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# Danazol-β-Cyclodextrin Binary System: A Potential Application in Emergency Contraception by the Oral Route

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Ganesh S. Jadhav,[1] Pradeep R. Vavia,[1] and Tarala D. Nandedkar[2]

[1]Pharmaceutical Division, Mumbai University Institute of Chemical Technology, Matunga, Mumbai – 400 019, India
[2]Department of Cell Biology, National Institute of Research in Reproductive Health (Indian Council of Medical Research), Parel, Mumbai – 400 012, India

## ABSTRACT

This study explored the potential of β-cyclodextrin to improve the aqueous solubility and dissolution of danazol, investigated a simple and less expensive method for preparation of a danazol-β-cyclodextrin binary system, and explored the potential application of a danazol-β-cyclodextrin binary system as a single-dose emergency contraceptive. Phase solubility analysis indicated formation of a first-order soluble complex with stability constant 972.03 $M^{-1}$, while Job's plot affirmed 1:1 stoichiometry. The hyperchromic shift in the UV-Vis spectrum of danazol in the presence of β-cyclodextrin indicated solubilization capability of β-cyclodextrin for danazol. The extrinsic Cotton effect with a negative peak at 280.7 nm confirmed the inclusion of danazol in the asymmetric locus of β-cyclodextrin. $^1$H–nuclear magnetic resonance analysis suggested that the protons of the steroidal skeleton of danazol display favorable interactions with the β-cyclodextrin cavity. The danazol-β-cyclodextrin binary system was prepared by kneading, solution, freeze-drying, and milling methods. The extent of the enhancement of dissolution rate was found to be dependent on the preparation method. Dissolution studies showed a similar relative dissolution rate (2.85) of the danazol-β-cyclodextrin binary system prepared by the freeze-drying and milling (in the presence of 13% moisture) methods. In a mouse model, the danazol-β-cyclodextrin binary system at 51.2 mg/kg (equivalent to a 400-mg human dose) showed 100% inhibition of implantation when given postcoitally. Moreover, the danazol-β-cyclodextrin binary system is safe up to 2000 mg/kg in the mouse (15.52 g/70 kg human) as a single oral dose. Thus, the danazol-β-cyclodextrin binary system could serve as a new therapeutic application: an oral emergency contraceptive at a physiologically acceptable single dose.

**KEYWORDS:** Danazol, β-cyclodextrin, solubility, dissolution studies, anti-implantation activity.

**Corresponding Author:** Pradeep R. Vavia, Pharmaceutical Division, Mumbai University Institute of Chemical Technology, Matunga, Mumbai 400 019, India. Tel: +91-22-24145616; Fax: +91-22-24145614; E-mail: vaviapradeep@yahoo.com

## INTRODUCTION

Endometriosis is one of the most common gynecological problems in adult women.[1] Danazol (DAN) (Figure 1) is used in a number of clinical situations,[2] including the therapy of endometriosis,[3] cystic mastitis,[4] precocious puberty,[5] and menorrhagia.[6] However, DAN is very poorly soluble in water and exhibits dissolution rate–limited absorption.[7] Moreover, a potential application of the antigonadotropic properties of DAN in postcoital contraception has been investigated, but oral administration of 600 mg DAN repeated after 12 hours had an inconsistent effect.[8] Oral administration of DAN above 1200 mg could be effective as a postcoital contraceptive with some degree of certainty, but the adverse effects at such doses could be unacceptable to many women. The side effects include weight gain, virilism, headache, breast tenderness, and pelvic pain.[9] It seemed unlikely that the drug could find clinical application as a contraceptive agent in females.[2]

A limiting factor to the in vivo performance of drugs like DAN after oral administration is their inadequate ability to be wetted by and dissolved in the gastrointestinal fluid. Several approaches (salt formation, prodrug, micronization, solid dispersions, solvates, adsorbates, or complexes) are employed to overcome the limiting aqueous solubility.[10] Cyclodextrins (CDs) have attracted growing interest in the pharmaceutical industry as complexing agents because of their low toxicity and ability to produce stable inclusion complexes.[11,12] CDs have been used extensively to increase the aqueous solubility, stability, and bioavailability of drugs.[13]

β-cyclodextrin (BCD), despite its limited aqueous solubility, was chosen to enhance the solubility of DAN because of its 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 because of 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.[14]

The most commonly used methods for preparation of drug-cyclodextrin complexes are the coprecipitation, slurry, paste, dry-mixing, spray-drying, and freeze-drying methods.[15]

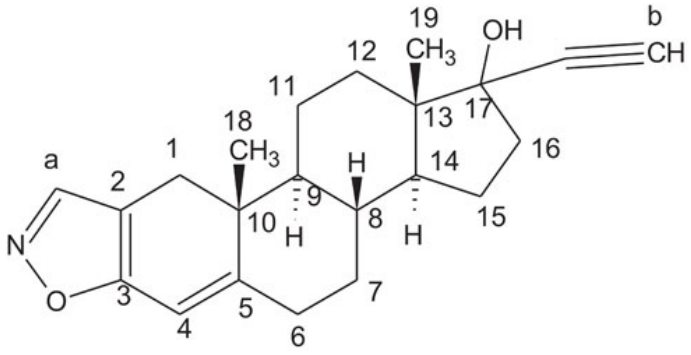


**Figure 1.** Chemical structure of danazol.

Freeze-drying and spray-drying are the methods most commonly used in industry for complexation. However, these methods involve multistage procedures that require considerable expenditure of time and resources. Some of these methods use organic solvents, and the residue of such solvents in the inclusion complex may be difficult to eliminate or may cause environmental problems. The dry-milling process is also used for the preparation of solid dispersions and complexation.[16,17] Cogrinding drugs with some additives, such as cholic acid and CDs, has been used as a method for complexation.[17,18]

The present work investigated the feasibility of using BCD to improve the aqueous solubility and dissolution rate of DAN. The work also investigated the effect of moisture on the dissolution profile of a DAN-BCD binary system. Moreover, a potential application of a DAN-BCD binary system as an oral emergency contraceptive at a physiologically acceptable dose was investigated in a mouse model. An acute oral toxicity study was also performed to determine the Lethal $Dose_{50}$ ($LD_{50}$) cutoff value of DAN and the DAN-BCD binary system.

## Materials and Methods

### *Materials*

DAN was used as supplied by Ar-Ex Lab Ltd (Mumbai, India). BCD was generously donated by SA Chemicals (Mumbai). Sodium carboxymethylcellulose (Akucell AF 0305) was a gift from Signet Chemicals (Mumbai). All other materials and solvents were of analytical reagent grade. The analysis of plasma samples for progesterone content was performed by radio immunoassay (RIA) using a Progesterone RIA kit from Diagnostic Systems Laboratories (Webster, TX).

### *Preliminary Studies*

#### *Phase Solubility Studies*

Solubility studies in water were performed according to the method reported by Higuchi and Connors.[19] The samples were filtered, suitably diluted, and analyzed using a UV spectrophotometer (Shimadzu 160A UV-Vis spectrophotometer, Shimadzu Corp, Kyoto, Japan) at 287 nm.

#### *Continuous Variation Method (Job's Plot)*

In the continuous variation method (Job's Plot),[20] equimolar (0.05 mM) solutions of DAN and BCD were added in varying quantities (mL) so as to obtain different *r* values. The samples were analyzed using a UV spectrophotometer at 287 nm.

### *Characterization of Binary System (Solution State)*

#### *UV-Vis Spectroscopy*

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

#### *Circular Dichroism Spectroscopy*

The circular dichroism spectra were obtained using a Jasco J-600 Spectropolarimeter (Tokyo, Japan). The signal-to-noise ratio was improved by superimposition of 5 different scans.

#### *Nuclear Magnetic Resonance Spectroscopy*

$^1$H–nuclear magnetic resonance (NMR) spectroscopic analysis was performed on a Bruker AMX-500 Fourier transform NMR spectrophotometer (Madison, WI) at 298°K. The 5-mM samples of DAN, BCD, and a mixture of DAN and BCD (5 mM:5 mM) were recorded in the $CD_3OD$:$D_2O$ (50:50 vol/vol) solvent system.

### *Preparation of Binary Systems*

#### *Physical Mixture*

DAN and BCD were passed individually through a 40-mesh sieve. A physical mixture (PM) in 1:1 M ratio was prepared by mixing the individual components together in a geometric proportion. This mixture was then passed through a 60-mesh sieve.

#### *Kneading Method*

In the kneading method (KM), the physical mixture of DAN and BCD in 1:1 M was triturated in a mortar with a small volume of water-methanol (1:1 vol/vol) solution. The thick slurry was kneaded for ~45 minutes and then dried at 45°C. The dried mass was pulverized and passed through a 60-mesh sieve.

*Solution Method*

In the solution method (SM), the aqueous solution of BCD (1M) was added to the alcoholic solution of DAN (1M) to obtain a complex of 1:1 stoichiometry. The resulting mixture was stirred for 24 hours and evaporated under vacuum at 45°C. The dried mass was pulverized and passed through a 60-mesh sieve.

*Freeze-Drying Method*

In the freeze-drying method (FD), the required 1:1 M quantity of DAN was dispersed in an aqueous solution of BCD at room temperature. After agitation for 7 days, the clear solution was lyophilized using LABCONCO (Freezone 4.5), (Kansas City, MS). The dried powder was passed through a 60-mesh sieve and then stored in a desiccator.

*Milling Method*

The ball-milling (BM) process was investigated in detail for preparation of the drug-BCD binary system. A ball mill of 1.25-L capacity with a height of 16 cm and a diameter of 14.4 cm (external)/11.1 cm (internal) was used. The ceramic pebbles (balls) used had average diameters of 2.2 ± 0.13 cm and average weights of 15.728 ± 0.49 g.

The effect of the presence of moisture during milling on the dissolution rate of DAN in the DAN-BCD blend was investigated. To control the moisture conditions during milling, various quantities of water (0% [ie, no water added], 5%, 10%, 13%, 15%, and 20% vol/wt) were added to the dry blend (PM). Each mixture was ground at a speed of 46 rpm for a specific time interval. At the end of the specific time interval, the ground material was unloaded from the ball mill and sieved through a 60-mesh sieve. The optimization of milling time was achieved by grinding each mixture for 4, 5, 6, and 7 hours.

***In Vitro Dissolution Studies***

The dissolution studies were performed in US Pharmacopeia XXIII <711> Apparatus 2. Accurately weighed binary systems equivalent to 50 mg of DAN were spread over 900 mL of dissolution medium (distilled water containing 0.02% wt/vol Tween 80). The stirring speed employed was 75 rpm, and the temperature was maintained at 37 ± 0.5°C. The filtrates of samples were analyzed for DAN content by the UV-Vis spectrophotometer at 287 nm. The dissolution profiles were evaluated on the basis of dissolution efficiency (DE)[21] at 20 and 60 minutes and dissolved percentage (DP) at 20 and 60 minutes. One-way analysis of variance (ANOVA) was used to test the significance of the differences between pure samples and other samples.

***Anti-Implantation Activity***

The activity was carried out as described by Reel et al.[22] The aqueous solution of DAN and DAN-$BCD_{BM}$ was prepared in an aqueous solution of 0.2% wt/vol sodium carboxymethylcellulose (as a vehicle). The mouse dose was calculated on the basis of its body surface area.[23] The value of the dose of DAN-$BCD_{BM}$ is not the value of the weight of powder of DAN-$BCD_{BM}$ but represents the active content (DAN) present in the binary system. Swiss albino mice (20-25 g) of both sexes were used in this study. They were raised in the animal house facility of the National Institute of Research in Reproductive Health (NIRRH; Parel, Mumbai). The mice were numbered, randomly selected, and kept in polycarbonate cages with a bedding of husks, and 12-hour light/dark cycles were maintained throughout the study period. Feed and water were given ad libitum. All animal manipulations were performed according to the ethical principles for animal care and management recommended by NIRRH's ethical committee. Proestrous female mice with a 4- or 5-day estrous cycle were cohabited overnight with proven fertile males (in the ratio of 2 females:1 male in 1 cage). Females found to have vaginal sperm the next morning were considered to have successfully mated (day 0 of pregnancy). Mated females were separated into 3 groups, each of which had 8 animals. One group served as a control, and the remaining 2 groups were treatment groups. On the morning of day 0 of pregnancy, the test substances and vehicle were administered orally to mice as a single dose, using a ball-tipped intubation needle fitted onto a syringe. The treatment groups were subdivided into 8 groups for various dose levels. The mice in the treatment groups received 6.4 mg/kg (equivalent to a 50-mg human dose), 12.8 mg/kg (equivalent to a 100-mg human dose), 25.6 mg/kg (equivalent to a 200-mg human dose), or 51.2 mg/kg (equivalent to a 400-mg human dose) of DAN or a respective equivalent amount of DAN-$BCD_{BM}$. On the ninth day of pregnancy, 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 minutes. The plasma samples were stored at –20°C in a deep freezer until the analysis of hormonal content was performed. The uterus of each mouse was examined to count the number of normal implantation sites and resorbing implantation sites. The percent inhibition of implantation was calculated by the following formula:

$$\%\ \textit{Inhibition of implantation} = \frac{\textit{Implantation sites in control group} - \textit{Implantation sites in treatment group}}{\textit{Implantation sites in control group}} \times 100 \quad (1)$$

The plasma level of progesterone was calculated using the percentage of bound/binding of zero (ng/mL) standard method.

The percent inhibition of progesterone content was calculated by the following formula:

$$\%\ Inhibition\ of\ progesterone\ content = \frac{Progesterone\ of\ control\ group - Progesterone\ of\ treatment\ group}{Progesterone\ of\ control\ group} \times 100 \quad (2)$$

The mean values of the number of normal implantation sites and the progesterone content of treated groups were compared with those of the control group using 1-way ANOVA followed by Dunnett's test and Bonferroni's multiple comparison test.

### *Acute Toxicity Studies*

Acute oral toxicity studies were performed to determine the $LD_{50}$ cutoff value of DAN in the plain and binary form made by BM. The acute toxic class method (423—Organization for Economic Cooperation and Development Guideline)[24] was adopted. Fresh (unused and healthy) Swiss albino mice (each weighing 20-25 g) of both sexes were used. A new group of mice was used for each dosage level (300 mg/kg, 50 mg/kg and 2000 mg/kg). The animals were quarantined in polypropylene cages in a well-ventilated room with controlled temperature, humidity, and lighting. The cages were lined with a rice husk bed and covered with mesh lids. Pelleted food was fed to the rats daily. Drinking water was administered through the mesh lid by keeping a 100-mL plastic bottle (with a suitable nozzle) in an inverted position.

The female mice were observed for pregnancy. If a mouse was found to be pregnant, it was excluded from the study. The animals were deprived of food for 4 hours prior to oral treatment and 2 hours after treatment. On the day of the experiment, the animals were divided into 2 groups, with 3 animals in each group. The animals of group 1 and group 2 received DAN and DAN-$BCD_{BM}$, respectively. The study was performed at a starting dose level of 300 mg/kg. The test solutions were prepared in 0.2% wt/vol of sodium carboxymethylcellulose. The mouse dose was calculated on the basis of its body surface area.[23] The value of the dose of DAN-$BCD_{BM}$ is not the value of the weight of the powder of DAN-$BCD_{BM}$ but represents the active content (DAN) present in the binary system. The study was performed by administering a single oral dose of either DAN or DAN-$BCD_{BM}$. Detailed observations were made of the effect of dose upon important physiological functions of animals such as locomotion, behavior, respiration, and pharmacotoxic signs like convulsions and vomiting. The decision regarding the repetition of the same dose level or the use of the next dose level (50 mg/kg or 2000 mg/kg) was based on the results of the starting dose level.

## Results and Discussion

### *Phase Solubility Studies*

The phase solubility profile for the complex formation between DAN and BCD at 25°C is shown in Figure 2. There is a linear increase in the solubility of DAN with increasing concentrations of BCD, exhibiting an $A_L$ type of solubility curve (with a slope less than 1) showing that first-order soluble complexes were formed and no precipitation was observed.[19] The apparent 1:1 stability constant $K_C$ was calculated from the straight line of the phase solubility diagram by using the following equation:

$$K_c = \frac{Slope}{Intercept \times (1 - Slope)} \quad (3)$$

The $K_C$ value for the DAN-BCD complex was found to be 972.03 $M^{-1}$ (within 100-5000 $M^{-1}$), which is considered adequate for improvement of the bioavailability of poorly soluble drugs.[11]

### *Continuous Variation Method (Job's Plot)*

The continuous variation method gives direct evidence of the stoichiometric ratios of inclusion phenomena. The continuous variation plot demonstrates (Figure 3) that the complex has 1:1 stoichiometry, since the ratio, r, has a maximum value at 0.5.

### *UV-Vis Spectroscopy*

UV spectra of DAN and DAN in the presence of BCD are shown in Figure 4. DAN-1 represents the spectrum of DAN with dilution, and DAN-2 represents the spectrum of DAN without dilution. The comparison of DAN-BCD should be made with DAN-1 since the spectra of both solutions contain the same concentration of DAN ($9.74 \times 10^{-6}$ M). There

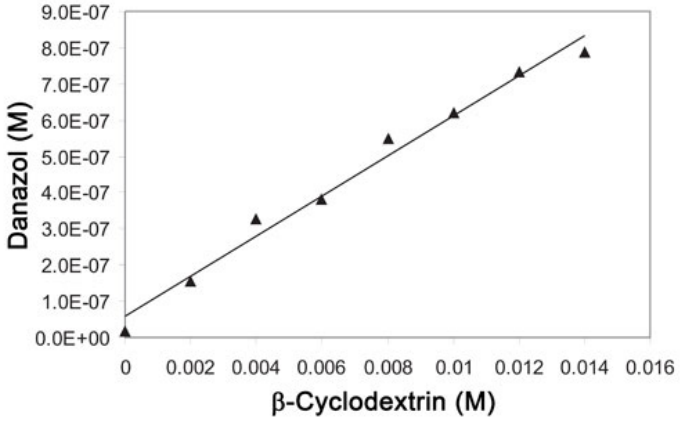


**Figure 2.** Phase solubility diagram of the danazol-β-cyclodextrin system in water at 25ºC.

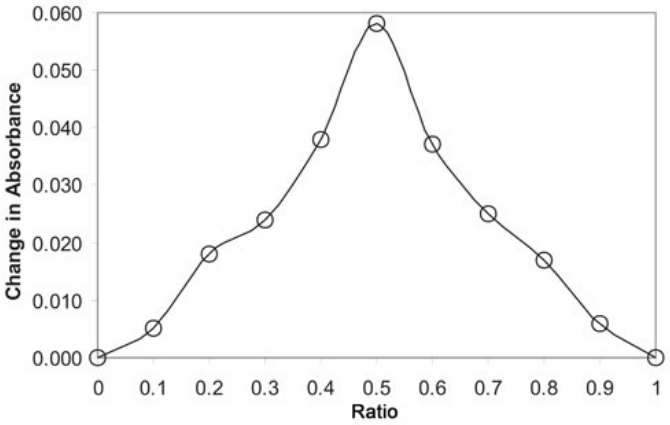


**Figure 3.** Continuous variation plot of the danazol-β-cyclodextrin system in water.

is a 49 170% increase in intensity (hyperchromic shift) of DAN in the presence of BCD, indicating the solubilization capability of BCD for DAN.

### *Circular Dichroism Spectroscopy*

The circular dichroism spectrum of the DAN ($3.36 \times 10^{-6}$ M)-BCD ($1.40 \times 10^{-2}$ M) system in aqueous solution is shown in Figure 5. In the presence of BCD, the optical activity was induced at 215.90 nm and 280.7 nm with positive and negative peak, respectively. These changes in optical activity could be attributed to the perturbation of the electronic transition of the drug caused by the inclusion in the cavity of BCD following complexation.[25,26] It is well known that the intrinsic Cotton effects of CDs are observed below 220 nm,[27] and inclusion of optically inactive compounds within the CD cavity generates an extrinsic Cotton effect in the wavelength region of the drug chromophore. Thus, circular dichroism spectroscopy reveals that DAN is embedded in the asymmetric locus of the BCD cavity.

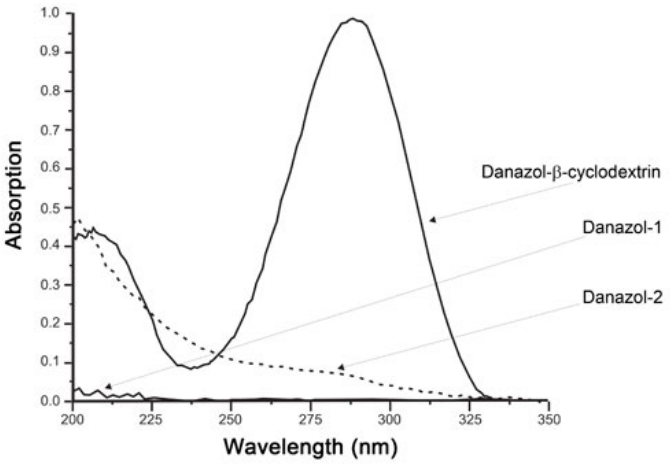


**Figure 4.** UV spectra of danazol ($9.74 \times 10^{-6}$ M) and danazol ($9.74 \times 10^{-6}$ M) in the presence of β-cyclodextrin ($1.40 \times 10^{-2}$ M).

### *NMR Spectroscopy*

In this study, owing to the extremely poor aqueous solubility of DAN, water could not be used. $^{1}$H-NMR signals for DAN and BCD were assigned according to NMR information provided by Balogh et al[28] and Schneider et al,[29] respectively. The chemical shift values of BCD and DAN are summarized in Table 1 as the complexed state ($\delta_c$), the free BCD state ($\delta_0$), and the chemical shift change $\Delta\delta$ (ppm) ($\Delta\delta = [\delta_c - \delta_0]$). All the protons of DAN showed significant chemical shift changes in the presence of BCD, but we could not assign most of the protons (-$CH_2$ and -CH-) of DAN exactly, because of the presence of too many protons with a complex pattern. However, it is evident from the chemical shift data that these protons also showed a significant chemical shift in the presence of BCD. Moreover, as DAN is a comparatively large molecule with rigidity in its structure, it might not be able to enter the inner cavity of BCD completely. In this study, relatively modest downfield (positive) chemical shift changes were observed for H3′ and H5′ protons of BCD. Moreover, downfield (positive) chemical shifts in steroidal protons of DAN suggested the 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 the steroidal skeleton of DAN are involved in the complexation with BCD.

### *In Vitro Dissolution Studies*

We attempted to improve the dissolution rate of DAN by milling it with BCD in different moisture conditions. Table 2 shows the optimization of milling parameters for a 1:1 M ball-milled binary system of DAN and BCD. It had been

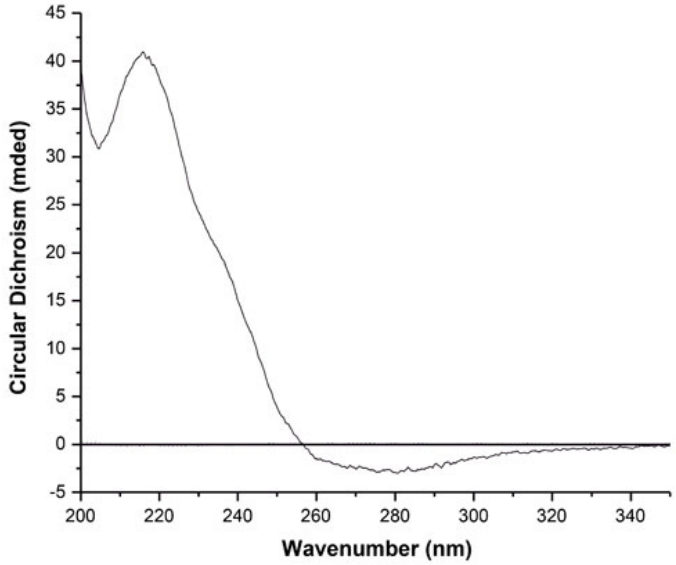


**Figure 5.** Circular dichroism spectrum of danazol ($3.36 \times 10^{-4}$ M) in the presence of β-cyclodextrin ($1.40 \times 10^{-2}$ M).

**Table 1.** Chemical Shift Changes of BCD and DAN Protons in $CD_3OD:D_2O$ (5:5 vol/vol)*

| Proton of BCD | $\delta_C$† | $\delta_0$‡ | $\Delta\delta$§ | Proton of DAN | $\delta_C$† | $\delta_0$‡ | $\Delta\delta$§ |
|---|---|---|---|---|---|---|---|
| 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 | | | | |

*BCD indicates β-cyclodextrin; DAN, danazol.
†Complexed state.
‡Free state.
§($\delta_c$ - $\delta_0$).

seen that in the case of the 1:1 M no-water-added form, the maximum release was achieved at 6 hours of milling; thereafter, at 7 hours of milling, it decreased, but not appreciably. However, it should be noted that a sharp increase in the dissolution rate of DAN was observed from 5% to 10% water content. Figure 6 clearly shows that 100% DAN was released at 13% moisture and that the drug release decreased when the moisture content exceeded 13%. At 20% it decreased to as low as 73%.

Thus, on the basis of in vitro dissolution data, we could speculate that the water molecules are acting not only as a lubricant at the molecular level during the comilling process but also as a binder for drug and BCD molecules, which might have caused inclusion of the drug at the surface of BCD.[30] Moreover, BCD molecules and a suitable amount of water would help stabilize the submicron particles of drug, possibly by forming a CD network covering the drug particles and preventing aggregation among them.[31] In higher moisture conditions, the affinities between BCD molecules would have increased during the milling, resulting in recrystallization of BCD molecules, lower affinities between BCD and DAN molecules, and, consequently, decreased comilling efficiency.[32] However, a detailed analysis involving particle size determination and solid state characterization (eg, Powder [P]-XRD) would help to identify the possible reasons for the comparable dissolution rate of DAN-$BCD_{BM}$ (13% moisture) and FD.

**Table 2.** Optimization of Milling Time and Moisture Content for 1:1 M Danazol-β-Cyclodextrin Binary System During Milling Method

| Added Moisture Content (% vol/wt) | % Cumulative Release at 10 Minutes | | | |
|---|---|---|---|---|
| | Milling Time (hr) | | | |
| | 4 | 5 | 6 | 7 |
| 0 | 23.12 | 30.82 | 34.88 | 34.21 |
| 5 | 29.16 | 33.12 | 36.11 | 35.12 |
| 10 | 35.68 | 41.22 | 41.12 | 41.18 |
| 13 | 39.10 | 45.40 | 45.08 | 45.12 |
| 15 | 35.87 | 42.80 | 42.22 | 42.54 |
| 20 | 32.12 | 35.21 | 35.10 | 35.18 |

The dissolution profiles of pure DAN and of the DAN-BCD complexes formed by the PM, KM, SM, FD, and BM (5 hours, 13% moisture) methods are illustrated in Figure 7. The dissolution profile of all components exhibited first-order release rate kinetics (Table 3). The results in terms of DE, DP, and relative dissolution rate are collected in Table 4. One-way ANOVA showed a significant ($P < .05$) increase in the dissolution rates in FD and BM as compared with PM and pure DAN. The dissolution rate expressed by $t_{50\%}$ of FD ($t_{50\%}$ = 11.41 minutes) and BM ($t_{50\%}$ = 11.92 minutes) was faster than that of DAN alone ($t_{50\%}$ = 97.60 minutes), while PM had a $t_{50\%}$ of 79.25 minutes.

It was evident from the dissolution data (Table 4) that almost 70% of the drug was released in 20 minutes from FD and BM, while for drug alone, 60 minutes was required to dissolve a mere 35% of the drug. It is also evident that FD, BM, SM, and KM exhibited higher dissolution rates than PM and pure DAN.

The extent of the enhancement of dissolution rate was found to depend on the preparation method of the complex. The

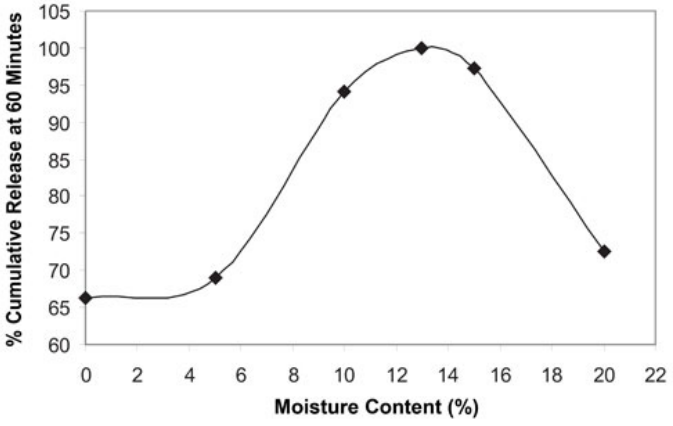


**Figure 6.** Effect of moisture content of 5-hour milled samples of the danazol-β-cyclodextrin binary system on release rate at 60 minutes.

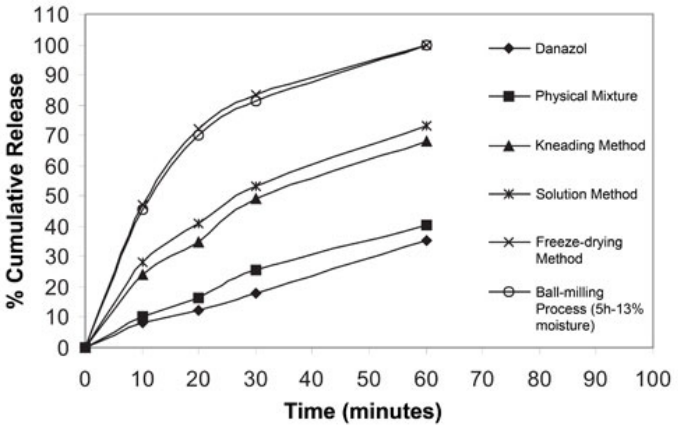


**Figure 7.** Comparative dissolution profile of the danazol-β-cyclodextrin binary system prepared by various methods.

improved dissolution of FD and BM may be due to the formation of an inclusion complex of the drug with BCD[33] and/or the conversion of the drug to an amorphous state or nearly amorphous state.[34] FD DAN had a slightly lower dissolution rate than did pure DAN at 50 and 60 minutes, which may be attributed to agglomerate formation and/or recrystallization. PM had a better dissolution rate than did DAN alone. Because BCD dissolves more rapidly in the dissolution medium than the pure drug does, it can be assumed that, in the early stages of the dissolution process, the BCD molecules operate locally on the hydrodynamic layer surrounding the particles of drug. This action results in an in situ inclusion process, which produces a rapid increase in the amount of dissolved drug.[35]

The dissolution rate improvement for PM and KM may be due to the wetting effect of BCD[36]; in fact, this effect is more pronounced in the case of KM, where the process of mixing the 2 components is more intensive, which may result in formation of the metastable state of the drug. SM showed slightly lower dissolution as compared with FD, which may be due to precipitation of the drug during complexation.[37]

### *Anti-Implantation Activity*

It was of interest to determine whether DAN and the DAN-BCD binary system would inhibit implantation when given postcoitally (within 4-5 hours of conception) as a single oral dose to the mated female mice. We also sought to determine the dose of the DAN-BCD binary system that would result in complete inhibition of implantation.

When given as a single oral dose on day 0 of pregnancy, both DAN and the DAN-BCD binary system showed dose-dependent anti-implantation activity. Table 5 shows the mean number of normal implantation sites of control group mice and treatment group mice.

Figure 8 shows a bar graph of the progesterone content of control group mice and treatment group mice on the ninth day of pregnancy.

Emergency contraception or postcoital contraception is defined as the use of a drug or device to prevent pregnancy after intercourse. We have compared the dose-dependent postcoital anti-implantation activity of DAN and the DAN-BCD binary system to determine the effect of enhanced aqueous solubility on the pharmacodynamic activity of the poorly water soluble drug DAN.

The results of the study are conclusive: the antifertility activity of both treatments (DAN and the DAN-BCD binary system) was because of anti-implantation activity and not antiovulatory activity. In mice, ovulation and fertilization of oocytes occurs during the same night in the estrous cycle in which the females are receptive to males, and it was inferred that the oocytes would have been fertilized by the time that vaginal plugs were seen. The DAN-BCD binary system at 51.2 mg/kg showed 100% inhibition of implantation (postcoital contraception). Moreover, in both treatment groups (DAN and the DAN-BCD binary system), dose-dependent (6.4-51.2 mg/kg) anti-implantation activity was observed. However, the ability to inhibit implantation was more pronounced for the DAN-BCD binary system than for DAN at all dose levels. This is because of the presence of a higher concentration of DAN in the blood when DAN is given in the more water-soluble form. Moreover, the anti-implantation activity of 6.4 mg/kg of the DAN-BCD binary system was similar to that of 25.6 mg/kg of DAN, indicating the superiority of the DAN-BCD binary system. The percent inhibition of implantation values revealed that 51.2 mg/kg of the DAN-BCD binary system was 2.16 times better than 51.2 mg/kg of DAN at preventing implantation. The absence of resorbing implantation sites along with the absence of normal implantation sites at 51.2 mg/kg of the DAN-BCD binary system indicates alteration in the high levels of progesterone that are required for maintenance of normal pregnancy.

**Table 3.** Dissolution Rate Data in Water for Drug and Binary Systems Made by Different Methods*

| | Time (min) | | | |
|---|---|---|---|---|
| Samples | $t_{25\%}$ | $t_{50\%}$ | $t_{75\%}$ | $t_{90\%}$ |
| DAN | 40.65 | 97.60 | 194.90 | 323.60 |
| PM | 31.96 | 79.25 | 160.00 | 266.90 |
| KM | 12.40 | 34.09 | 71.16 | 120.10 |
| SM | 10.02 | 29.07 | 61.62 | 104.60 |
| FD | 6.46 | 11.41 | 19.86 | 31.04 |
| $DAN_{FD}$ | 49.74 | 133.30 | 276.10 | 465.00 |
| BM (13% moisture) | 6.98 | 11.92 | 20.37 | 31.54 |
| $DAN_{BM\text{-}5h}$ | 30.19 | 83.91 | 179.70 | 297.00 |

*DAN indicates danazol; PM, physical mixture; KM, kneading method; SM, solution method; FD, freeze-drying method; BM, ball-milling process.

**Table 4.** Dissolution Profile Data in Water for Drug and Binary Systems Made by Different Methods*

| System | $DE_{20\ mins}$ (%) ± SD | $DP_{20\ mins}$ ± SD | $RDR_{20\ mins}$ | $DE_{60\ mins}$ (%) ± SD | $DP_{60\ mins}$ ± SD | $RDR_{60\ mins}$ |
|---|---|---|---|---|---|---|
| DAN | 7.04 ± 0.9 | 12.26 ± 1.1 | | 18.19 ± 1.0 | 35.13 ± 1.1 | |
| PM | 9.15 ± 0.8 | 16.12 ± 1.0 | 1.31 | 23.05 ± 0.7 | 40.37 ± 0.9 | 1.15 |
| KM | 20.77 ± 0.6 | 34.89 ± 0.9 | 2.85 | 43.24 ± 0.5 | 68.12 ± 0.7 | 1.94 |
| SM | 24.27 ± 0.5 | 40.82 ± 0.7 | 3.33 | 47.49 ± 0.9 | 73.12 ± 1.2 | 2.08 |
| FD | 41.64 ± 0.3 | 72.32 ± 0.6 | 5.89 | 72.65 ± 0.2 | 100.0 ± 0.5 | 2.85 |
| BM (13% moisture) | 40.27 ± 0.5 | 70.28 ± 0.7 | 5.73 | 71.41 ± 0.4 | 100.0 ± 0.6 | 2.85 |

*DE indicates dissolution efficiency; DP, dissolved percentage; RDR, relative dissolution rate (with reference to pure drug); DAN, danazol; PM, physical mixture; KM, kneading method; SM, solution method; FD, freeze-drying method; BM, ball-milling process.

Plasma progesterone analysis revealed a dose-dependent decrease (6.4-51.2 mg/kg) in progesterone content in the DAN-BCD binary system treatment mice. The lowest dose of the DAN-BCD binary system (6.4 mg/kg) was more efficient in reducing the plasma content of progesterone than the highest dose of DAN (51.2 mg/kg), indicating once again the superiority of the DAN-BCD binary system. However, the ability of DAN to reduce the progesterone content was not much different at higher doses (12.8, 25.6, and 51.2 mg/kg). This indicated that the anti-implantation activity of DAN was not increased as the dose was increased. There was a significant difference in the reduced values of progesterone content along with a significant difference in the reduced number of implantation sites for all dose levels (6.4-51.2 mg/kg) of the DAN-BCD binary system. The decrease in progesterone levels (on the ninth day of pregnancy) in all the treatment group mice as compared with the control group mice ruled out the possibility of pseudopregnancy. If pseudopregnancy had occurred, the progesterone level of the treatment animals on the ninth day of pregnancy would have been similar to that of the control group animals. Moreover, the observed decrease in progesterone level (on the ninth day of pregnancy) in treatment group mice as compared with control group mice indicated that the animals in treatment groups started cycling normally, even after the administration of DAN and the DAN-BCD binary system. In the mouse model, the DAN-BCD binary system showed dose-dependent postcoital anti-implantation activity in the range of 6.4 mg/kg to 51.2 mg/kg (equivalent to 50-400 mg human dose). DAN at 51.2 mg/kg in the mouse failed to show complete inhibition of implantation postcoitally. However, the DAN-BCD binary system at 51.2 mg/kg as a single oral dose showed complete inhibition of implantation when given postcoitally. The reason for such a difference in the activity could be related to the bioavailability of DAN when given orally, which in turn depends on the aqueous solubility of the drug.

### *Acute Toxicity Studies*

A single oral dose of 300 mg/kg of pure DAN to 3 mice did not produce any change in general behavior or autonomic signs and also did not lead to any mortality up to 14 days from the day of administration. The same dose given to another set of 3 mice also did not lead to any mortality up to 14 days, and all 3 mice were healthy. To the next set of 3 mice, 2000 mg/kg was administered. All 3 animals remained healthy even after 14 days. The same dose was repeated for another set of 3 mice. One mouse out of 3 showed abnormal gait and posture after 4 hours and stretching of hind legs after 6 hours. The same animal died within 48 hours of administration,

**Table 5.** Effect of DAN and DAN-BCD Binary System on Postcoital Implantation and Progesterone Content of Mice After Single-Dose Administration by Oral Route*

| Group | Number of Normal Implantation Sites, Mean ± SEM† | Number of Resorbing Implantation Sites, Mean ± SEM | % Inhibition of Implantation | % Inhibition of Progesterone Content |
|---|---|---|---|---|
| $G_A$ | 10.00 ± 0.267 | 0.25 ± 0.163 | NA | NA |
| $G_B$ | 9.25 ± 0.163 | 0.75 ± 0.163 | 7.5 | 43.8 |
| $G_C$ | 7.38 ± 0.183 | 2.38 ± 0.183 | 26.2 | 54.1 |
| $G_D$ | 6.25 ± 0.163 | 3.63 ± 0.183 | 37.5 | 60.3 |
| $G_E$ | 5.38 ± 0.183 | 4.88 ± 0.226 | 46.2 | 67.7 |
| $G_F$ | 6.38 ± 0.183 | 2.00 ± 0.267 | 36.2 | 73.6 |
| $G_G$ | 4.25 ± 0.163 | 1.63 ± 0.323 | 57.5 | 82.9 |
| $G_H$ | 2.38 ± 0.183 | 0.88 ± 0.295 | 76.2 | 96.5 |
| $G_I$ | 0 | 0 | 100 | 99.2 |

*DAN indicates danazol; BCD, β-cyclodextrin; NA, not applicable.

†One-way analysis of variance: The mean values of the number of implantation sites of all treatment groups are significantly different than the mean value of the number of implantation sites of the control group at $P \leq .05$ (confirmed by Dunnett's multiple comparison test).

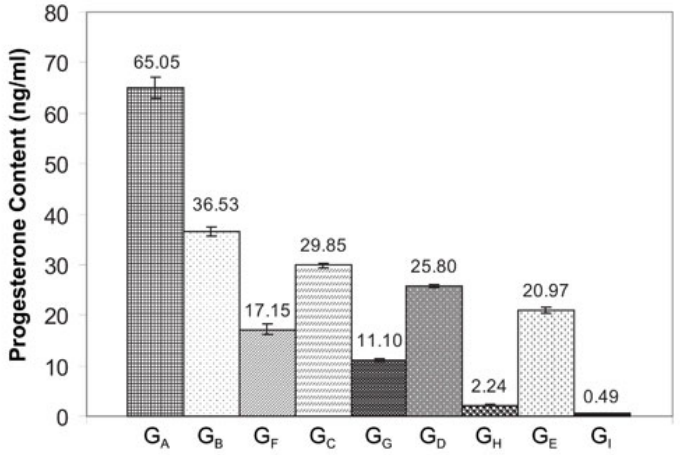


**Figure 8.** Effect of danazol and the danazol-β-cyclodextrin binary system on the plasma progesterone content of mice after postcoital single-dose oral administration. Mean SEM (Standard Error of Mean). One way ANOVA: Mean values of progesterone content of all treatment groups are significantly different than the mean value of progesterone content of control group at $p \leq 0.01$ (confirmed by Dunnett's multiple comparison test). $G_A$ indicates control; $G_B$, 6.4 mg/kg DAN; $G_C$,12.8 mg/kg DAN; $G_D$, 25.6 mg/kg DAN; $G_E$, 51.2 mg/kg DAN; $G_F$, 6.4 mg/kg DAN-BCD; $G_G$, 12.8 mg/kg DAN-BCD; $G_H$, 25.6 mg/kg DAN-BCD; and,$G_I$, 51.2 mg/kg DAN-BCD.

while the other 2 remained healthy even after 14 days. The observations revealed that the single oral dose of 300 or 2000 mg/kg of DAN is quite safe. Thus, according to the acute toxic class method, DAN can be classified as a GHS category 5 drug (>2000-2500) and has an $LD_{50}$ cutoff value of 2500 mg/kg of the body weight of a mouse (equivalent to 19.40 g in humans).

A single oral dose of 300 mg/kg of the DAN-BCD binary system to 3 mice did not produce any change in general behavior or autonomic signs and also did not lead to any mortality up to 14 days from the day of administration. The same dose given to another set of 3 mice also did not lead to any mortality up to 14 days, and all 3 mice were healthy. To the next set of 3 mice, 2000 mg/kg was administered. One mouse out of 3 showed abnormal gait and posture after 6 hours and stretching of hind legs after 8 hours. The same animal died within 24 to 48 hours, while the other 2 remained healthy even after 14 days. The same dose of 2000 mg/kg was repeated in another set of 3 animals. Two mice out of 3 died within 24 to 48 hours, while the third remained healthy even after 14 days.

The observations revealed that the single oral dose of 300 mg/kg of the DAN-BCD binary system is quite safe, while 2000 mg/kg is not safe. Moreover, the toxicity observed at the dose of 2000 mg/kg of the DAN-BCD binary system is due to DAN only and not due to BCD, since the dose of BCD administered is 6.73 g/kg, which is reported to be quite safe (rat $LD_{50}$ oral is 18.8 g/kg, equivalent to 15.04 g/kg in a mouse).[38] Thus, the DAN-BCD binary system can be classified as GHS category 4 (>300-2000) and has an $LD_{50}$ cutoff value of 2000 mg/kg of the body weight of a mouse (equivalent to 15.52 g in humans). The results in the mouse model revealed that although the DAN-BCD binary system showed a low $LD_{50}$ cutoff value as compared with DAN, the BCD binary system of DAN is likely to be safe up to 15.52 g in humans (for 70 kg), given as a single dose, which is ≈38.8 times higher than the intended single dose of DAN (400 mg) for emergency contraception.

## Conclusion

The present study clearly demonstrated that the aqueous solubility of DAN can be substantially increased by making a DAN-BCD binary system. Comilling of DAN with BCD in the presence of optimum moisture could serve as a simple and less expensive method for preparation of the binary system. Moreover, from the extrapolation of mouse studies we can conclude that the pharmaceutical preparation containing the DAN-BCD binary system could serve in humans as an oral emergency contraceptive at a physiologically acceptable single dose.

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