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Particle size reduction for improvement of oral bioavailability of hydrophobic drugs: I. Absolute oral bioavailability of nanocrystalline danazol in beagle dogs

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Abstract

Danazol is a poorly water soluble compound (10 μg/ml) that demonstrates poor bioavailability. The impact on bioavailability of increasing the area for dissolution by decreasing drug crystal particle size to less than 200 nm and stabilizing the particles to prevent agglomeration in the GI tract has been evaluated. A randomized three-way crossover study was conducted in fasted male beagle dogs to compare absolute oral bioavailability of danazol from three formulations. The three formulations examined were: A, an aqueous dispersion of nanoparticulate danazol (mean particle size 169 nm); B, danazol-hydroxypropyl-β-cyclodextrin (HPB) complex; C, an aqueous suspension of conventional danazol particles (mean particle size 10 μm). The three formulations were administered (200 mg) at 1 week intervals, and a fourth leg was conducted using intravenous danazol-HPB at a dose of 3 mg/kg. Plasma samples were obtained over the course of 24 h and analyzed by SPE-HPLC. Absolute oral bioavailability of each formulation was determined by comparison of oral AUC values to intravenous AUC values in the same dog, normalized to a 20 mg/kg dose. Absolute bioavailabilities of the three formulations were: nanoparticulate danazol, 82.3 $\pm$ 10.1%; cyclodextrin complex, 106.7 $\pm$ 12.3%; conventional danazol suspension, 5.1 $\pm$ 1.9%. The bioavailabilities of nanoparticle dispersion and cyclodextrin complex are not significantly different ($P = 0.05$) suggesting that the nanoparticle dispersion had overcome the dissolution rate limited bioavailabilty observed with conventional suspensions of danazol. This approach should have general applicability to many poorly soluble drugs with dissolution rate-limited absorption.

Keywords: Danazol; Bioavailability; Particle size; Nanoparticle; Poor water solubility; Dissolution; Dog

1. Introduction

The factors that can limit the oral bioavailability of drugs include first-pass metabolism, chemical stability, transport, solubility, and dissolution. These factors are generally addressed in discovery programs through chemical modifications of a molecule to improve bioavailability. However, this approach can result in significant alterations in the biological effect of the pharmacophor. Formulation approaches to modify bioavailability of

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a molecule are attractive alternatives but have met with modest success. Poorly soluble compounds tend to be eliminated from the GI tract before they have had opportunity to fully dissolve and be absorbed into the circulation. This results in low and erratic bioavailability and poor dose proportionality. These drawbacks have limited the development of poorly soluble molecules. A generally applicable approach to enhance the bioavailability of poorly water soluble molecules that are dissolution rate limited is presented herein.

The dissolution rate of a drug is a function of its intrinsic solubility and its particle size. Previous studies with a number of poorly soluble drugs have demonstrated that particle size reduction can lead to an increased rate of dissolution and higher oral bioavailability, including progesterone (Hargrove et al., 1989), nitrofurantoin (Watari et al., 1983), estradiol and estrone (Englund and Johansson, 1981), oxfendazole (Shastri et al., 1980) and proquazone (Nimmerfall and Rosenthaler, 1980). The majority of such studies have involved mechanical size reduction to particles larger than 1 μm. Kondo et al. (1993a) have reported a modest doubling in bioavailability for HO-221 when the mean particle size is reduced from 4.15 to 0.45 μm. This size reduction should lead to an increase in specific area and consequently dissolution of approx. 10-fold for the drug particles. Thus, a much greater improvement in bioavailability would be expected for such a change in particle size if dissolution was rate limiting, assuming that the particles remain discrete. If agglomeration of particles occurs in the GI tract then the effective surface area for dissolution will be reduced and concomitantly bioavailability. In subsequent publications Kondo et al. (1993b) reported that the 0.45 μm formulation of HO-221 was approx. 5–20% bioavailable, showed fed vs fasted variability and was not dose proportional, whereas in Kondo et al. (1994) coprecipitates with HPMCP (hydroxypropyl methylcellulose phthalate) were almost totally bioavailable. The data from these publications (Kondo et al., 1993b, 1994) are suggestive that HO-221 has dissolution rate limited bioavailability.

The above-cited studies demonstrate that there is still considerable potential for substantially enhancing bioavailability by particle size reduction in the submicron range. The present study was designed to evaluate the applicability of this approach to danazol, which has a low water solubility (10 μg/ml) and poor bioavailability. Accurate evaluation of this approach required comparison of a nanoparticulate danazol dispersion with a suspension of conventional danazol particles in the presence of the same stabilizing agent. The stabilizing agent was intended to prevent agglomeration of the nanoparticles both in the formulation and following addition to simulated gastric and intestinal fluids. A measure of the maximum oral bioavailability achievable by particle size reduction can be provided by a cyclodextrin-danazol complex, in which danazol is dispersed on a molecular level. Hydroxpropyl-β-cyclodextrin was chosen as the complexing agent, since this is the least toxic of available cyclodextrins. Dog was chosen as the species for bioavailability determinations, since this is the accepted model for preclinical bioequivalence studies, and data were available on the oral availability of the commercial danazol dosage form (Danocrine) in the same species.

2. Materials and methods

2.1. Materials

Danazol (17-α-pregna-2,4-dien-20-yno[2,3-*d*]isoxazol-17-ol) was obtained in micronized form (mean particle size, 10 μm; particle size range, 5–30 μm) from Sterling Drug Inc., New York, NY. Polyvinylpyrrolidone K-15 (PVP) was purchased from GAF, Wayne, NJ. Hydroxpropyl-β-cyclodextrin (HPB) was obtained from Pharmatec, Alachua, FL. Zirconium oxide grinding spheres 0.85–1.18 mm diameter were purchased from Zircoa Inc., Solon, OH.

2.2. Preparation of formulations

An intravenous formulation of danazol was prepared as by dissolving 10 mg/ml danazol in 50% w/w HPB, and sterilizing by filtration.

Three oral formulations of danazol were prepared as follows:

1. A hydroxypropyl-β-cyclodextrin (HPB)/danazol complex containing 10 mg/ml danazol in 50% w/w in HPB.
2. An aqueous suspension of conventional danazol particles containing 5% w/w danazol and 1.5% w/w PVP.
3. A nanoparticulate dispersion of danazol was prepared using a ball milling process. A 600 ml cylindrical vessel (inside diameter 7.6 cm) was filled approximately halfway with zirconium oxide grinding spheres. The following ingredients were added directly to the glass vessel: danazol, 10.8 g; polyvinylpyrrolidone K-15, 3.24 g; high purity water, 201.96 g. The cylindrical vessel was rotated horizontally about its axis at 57% of the critical speed. The critical speed is defined as the rotational speed of the grinding vessel when centrifuging of the grinding media occurs. After 5 days of ball milling, the slurry was separated from the grinding media through a screen and evaluated for particle size by sedimentation field flow fractionation and SEM and under light microscopy for particle agglomeration.

2.3. Bioavailability studies

Animal experiments were conducted in accordance with the National Institutes of Health, Guide for the Care and Use of Laboratory Animals and Institutional Animal Care and Use Committee.

The three oral formulations of danazol were administered in a randomized three-way crossover study to fasted male beagle dogs with 1 week between doses. An intravenous leg was conducted in all five dogs on the fourth week. The doses were:

1. Aqueous dispersion of nanoparticulate danazol (169 nm), 4.0 ml (200 mg) orally.
2. Hydroxypropyl-β-cyclodextrin/danazol complex, 20 ml (200 mg) orally.
3. Aqueous suspension of conventional danazol particles, 4.0 ml (200 mg) orally.
4. Hydroxypropyl-β-cyclodextrin/danazol complex, sterilized by filtration, 3 mg/kg intravenously.

Five dogs received each of the three oral formulations during the first 3 weeks of the protocol, with 1 week between administrations. Dogs were fasted for 12 h prior to oral dosing. The cephalic vein of each dog was cannulated and formulations were administered by oral gavage. Blood samples were withdrawn from the cephalic cannula at 0 (pre-medication), 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, and 24 h after administration and collected in heparinized tubes. Plasma (0.5 ml) was obtained immediately and rapidly frozen (dry ice/acetone) prior to analysis. Food was returned to each animal at 12 h post administration.

All five dogs received the intravenous formulation in the fourth week. Dogs were fasted as for oral administration and cannulated in the cephalic vein. The intravenous formulation was administered via the cephalic cannula and blood samples were withdrawn at 0 (pre-medication), 2, 4, 8, 15, 30 min and 1, 2, 4, 8, 12 and 24 h after administration, and collected into heparinized tubes. Plasma (0.5 ml) was obtained immediately and rapidly frozen (dry ice/acetone) prior to analysis. Food was returned to each animal at 12 h post administration.

2.4. Bioanalytical methods

Plasma samples were prepared by solid-phase extraction prior to analysis. Plasma (250 μl) was mixed with 25 μl water and 50 μl of internal standard (testosterone propionate, 220 μg/ml in methanol). Samples were mixed by vortex and applied to 1 ml C18 SPE cartridges (J.T. Baker) prepared by application of 1 column volume each of methanol and water. Following application of samples, the columns were washed with 0.5 ml water and 0.5 ml 10% acetonitrile. Danazol and internal standard were eluted in 0.5 ml acetonitrile and eluent was evaporated to dryness under nitrogen. Samples were reconstituted in 100 μl of mobile phase (0.01 M ammonium phosphate, pH 6.8/60% acetonitrile) and transferred to WISP mini-vials for HPLC analysis. Recovery of danazol and testosterone propionate by this method was > 85%. Standard curves were linear over the

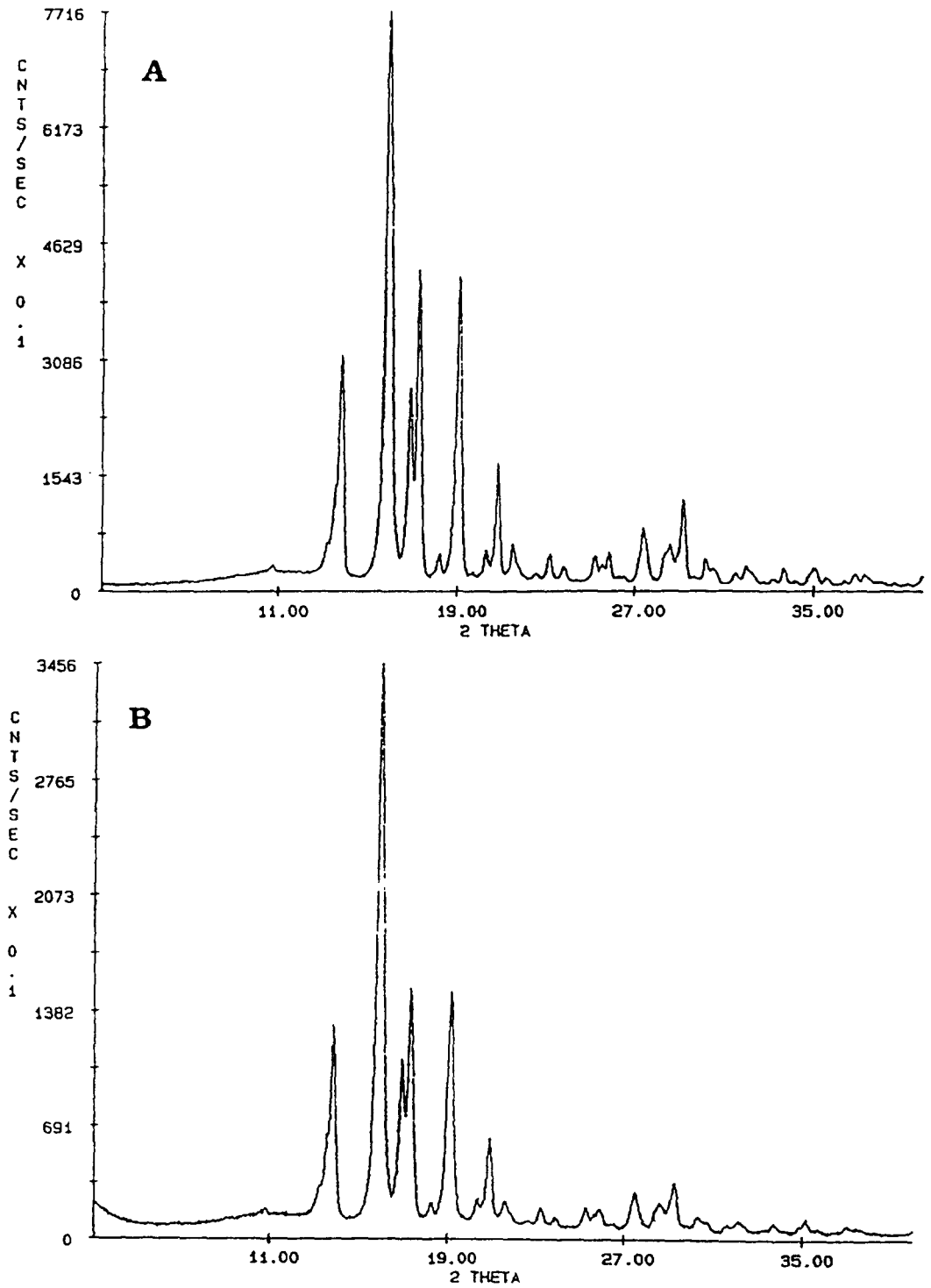

Fig. 1. X-ray diffraction patterns: (A) danazol before milling; (B) aqueous nanoparticulate danazol dispersion.

range 20 to 4000 ng/ml. The minimum quantifiable level of danazol in dog plasma was 10 ng/ml.

Samples were analyzed by HPLC using a published method (Nygard et al., 1987). The column was a Zorbax C8 (3 cm × 4 mm) with a Zorbax C8 guard cartridge. The mobile phase was A: 0.01 M ammonium phosphate, pH 6.8/40% acetonitrile, and B: 0.01 M ammonium phosphate, pH 6.8/60% acetonitrile, with a linear gradient from 50% B to 100% B over 7 min. The flow rate was 2.5 ml/min with a run time of 15 min.

3. Results and discussion

The use of particle size reduction to increase the surface area for dissolution and thereby increase bioavailability of poorly water soluble molecules has met with modest success, particularly in the submicron size range (Kondo et al., 1993b). Decreasing particle size increases the surface-to-volume ratio and size is inversely proportional to specific surface area for spherical particles (Schott, 1985), e.g., a 10-fold reduction in mean particle size results in a 10-fold increase in specific surface area. Thus, for drugs with dissolution rate limited bioavailability a 10-fold reduction in particle size would be anticipated to increase dissolution rate by 10-fold and subsequently increase bioavailability by 10-fold. The modest gains in bioavailability observed with particle size reduction have been attributed to agglomeration of particles in the milieu of the GI tract, resulting in a decrease in the effective surface area for dissolution. Agglomeration is of particular concern for particles in the submicron range (Higuchi et al., 1985) necessitating the use of stabilizers.

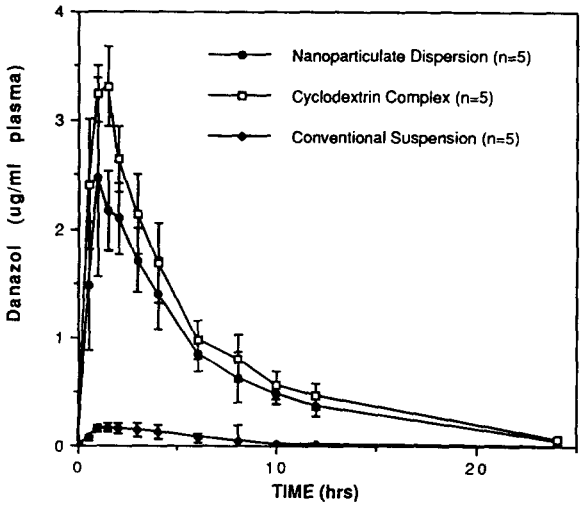

Fig. 2. Effect of formulation on mean ± SE plasma concentrations of danazol following oral administration of three formulations to fasted male beagle dogs.

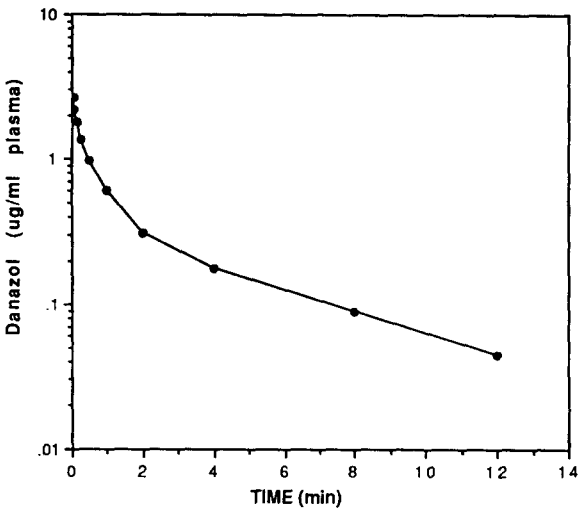

Fig. 3. Mean plasma concentrations of danazol following intravenous administration of cyclodextrin/danazol complex to fasted male beagle dogs (SE values lie within mean data point).

Stabilization of a nanoparticulate dispersion can be achieved by steric or charge stabilization methods. The variation in pH during transit through the GI tract may preclude the use of a charge stabilizing suspending agent, such as sodium lauryl sulfate. The low pH of the stomach can result in neutralization of cationic surfactant stabilizer's charge leading to irreversible agglomeration of nanoparticles. Nanoparticles of danazol stabilized with sodium lauryl sulfate agglomerate when exposed to simulated gastric fluid. Generally, anionic surfactant stabilizers are too toxic to be considered for oral application. Charged surfactant stabilizer performance can be

dramatically impacted by the ionic strength of the medium. However, dispersions prepared with steric stabilizers would not be expected to demonstrate pH and ionic strength dependent stability.

A nanoparticulate formulation of danazol (5% w/w) stabilized with PVP (1.5% w/w) was prepared as outlined in the methods section. The resultant formulation was sized using a sedimentation field flow fractionator and had a number average particle diameter of 84.9 nm. and a weight average particle diameter of 169.1 nm. The particles varied in size from 26 to 340 nm. The crystal structure was determined by X-ray diffraction to be unchanged by the dispersion process (see Fig. 1). SEM of the formulation supported the size range reported above. The formulation did not flocculate or agglomerate when added to simulated gastric fluid or intestinal fluid USP. The formulation was physically and chemically stable for at least 6 months at room temperature.

The concentration of 1.5% w/w PVP was found to be critical in conferring stability of the nanoparticles. A nanoparticulate formulation of danazol (5% w/w) stabilized with PVP (1.0% w/w) was prepared as outlined in section 2. The resultant formulation was observed under light microscopy. The slurry was partially aggregated with aggregates of up to 10 μm in diameter. The amount of stabilizer (PVP 1%) was apparently insufficient to confer stability.

The mean plasma levels obtained for the three oral formulations are presented in Fig. 2. The mean plasma levels obtained for the intravenous administration are presented in Fig. 3.

Model independent pharmacokinetic parameters C_{max} and t_{max} are presented in Table 1 for the oral formulations. Values of the area under individual plasma time curves were calculated by computer using NONLIN84 analytical methods. The area under the curve for all animals was normalized to a 20 mg/kg dose using the actual dose administered and the weight of each dog at the time of administration, as follows:

$$\text{AUC for 20 mg/kg dose} = \frac{\text{AUC} \times \text{dog weight (kg)} \times 20}{\text{actual dose (mg)}}$$

Normalized AUC values are presented in Table 1. Absolute oral bioavailabilities of each formulation were calculated using the AUC values from intravenous administration in the same dog. The absolute oral bioavailabilities of the three oral formulations are presented in Table 1. The nanoparticulate dispersion and cyclodextrin complex were both significantly different from the conventional danazol suspension (unpaired Student's t-test). However, the nanoparticle dispersion and cyclodextrin complex are not significantly different at $P = 0.05$. The cyclodextrin complex given orally was not significantly different at $P = 0.05$ from when given intravenously.

The size reduction of danazol crystals from a mean weight average particle size of 10 μm to 169 nm should result in an approx. 59-fold increase in specific surface area based on the assumption that crystals are essentially spherical. SEM evaluation of the crystals supports this assumption. Thus, if discreet nanoparticles are maintained in the GI tract a significant enhancement in bioavailability of up to 59-fold compared to a conventional dispersion would be anticipated, subject to a boundary condition of 100% bioavailability assuming no permeation rate limitation or metabolic or chemical instability losses. The bioavailability data obtained with the dana-

Table 1
Pharmacokinetic parameters following oral and intravenous administration of danazol formulations to fasted male beagle dogs ($n = 5$)

Formulation	C_{max} (μg/ml)	t_{max} (h)	AUC [a] (μg h ml^{-1})	Absolute bioavailability
Cyclodextrin oral	3.94 ± 0.14	1.2 ± 0.2	20.4 ± 1.9	106.7 ± 12.3
Nanoparticle dispersion	3.01 ± 0.80	1.5 ± 0.3	16.5 ± 3.2	82.3 ± 10.1
Conventional suspension	0.20 ± 0.06	1.7 ± 0.4	1.0 ± 0.4	5.1 ± 1.9
Cyclodextrin i.v.			19.8 ± 0.6	100

[a] Based on NONLIN84 AUC values normalized to a dose of 20 mg/kg.

zol/cyclodextrin complex suggest that the influence of permeation, metabolic and chemical instabilities on bioavailability of danazol are minor. The cyclodextrin complex is a molecular dispersion of danazol and represents a formulation in which there is no dissolution step to limit bioavailability. This formulation may be considered the standard to which other oral formulations should be compared. The bioavailability of the nanoparticle dispersion and cyclodextrin complex are not significantly different ($P = 0.05$) suggesting that the nanoparticle dispersion had overcome the dissolution rate limited bioavailabilty observed with conventional suspensions of danazol.

The approach of reducing particle size to the low nanometer range in the presence of a steric stabilizer for enhancing oral bioavailability is anticipated to have general applicability to many poorly soluble drugs with dissolution rate-limited absorption. The specific use of a steric stabilizer (e.g., PVP) to stabilize the nanoparticulate dispersion against agglomeration in gastric and intestinal environments is a key aspect in preserving the benefits of particle size reduction in vivo, i.e., greatly increased available surface area for dissolution.

4. Conclusions

An aqueous nanoparticle dispersion of danazol (5% w/w) and PVP (1.5% w/w) was prepared using wet milling techniques to achieve a weight average particle size of 169 nm. The formulation did not agglomerate or flocculate when added to simulated gastric or intestinal fluid.

The absolute bioavailability in fasted male beagle dogs of the nanoparticulate danazol formulation ($82.3 \pm 10.1\%$), danazol-hydroxypropyl-β-cyclodextrin complex ($106 \pm 12.3\%$) and an aqueous suspension of conventional danazol particles ($5.1 \pm 1.9\%$) were determined. The nanoparticle dispersion and cyclodextrin complex were not significantly different ($P = 0.05$) suggesting that the nanoparticle dispersion has overcome the dissolution rate limited bioavailability observed with conventional suspensions of danazol.

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