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Physicochemical, in silico and in vivo evaluation of a danazol–β-cyclodextrin complex

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Abstract

Inclusion complexation of danazol with β-cyclodextrin (BCD) in aqueous solution, in solid state and in silico state was investigated to examine the interactions of danazol with BCD. The study also explored the potential application of danazol–β-cyclodextrin complex as an oral antiovulatory agent. Phase solubility analysis suggested formation of first-order soluble complex with stability constant 972.03 M^{-1} while Job's plot affirmed 1:1 stoichiometry. Solution state complexation in water was studied by ultra violet absorption, circular dichroism and nuclear magnetic resonance (^{1}H NMR) spectroscopy. The solid state complexes were evaluated by differential scanning calorimetry, powder X-ray diffractometry, fourier transform infrared spectroscopy and scanning electron microscopy. Thermodynamic studies in water indicated exothermic nature of inclusion complexation. Molecular modeling was used to help establish the mode of interaction of BCD with danazol. ^{1}H NMR analysis suggested that the protons of steroidal skeleton of danazol are preferably involved in the complexation with BCD, which was confirmed by molecular dynamic simulations. An inclusion complex model has been established for explaining the observed enhancement of solubility of danazol in water by BCD. Moreover, in mouse model, danazol–β-cyclodextrin complex at 51.2 mg/kg (equivalent to 400 mg human dose) showed 100% anovulation when given orally.

Keywords: Danazol; β-Cyclodextrin; Solubility; Molecular modeling; Antiovulatory activity

1. Introduction

Cyclodextrins (CDs), cyclic oligosaccharides derived from starch, have been used extensively to increase the aqueous solubility, stability, and bioavailability of drugs (Loftsson and Brewster, 1996). An important prerequisite for the preparation and use of inclusion complex is understanding the interactions between guest and CD during complexation. Structural information, such as the stoichiometry and geometry of the complex, and thermodynamic information, such as the stability constant and the changes in enthalpy and entropy are necessary to clarify the complexation mechanism and driving forces governing the interaction. The balance among several forces such as hydrophobic interactions, van der Waal interactions, electrostatic interactions, hydrogen bond formation, and the changes in environment of water molecules in both the host and hydrated guest molecules contribute heavily to the overall stability of the complex (Inoue et al., 1993; Rekharsky et al., 1994; Connors, 1996; Junquera and Aicart, 1999).

Endometriosis is one of the most common gynecological problems in adult women (Kistner, 1978). Danazol (DAN) (Fig. 1) is used in number of clinical situations, including the therapy of endometriosis (Dmowski and Cohen, 1978), cystic mastitis (Laucrsen and Wilson, 1976), precocious puberty (Lee et al., 1975) and menorrhagia (Chimbira et al., 1979). However, DAN is very poorly soluble in water and exhibits dissolution rate-limited absorption (Chen et al., 2004). Danazol inhibits the pituitary response to luteinizing hormone-releasing hormone, resulting in suppression of follicular stimulating hormone (FSH) and luteninzing hormone (LH) release, but does not suppress the pituitary FSH and LH content (Dmowski, 1979). A clinical study had explored the therapeutic efficacy of DAN (7 day administration) in producing an inadequate luteal phase (Wentz et al., 1976). Short term administration of DAN was proposed because of androgenic effects and striking suppression of estrogen output when administered on a chronic basis.

β-Cyclodextrin (BCD), although it has limited aqueous solubility, was chosen to enhance the solubility of DAN due to its

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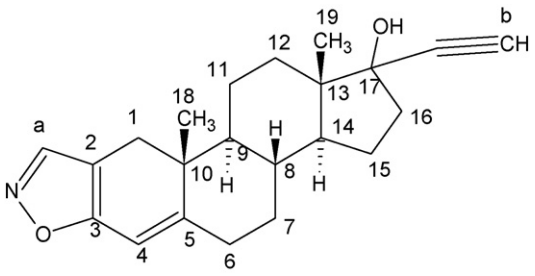

Fig. 1. Chemical structure of danazol.

central cavity diameter (6–6.5 Å, appropriate to accommodate most aromatic rings), efficiency of drug complexation, availability in pure form and relatively low cost. Absorption of BCD in an intact form is limited because of its bulky nature, and due to this, it acts as a true carrier by keeping the hydrophobic drugs in solution and delivering them to the surface of the biological membrane such as gastrointestinal mucosa, where they partition into the membrane (Loftsson, 1999).

The purpose of present study was to investigate the inclusion complex formation of DAN with BCD in order to gain insight into the mode of interaction in solution as well as in solid state. Molecular modeling has been used as a complementary tool for characterizing the interactions between DAN and BCD. Moreover, a potential application of danazol–β-cyclodextrin (DAN–BCD) as an oral antiovulatory agent at physiologically acceptable dose has been explored in mouse model.

2. Materials and methods

2.1. Materials

Danazol was obtained as a gift sample from Ar-Ex Lab. Ltd., Mumbai, India. BCD was generously donated by SA Chemicals, Mumbai, India. Sodium carboxymethylcellulose (Akucell AF 0305) was gifted by Signet Chemicals, Mumbai, India. All other materials and solvents were of analytical reagent grade. The analysis of plasma samples for luteninzing hormone (LH) content were carried out by radio immuno assay (RIA) using LH-IRMA kit from Diagnostic Systems Laboratories, USA.

2.2. Stoichiometry and stability constant of complex

2.2.1. Continuous variation method (Job's plot) (Job, 1928)

Equimolar (0.05 mM) solutions of DAN and BCD were added in varying quantities (mL) so as to get different *r*-values. This was accomplished by keeping the final total volume constant in all solutions (10 mL). The samples were analyzed using UV spectrophotometer (Shimadzu 160 A UV–vis spectrophotometer, Shimadzu Corp., Kyoto, Japan) at 287 nm.

2.2.2. Solubility method (phase solubility analysis)

Solubility studies were carried out according to the method reported by Higuchi and Connors (1965). Excess amounts of DAN were added to aqueous solutions (pH 6.2 ± 0.1) containing increasing concentrations of BCD (2.0×10^{-3} to 14.0×10^{-2} M) and the samples were shaken on a rotary shaker at 25 ± 0.3 °C, until solubility equilibria were reached. After equilibration, aliquots of the supernatant were filtered through a membrane filter (0.22 μm, Sartorious cellulose nitrate filter, Germany). The filtrates were then suitably diluted with water and analyzed using UV spectrophotometer at 287 nm.

2.2.3. Spectroscopic method (Scott equation) (Scott, 1956)

UV absorption changes of DAN (1.0×10^{-5} M) in the presence of BCD (varied from 1.5×10^{-3} to 1.25×10^{-2} M) were measured at 287 nm. The apparent stability constant, K_C was calculated according to conventional Scott's equation.

2.2.4. Spectral shift method (Benesi–Hildebrand equation) (Benesi and Hildebrand, 1949)

One milliliter of DAN solution containing 1.0×10^{-5} M was added to aqueous solutions of BCD of graded concentrations (3.0×10^{-3} to 1.25×10^{-2} M). The final volume of the system was kept constant to 10 mL with distilled water. The solutions were agitated at 200 rpm for 8 h on a rotary shaker at 25 ± 0.3 °C. The mixtures thus equilibrated were scanned for their absorbance at 287 nm and the stability constant, K_C was computed by Benesi–Hildebrand equation.

2.3. Solution state characterization

2.3.1. UV–vis spectroscopy

The UV spectra of DAN (9.74×10^{-6} M) and DAN (9.74×10^{-6} M) in presence of BCD (1.40×10^{-2} M) were recorded in the region of 200–400 nm at 1 nm slit width.

2.3.2. Circular dichroism spectroscopy

The circular dichroism spectra were obtained using a Jasco J-600 Spectropolarimeter. Absorbance of the sample was kept below 2 in the whole wavelength range explored (200–350 nm). The signal to noise ratio was improved by superimposition of five different scans.

2.3.3. Nuclear magnetic resonance (NMR) spectroscopy

^{1}H NMR spectroscopic analysis was performed on Bruker AMX-500 Fourier transform nuclear magnetic resonance (FT-NMR) spectrophotometer at 298 K. The 5 mM samples of DAN, BCD, and mixture of DAN–BCD (5:5 mM) were recorded in CD_3OD:D_2O (50:50, v/v) solvent system. The proton signal of CH_3 (of CD_3OD) was used for referencing.

2.4. Preparation of solid complexes

DAN–BCD complexes of 1:1 M ratios were prepared by physical mixture (PM), kneading method (KM), solution method

(SM), freeze-drying method (FD) and less expensive, industrially feasible ball milling method (BM) (Jadhav et al., 2007).

2.5. Solid state characterization

2.5.1. Differential scanning calorimetry (DSC)

Thermograms of pure materials (DAN, BCD), PM and FD complex were recorded on a Perkin-Elmer DSC 7 model. About 10 mg samples were sealed in aluminium pans and heated at a rate of 10 °C/min from 30 to 310 °C.

2.5.2. Powder X-ray diffractometry (P-XRD)

The powder X-ray diffraction patterns of pure materials (DAN, BCD), PM and FD complex were recorded on a Philips X-ray diffractometer (PW 1710, Philips Analytical, Almelo, The Netherlands) with a copper target, voltage 40 kV, current 30 mA, and a scanning rate of 1°/min.

2.5.3. Fourier transform infrared (FTIR) spectroscopy

FTIR spectra of pure materials (DAN, BCD), PM and FD complex were obtained in the range of 400–4600 cm^{-1} using a Jasco-FTIR spectrophotometer (Jasco, Essex, UK) by KBr disc method.

2.5.4. Scanning electron microscopy (SEM)

The Environmental SEM (E-SEM) analysis of DAN, BCD, PM and FD complex were recorded on Jeol JSM-840 scanning electron microscope.

2.6. Mechanism of complexation

2.6.1. Thermodynamics of complexation

Phase solubility studies (as described in Section 2.2.2) were carried out at 25, 40, 50 and 60 ± 0.3 °C, until solubility equilibria were reached. After equilibration, aliquots of the supernatant were filtered through a membrane filter. The filtrates were suitably diluted with water and analyzed using UV spectrophotometer at 287 nm.

2.6.2. Molecular modeling studies

The use of molecular mechanics (Madrid et al., 1998) and dynamic simulations (Melani et al., 1998; Jadhav et al., 2006) has previously been reported in the study of CD complexes. Docking programs have also been used to study inclusion complexes for qualitative purposes (Otero-Espinar et al., 1992). Molecular mechanics and dynamics calculations were carried out on a Pentium 2.8 GHz PC with Linux OS (Red Hat Enterprise WS 3.0) using the Discover (Accelrys, Inc., USA) molecular modeling software. The initial coordinates for BCD were obtained from the Protein Data Bank with the accession code 1BFN (Adachi et al., 1998). The partial charges and potentials were calculated using the consistent valence force field (CVFF). A single drug molecule was docked manually into the cavity formed by secondary hydroxyls (2′-OH) on one side of BCD. The resulting complex was minimized using steepest descents followed by conjugate gradients to a gradient convergence of 0.001 kcal/(mol Å). The complex was then subjected to a 5 ps molecular dynamics (MD) simulation at 298 K with a step size of 1 fs. The trajectory was analyzed to locate the minimum energy complex and it was further minimized as above said protocol. The resulting complex was analyzed for intermolecular interactions, interaction energies and mode of drug binding. Jurs descriptors, namely total polar surface area (TPSA) and total hydrophobic surface area (THSA) were calculated for DAN, BCD and DAN–BCD complex.

2.6.3. Antiovulatory activity (Nandedkar et al., 2001)

Comparative antiovulatory studies of DAN and BM complex of danazol–β-cyclodextrin (DAN–BCD_{BM}) were carried out to evaluate the effect of BCD complexation on in vivo efficacy of DAN.

All experiments for animal testing were approved by Institutional Ethical Committee for Animal Use and Care and all animal experimentations were performed in compliance with the Guidelines for the Care and Use of Laboratory Animals in National Institute of Research in Reproductive Health (NIRRH), Parel, Mumbai.

The aqueous solution of DAN and DAN–BCD_{BM} was prepared in 0.2% (w/v) sodium carboxymethylcellulose (as a vehicle). The mouse dose was calculated on the basis of its body surface area (Paget and Barnes, 1964). The values of dose of DAN–BCD_{BM} are not the value of weight of powder of DAN–BCD_{BM} but they represent the active content (DAN) present in the binary system. Swiss albino adult female mice (20–25 g) were used in this study. They were raised in the animal house facility of NIRRH. The mice were numbered, randomly selected and kept in polycarbonate cages with a bedding of husk, and 12-h light/dark cycles were maintained throughout the study period. Feed and water were given *ad libitum*. The estrous cycles of mice were monitored daily for 2 weeks by microscopic examination of vaginal smears. The animals in metestrus were selected and grouped into three groups keeping eight animals in each group (maximum three animals in each cage). Amongst three groups, one group served as a control and the remaining two groups were treatment groups. On the day of metestrus, test substances diluted with vehicle and vehicle were administered orally to mice as a single dose, using ball-tipped intubation needle fitted onto a syringe. The treatment groups were subdivided into eight groups for various dose levels. The mice in treatment group received 6.4 mg/kg (equivalent to 50 mg of human dose), 12.8 mg/kg (equivalent to 100 mg of human dose), 25.6 mg/kg (equivalent to 200 mg of human dose) and 51.2 mg/kg (equivalent to 400 mg of human dose) of DAN and respective equivalent amount of DAN–BCD_{BM}. On the estrus day, the animals were sacrificed under ether anesthesia. Blood was collected from the heart of all sacrificed mice and plasma was separated by centrifugation at 5000 rpm for 5 min. The plasma samples were stored at −20 °C in deep freezer until the analysis of hormonal content was carried out. The ampulla of each oviduct was observed under microscope (100×, Zeiss, Axiolab) and numbers of oocytes were counted using image analyzer (Vicon Image Analyzer, VC2820A) fitted to microscope. Percent inhibition of oocytes was calculated by the following

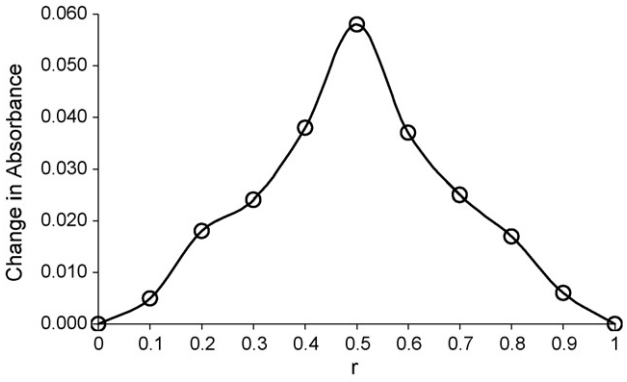

Fig. 2. Continuous variation plot of danazol–β-cyclodextrin system in water at 25 °C.

formula:

$$\%\text{inhibition of oocytes} = \frac{\text{oocytes of control group} - \text{oocytes of treatment group}}{\text{oocytes of control group}} \times 100 \quad (1)$$

The plasma level of LH was calculated using % of bound/bound maximum method. Percentage inhibition of LH content was calculated by the following formula:

$$\%\text{inhibition of LH content} = \frac{\text{LH of control group} - \text{LH of treatment group}}{\text{LH of control group}} \times 100 \quad (2)$$

The mean values of number of oocytes and LH content of treated groups were compared with those of control group using one-way analysis of variance (ANOVA) followed by Dunnett's test and Bonferroni's multiple comparison test.

3. Results

3.1. Stoichiometry and stability constant of complex

3.1.1. Continuous variation method (Job's plot)

The r ratio was plotted against the difference of the absorbance of drug in presence of BCD and in absence of BCD. The results of Job's Plot are shown in Fig. 2.

3.1.2. Solubility method (phase solubility analysis)

The phase solubility profile for the complex formation between DAN and BCD at 25 °C is shown in Fig. 3. There is linear increase in the solubility of DAN with increasing concentrations of BCD. The apparent 1:1 stability constant, K_C was calculated from the straight line of the phase solubility diagram by using the following equation:

$$K_C = \frac{\text{slope}}{\text{intercept} \times (1 - \text{slope})} \quad (3)$$

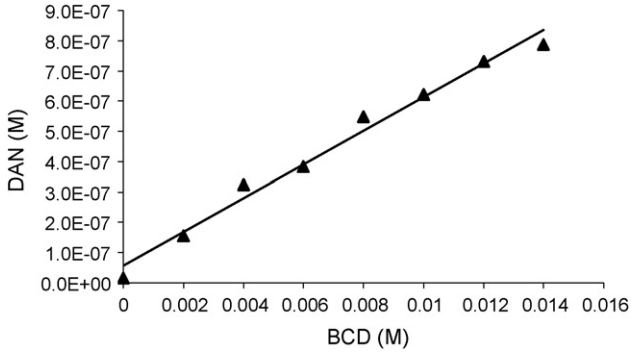

Fig. 3. Phase solubility diagram of danazol–β-cyclodextrin system in water at 25 °C.

The K_C value is a useful index to estimate the degree of binding strength of the complex. The K_C value for DAN–BCD was found to be 972.03 M^{-1}. The complexation efficiency of BCD for 1:1 DAN–BCD complex was found to be 1.50×10^{-5}.

3.1.3. Spectroscopic method (Scott equation)

The typical Scott plot for DAN–BCD system is shown in Fig. 4. The K_C computed by spectroscopic method was found to be 118.75 M^{-1}.

3.1.4. Spectral shift method (Benesi–Hildebrand equation)

It was possible to determine K_C from double reciprocal plot since the molar absorptivities of the complex and drug differed at the same wavelength (Connors and Mollica, 1966). The Benesi–Hildebrand plot (Fig. 5) is linear indicating the presence of 1:1 M complex. The apparent K_C was found to be 200.59 M^{-1}.

3.2. Solution state characterization

3.2.1. UV–vis spectroscopy

UV spectra of DAN and DAN in presence of BCD are shown in Fig. 6. DAN-1 represents the spectrum of DAN with dilution and DAN-2 represents the spectrum of DAN without the dilution. The comparison of DAN–BCD should be made with DAN-1 since the spectrums of both solutions contain same con-

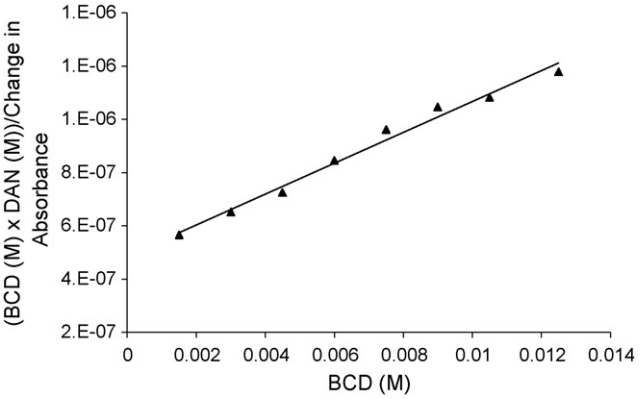

Fig. 4. Scott plot for danazol–β-cyclodextrin system in water at 25 °C.

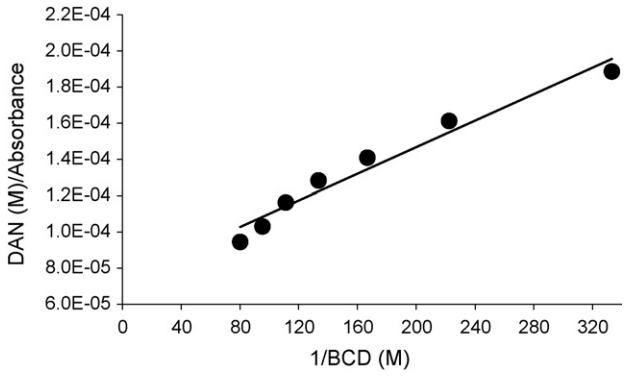

Fig. 5. Benesi–Hildebrand plot for danazol–β-cyclodextrin system in water at 25 °C.

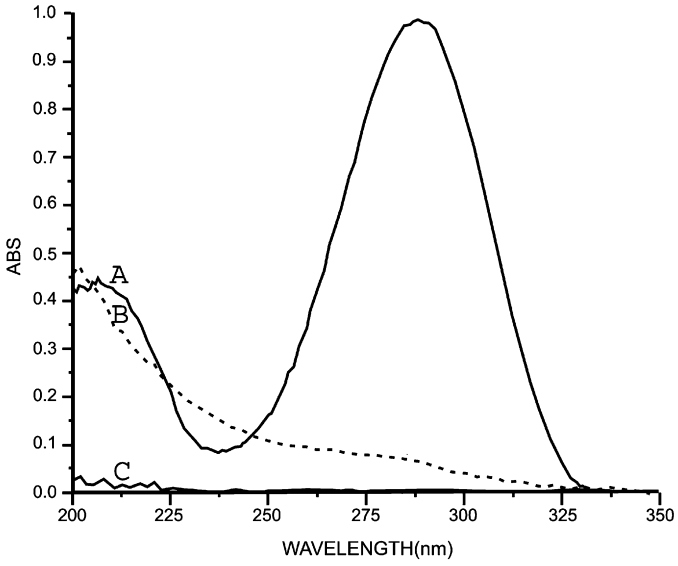

Fig. 6. UV spectrum of DAN (9.74×10^{-6} M) and DAN (9.74×10^{-6} M) in presence of BCD (1.40×10^{-2} M). (A) DAN–BCD, (B) DAN-2, (C) DAN-1.

centration of DAN (9.74×10^{-6} M). There is 49,170% increase in intensity of DAN in presence of BCD.

3.2.2. Circular dichroism spectroscopy

The circular dichroism spectra of DAN and DAN–BCD system in aqueous solution are shown in Fig. 7. In presence of BCD the optical activity was induced at 215.90 nm and 280.70 nm with positive and negative peak, respectively.

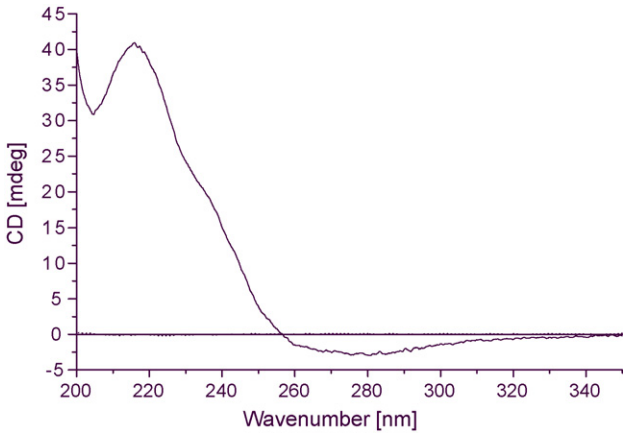

Fig. 7. Circular dichroism spectrum of DAN (3.36×10^{-4} M) in presence of BCD (1.40×10^{-2} M).

3.2.3. Nuclear magnetic resonance (NMR) spectroscopy

In this study, owing to poor aqueous solubility of DAN, water could not be used. ^{1}H NMR signals of DAN (Balogh et al., 1995) and BCD (Schneider et al., 1998) were assigned according to NMR information given in the references. The chemical shift values of BCD and DAN are summarized in Table 1 as complexed state (δ_c), free BCD state (δ_0) and chemical shift change $\Delta\delta$ (ppm) ($\Delta\delta = (\delta_c - \delta_0)$).

3.3. Solid state characterization

3.3.1. Differential scanning calorimetry (DSC)

The DSC thermograms of pure DAN, BCD, PM and FD complex are shown in Fig. 8. A DSC thermogram of DAN exhibited a sharp endothermic peak at 230.84 °C, corresponding to its melting point. The DSC thermogram of BCD showed a broad endotherm in the range of 65–115 °C, which can be attributed to desolvation of water molecules present in BCD cavity. The endothermic peak of DAN was retained at 229.0 °C along with a broad endotherm in the range of 65–110 °C in PM. This may be attributed to presence of less or no interaction between the pure components in the PM. The endothermic peak of DAN was absent in FD complex. The thermal behavior data of the systems studied are collected in Table 2. In PM there is only 17.48% reduction in the height of DAN peak indicating absence of any interactions. FD complex did not show presence of DAN

Table 1
Chemical shift changes of BCD and DAN protons in CD_3OD:D_2O (5:5, v/v)

Proton of BCD	δ_C[a]	δ_0[b]	$\Delta\delta$[c]	Proton of DAN	δ_C[a]	δ_0[b]	$\Delta\delta$[c]
H1′	5.044	5.032	+0.012	a-H	8.189	8.061	+0.128
H2′	3.610	3.603	+0.007	1-H	2.965	2.816	+0.149
H3′	3.917	3.897	+0.020	4-H	6.305	6.147	+0.158
H4′	3.557	3.554	+0.003	18-H	0.897	0.823	+0.074
H5′	3.770	3.750	+0.020	19-H	1.060	0.985	+0.075
H6′	3.879	3.869	+0.010	–	–	–	–

[a] Complexed state.
[b] Free state.
[c] $(\delta_c - \delta_0)$.

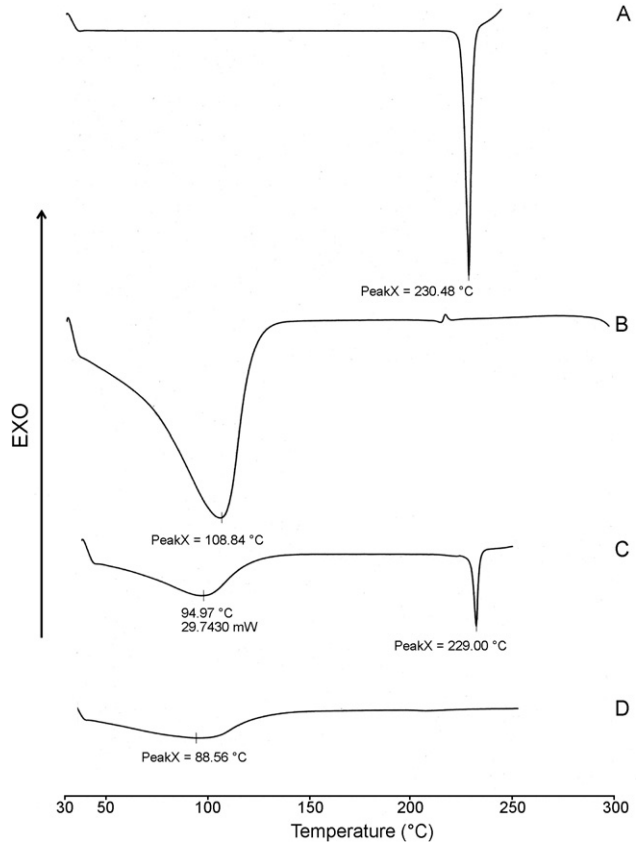

Fig. 8. DSC thermograms of danazol, β-cyclodextrin, physical mixture and freeze-dried complex. (A) DAN, (B) BCD, (C) PM, (D) FD.

peak (100% difference) indicating strong interactions of DAN and BCD.

3.3.2. Powder X-ray diffractometry (P-XRD)

Crystallinity was determined by comparing some representative peak heights in the diffraction patterns of FD with those of a reference (DAN). The relationship used for the calculation of crystallinity was relative degree of crystallanity (RDC) = I_{sam}/I_{ref}, where I_{sam} is the peak height of the sample under investigation and I_{ref} is the peak height at the same angle for the reference (Ryan, 1986).

The P-XRD patterns of pure DAN, BCD, PM and FD are depicted in Fig. 9. The diffractogram of BCD exhibited characteristic peaks at 9.51°, 10.92°, 12.84°, 13.44°, 14.48°, 16.66°, 18.18°, 18.72°, 19.06°, 22.08°, 24.06° and 24.66° due to its crystalline nature. DAN exhibited a series of intense peaks at 15.78°, 16.70°, 17.08°, 18.94°, 20.22°, 20.74°, 23.12°, 25.13° and 29.00°, which were indicative of crystalline nature of DAN. Most of the principal peaks of DAN and BCD were present in the diffraction patterns of PM. This indicated that there was no interaction between the pure components of PM. In contrast to these observations, FD showed disappearance of characteristic peaks of DAN at 20.22°, 21.44° and 41.14° while the RDC values were 0.1528 (at 15.78°), 0.2565 (at 17.08°), 0.1653 (at 18.94°), 0.1751 (at 20.74°) and 0.1781 (at 25.13°). Moreover, the diffraction pattern of the peaks of FD was more diffused as compared to pure components (DAN and BCD). These observations were indicative of the transformation of DAN from crystalline to amorphous state, which might be because of inclusion of DAN into BCD cavity.

Table 3
Changes of IR absorption frequencies of DAN and BCD in FD complex

Group assignment	Wave number (cm^{-1})		Δ (cm^{-1})[a]
	DAN	DAN in FD	
C≡CH stretch	2099.0	2099.3	+0.3
C=N stretch	1631.7	1638.9	+7.2
C=C stretch	1600.2	1601.6	+1.4

Group assignment	Wave number (cm^{-1})		
	BCD	BCD in FD	Δ (cm^{-1})[a]
O–H (primary sugar stretch)	1027.8	1028.3	+0.5

[a] Difference in wave number.

3.3.3. Fourier transform infrared (FTIR) spectroscopy

Supporting evidence for complexation of a guest molecule with the BCD can be obtained by IR spectroscopy. FTIR spectrum of DAN showed characteristic absorption band at 2099.0 cm^{-1} which may be attributed to C≡CH stretch of acetylene group. It also showed absorption bands at 1631.7 which may be assigned to C=N stretch of isooxazole ring. FTIR spectrum of BCD showed a broad absorption bands at 3376.9 cm^{-1} (O–H stretching), 2924.5 cm^{-1} (C–H aliphatic stretch) and 1027.8 cm^{-1} (O–H primary sugar stretch). The absorption band of O–H stretch of DAN got merged in O–H stretching of BCD (3376.9 cm^{-1}, broad), in samples of PM and FD complex. The spectral pattern of PM corresponds simply to the superimposition of the IR spectra of the individual components. The changes of IR absorption frequencies of DAN and BCD in FD complex are collected in Table 3.

3.3.4. Scanning electron microscopy (SEM)

The scanning electron micrographs of DAN, BCD, PM and FD are shown in Fig. 10. DAN appeared as irregular and three-dimensional particles while BCD also appeared as three-dimensional particles with parallelogram shape. The PM clearly depicted the crystalline nature of both DAN and BCD. In FD

Table 2
Thermal data of DAN, PM and FD complex

Sample	Quantity of DAN in sample (mg)	Observed peak height, Δ (J/g)	Theoretical peak height, Δ (J/g)	% Difference
DAN	3.00	46.36	–	–
PM	0.80	10.20	12.36	17.48
FD	1.83	–	28.28	100

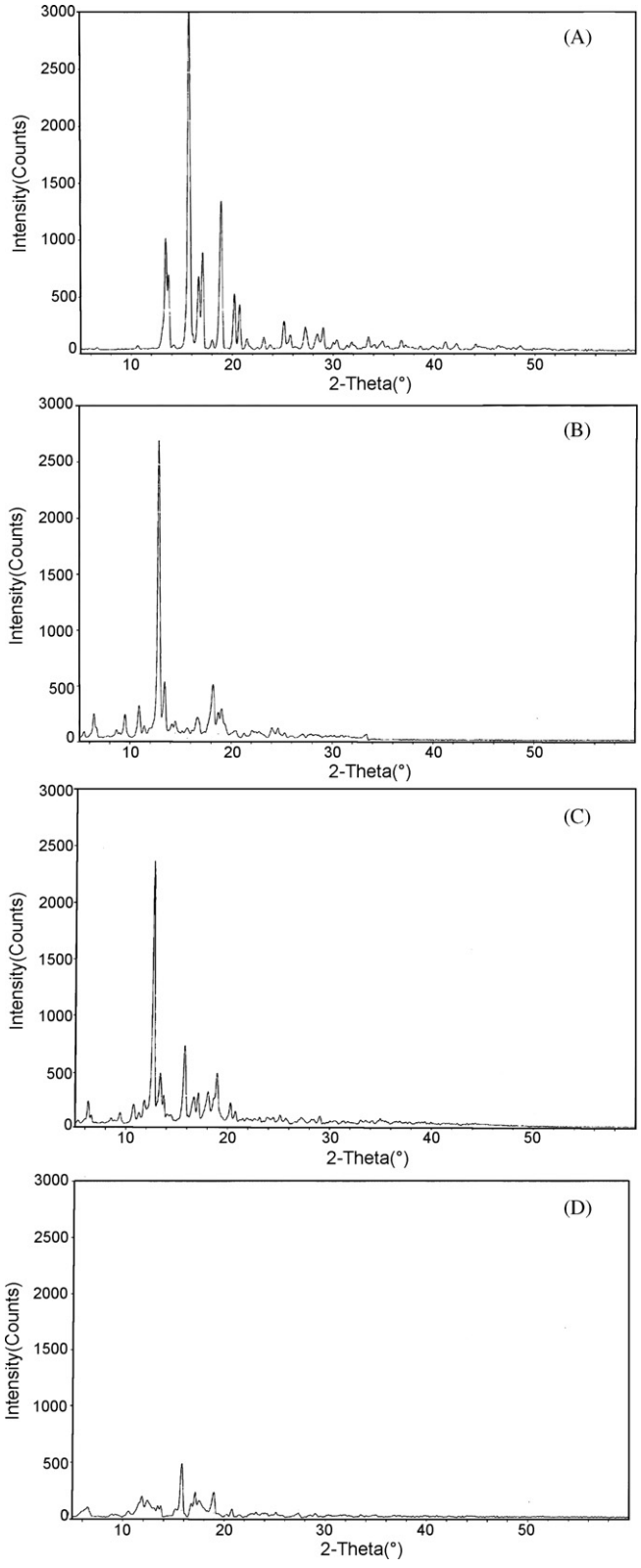

Fig. 9. P-XRD diffractograms of danazol, β-cyclodextrin, physical mixture and freeze-dried complex. (A) DAN, (B) BCD, (C) PM, (D) FD.

Table 4
Thermodynamic parameters for the complexation of DAN with BCD

Temperature (°C)	K_C	ΔG° (kcal/mol)	ΔH° (kcal/mol)	ΔS° (cal/° mol)
25	972	−4.0763	−1.5850	+8.3603
40	884	−4.2225		+8.4265
50	800	−4.2932		+8.3845
60	735	−4.3700		+8.3635

complex the original morphology of both individual components (DAN and BCD) disappeared and it was not possible to distinguish the presence of either DAN or BCD.

Comparative in vitro dissolution study of DAN and DAN complexes made by various methods were performed in a USP XXIII dissolution apparatus number II and extent of the enhancement of dissolution rate was found to be dependant on the preparation method of complex (Jadhav et al., 2007).

3.4. Mechanism of complexation

3.4.1. Thermodynamics of complexation

The aqueous solubility of DAN, in presence of BCD, increased with elevation of temperature (25, 40, 50 and 60 °C), resulting in an increase in the slope of the solubility curve. This increase in the slope of the solubility curve may be related to the liberation of water molecules bound in the cavity of CDs on elevation of temperature (Shinoda and Fujihira, 1968). The decrease in K_C values with increasing temperatures indicates the exothermic nature of inclusion complexation. Typical van't Hoff plots confirmed fairly well to linear behavior over the temperature range of 25–60 °C (Fig. 11). Thermodynamic parameters were determined from the dependence of the K_C values on temperature in water medium (Table 4).

3.4.2. Molecular modeling studies

The final energy minimized DAN–BCD complex is shown in Fig. 12. The steroidal skeleton of DAN is partially buried into the BCD cavity formed by 2′-hydroxyls (secondary face). The methyl groups present at the junctions of A/B and C/D (18 and 19 protons) rings show favorable interaction with BCD. The isooxazole ring, part of ring A and acetylene groups are exposed to the solvent. The intermolecular interaction energies are shown in Table 5. The major contribution to the stabilization of the complex is dispersive (attractive) van der Waals interaction energy. Table 6 summarizes changes in the solvent-accessible polar and hydrophobic surface area, as calculated from Jurs descriptors, of DAN upon complexation with BCD. There is no significant change in the total polar surface area, but the total hydrophobic surface area of DAN decreases by 55% upon complexation with BCD. Since DAN is conformationally restricted molecule, there are no changes in torsion angles upon complexation with BCD.

3.5. Antiovulatory activity

It was of interest to determine whether DAN and DAN–BCD_{BM} given orally on the day of metestrus would block

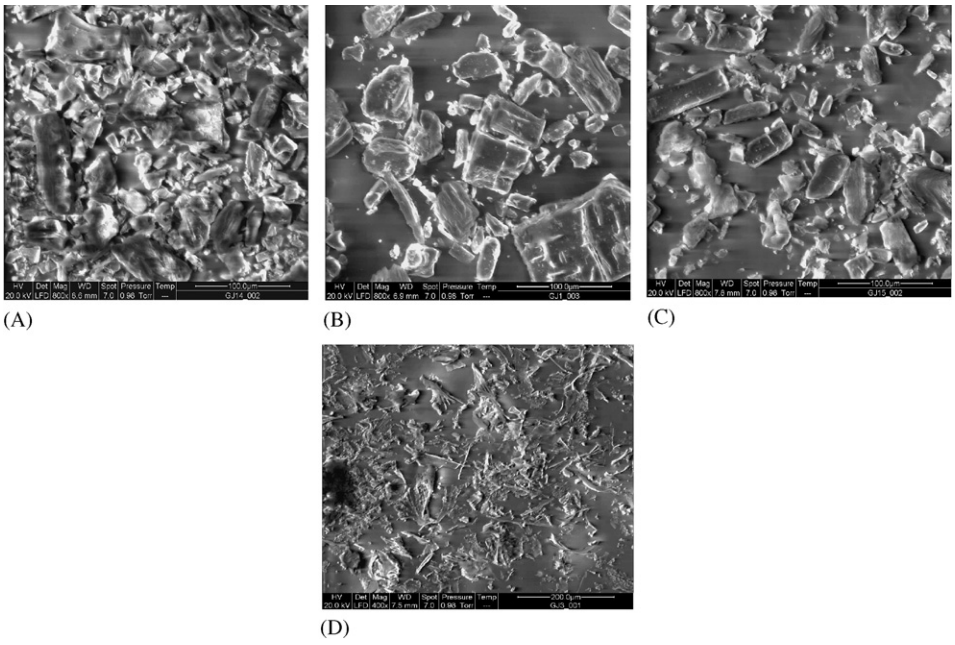

Fig. 10. SEM of danazol, β-cyclodextrin, physical mixture and freeze-dried complex. (A) DAN, (B) BCD, (C) PM, (D) FD.

Table 5
Intermolecular interaction energies of DAN, BCD and DAN–BCD complex

Molecule	Internal energy	VdW repulsion	VdW dispersion	Electrostatic	Total non-bond energy	Total energy
BCD	63.3	255.0	−203.8	99.9	151.1	214.4
DAN	57.5	115.4	−70.1	−30.8	14.5	71.8
BC[a]	120.7	370.4	−273.9	69.1	165.6	286.2
AC[b]	117.9	384.8	−318.5	54.8	121.1	239.0
AC–BC[c]	−2.8	14.4	−44.6	−14.3	−44.5	−47.2

[a] Before complexation.
[b] After complexation.
[c] Difference between after complexation and before complexation.

ovulation in normal cycling female mice when administered as single dose. The study was also focused to determine the dose of DAN–BCD_{BM} that would result in complete anovulation. When administered on the day of metestrus both DAN and DAN–BCD_{BM} showed dose-dependent antiovulatory activity. Table 7 shows the mean number of oocytes of control group mice and treatment group mice. Fig. 13 shows the comparative bar graph of LH content of control group mice and treatment group mice at estrus day.

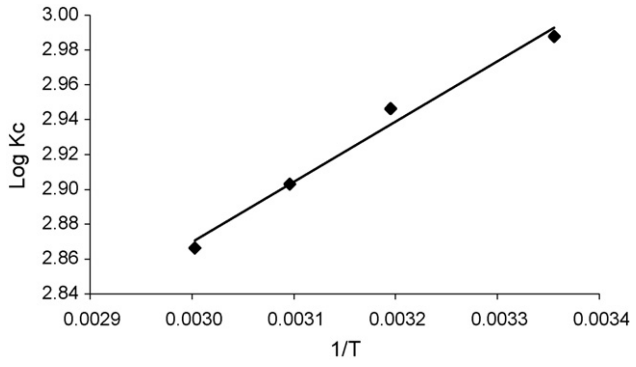

Fig. 11. van't Hoff plot of danazol–β-cyclodextrin system at 25, 40, 50 and 60 °C.

Table 6
Changes in solvent-accessible polar and hydrophobic surface area of DAN upon complexation with BCD

Molecule	TPSA[a]	THSA[b]	% Decrease in THSA
BCD	656.6	554.6	
DAN	146.0	413.3	55
DAN–BCD	675.5	534.5	

[a] Total polar surface area.
[b] Total hydrophobic surface area.

4. Discussion

The continuous variation method gives direct evidence of stoichiometric ratios of inclusion phenomenon and Fig. 2 demonstrated that the complex has 1:1 stoichiometry since the ratio, *r*, has a maximum value at 0.5.

Phase solubility profile (Fig. 3) exhibits A_L-type of solubility curve (with a slope less than 1) suggesting that soluble complexes of first-order could have been formed and no precipitation

Table 7
Effect of DAN and DAN–BCD complex on ovulation of mice after single dose administration by oral route

Group	Number of oocytes in both oviduct (Mean ± S.E.M.)	% Inhibition of oocytes	% Inhibition of LH content
G_A[a]	11.63 ± 0.263	–	–
G_B[b]	9.38 ± 0.183	19.35	17.50
G_C[c]	7.50 ± 0.267	35.48	33.20
G_D[d]	6.63 ± 0.183	43.01	44.49
G_E[e]	6.13 ± 0.295	47.31	51.97
G_F[f]	6.88 ± 0.295	40.86	57.22
G_G[g]	4.38 ± 0.183	62.37	62.78
G_H[h]	2.38 ± 0.183	79.57	70.39
G_I[i]	0	100.00	89.55

Eight animals in each group, mean ± S.E.M. (standard error of mean). One-way ANOVA: mean values of oocytes count of all treatment groups are significantly different than the mean of oocytes count of control group at $p < 0.01$ (confirmed by Dunnett's multiple comparison test).

[a] Control.
[b] 6.4 mg/kg danazol.
[c] 12.8 mg/kg danazol.
[d] 25.6 mg/kg danazol.
[e] 51.2 mg/kg danazol.
[f] 6.4 mg/kg danazol–β-cyclodextrin complex.
[g] 12.8 mg/kg danazol–β-cyclodextrin complex.
[h] 25.6 mg/kg danazol–β-cyclodextrin complex.
[i] 51.2 mg/kg danazol–β-cyclodextrin complex.

was observed (Higuchi and Connors, 1965). The K_C value calculated by solubility method, spectroscopic method and spectral shift method was found to be 972.02, 118.75 and 200.59 M^{-1}, respectively. The stability constant estimated by spectroscopic method and spectral shift method was relatively less compared to that obtained by phase solubility studies. It should be noted that the use of the spectroscopic and spectral shift techniques is certainly not the method of first choice when the drug has low aqueous solubility, because the difference in the absorption will be too small to allow for a reliable determination. In contrast, the phase solubility method provides much better estimation in these cases (Frijlink et al., 1989).

The hyperchromic shift in the UV spectrum of DAN in presence of BCD indicated solubilization capability of BCD for DAN.

The changes in optical activity of DAN could be attributed to the perturbation of electronic transition of drug caused by the inclusion in the cavity of BCD following complexation (Engle et al., 1994; Ventura et al., 1998). It is well known that the intrinsic Cotton effects of CDs are observed below 220 nm (Otagiri et al., 1975) and inclusion of optically inactive compounds within CD cavity generates extrinsic Cotton effect in the wavelength region of drug chromophore. Thus, the result of circular dichroism spectroscopy reveals that DAN is embedded in the asymmetric locus of BCD cavity.

All the protons of DAN showed significant chemical shift changes in presence of BCD but we could not assign most of the protons (–CH_2 and –CH–) of DAN exactly, due to presence of too many protons with complex pattern. However, it is evident from the chemical shift data that these protons also showed significant chemical shift in presence of BCD. Moreover, as DAN is a comparatively large molecule with rigidity in the structure, it might not be able to enter completely into the inner cavity of BCD. In this study, relatively modest down field (positive) chemical shift changes were observed for H3′ and H5′ protons of BCD. Moreover, downfield (positive) chemical shifts in steroidal protons of DAN suggested presence of van der Waals interaction energies in stabilization of the complex. Thus, from the results of ^{1}H NMR studies we hypothesized that the protons of steroidal skeleton of DAN are preferably involved in the complexation with BCD.

DSC studies showed absence of an endothermic peak of DAN at 230 °C in FD, which suggested the absence of a free drug. This could be attributed to the formation of an amorphous solid product, encapsulation of the drug inside the BCD cavity, or both (Esclusa-diaz et al., 1994; Mura et al., 1999).

P-XRD analysis revealed that the FD complex has a completely different pattern compared to the pure raw materials. It was no longer possible to distinguish some of the characteristic peaks of DAN, thus confirming the existence of new amorphous form in FD.

FTIR spectrum of FD showed very negligible change in the IR wave number value of C≡CH stretch (of acetylene group of DAN) and C=N stretch (of isooxazole ring) suggesting that these part of DAN might not be interacting with the hydrophobic cavity of BCD.

SCM photographs clearly demonstrated the drastic change in surface morphology of FD complex indicating formation of a new solid phase, which might have occurred due to molecular encapsulation of DAN in BCD.

Thermodynamic parameters (Table 4) showed that the inclusion complex phenomenon is predominantly due to favorable enthalpy changes, which could still compensate more for the unfavorable entropy changes. In other words, negative ΔH value suggested that inclusion of DAN into BCD is an enthalpy driven process. While positive value of ΔS suggested the role of hydrophobic interactions, which involves breakdown and removal of the structured water molecules inside the BCD cavity and around the non-polar substrate (Chun and Yun, 1993). The magnitude of ΔS is little higher

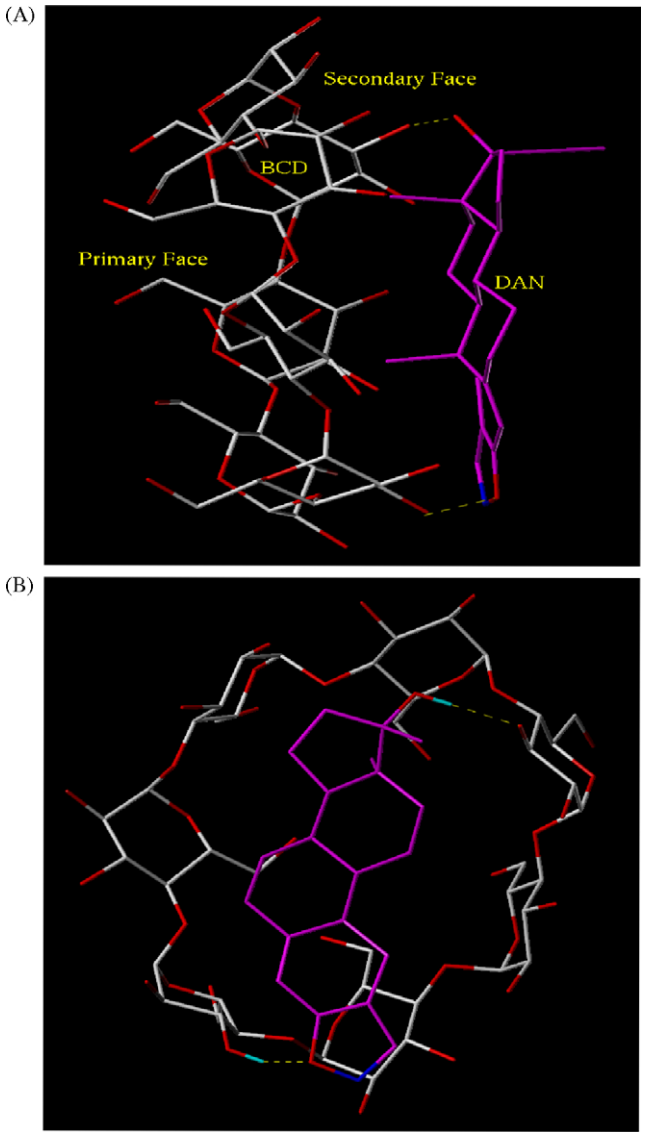

Fig. 12. Orientation of danazol molecule in β-cyclodextrin cavity after molecular dynamics simulation. (A) Side view and (B) top view.

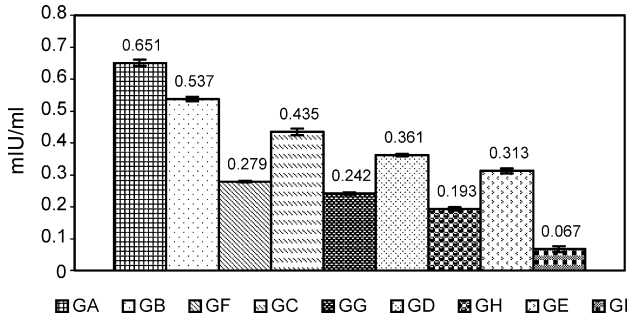

Fig. 13. Effect of DAN and DAN–BCD binary system on plasma luteinizing hormone content of mice after single dose oral administration. G_A: Control, G_B: 6.4 mg/kg danazol, G_C: 12.8 mg/kg danazol, G_D: 25.6 mg/kg danazol, G_E: 51.2 mg/kg danazol, G_F: 6.4 mg/kg danazol–β-cyclodextrin complex, G_G: 12.8 mg/kg danazol–β-cyclodextrin complex, G_H: 25.6 mg/kg danazol–β-cyclodextrin complex, G_I: 51.2 mg/kg danazol–β-cyclodextrin complex. Eight animals in each group, mean ± S.E.M. (standard error of mean). One-way ANOVA: mean values of LH content of all treatment groups are significantly different than the mean value of LH content of control group at $p < 0.05$ (confirmed by Dunnett's multiple comparison test).

suggesting role of steric interactions in stabilization of complex.

The modeled complex revealed that dispersive (attractive) van der Waals interaction energy is major contributor for stabilization of complex which is complementary with the thermodynamic data, which indicated role of non-bonded interactions (along with steric interactions) in the process of complex formation, and is also complementary with ^{1}H NMR data of DAN–BCD system, which suggested the presence of van der Waals interaction energy based on downfield shift of steroidal protons of DAN. There is no change in conformation of DAN molecule after complexation with BCD due to its rigidity. However, more rigid guests are known to have better complexation ability than flexible molecules, because the host–guest interaction is better defined in the case of rigid guests (Klein et al., 2000). This is also in accord with data of phase solubility studies at various temperatures, which indicated decrease in stability constant values (from 972 M^{-1} at 25 °C to 735 M^{-1} at 60 °C) as the temperature is increased; this could be because of decrease in rigidity of DAN molecule at elevated temperatures.

^{1}H NMR and molecular modeling study revealed that protons of steroidal skeleton are buried in the BCD cavity while isooxazole and acetylene group do not insert in the cavity which was also revealed in FTIR data showing absence of interaction of acetylene group (of DAN) with hydrophobic cavity of BCD. Significant decrease in the total hydrophobic surface area (55%) of DAN upon complexation with BCD might be responsible for increased solubility of DAN in the inclusion complex form. Thus, the data of modeled drug corroborate well with the experimental data suggesting that the molecular modeling can be used as a complementary tool for characterizing the inclusion complexes of drug with CDs.

We have carried out comparative dose-dependent antiovulatory activity of DAN and DAN–BCD_{BM} for determining the effect of enhanced aqueous solubility on pharmacodynamic activity of poorly water-soluble drug, DAN. DAN–BCD_{BM} at 51.2 mg/kg showed 100% inhibition of ovulation. Moreover, in both treatments group (DAN and DAN–BCD_{BM}) dose-dependent (6.4–51.2 mg/kg) antiovulatory activity was observed. However, the ability to inhibit ovulation was more pronounced in case of DAN–BCD_{BM} as compared to DAN at all dose levels. This would be because of presence of higher concentration of DAN in blood when given in the more water-soluble form. Moreover, Bonferroni's multiple comparison test (at $p < 0.05$) showed that there is no significant difference between mean oocytes counts of 6.4 mg/kg DAN–BCD_{BM} treated mice and 12.8 mg/kg, 25.6 mg/kg and 51.2 mg/kg DAN treated mice, indicating superiority of DAN–BCD_{BM} complex. The percent inhibition of oocytes values revealed that 51.2 mg/kg of DAN–BCD_{BM} complex is 2.11 times better than 51.2 mg/kg of DAN for preventing ovulation.

Plasma progesterone analysis revealed dose-dependent decrease (6.4–51.2 mg/kg) in LH content in DAN–BCD_{BM} treatment mice. The percent inhibition of LH content values revealed that 51.2 mg/kg of DAN–BCD_{BM} complex is 1.92 times better

than 51.2 mg/kg of DAN for preventing ovulation. Moreover, the lowest dose of DAN–BCD_{BM} (6.4 mg/kg) was more efficient in reducing the plasma content of LH than the highest dose of DAN (51.2 mg/kg) indicating once again the superiority of DAN–BCD_{BM}.

There was significant difference in reduced values of LH content along with significant difference in reduced number of oocytes amongst all dose levels (6.4–51.2 mg/kg) of DAN–BCD_{BM}.

In mouse model, DAN–BCD_{BM} showed dose-dependent antiovulatory activity in the range of 6.4–51.2 mg/kg (equivalent to 50–400 mg human dose). DAN at 51.2 mg/kg in mouse failed to show complete inhibition of ovulation but DAN–BCD_{BM} at 51.2 mg/kg as oral single one time administration has shown complete anovulation. The reason for such difference in the activity could be related to solubility dependant oral bioavailability of DAN.

5. Conclusions

The data of circular dichrohism, NMR, DSC, P-XRD, and SEM studies showed that β-cyclodextrin encapsulates danazol by inclusion phenomenon. The results of molecular modeling are in good accord with the experimental data. Moreover, antiovulatory studies in mouse model showed superiority of DAN–BCD_{BM} complex over DAN alone. Thus, pharmaceutical preparation containing DAN–BCD_{BM} complex at acceptable physiologically dose level (400 mg human dose) is likely to have a great potential as an oral antiovulatory agent.

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