives can be either similar [22] or different [25, 26]. The proposed structured of danazol–β-CD complex described by Jadhav and Vavia did not show any parts of danazol residing inside the CD cavity, although the authors concluded that β-CD encapsulated danazol by inclusion. In contrast to our findings, the authors stated that the isooxazole ring did not interact with the hydrophobic cavity of β-CD. Instead, the authors proposed that the methyl groups present at the junctions of A/B and C/D rings of danazol showed favorable interactions with β-cyclodextrin. The isooxazole ring, part of the A ring and the acetylene groups were not included in interactions with the cyclodextrin, but were exposed to the solvent. However, some of the data in their paper seem to contradict this assumption. First, a substantial change in C=N stretch (in the isooxazole) (7.2 cm^{-1}) can be observed in their FTIR experiment. The authors' decision to consider this change as negligible is, in our opinion, questionable. Moreover, in their ^{1}H-NMR data, significant changes in chemical shift can be perceived for H-A in the isooxazole ring and H-1 and H-4 in the A-ring of danazol. In our view, when taken together, these two facts do point at the isooxazole and A-ring entering the CD cavity, similar to the conclusion in this paper.

Table 2 Estimated distances between the protons of danazol and HP-β-CD and HP-γ-CD, which have interactions with each other

Danazol protons	CD proton	Distances (Å) (HP-β-CD)	Distances (Å) (HP-γ-CD)
H-A	H3′	4.25	4.31
H-A	H5′	3.83	NA
H-A	H2HP	4.70	4.28
H-1a	H3′	NA	3.80
H-1a	H5′	4.25	NA
H-1a	H2HP	NA	3.93
H-1b	H3′	4.00	3.59
H-1b	H5′	4.28	3.93
H-1b	H2HP	4.12	3.72
H-4	H3′	3.77	4.17
H-4	H5′	3.61	4.42
H-4	H2HP	4.02	4.15
H-8/14	H3′	NA	4.19
H-8/14	H5′	NA	4.19
H-11a	H3′	4.68	NA
H-15a	H3′	NA	3.80
H-15a	H5′	NA	3.63
H-15a	H2HP	NA	4.34
H-18	H3′	NA	3.92
H-18	H5′	NA	3.85
H-18	H2HP	NA	4.47
H-19	H3′	4.92	4.53
H-19	H5′	NA	4.74
H-19	H2HP	4.61	4.89
H-20	H3′	NA	3.49
H-20	H5′	NA	3.75
H-20	H2HP	NA	4.19

Danazol:HP-γ-CD An expansion of the 2D ROESY ^{1}H spectrum of the danazol:HP-γ-CD complex is reported in Fig. 6 and the estimated distances (<5 Å) between the danazol and HP-γ-CD protons, which have interactions with each other, are presented in Table 2. Like HP-β-CD, H-A, H-4, H-1 and H-19 also have cross-peaks with the inside cavity protons H-3′ and H-5′, though the estimated distances are different. For H-A, there is no cross-peak with H-5′, while the distance to the H-2HP at the substitution hydroxypropyl group is shorter. For H-4, the distances to H-3′ and H-5′ are longer. This result is consistent with the other results for HP-γ-CD, where a lower magnitude in stability constant was found in the phase solubility analysis, indicating a looser complex when the cavity of CD is wider. By contrast, it is not clear why the distances between H-1b and H-3′, H-5′ are shorter compared to those in HP-β-CD.

Besides the similarity in cross-peaks with H3′ and H5′ as mentioned above, with HP-γ-CD, danazol has more protons interacting with H3′ and H5′. The most intensive new cross-peaks are peaks between H-3′ and H-5′ with H-20, H18, H-15. This indicates an additional binding site for HP-γ-CD, in which the D and C rings of danazol are also inside the cavity of HP-γ-CD. Because the binding stoichiometry between danazol and HP-γ-CD was determined as 1:1 and the size of danazol molecule and HP-γ-CD does not allow the whole danazol molecule to fit into

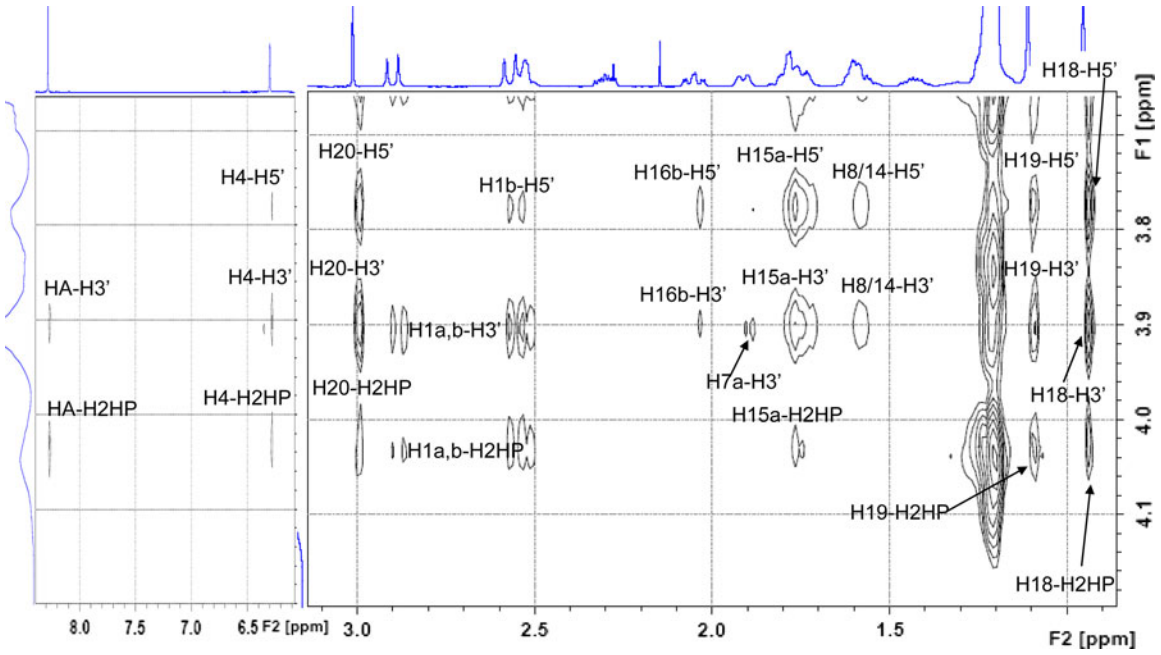


Fig. 6 Expanded region of the 2D ^{1}H-NMR spectrum of danazol:HP-γ-CD mixture showing cross-peaks of danazol protons and HP-γ-CD cavity protons

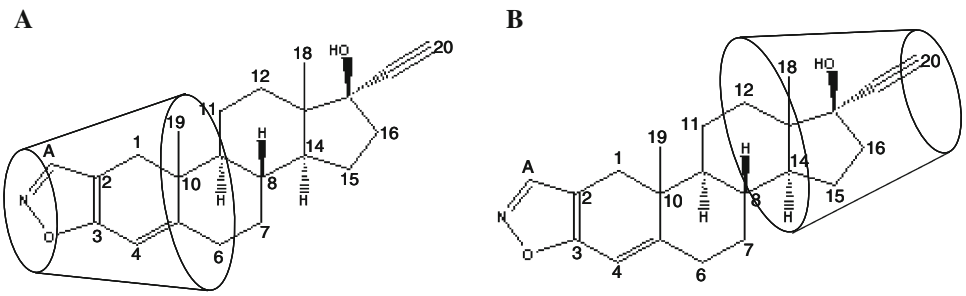


Fig. 7 Possible structures of danazol–HP-β-CD (**a**) and danazol-HP-γ-CD (**a**, **b**) inclusion complexes

the cavity of HP-γ-CD, two different structures of the inclusion complex danazol:HP-γ-CD have to be present in solution. The distances between danazol protons and the H-3′ and H-2HP except for H-15, H-16 and H-18 are shorter compared to those of the H-5′ proton, indicating that the guest molecule is introduced within HP-γ-CD through the wider rim of the cavity. The structure of the two 1:1 danazol:HP-γ-CD inclusion complexes can therefore be presented as shown in Fig. 7 a, b.

Determination of the aggregation in the CD solutions

It is stated in a recent review of Messner et al. [27] that dissolved CDs self-assemble to form nanosized aggregates, and that the aggregation gradually increases with increasing CD concentration. In this study, the concentration of CDs used during NMR study (17 mM) was relatively low compared to the maximum CDs concentrations in phase solubility analysis (72 and 64 mM for HP-β-CD and HP-γ-CD, respectively). Thus, a validation is needed in order to use the NMR results to explain phenomena occurring in higher concentration CD solutions.

For HP-β-CD, two solutions, containing 10% (72 mM) CD and with or without danazol (prepared as described in the phase solubility analysis), were measured by DLS. The normalized intensity correlation functions of these two solutions are shown in Fig. 8. In both solutions, one fast correlation was observed, indicating only one size distribution of particles. The hydrodynamic radii of these particles are 0.9 and 1.0 nm for 10% solution of HP-β-CD and a 10% solution of HP-β-CD 10% saturated with danazol, respectively. This size is about the size of a single CD molecule, indicating the absence of aggregates in these solutions. This result together with the linear increase of danazol solubility in the presence of HP-β-CD (A_L profile) and the proved 1:1 stoichiometry of the inclusion complex

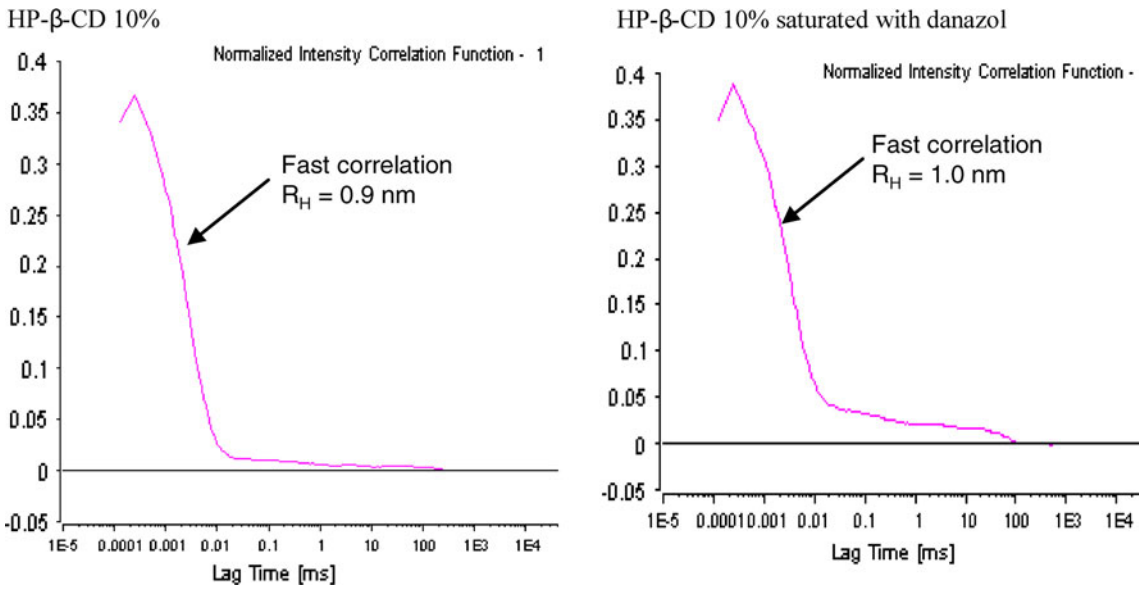


Fig. 8 Normalized intensity correlation function of solutions of 10% HP-β-CD and 10% HP-β-CD 10% saturated with danazol

at lower concentration by NMR experiments leads to the conclusion that 1:1 inclusion complex between danazol:HP-β-CD is responsible for the increased solubility of danazol.

For HP-γ-CD, the same experiments were carried out, but with two different CD concentrations, namely 5% (32 mM) and 10% (64 mM). The normalized intensity correlation functions are shown in Fig. 9. In these solutions, two correlations, one fast and one slower, were observed, indicating two size distributions of particles. The hydrodynamic radii of these particles are 0.8 nm (the size of a single CD molecule) and about 70 nm. This indicates aggregates existing in both 5 and 10% HP-γ-CD solutions. Interestingly, in the solution of 5% HP-γ-CD saturated with danazol, only one fast correlation was observed and the hydrodynamic radius is 0.9 nm. This means that the inclusion complex between HP-γ-CD and danazol does not form aggregates and that the concentration of free HP-γ-CD that remains in solution is not sufficient for self-aggregation. In the 10% HP-γ-CD solution saturated with danazol, a similar correlation profile was observed as in the pure HP-γ-CD solution. However the slower second correlation, representing the aggregates, is smaller in the 10% HP-γ-CD solution containing danazol than the CD solution devoid of danazol, indicating that aggregation is somewhat less in the presence of danazol. The observed aggregates might be formed by either only the free HP-γ-CD in the solution or only the inclusion complex danazol:HP-γ-CD or both. Unfortunately, with current techniques, it is not possible to clarify the constitution of these aggregates. Hence, the conclusion that can be drawn from these results is that for HP-γ-CD concentration up to 5%, the formation of an 1:1 inclusion complex between danazol and HP-γ-CD is responsible for the increased solubility of danazol and no aggregates are present. More advanced technologies that are able to discriminate between differently constituted aggregates, are required to elucidate whether this also the case for higher HP-γ-CD concentration.

Conclusions

The solubility of danazol in water increased in the presence of HP-β-CD or HP-γ-CD. Danazol forms a soluble 1:1 complex with HP-β-CD and HP-γ-CD (A_L type phase solubility diagram). The apparent stability constants were determined and $K_{1:1}$ of HP-β-CD was found to be greater than that of HP-γ-CD, likely due to the fact that the larger cavity of γ-CD forms a looser complex with danazol compared to β-CD.

The ^{1}H-NMR analysis of danazol in the presence of two CDs confirmed the formation of inclusion complexes. Analysing 2D ROESY spectra, we could propose that the inclusion complex between danazol and HP-β-CD is formed by entrance of the isooxazole and A rings of danazol into the CD cavity from its wider rim. For the danazol–HP-γ-CD complex, there are two different complex structures simultaneously in solution: (1) the same as in the case of HP-β-CD, the isooxazole and A ring in the cavity and (2) the C and D ring enter inside the cavity of HP-γ-CD also from the wider rim. DLS indicated the absence of self-aggregation in solutions of the CD's and danazol up to 10% CD in the case of HP-β-CD and up to 5% in the case of HP-γ-CD.

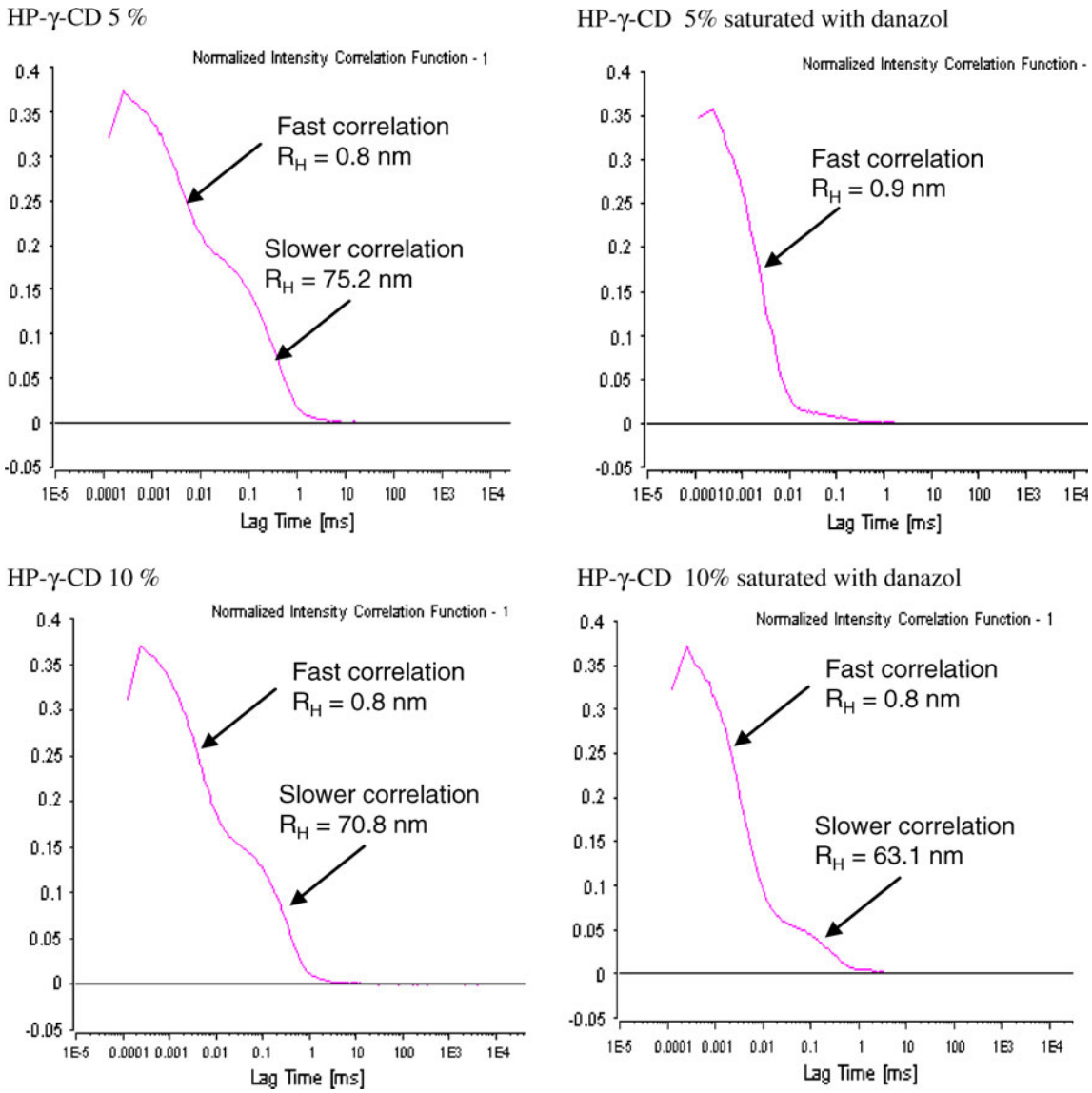


Fig. 9 Normalized intensity correlation function of solutions of 5 and 10% of HP-γ-CD in the absence or presence of danazol

Acknowledgments A K.U. Leuven Industrial Research Fund (IOF) provided financial support for this study. MVS acknowledges the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT-Vlaanderen) for a PhD grant.

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