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Supersaturation Produces High Bioavailability of Amorphous Danazol Particles Formed by Evaporative Precipitation into Aqueous Solution and Spray Freezing into Liquid Technologies

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ABSTRACT The bioavailability of high surface area danazol formulations was evaluated in a mouse model to determine what effect high supersaturation, as measured in vitro, has on the absorption of a poorly water soluble drug. Danazol, a biopharmaceutics classification system II (BCS II) compound, was used as the model drug. Evaporative precipitation into aqueous solution (EPAS) and spray freezing into liquid (SFL) technologies were used to prepare powders of danazol/PVP K-15 in a 1:1 ratio. The evaporative precipitation into aqueous solution (EPAS) and SFL compositions, physical mixture and commercial product were dosed by oral gavage to 28 male Swiss/ICR mice for each arm of the study. Samples were taken at time points ranging from 0.5 to 24 h. Pooled mouse serum was analyzed for danazol by high performance liquid chromatography (HPLC). Powders were analyzed for their ability to form supersaturated solutions through dissolution at concentrations of 1 mg/mL which was the dose delivered to the mouse models. Spray freezing into liquid (SFL) and EPAS compositions displayed higher C_{max} at 392.5 ng/mL and 430.1 ng/mL, respectively, compared to the physical mixture (204.4 ng/mL) and commercially available danazol (199.3 ng/mL). The T_{max} for all compositions studied was near the 1 h time point. The area under the curve (AUC) for the SFL composition was 2558 ng.h/mL compared to EPAS composition at 1534 ng·h/mL. The area under the curve (AUC) for the physical mixture and commercially available danazol were 672 ng.h/mL and 1519 ng.h/mL, respectively. The elimination rate constants for the EPAS composition, SFL composition, and physical mixture were similar at ~0.15 h^{-1} where as the commercially available danazol capsules displayed an elimination rate constant of 0.103 h^{-1}. The extent of danazol absorption in the mouse model was higher for SFL composition compared to the less amorphous EPAS composition, physical mixture, and commercially available danazol powders. Both EPAS and SFL compositions were able to form supersaturated solutions. However, the SFL composition displayed a supersaturation of 33% above

control and was able to maintain supersaturation for 90 min compared to the EPAS composition (27% supersaturation above control for 60 min). Through the use of a testing method for supersaturation, it was found that EPAS and SFL compositions achieve higher apparent solubilities when compared to the physical mixture and commercially available danazol capsules. Because of the greater extent of dissolution of the SFL composition, the bioavailability was enhanced in a mouse model.

INTRODUCTION

Bioavailability enhancement of poorly water soluble active pharmaceutical ingredients (API) is key for improving existing therapies and allowing for formulation of certain new chemical entities. Active pharmaceutical ingredients (API) described by the biopharmaceutics classification system (BCS) as class II display poor water solubility with high mucosal permeability. The rate limiting step for absorption of these APIs is the dissolution rate and the apparent solubility. Active pharmaceutical ingredients (API) which are poorly water soluble may be poorly absorbed due to a lack of sufficient concentrations of solubilized drug within the lumen of the gastrointestinal tract following oral dosing. Improved bioavailability of poorly water soluble API has been achieved by several means. Particle size reduction through milling (Liversidge & Cundy, 1995) or a solution formulation filled into a soft gelatin capsule (Erlich et al., 1999) have shown improved bioavailability. Other methods include formulation with cyclodextrins (Muraoka et al., 2004; Jambhekar et al., 2004; Wong & Yuen, 2001; Savolaimen et al., 1998), microemulsions (Kawakami et al., 2002; Kang et al., 2004; Wang et al., 2004), solid solution formation (Kapsi & Ayres, 2001), and solid lipid nanoparticles (Sznitowska et al., 2001). Our laboratories have introduced two technologies, spray freezing into liquid (SFL) (Rogers et al., 2002, 2002, 2003, 2003, 2003; Yu et al., 2002; Hu et al., 2002, 2003, 2004, 2000) and evaporative precipitation into aqueous solution (EPAS) (Chen et al., 2002, 2004, 2004; Sarkari et al., 2002), for improving bioavailability by creating nanostructured particles which show rapid dissolution and enhanced apparent solubilities.

The spray freezing into liquid (SFL) technology produces powders with surface areas from 12 to 83 m^2/g and thus enhanced dissolution rates. In this particle engineering process, a feed solution containing an API and dissolution enhancing excipient(s) is atomized directly into a cryogenic liquid such as nitrogen. The resulting dried powder is composed of discrete microparticles where the API is molecularly dispersed with a polymer in a porous matrix (Vaughn et al., 2005). This molecular dispersion is achieved by rapid freezing in liquid nitrogen which prevents phase separation. In previous studies, it was found that enhanced dissolution is due to the amorphous morphology, high surface area, and enhanced wettability of the SFL nanostructured particles (Hu et al., 2003). A schematic representation of the SFL apparatus has been reported (Rogers et al., 2002). With this technology, the API loaded organic solvent is pressurized and atomized via a poly ether-ether ketone (PEEK) nozzle below the surface of liquid nitrogen. This type of atomization may be utilized for other cryogens, for example liquid CO_2 (Young et al., 1999). Because of the rapid flow rate, liquid–liquid impingement, and marked temperature drop in the jet, the emitted solvent is atomized into fine, high surface area microdroplets that are frozen rapidly. The suspension of frozen microdroplets is lyophilized to remove solvent resulting in an amorphous, micronized powder.

In the evaporative precipitation into aqueous solution (EPAS) process, the API precipitates due to evaporation of the organic solvent near or above the boiling point and contact with an aqueous solution. A schematic representation of the EPAS process has been reported (Sarkari et al., 2002). For this technology, the API loaded organic solvent is pressurized, heated, and atomized through a nozzle (e.g., an elliptical conical stainless steel nozzle) into a heated water bath containing stabilizing excipients. The large pressure drop across the small nozzle orifice creates intense atomization with rapid evaporation of the primary organic solvent due to the high temperature of the feed solution and aqueous receiving solution. The rapid evaporation of the feed solvent results in supersaturation, nucleation, and precipitation of the API. The excipients within the organic feed solution and/or aqueous receiving vessel stabilize the particles by preventing particle growth and agglomeration of the API precipitate. In addition, the excipients also enhance the API particle dissolution rate and long term storage stability (Sarkari et al., 2002).

Amorphous API is metastable and upon dissolution in aqueous media can form a solution with a high degree of supersaturation. Solid dispersions of amorphous API have been shown to form a supersaturated dispersion in vitro (Yamada et al., 1999; Kohri et al., 1999; Suzuki et al., 1997, 1998) leading to significant improvement in bioavailability (Yamashita et al., 2003). However, very few studies have related bioavailability of high surface area of nanoparticle formulations or nanostructured aggregates to in vitro studies of dissolution and supersaturation. Nanoparticles of API also display the ability to form supersaturated dispersions due to an increase in chemical potential due to the high curvature and large fraction of surface molecules as described by the Kelvin equation (Muller & Keck, 2004; Grant & Brittian, 1995). Because the bioavailability of a poorly soluble API is dependent on the solubility and concentration of drug available for absorption within the lumen of the gastrointestinal tract, the ability to increase the apparent solubility of the drug, above the equilibrium value for the crystalline form, for an extended time period will improve its bioavailability.

Danazol, which is a BCS class II compound, was utilized as a model drug to evaluate particle formation processes, supersaturation (apparent solubility enhancement), and bioavailability improvement from nanoparticles formed by EPAS and SFL. In vitro–in vivo correlations have been evaluated previously with danazol (Sunesen et al., 2005; Dressman & Reppas, 2000). Also, the bioavailability of nanoparticle danazol formulations (Liversidge & Cundy, 1995), solubilized danazol (Erlich et al., 1999) and cyclodextrin complexed danazol (Farag et al., 1996) has been evaluated. The effect of physiological conditions on the bioavailability of danazol has also been studied (Sunesen et al., 2005).

The objective of this study was to determine the level and duration of supersaturation (apparent solubility) of a poorly water soluble API, to measure bioavailability in a mouse model, and to relate the in vitro and in vivo results. The enhancement of the apparent solubility of danazol is expected to improve the level and duration of danazol absorption in vivo. The bioavailability of nanoparticle danazol formulations was evaluated in a mouse model to determine what effect the improved solubility has on the absorption of a poorly water soluble drug. The nanoparticle formulations (EPAS and SFL) were compared with the physical mixture and commercially available danazol as controls. The bioavailability is described in terms of the new in vitro measurements of supersaturation, as well as other properties of the particles reported recently (Vaugn et al., 2005). Briefly, the SFL composition displayed amorphous character, a primary danazol particle size of 30 nm, and was consistent with a solid solution. The evaporative precipitation into aqueous solution (EPAS) composition was mostly amorphous with slight crystallinity, a primary danazol particle size of 500 nm, and was consistent with a solid dispersion. The effects of the differences in the properties of these two types of particles on supersaturation and bioavailability is examined. Dissolution in the previous work was conducted at sink conditions, whereas in the current work has been performed above the equilibrium solubility to evaluate supersaturation. The ability to relate bioavailability to particle properties, in vitro studies of dissolution rates, and supersaturation is highly useful for particle engineering of poorly water soluble drugs to achieve high bioavailabilities.

MATERIALS AND METHODS

Materials

Micronized danazol, polyvinylpyrrolidone (PVP) K-15, and 1.0 N hydrochloric acid (HCl) solution were purchased from Spectrum Chemicals (Gardena, CA). Hexanes, dichloromethane, and high performance liquid chromatography (HPLC) grade acetonitrile were obtained from EM Science (Gibbstown, NJ). Danocrine® 50 mg Capsules (Sanofi-Synthelabo Inc., New York, NY) were purchased from University Health Services Pharmacy (Austin, TX).

Preparation of SFL Micronized Powder

A solution of 0.2% w/v danazol and 0.2% w/v PVP K15 was prepared in acetonitrile. Aliquots of the solution were loaded into a high pressure solution cell and atomized beneath the liquid nitrogen surface at 50 mL/min constant flow through a 127 μm I.D. PEEK nozzle. The poly ether-ether ketone (PEEK) tubing acted as an insulating nozzle that prevented freezing within the nozzle orifice. The constant pressure was supplied by an ISCO syringe pump (Model 100DX; ISCO, Inc., Lincoln, NE). Because of the low viscosity of the acetonitrile API solution, a pressure of only 3000 psi was

required to produce a flow rate of 50 mL/min. The frozen microparticles were collected and dried by a VirTis Advantage Tray Lyophilizer (The VirTis Company, Inc., Gardiner, NY). The final powder was protected from moisture and stored under vacuum.

Preparation of EPAS Micronized Powder

A solution of 2% w/v danazol and 1% w/v PVP K-15 was prepared in dichloromethane. This solution was pumped via an HPLC pump at 2 mL/min through a heat exchange coil set at 80°C. The heated solution was sprayed under constant pressure of 5000 psi through a fine, elliptical, conical nozzle (formed by crimping 0.030 inch I.D. stainless steel tubing) into a heated water bath (80°C) containing 1% w/v PVP K-15 of equal volume to that of the organic phase. The resultant dispersion (danazol:PVP K-15 1:1) was quenched by injecting it into liquid nitrogen via a syringe and needle and lyophilized to form powder. The final powder was protected from moisture and stored under vacuum.

Preparation of Co-ground Physical Mixture

A co-ground physical mixture consisting of 3.0 g danazol and 3.0 g PVP was mixed and ground using a mortar and pestle.

Oral Dosing of a Murine Model

An animal study was designed and approved by the University of Texas at Austin Institute of Animal Care and Use Committee (IACUC). Four groups of 28 mice (male, 32 g ICR mice) were dosed with 12.5 mg/kg danazol by oral gavage of a 1 mg/mL danazol dispersion. The danazol formulations included an nanoparticle SFL composition, nanoparticle EPAS composition, physical mixture, or commercially available danazol. Mice were sacrificed by CO_2 narcosis at 0.5, 1, 2, 4, 6, 12, and 24 h following oral dosing. Blood was drawn by cardiac puncture, allowed to clot, centrifuged, and serum was collected for HPLC analysis. A non-compartmental model was used to determine pharmacokinetic parameters for danazol absorption and clearance using WinNonlin version 4.1 (Pharsight Corporation, Mountain View, CA).

Supersaturation In Vitro Analysis

The apparatus used for supersaturation testing consisted of a VanKel VK 6010 Dissolution Tester with a Vanderkamp VK650A heater/circulator (Varian, Inc., Palo Alto, CA) USP IV paddle dissolution apparatus connected via 3 mm Teflon tubing to a Cole-Parmer Masterflex peristaltic pump (Cole Parmer Instruments Company, Vernon Hills, IL) followed by a QS 10 mm path length flow cell (Agilent Technologies, Palo Alto, CA) inside a Cary 3E UV-Visible spectrophotometer (Varian, Inc., Palo Alto, CA). The pump circulates a small portion of the 50 mL of medium (0.75% SDS, 1.2% Tris buffer adjusted to pH 9 with 1N HCl) from the 100 mL dissolution vessel through the flow cell and returns the fluid to the vessel at a rate of 30 mL per minute. Under these flow conditions, the time required for the transport of a fluid element from the dissolution vessel to the detector flow cell was 10 sec. Measurement of the drug concentration in solution was done by adding 50 mg danazol equivalent drug powder to dissolution medium containing dissolution medium and monitoring the danazol peak absorbance wavelength (264 nm) to determine the concentration of danazol in solution. A 1 mg/mL dispersion was formed during supersaturation analysis compared to the equilibrium solubility of danazol in the medium which is 0.47 mg/mL (data not shown). During measurement of the supersaturated concentration, an inline membrane filtration unit was placed between the pump and flow cell to retain suspended solids that would scatter light and introduce error into the measurement. The filter unit was comprised of an aluminum housing inside which a medium porosity glass frit supports a 90 mm nylon membrane with a mean pore size of 200 nm. Data sampling of the flow cell concentration was done at a rate of 3 Hz.

Chromatographic Method

Calibration standards and serum were analyzed using a method previously published (He et al., 1988). Briefly, pooled serum from the four mice at each time point (1 mL) was analyzed for danazol by liquid–liquid extraction by reversed phase high performance liquid chromatography (RP-HPLC). To the 1 mL mouse serum, 8 mL of hexanes was added and vortex mixed for 1.5 min, followed by centrifugation at 3000 G (15 min). The supernatant was transferred to separate centrifuge tubes and dried under a stream of nitrogen at 60°C.

Samples were then reconstituted with 250 μL mobile phase (70% acetonitrile: 30% water) and vortex mixed (1 min) before transferring into HPLC injection vials with low volume inserts (150 μL). Each sample was analyzed using a Shimadzu LC-10 liquid chromatograph (Shimadzu Corporation, Kyoto, Japan) equipped with a heated (37°C) C-18 base-deactivated column (5 μm, 50 × 4.6 mm) protected by a C-18 guard column (5 μm, 7.5 × 4.6 mm) (Alltech Associates, Inc., Deerfield, IL). The mobile phase eluted a danazol peak (263 nm) at 7 min at a flow-rate of 1.0 mL/min and an absorption wavelength of 288 nm (λ_{max}).

RESULTS

Danazol Supersaturation

The results from dissolution of a supersaturated dispersion of danazol are shown in Fig. 1. From the figure it can be seen that the SFL composition displayed 33% supersaturation relative to the physical mixture (crystalline microparticles and PVP K-15) at its maximum absorbance and remained above saturation for 90 min. The evaporative precipitation into aqueous solution (EPAS) composition dissolved to form a supersaturated dispersion which was 27% above the control physical mixture at its maximum absorbance and remained supersaturated for 60 min. The equilibrium solubility of crystalline danazol in the dissolution medium is 0.47 mg/mL. The physical mixture displayed higher apparent solubility compared to the commercially available danazol capsule powder within the time frame of analysis which was due to the addition of PVP within the physical mixture formulation.

Danazol Bioavailability

The danazol pharmacokinetic data are shown in Fig. 2 and Table 1. Spray freezing into liquid (SFL) and EPAS compositions displayed higher C_{max} at 392.5 ng/mL and 430.1 ng/mL, respectively, compared to the physical mixture (204.4 ng/mL) and commercially available danazol (199.3 ng/mL). The T_{max} for each of the compositions studied was at the 1 h time point. The area under the curve (AUC) (Table 1) for the SFL composition was 2558 ng.h/mL compared to the EPAS processed composition at 1534 ng·h/mL. The area under the curve (AUC) for the physical mixture and commercially available danazol were 672

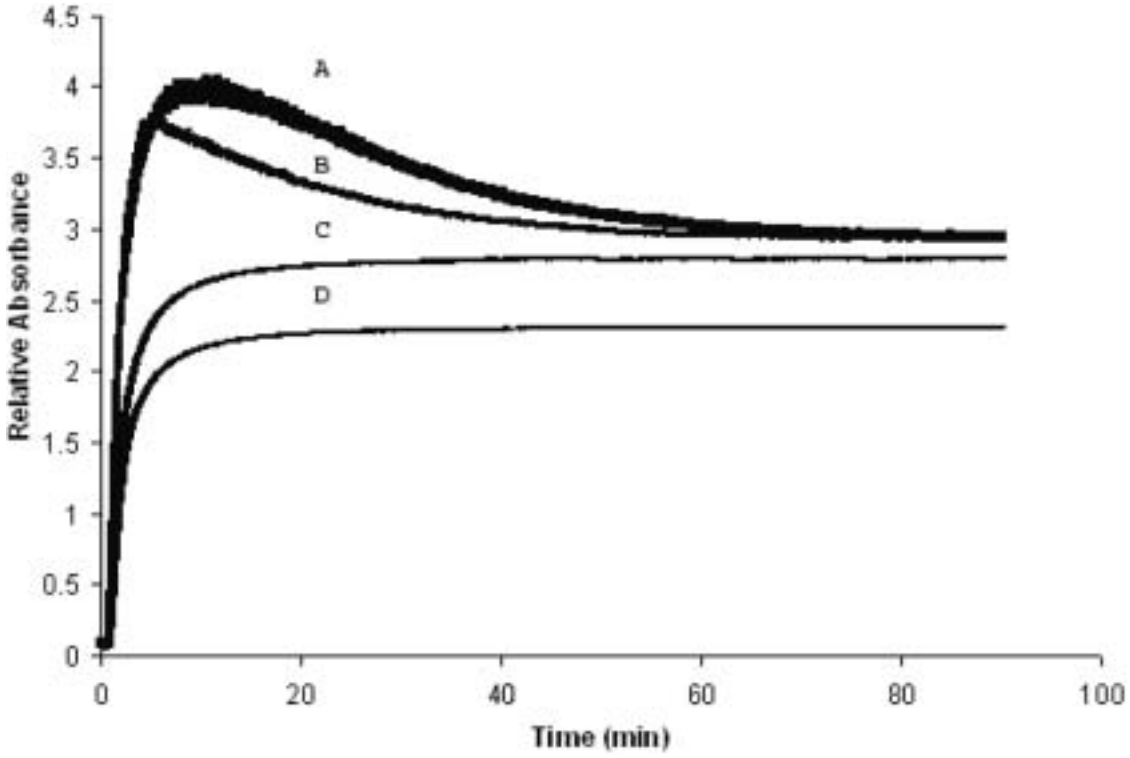

FIGURE 1 **Supersaturated Dissolution in 0.75% SDS. 1.21% Tris Buffer at pH 9 for the SFL Composition (Danazol:PVP-K15 1:1) (A), EPAS Composition (Danazol:PVP-K15 1:1) (B), Physical Mixture (Danazol:PVP-K15 1:1) (C), and Commercially Available Danazol (D).**

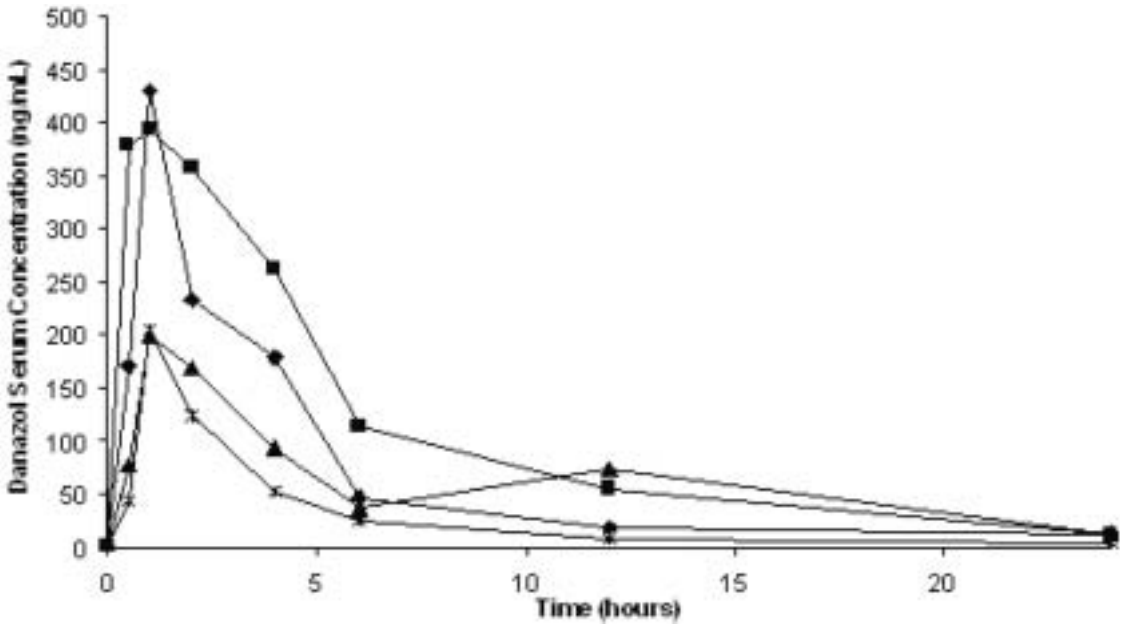

FIGURE 2 **Oral Bioavailability of Danazol in a Mouse Model for the SFL Composition (Danazol:PVP-K15 1:1) (■), EPAS Composition (Danazol:PVP-K15 1:1) (♦), Physical Mixture (Danazol:PVP-K15 1:1) (✱), and Commercially Available Danazol (▲).**

TABLE 1 **Pharmacokinetic Parameters Calculated Using Noncompartmental Analysis in Win-Nonlin of the Mice Dosed with the EPAS and SFL Compositions, Physical Mixture, and Commercially Available Danazo l**

ITZ Formulation	C_{max} (ng/mL)	T_{max} (hrs)	$T_{1/2}$ (hrs)	K_{el} (hrs-1)	AUC (0–24) (ng·h/mL)	AUC_{inf} (ng·h/mL)
Commercially Available Danazol	199.3	1.0	6.7	0.103	1519	1648
Physical Mixture (danazol:PVP-K15 1:1)	204.4	1.0	4.5	0.154	672	703
EPAS Composition (danazol:PVP-K15 1:1)	430.1	1.0	4.6	0.150	1534	1613
SFL Composition (danazol:PVP-K15 1:1)	392.5	1.0	4.4	0.158	2558	2626

ng.h/mL and 1519 ng.h/mL, respectively. The elimination rate constants for the EPAS composition, SFL composition, and physical mixture were similar at ~0.15 h^{-1} whereas the commercially available danazol capsules displayed an elimination rate constant of 0.103 h^{-1}. The extent of danazol absorption in the mouse model was higher for the SFL composition compared to the EPAS composition, physical mixture, and commercially available danazol powders.

DISCUSSION

In the current study, danazol was used as a model drug to compare the ability of nanoparticle danazol formulations to form supersaturated solutions in vitro and how this affects its bioavailability in vivo. Danazol formulated with PVP K-15 and processed using both SFL and EPAS technologies were evaluated in our laboratories in a previous study (Vaugn et al., 2005). The spray freezing into liquid (SFL) powders collected displayed complete amorphous character as determined by x-ray powder diffraction (XRD). Thus, the SFL composition was shown to form a solid dispersion on the molecular level through the ability of the freezing process to prevent nucleation and growth. Also, the SFL composition displayed the highest mixing and miscibility within the PVP polymer compared to the EPAS composition and physical mixture powders as determined from the glass transition temperature (Tg) measured by differential scanning calorimetry (DSC) and interpreted with the Gordon-Taylor equation. This aspect improves the ability of the formulation to prevent re-crystallization and particle growth when in the solid state.

In contrast, the EPAS composition displayed partially crystalline character (XRD) and larger primary

particle sizes compared to the SFL composition. The nucleation and growth of particles during the EPAS process allows for nanoparticle formation which may be crystalline or amorphous depending on the stabilizer used and processing conditions. The particles used in this study were partially crystalline with a 500 nm primary particle size. The level of miscibility with the PVP was less than the SFL composition according to the Tg measured by DSC although higher than the physical mixture.

Active pharmaceutical ingredients (API) in BCS II are poorly absorbed due to a lack of sufficient concentrations of solubilized drug. Formulations which are amorphous can overcome this by supersaturation of the dissolution medium allowing for greater absorption of the delivered dose as well as improving the dissolution rate (Yu, 2001).

The dissolution rate of a particle can be described by the Noyes-Whitney Eq. (1):

$$\frac{dC}{dt} = \frac{AD(C_s - C)}{h} \qquad (1)$$

where dC/dt is the rate of dissolution, A is the surface area of the particle, D is the diffusion coefficient, C_s is the apparent solubility of the drug in the dissolution medium, C is the concentration of the drug in the dissolution medium at time t, and h is the thickness of the diffusion boundary layer. The diffusion coefficient is dependent on the drug in question and cannot be adjusted. Also, the thickness of the boundary layer can be manipulated in vitro through increased agitation; however, this proves to be difficult in vivo. The rate of dissolution can be improved (increased) by creating particles which have a high surface area, improved wettability (higher surface area in contact with dissolution medium), or by increasing the apparent solubility of the drug. Through the production of nanoparticles or aggregates of nanoparticles with high surface area, the surface area of the particle increases significantly thereby increasing the rate of dissolution. Nanoparticles can also form supersaturated dispersions, although not to the same extent as amorphous particles. According to the Ostwald-Freundlich/Kelvin equations, small droplets of liquid dispersed in a gas–liquid medium evaporate more quickly and to a greater extent than do large droplets due to increased curvature at the droplet surface which raises the vapor pressure of the liquid (Grant & Brittian, 1995). This theory is also applicable to solid–liquid interfaces as well. By decreasing the size of solid particles to the nanometer scale, the high energy state which is achieved will increase the extent to which the particles can dissolve due to an increase in dissolution pressure. In turn, this will increase the apparent solubility of the dissolving particles. A decrease in the interfacial tension between the particle and water, due to coating by a stabilizing excipient, will decrease the supersaturation due to curvature.

The amorphous nature and small particle size of the nanoparticle formulations (EPAS and SFL) allows for higher apparent solubilities, thereby increasing the dissolution rate and increasing the concentration of drug available for absorption. In this study, the amorphous SFL composition was able to attain a concentration which was 33% above the apparent solubility of the physical mixture, which was made up of microparticulate crystalline danazol. The evaporative precipitation into aqueous solution (EPAS) composition was able to achieve supersaturation which was 27% above the control physical mixture. The ability of the nanoparticle formulations to achieve higher apparent solubilities translated to higher C_{max} values in the in vivo study because more API was available for absorption. Yamashita et al formed solid dispersions of tacrolimus in various stabilizers to evaluate their ability to form supersaturated solutions (Yamashita et al., 2003). In their study, solid dispersions of tacrolimus were formed by solvent evaporation and were amorphous. The dispersions formed supersaturated solutions at nearly the same initial concentration. However, the time above supersaturation varied significantly. In vivo data from the current study found that the supersaturating formulations displayed significantly higher and extended absorption of the API. In the current study, the maximum level of supersaturation translated to an increased C_{max}, although the time above saturation improved the AUC, or the total amount of drug absorbed, as in the case of SFL.

Partially crystalline and nanoparticulate danazol formed by the EPAS process was also able to enhance the apparent solubility of the drug over a 60 min time period with a Cs-max of 27% above the physical mixture. Although not completely amorphous, the EPAS composition did display some level of amorphous character as well as being composed of nanoparticle

domains of danazol. As with the SFL composition, the amorphous regions of the danazol domains would display similar abilities to supersaturate the dispersion in vitro and in vivo although the crystalline regions can cause a premature reduction in the solubility due to seeding of the dissolved API (Otsuka et al., 2002). This phenomenon was seen in the in vitro supersaturation analysis as well as the in vivo study. The time course for supersaturation was shorter for the EPAS composition and the extent of absorption was much lower than the SFL composition in vivo. Precipitation of the drug within the GI tract could significantly reduce the extent of absorption of the EPAS composition. Both the commercially available danazol capsule powder and physical mixture showed relatively low C_{max} values and lower AUC values compared to the SFL processed danazol. Because these formulations are microparticulate and crystalline, the degree and extent of absorption is limited by the equilibrium solubility of the drug, rather than an enhanced apparent solubility which was achieved through the EPAS and SFL compositions.

CONCLUSION

Quantitation of degree and extent of supersaturation, both level and time duration, correlated well with in vivo results obtained for danazol. Through the use of a testing method for supersaturation, it was found that EPAS and SFL compositions achieve higher apparent solubilities when compared to the physical mixture and commercially available danazol capsules. This improvement in solubility allowed for more danazol to be available for absorption in vivo. Because of the greater extent of dissolution of the SFL composition, the bioavailability was enhanced in a mouse model.

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