Original Research Article

Enhanced bioavailability of danazol nanosuspensions by wet milling and high-pressure homogenization

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Abstract

Introduction: The majority of drugs obtained through synthesis and development show poor aqueous solubility and dissolution velocity, resulting in reduced bioavailability of drugs. Most of these problems arise from formulation-related performance issues, and an efficient way to overcome these obstacles and to increase dissolution velocity is to reduce the particle size of drug substances to form drug nanosuspensions. **Materials and Methods:** Danazol nanosuspensions were prepared by wet milling (WM) and high-pressure homogenization (HPH) methods. The nanosuspensions obtained using these fabrication methods were analyzed for their particle size, surface charge, and the crystallinity of the product was assessed by X-ray diffraction (XRD) and differential scanning calorimetry techniques. To determine *in vitro* and *in vivo* performances of the prepared nanosuspensions, dissolution velocity, and bioavailability studies were performed. **Results:** Particle size and zeta potential analysis showed the formation of nanosized particles with a negative charge on the surface. XRD depicted the nanocrystalline nature of danazol with low diffraction intensities. With increased surface area and saturation solubility, the nanosuspensions showed enhanced dissolution velocity and oral bioavailability in rats when compared to the bulk danazol suspension. **Conclusions:** The results suggest that the preparation of nanosuspensions by WM or HPH is a promising approach to formulate new drugs or to reformulate existing drugs with poorly water-soluble properties.

Key words: Danazol, high-pressure homogenization, nanosuspensions, wet milling

INTRODUCTION

The introduction of new techniques such as combinatorial chemistry combined with high-throughput screening has accelerated the synthesis of new drug candidate compounds. However, one of the major problems facing the industry today is the tremendous increase in the synthesis of poorly water-soluble drugs. Around 60% of drugs obtained from synthesis and 40% of drugs obtained from the development, pipeline shows poor aqueous solubility.[1-3] Following oral administration, the major limitation of these poorly water-soluble drugs is their inability to dissolve in the gastrointestinal tract fluid, which results in decreased bioavailability of the drugs. It is possible to increase the bioavailability of these drugs by increasing their dissolution velocity. Several strategies have been introduced, for example, the use of solvent mixtures, inclusion complexes such as cyclodextrins,[4] oil/water or water/oil/water emulsions,[5-7] micronization,[8,9] liposomes,[10] and surfactant-assisted dispersions, for increasing the dissolution velocity of these poorly soluble drugs. However, there are several limitations to these approaches such as the solubility of the drug in solvents or oils (oil/water emulsions) or formulation constraints such as the need to have the right structure and size of drug molecules to fit into a cyclodextrin structure. Hence, it is important to develop

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Access this article online

Quick Response Code:

Website: www.jpionline.org

DOI: 10.4103/2230-973X.195931

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How to cite this article: Kanthamneni N, Valiveti S, Patel M, Xia H, Tseng YC. Enhanced bioavailability of danazol nanosuspensions by wet milling and high-pressure homogenization. Int J Pharma Investig 2016;6:218-24.

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a universal engineering technology that can be applied to the majority of poorly water-soluble drugs.

One of such formulation approaches is the nanosizing of drugs to create particles with a mean diameter of <1 μm. Nanosizing of drug molecules enhances the dissolution velocity due to increased surface area, increased saturation solubility, and decreased diffusion distance of the drug particles.[11,12] Reduction in particle size can be performed using a variety of techniques, and the use of technologies has emerged as a promising method. Nanosuspensions are sub-micron colloidal dispersions of drug particles in an outer liquid phase; the dispersion medium can be aqueous, nonaqueous (oils, polyethylene glycol) or a mixture of both (water-ethanol mixtures) and with or without stabilizing agents.[13] Drugs, which are insoluble in both water and oils, can be used to formulate nanosuspensions with enhanced physical stability and have high batch reproducibility.[14] Nanosuspensions obtained from solid pure drug particles can be crystalline, partially crystalline or completely amorphous in nature,[15] and they enhance the bioavailability of drugs because of the additional interactive forces with the surface of mucosal membranes.[16] Hence, it is a good approach to reformulate existing poorly water-soluble drugs to remove toxicologically less favorable solubilizing excipients without modifying the principle therapeutic effect.

There are several techniques to produce nanotechnology-based suspensions such as precipitation, wet milling (WM), and high-pressure homogenization (HPH).[12,17,18] Precipitation method, also described as "bottom-up technology," involves precipitation of drug nanoparticles by adding the solvent containing dissolved drug to a nonsolvent, in which the drug is not soluble. However, use of this method has some limitations in the areas of controlling the size of particles and the ability of the drug to dissolve in organic solvents.[19] The WM method was first developed and reported by Livesidge *et al*. in 1992 and later commercialized by Nanosystems.[12] In the WM method, the nanosizing of drugs was carried out using glass beads or media mills. Nanocrystals were generated as a result of high-energy shear forces generated by the impaction of milling media with the drug.[20] As the drug is suspended in aqueous media, the thermal energy generated is considered lower than dry milling techniques and is also considered an efficient way to generate drug crystals.[21] Some products already available in the market and developed with this technique are Rapamune and Emend, launched in 2002 and 2003, respectively. Another method of producing nanocrystalline suspensions is by means of the HPH method, developed by Muller in 1999.[22] It is a fluid mechanical process that involves the breaking of particles into micro- or nano-scale to create a stable dispersion or emulsion for further processing. Particle reduction takes place due to cavitation with high shear forces and collision of particles against each other. HPH works by creating high shear and turbulence by a homogenizing valve on compression, thus inducing disintegration of particles and resulting in a formulation of uniformly sized particles.[22]

We evaluated the WM and HPH methods in the preparation of danazol nanosuspensions and tested the hypothesis of dissolution enhancement at the nanoscale range. Danazol was selected as a model drug as it is a Biopharmaceutical Classification System Class II compound whose oral bioavailability is limited by low dissolution velocity and water solubility (<0.001 mg/ml, logP = 4.53).[23,24] Danazol has a C_{max} and T_{max} of 53.2 ng/ml and 2.5 h, respectively, after administering a single dose of 200 mg.[25] The physicochemical characterization of the nanosized particles such as thermal behavior and crystalline nature were determined using differential scanning calorimetry (DSC) and X-ray diffraction (XRD), respectively. Furthermore, to evaluate any correlation with the *in vitro* dissolution results, the *in vivo* data were generated following oral administration in rats.

MATERIALS AND METHODS

Materials

Danazol was purchased from Spectrum Chemicals. Sodium glycocholate was obtained from ACROS Organics. Type 2 glass beads used for the technique were purchased from Glenmills Inc. Capsules (size 2) were obtained from Capsugel, USA. All materials, reagents, and solvents were purchased from commercial sources and used without further purification.

Methods

Preparation of nanosuspensions

High-pressure homogenization

HPH was performed using a piston-gap High-Pressure Homogenizer (AVESTIN, Inc., Canada). To prevent blocking of the homogenizer valve, premilling of the aqueous suspension (100 mg of danazol in 7 ml of 0.2% w/v sodium glycocholate solution) was performed in a Covaris AFA System (E-Series, Covaris, Inc., USA) for 120 s. The premilled suspension was then passed through a C-3 high-pressure homogenizer for 80 cycles at 30,000 psi. The resulting nanosuspension was collected and mannitol was added in a 1:1 ratio before lyophilization for 24 h.

Wet milling

In the WM process, 100 mg of drug was suspended in 1.9 ml of sodium glycocholate solution (0.2% w/v) and loaded into a 4 ml propylene tube containing 2.1 g of grinding media (zirconium, 0.68 mm). The pearls were stirred with the help of a magnetic stirrer on a magnetic plate (VP 706-7, V and *P* Scientific, Inc., USA) at 1600 rpm for 4 h. Samples were drawn and filtered, followed by lyophilization by adding a 1:1 ratio of danazol: Mannitol.

Particle size analysis

The size and size distribution of the prepared nanosuspensions were measured using a dynamic light scattering size analyzer. The analyses were performed at a scattering angle of 90° and a temperature of 25°C. Particle size analysis of dispersions was carried out before and after lyophilization. The lyophilized powders were dispersed in distilled water at a concentration

of 0.1 mg/ml before analysis. Inactive ingredients (sodium glycocholate, mannitol) constituting lyophilized powders become dissolved when dispersed in water, resulting in dispersion and measurement of only drug particles. The diluted samples were placed in cuvettes and analyzed using a Malvern Zetasizer (Malvern Instruments Ltd., Worcestershire, UK). The obtained homogeneous dispersions were assessed for mean diameter (nm) and size distribution (%). The data presented are the mean values of three measurements produced under identical conditions.

Zeta potential

Zeta potential of the nanosuspension was carried out using a Zetasizer (Malvern Instruments Ltd., Worcestershire, UK). After lyophilization, each of the dispersions was subjected to zeta potential analysis by dispersing the lyophilized powders in distilled water at a concentration of 0.1 mg/ml. The data presented are the mean values of four measurements produced under identical conditions.

X-ray diffraction

The crystalline nature of the drug was determined by XRD analysis. A Bruker X-Ray Powder Diffractometer D8 Advance (Bruker AXS Inc., Germany) was used to obtain the XRD pattern. The analysis was performed at 40 mA and 40 kV with CuKα radiation (1.54 Å) in parallel beam mode utilizing a sodium iodide scintillation detector. Samples were scanned over a range of 2θ values from 3° to 35° with a step size of 0.05° (2θ) and a counting time of 4 or 0.6 s.

Differential scanning calorimetry

DSC is a thermodynamic analytical technique used to assess the crystalline nature and thermal behavior of powders. This was obtained with a Differential Scanning Calorimeter (Q1000, TA Instruments, USA). DSC scans of empty and lyophilized danazol particles were performed using aluminum pans under dynamic N_2 atmosphere (100 mL/min) and a heating rate of 10°C/min between −30°C and 350°C. Danazol, surfactants, and mannitol alone were used as controls, and the empty closed aluminum pan served as a reference.

In vitro dissolution

In vitro dissolution studies were carried out using USP Type II paddle apparatus (Distek Dissolution System 2100C, Distek, USA), rotating at 100 rpm and a temperature of 37 ± 0.5°C. The drug release studies were conducted in 100 ml of phosphate buffer solution at pH of 7.4 for 2 h. Known weights of the lyophilized powders (equivalent to 3 mg of the drug) were filled into size 2 capsules. To avoid floating of the capsule, sinkers were used. The samples were collected at predetermined intervals (0, 5, 10, 20, 40, 60, 90, and 120 min), filtered through PDVF 0.45 μ filter and analyzed by HPLC (Agilent 1100 Series) fitted with a reverse phase column (C18, 5 μm, 4.6 mm × 150 mm). Samples were passed through the column at a flow rate of 1 mL/min in a mobile phase of 0.1% trifluoro acetic acid in water (A) and acetonitrile (B) and detected at 286 nm. Gradient was maintained at 5% B for 0.1 min, increased to 95% B in 7.0 min and held at this level for 1.5 min, before decreasing to the initial 5% B for the rest of the run. At each time point, 2 ml samples were withdrawn from the dissolution chamber and replaced with fresh media.

In vivo experiments

Male Sprague-Dawley rats weighing around 210 ± 15 g were obtained from Charles River Laboratories International, Inc., Wilmington, MA, USA. The animals were acclimated for 2 weeks before experimentation and were fed with a standard diet and water *ad libitum*. All experiments and procedures were performed under protocols approved by the Boehringer-Ingelheim Institutional Animal Care and Use Committee, and according to the United States Animal Welfare Act. Three rats for each suspension were dosed by oral gavage at 10 mg/kg. At predetermined time points (0, 0.25, 0.5, 1, 2, 4, 6, 8, 24 h), blood samples were collected from each rat into Eppendorf tubes containing 7.5% sodium ethylenediamine tetraacetate solution. The drug was extracted from blood and using LC/MS/MS analysis, danazol in rat plasma was determined. Chromatography was achieved by Agilent 1200 Series pump, Varian Polaris C18, 5 μm, 2.1 mm × 50 mm (Palo Alto, CA, USA) column and gradient elution. The mobile phase consisted of 10 mM ammonium acetate in water (A) and acetonitrile (B), at a flow rate of 0.5 mL/min. The gradient was maintained at 5% B for 0.1 min, increased to 95% B in 1.0 min, and held at this level for 1.0 min, finally decreasing to the initial 5% B within 0.3 min. There was a 0.5 min reequilibration with mobile phase between each run. Quantitation was handled by an API 5000 triple quadrupole mass spectrometer (Applied Bios stems, Toronto, Canada), set to electrospray positive ionization mode, with Analyst 1.4.2 (SCIEX, USA) operating software.

RESULTS AND DISCUSSION

Particle size and zeta potential

Particle size and size distribution play a major role in determining the dissolution velocity, saturation solubility, and biological performance of nanosuspensions.[12] Figure 1 shows the mean particle size of danazol nanosuspensions prepared by either the WM or HPH method. The particle size analysis was determined before and after lyophilization of nanosuspensions. Lyophilized powders with mannitol were easily dispersed, and the suspension showed similar particle size before and after lyophilization. However, the nanosuspension produced by WM (400 nm) had relatively smaller particles when compared to HPH (600 nm). The number of contact points between the particles and media increased exponentially with small sized beads used in the WM method, resulting in more efficient grinding of particles and smaller sized particles when compared to breaking down of drug crystals at crystal imperfections with the HPH method.[26]

As explained by Merisko-Liversidge *et al*., the decrease of particle size increases surface area and surface energy.[20] Unless carefully monitored, this high surface energy might agglomerate the nanometer-sized particles to thermodynamically stable aggregates,

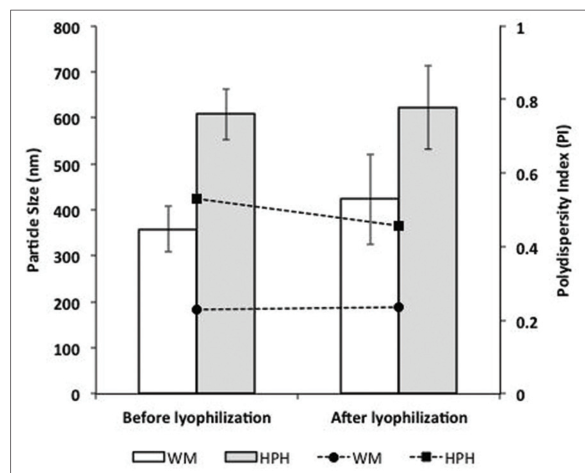

Figure 1: Mean particle size (bars) and polydispersity index (dotted line) values of danazol nanosuspensions prepared by wet milling and high-pressure homogenization methods. Results show the difference in particle size before and after lyophilization of nanosuspensions with mannitol. Each data point is represented as mean ± standard deviation (n = 3)

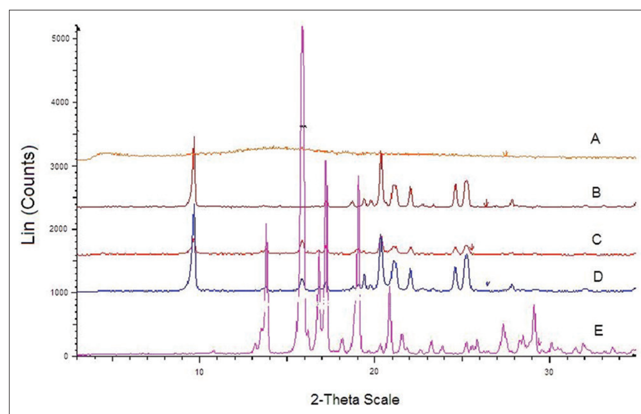

Figure 2: X-ray diffraction thermograms. A: Sodium glycocholate, B: Lyophilized danazol nanoparticles prepared by high-pressure homogenization, C: Lyophilized danazol nanoparticles prepared by wet milling, D: Mannitol, E: Bulk danazol

resulting in Ostwald ripening. Ostwald ripening results in particle size growth of active drug due to uncontrolled precipitation or crystallization.[27] Danazol nanosuspensions produced without addition of surfactants exhibited agglomeration (data not shown) and to prevent the agglomeration, sodium glycocholate was added as a surfactants. Surfactants are believed to impart steric or ionic stabilization to the surface of the nanoparticles and prevent agglomeration.[28] In addition, polydispersity index values (PDI) should be as low as possible to prevent the occurrence of different saturation solubilities and concentration gradients produced by a wide range of particle sizes.[12,29] The PDI values of the danazol nanosuspensions were <0.3 prepared by WM and 0.45 by HPH, respectively, indicating the WM method produces more homogeneous particles compared to the HPH method.

Zeta potential (mV) is a measure of the surface charge of an entity and gives an indication of the physical stability of nanosuspensions.[12] Both the drug and the stabilizer govern the zeta potential value. Values of ± 30 mv are required for electrostatically stabilized nanosuspensions.[30] Zeta potential values for lyophilized danazol nanosuspensions prepared by WM and HPH methods are shown in Table 1. There is no significant difference in zeta potential values between both methods, and each has values above −30 mV. The high charge on the surface of particles makes them able to repel one another and prevents agglomeration of the particles.

X-ray diffraction

During nanosizing, the crystalline state of drug particles may be converted to an amorphous state. Changes in the physical state of drug particles can be determined using XRD analysis and can be supported with DSC data.[12] Figure 2 shows the results of XRD peaks for bulk danazol and lyophilized danazol nanoparticles prepared by the HPH and WM methods. The bulk danazol showed XRD peaks similar to those obtained by Liversidge and Cundy.[31,32] The characteristic diffraction peaks of danazol were at 15.8, 17.2, and 19.0 (2θ) degrees. The danazol nanoparticles showed similar characteristic diffraction peaks but with weaker diffraction intensity. The peak intensity of the nanoparticles reduced to less than half compared with that of the bulk danazol, suggesting that there was no polymorphic transition of danazol by the preparation methods. This weaker diffraction intensity may be attributed to the reduction of particle size to the nanoscale range, which facilitates better X-ray transmission and weaker diffraction intensities.[21,33]

Table 1: Zeta potential values for danazol nanosuspensions prepared by high-pressure homogenization and wet milling

	Zeta potential (mV)
Danazol nps - HPH	−35.8±4.7
Danazol nps - WM	−42.7±5.66

Values are presented as mean±SD (n=3). HPH: High-pressure homogenization, WM: Wet milling, SD: Standard deviation, nps: Nanoparticles

Differential scanning calorimetry

The crystalline nature and changes to the polymorphic nature of a drug during nanosizing can also be identified by DSC.[34] Figure 3 provides the DSC thermograms of bulk danazol, lyophilized danazol particles (prepared by WM and HPH), mannitol, and sodium glycocholate. Bulk danazol showed a characteristic endothermic peak at 227°C, similar to one provided by Tanaka *et al*. and Rogers *et al*.[21,35] Mannitol had a characteristic peak at 165°C,[31] and sodium glycocholate showed amorphous nature with no characteristic peaks. However, DSC thermograms of lyophilized danazol particles produced by both methods showed no visible endothermic danazol peak (at 227°C) but showed a characteristic mannitol peak. Absence of the endothermic melting peak of danazol may indicate the conversion of crystalline state to semi-crystalline or amorphous state of danazol during nanosizing as there is a significant amount of grinding involved.[36-38] However, the hypothesis of phase conversion might not be prevalent with these nanosuspensions as it is in conflict with the

observation by XRD [Figure 2]. Although XRD cannot quantify the presence of crystalline material to about 3–5% or less,[39,40] it is considered to be more sensitive and provides greater clarity and more comprehensive understanding of events.[41] Because of its higher sensitivity, it is recommended by the FDA over other techniques such as FTIR/Raman/DSC in the determination of polymorphism.[42] Hence, the undetectable melting peak of danazol nanoparticles by DSC might be due to the insensitivity of our equipment to detect the nanosized crystalline danazol.

In vitro dissolution

Figure 4 shows the release profiles of danazol from the bulk danazol suspension and danazol nanosuspensions prepared by both WM and HPH methods. As shown in Figure 4, a significantly faster dissolution velocity was observed with the nanosuspensions when compared with the bulk. It took an hour for 50% of the bulk drug dissolved in the dissolution media compared with 15 min for the nanosuspensions. Furthermore, a significantly faster dissolution was observed from the nanosuspension prepared by HPH in comparison with WM. This increased dissolution velocity of the nanosuspension is attributed to several factors such as reduced particle size and increased surface area; along with increased saturation solubility caused by the vapor pressure effect.[22] Additionally, as explained by the Prandtl equation, the diffusional distance for the drug decreases by increasing curvature of nanosized particles, which contributes to the increase in the dissolution velocity.[12] The increased curvature can be attributed to the presence of sodium glycocholate around the particles. In addition, due to the presence of the surfactant, the particles were wetted immediately, resulting in fast release of danazol from the nanosuspensions when compared with the bulk suspension.

In vivo release

Oral bioavailability of danazol from the bulk danazol suspension and danazol nanosuspensions is shown in Figure 5, and the pharmacokinetic parameters are shown in Table 2. The increase in dissolution velocity of nanosuspensions and thereby increased bioavailability has demonstrated moderate success. Area under the curve (AUC) values for the nanosuspensions prepared by HPH and WM were around 681 and 915 nM.hr, respectively, when compared with 332 nM.hr for the bulk suspension, which represented a 2.1- and 2.8-fold increase in AUC, respectively. Similarly, C_{max} values for the HPH and WM nanosuspensions were 385 and 656 nM, respectively, compared with 97 nM for the bulk suspension, thus showing a 6.7- and 4-fold increase in C_{max}, respectively. This increased bioavailability is due to the increased dissolution velocity and saturation solubility of both nanosuspensions in the GI tract.[43] However, the observed faster *in vitro* dissolution of the HPH nanosuspension compared with that of the WM nanosuspension was not reflected in the *in vivo* results.

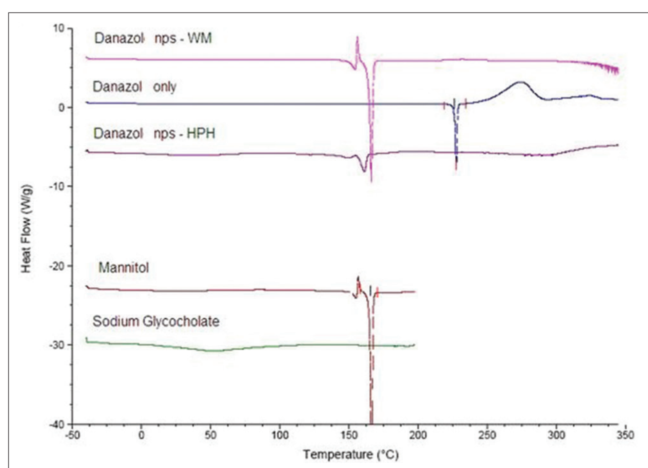

Figure 3: Differential scanning calorimetry thermograms for bulk danazol (Danazol), lyophilized danazol nanoparticles prepared by homogenization (Danazol nps-HPH), lyophilized danazol nanoparticles prepared by wet milling (Danazol rps-WM), mannitol and sodium glycocholate

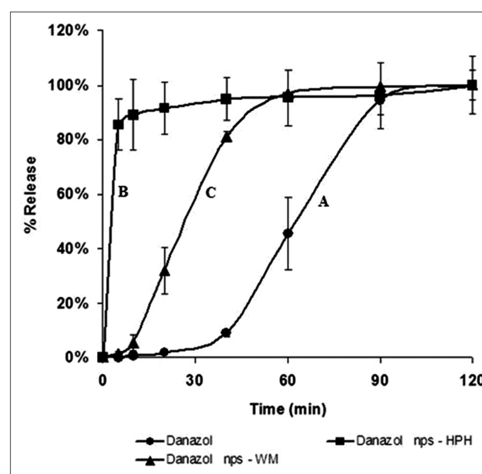

Figure 4: Release profiles. A: Bulk danazol suspension (Danazol), B: Danazol nanosuspensions prepared by high-pressure homogenization (Danazol nps-HPH), C: Danazol nanosuspensions prepared by wet milling (Danazol rps-WM). Each data point is represented as mean ± standard deviation (n = 3)

Table 2: Mean values of the pharmacokinetic parameters following oral administration of bulk danazol suspension, danazol nanoparticles – high-pressure homogenization (Danazole nps-HPH), and danazol nanoparticles – wet milling (Danazole nps-WM) at 10 mg/kg to rats (n=3)

Formulation	AUC* information (nM.h)	C_{max} (nM)	T_{max} (h)	T1/2 (h)	AUC* fold increase	C_{max} fold increase
Danazol	331.7±236.0	97.3±96.5	1.6±0.6	1.26±0.6	1	1
Danazole nps - HPH	680.5±381.5	385.0±205.9	0.7±0.3	0.98±0.4	2.1	4.0
Danazole nps - WM	914.7±211.6	655.8±252.6	0.5±0.0	13.14±7.9	2.8	6.7

*Area under curve

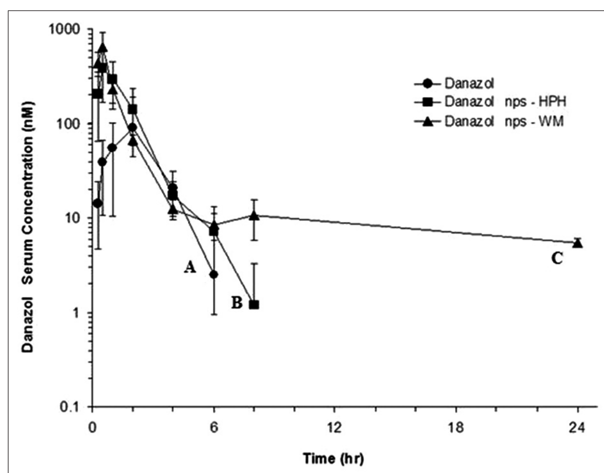

Figure 5: Mean plasma levels of danazol versus time after oral administration. A: Bulk danazol suspension, B: Danazol nanosuspensions prepared by high-pressure homogenization (Danazol nps-HPH), C: Danazol nanosuspensions prepared by wet milling (Danazol rps-WM) at 10 mg/kg to rats. Each data point is represented as mean ± standard deviation (*n* = 3)

CONCLUSIONS

To improve the dissolution velocity and thereby bioavailability of poorly soluble drugs, danazol nanosuspensions were prepared using WM and HPH methods with sodium glycocholate as a stabilizer. XRD results showed nanocrystalline nature of lyophilized nanosized danazol particles with low diffraction intensities. The lyophilized particles with mannitol showed good resuspendability. Dissolution studies showed increased dissolution velocity of the nanosuspenions when compared with the bulk suspension due to increased solubility, increased surface area, and decreased diffusional distance. On oral administration, the nanosuspensions showed greater C_{max} and AUC than the bulk suspension. Hence, it indicates that the development of nanosuspensions is a practical approach to improve bioavailability of poorly water-soluble drugs, and can be used to reformulate existing drugs for enhancing bioavailability.

Acknowledgments

The authors would like to thank Dr. Raj Nagaraja for his assistance in the conduct of the rat bioavailability studies, and Dr. Anupama Nagaraja for her help with the experiments. The authors would also like to thank Dr. Sreeram Potluri and Dr. Bryant Yung for their help. This work was supported by Boehringer Ingelheim Pharmaceuticals, Inc.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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