# An in vitro digestion test that reflects rat intestinal conditions to probe the importance of formulation digestion vs first pass metabolism in danazol bioavailability from lipid based formulations

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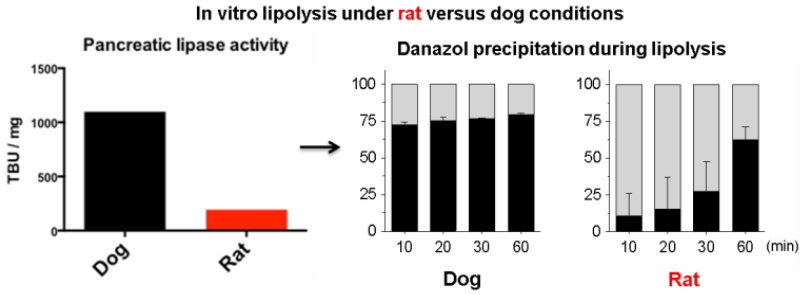
## ABSTRACT GRAPHIC




## ABSTRACT

The impact of gastrointestinal (GI) processing and first pass metabolism on danazol oral bioavailability (BA) was evaluated after administration of self-emulsifying drug delivery systems (SEDDS) in the rat. Danazol absolute BA was determined following oral and intraduodenal (ID) administration of LFCS class IIIA medium chain (MC) formulations at high ($SEDDS_H$-III) and low ($SEDDS_L$-III) drug loading and a lipid free LFCS class IV formulation (SEDDS-IV). Experiments were conducted in the presence and absence of ABT (1-aminobenzotriazole) to evaluate the effect of first pass metabolism. A series of modified in vitro lipolysis tests were developed to better understand the in vivo processing of SEDDS in the rat. Danazol BA was low (< 13%) following oral and ID administration of either SEDDS. Increasing drug loading, ID rather than oral administration, and administration of SEDDS IV rather than SEDDS III led to higher oral BA. After pre-treatment with ABT, however, danazol oral BA significantly increased (e.g. 60% compared to 2% after administration of $SEDDS_L$-III), no effect was observed on increasing drug loading, and differences between SEDDS III and IV were minimal. In vitro digestion models based on the lower enzyme activity and lower dilution conditions expected in the rat, resulted in significantly reduced danazol precipitation from SEDDS III or SEDDS IV on initiation of digestion. At the doses administered here (4-8 mg/kg), the primary limitation to danazol oral BA in the rat was first pass metabolism, and the fraction absorbed was > 45% after oral administration of SEDDS III or SEDDS IV. In contrast, previous studies in dogs suggest that danazol BA is less dependent on first pass metabolism and more sensitive to changes in formulation processing. In vitro digestion models based on likely rat GI conditions suggest less drug precipitation on formulation digestion when compared to equivalent dog models, consistent with the increases in in vivo exposure (fraction absorbed) seen here in ABT pre-treated rats.

## KEY WORDS

Absorption; Lipid-based drug delivery systems; in vitro digestion; supersaturation; danazol, first pass metabolism; Bioavailability

## ABBREVIATIONS

| | |
|---|---|
| 4-BPB | bromophenyl boronic acid |
| ABT | 1-aminobenzotriazole |
| AUC | area under the curve |
| BA | bioavailability |
| BS | bile salt |
| $C_{max}$ | peak plasma concentration |
| CrEL | cremophor EL |
| CYP | cytochrome P450 |
| *F* | absolute bioavailability |
| GI | gastrointestinal |
| HPLC | high performance liquid chromatography |
| ID | Intraduodenal |
| IV | intravenous |
| LBDDS | lipid-based drug delivery system |
| LCMS | liquid chromatography mass spectrometry |
| MC | medium-chain |
| NaTDC | sodium taurodeoxycholate |
| PL | phospholipid |
| SBA | serum bile acid |
| SEDDS | self-emulsifying drug delivery system |
| $t_{1/2}$ | half life |
| TBU | tributyrin units |
| $T_{max}$ | time of occurrence of peak plasma concentration |
| $Vd_{\beta}$ | volume of distribution |

## INTRODUCTION

Self-emulsifying drug delivery systems (SEDDS) provide a means to enhance the absorption of poorly water-soluble drugs (PWSD),[1-4] and their utility has been exemplified with a range of drugs including cyclosporine, halofantrine and danazol.[5-8] Drug administration as a SEDDS circumvents traditional dissolution since the drug is presented to the gastrointestinal (GI) tract in a molecularly dispersed state (i.e. in solution in the formulation). Incorporation of SEDDS into lipid digestion pathways also results in the generation of a series of mixed colloidal species in the GI tract, comprising exogenous (i.e. formulation derived) and endogenous (bile salts, phospholipids) species that collectively promote drug solubilisation.

In response to dilution, and interaction of the formulation with pancreatic and biliary fluids, the solubilisation capacity of SEDDS typically changes during GI processing and depending on the nature of the formulation, dispersion and digestion can result in a decrease in solubilisation capacity and the generation of transient supersaturation.[9-11] Supersaturation ultimately provides a driver for precipitation and where this is significant, drug absorption from SEDDS is typically compromised. Realisation that drug precipitation on digestion may provide an indication of formulation performance has led to increasingly common utilisation of in vitro models of lipid digestion to assist in the design and evaluation of candidate formulations.[12-15]

Where supersaturation is maintained for extended periods of time, drug absorption continues and may be enhanced by virtue of an increase in thermodynamic activity. The degree of supersaturation generated by either dispersion or digestion of SEDDS is expected to increase with increases in drug dose and the likelihood of precipitation similarly expected to increase as the quantity of drug in metastable solution reaches the critical point for nucleation.[16-18]

In a previous study from our laboratories, the impact of increasing drug dose on danazol bioavailability from SEDDS formulations in beagle dogs was explored in an attempt to delineate the potentially opposing effects of increasing dose leading to supersaturation (and an increase in thermodynamic activity and absorption) versus an increase in precipitation (and a reduction in absorption).[19] Interestingly, the data obtained varied as a function of the animal cohort.[19] In older animals increasing dose resulted in increases in bioavailability up to a critical point (consistent with increases in supersaturation and thermodynamic activity), above which further increases resulted in a reduction in

bioavailability (consistent with increased precipitation). In contrast, in a younger animal cohort linear increases in danazol exposure were evident with increases in dose and bioavailability was unchanged.

In the case of danazol, however, evaluation of the effects of dose on bioavailability is further complicated by the possibility of changes to first pass metabolism. Thus, increases in bioavailability with increases in dose might also reflect saturation of first pass effects. The prospect of first pass metabolism of danazol has been suggested previously[20], however, subsequent studies have also described oral bioavailabilities as high as 100% in beagle dogs[21] in seeming contradiction to the possibility of significant pre-systemic metabolic limitations to bioavailability.

In light of this, the objectives of the current studies were two fold. Firstly, we sought to further explore the role of drug dose on the bioavailability of danazol from SEDDS and in particular to evaluate the potential limitations of first pass metabolism. Studies were conducted in rats to allow more facile comparison of the impact of first pass metabolism versus gastric and intestinal processing on danazol bioavailability. The results of these studies, however, raised regarding the ability of previous in vitro lipid digestion models to accurately describe events in the rat. As such a second objective of the current studies was to identify modifications to the in vitro lipolysis model that allow better alignment with events in the rat GI tract. The data suggest that in the rat, lipid digestion may be less efficient than it is in the dog (or potentially in humans), that danazol absorption from SEDDS formulations is relatively high (> 50%) and that the principle limitation to danazol bioavailability is first pass metabolism.

## MATERIALS AND METHODS

### *Materials*

Danazol (pregna-2,4-dien-20-yno[2,3-d]isoxazol-17-ol) was supplied by Coral drugs PVT (New Delhi, India), progesterone and 1-aminobenzotriazole (ABT) was from Sigma-Aldrich (St Louis, MO, USA). Captex 300 and Capmul MCM EP were kindly donated by Abitec Corporation (Janesville, WI, USA). Soybean oil, Cremophor EL (polyoxyl 35 castor oil), sodium taurodeoxycholate > 95% (NaTDC), porcine pancreatin (8 x USP specification activity), glyceryl tributyrate, hydroxypropyl methylcellulose (HPMC E4M) and 4-bromophenylboronic acid (4-BPB) were from Sigma-Aldrich (St Louis, MO, USA). Lipoid E PC S, (Lecithin from egg, approximately 99% pure phosphatidylcholine (PC)) was

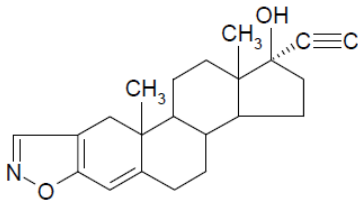
from Lipoid GmbH (Ludwigshafen, Germany). Sodium heparin (1000 IU/mL) was obtained from DBL (Mulgrave, VIC, Australia) and normal saline (0.9%) obtained from Baxter Healthcare (Old Toongabbie, QLD, Australia). Anaesthetics; Parnell ketamine (100 mg/ml) from Parnell Labs, NSW, Australia, Ilium xylazine-100 (100 mg/ml) from Troy Labs, NSW, Australia) and A.C.P 10 (13.5 mg acepromazine maleate equivalent to 10 mg acepromazine) from Ceva Delvet, NSW, Australia were used as received. Sodium hydroxide 1 M, which was diluted to obtain 0.6 M NaOH titration solution, was from Merck (Darmstadt, Germany) and water was purified with a Milli-Q (Millipore, Bedford, MA, USA) system. All other chemicals and solvents were of analytical purity or high performance liquid chromatography (HPLC) grade.

## *Formulation preparation*

### SEDDS formulations for oral/intraduodenal administration

All formulations were prepared as previously described[22] and danazol, molecular structure illustrated in Table 1 (aqueous solubility 0.59 µg/ml,[23] log *P* of 4.53[24]) was incorporated at low ($_L$) and high ($_H$) drug loading (40% or 80% of saturated solubility in the formulation based on measured values at equilibrium at 37°C). Drug solubility in each formulation was assessed in triplicate as previously described.[11, 25] The formulations are summarised in Table 1**.** After mixing, the formulations were vortexed for 30 s and equilibrated overnight at 37°C.

**Table 1** Composition (% w/w) of self-emulsifying lipid-based formulations containing danazol

| **Excipients (w/w) (%)** | **SEDDS-IV** | **SEDDS$_L$-III** | **SEDDS$_H$-III** | **Danazol** |
|---|---|---|---|---|
| **LCFS classification** [a] | **IV** | **IIIA** | **IIIA** | |
| Captex 300: Capmul MCM (1:1) | 0 | 60 | 60 |  |
| Cremophor EL | 65 | 30 | 30 | |
| Ethanol (100%) | 35 | 10 | 10 | |
| Target drug load in formulation [mg/g] | 17.9 [b] | 12.1 [b] | 24.3 [c] | |



**Captex 300** (medium-chain triglycerides); **Capmul MCM** (medium-chain mono-, di- and triglycerides); **Cremophor EL** (surfactant); **Ethanol** (co-solvent)

[a] Lipid Formulation Classification System (LFCS) as described by Pouton et al[26, 27]

[b] Equivalent to 40% of the saturated solubility in the formulation at 37°C

[c] Equivalent to 80% of the saturated solubility in the formulation at 37°C

## *Intravenous formulation*

An intravenous formulation of danazol (1.2 mg/mL) was prepared using 15% (w/v) hydroxypropyl-β-cyclodextrin (HP-β-CD). The binding constant for danazol to β-cyclodextrin is relatively low (9.72 x $10^2$ $M^{-1}$)[28] and no significant impact on PK parameters is therefore expected in spite of the relatively high cyclodextrin concentration employed.[29]

Danazol and HP-β-CD were dissolved in 0.9% saline using a magnetic stirrer (Teflon coated stirrer bar, 10 x 6mm) at ambient temperature and filtered through a 0.22 μm filter (Millix®-GV) before use.

## BIOAVAILABILITY STUDIES IN RATS

SEDDS formulations were evaluated in vivo to examine the impact of excipients on drug absorption. In some test groups, a non-specific cytochrome P450 inhibitor (1-aminobenzotriazole, ABT), that is commonly used in animal models to inhibit hepatic and intestinal CYP enzymes, was administered orally.[30, 31] Danazol bioavailability was explored following oral and intraduodenal (ID) administration to assess the impact of gastric processing on formulation performance.

### *Surgical and experimental procedures*

All surgical and experimental procedures were approved by the Monash Institute of Pharmaceutical Sciences animal ethics committee and were conducted in accordance with EC Directive 86/609/EEC for animal experiments and the Australian and New Zealand Council for the Care of Animals in Research and Teaching guidelines.

Experiments were conducted as a series of one-way parallel studies in male Sprague Dawley rats (250-320 g). Surgical and recovery procedures were as described previously.[32, 33] Briefly, polyethylene tubing cannulas (0.96 x 0.58) were surgically implanted into the right carotid artery to facilitate serial blood collection. In some treatment groups, cannulas were also inserted into the right jugular vein (for IV administration) or duodenum (for intraduodenal (ID) administration). Animals were allowed to recover overnight prior to formulation administration. At the conclusion of the experiments, rats were euthanised via a lethal dose of sodium pentobarbitone (100 mg/mL).

### *Formulation administration and sample collection*

Rats were fasted for 12 h prior to dosing and remained fasted until the conclusion of the study. Drinking water was provided *ad libitum*. In groups pre-treated with ABT (100 mg/kg), a single bolus dose (1.2 mL) of ABT in saline (25

mg/mL) was administered via oral gavage post surgery, approximately 14 h prior to IV, oral or ID dosing.[30, 31] A similar dosing protocol has been shown previously to provide almost complete inhibition (93%) of CYP-mediated antipyrine clearance.[30]

The intravenous formulation (2.3 mg/kg) was administered over 0.5 min by infusion pump (1 mL/min) via the indwelling jugular vein cannula. The cannula was subsequently flushed with 0.5 mL of saline. Blood samples (300 µL) were taken pre-dose and at 1, 5, 15, 30, 60, 120, 180, 240, 360 and 480 min after IV administration.

For the oral and ID treatment groups, SEDDS formulations were pre-dispersed in water (100 mg formulation + 400 mg MilliQ water) immediately prior to administration. For ID dosing, the pre-dispersed formulations were administered into the duodenum via the ID cannula over 30 min at a constant infusion rate (1 mL/hr) followed by infusion of 0.5 mL of MilliQ water to flush the cannula. Blood samples (300 µL) were taken at pre-dose, 15, 25, 35, 45, 60, 90 min and 2, 3, and 5 h after ID dosing. For oral administration, rats were lightly anaesthetised via inhalation of isoflurane (2.5% v/v), and the pre-dispersed formulations were dosed via oral gavage followed by 0.5 mL MilliQ water to rinse the gavage tube. Blood samples were taken at pre-dose, 15, 30, 45, 60, 90 min and 2, 3, 4 and 5 h after oral dosing. Blood samples were collected into 1.5 mL eppendorf tubes containing 10 µL of sodium heparin (10 IU/mL) and cannulas were flushed with heparinised saline (2 IU/mL) between samples to ensure patency of the cannula. Samples were centrifuged for 5 min at 6700 x *g* (Eppendorf minispin plus, Eppendorf AG, Hamburg, Germany) to separate plasma. Collected plasma samples were stored in eppendorfs at -80°C until analysis.

### *Quantification of danazol in plasma samples by LC-MS*

Plasma samples and calibration standards for danazol were prepared and quantified by LC-MS as previously described.[34]

### *Pharmacokinetic Data analysis*

The peak plasma concentrations ($C_{max}$) and the time for their occurrence ($T_{max}$) were noted directly from the individual plasma concentration vs. time profiles. The areas under the plasma concentration vs. time profiles to the last measured timepoint ($AUC_{0\text{-}tz}$) were calculated using the linear trapezoidal method. In the absent of ABT treatment, the AUC was in general very low and the terminal phase poorly defined. The mean terminal elimination rate constant

from the IV study was therefore used to extrapolate the AUC to infinity ($AUC_{0-\infty}$). In the presence of ABT, the AUCs were higher and extrapolated AUC was based on elimination rate constants obtained from individual plasma profiles. Clearance (Cl), volume of distribution ($Vd_{\beta}$) and bioavailability (*F*) were calculated using standard calculation methods. Statistically significant differences were determined by ANOVA followed by a Tukey test for multiple comparisons at a significance level of $\alpha = 0.05$. All statistical analysis was performed using SigmaPlot Statistics for Windows version 11.0.

## IN VITRO EXPERIMENTS

### *In vitro dispersion of SEDDS formulations*

Evaluation of the impact of gastric dispersion was performed in model gastric fluid using two dilution levels and pHs, and also in ex vivo rat gastric fluids. First, 1 g of lipid-formulation ($SEDDS_L$-III and SEDDS-IV) was dispersed in 36 mL simulated gastric fluid (0.1 N HCL, pH of 1.2). Experiments were performed in a glass vessel at 37°C with a thermostatically controlled water jacket and stirred magnetically (disc-shaped Teflon coated stirrer bar, 10 x 14 mm) with samples (200 µL) collected after 30 min.

Subsequently, a low dilution/ intermediate pH model was utilized to better reflect rat gastric conditions. Here, 100 mg of lipid-based formulation was dispersed in 900 µL of buffer (pH 5.5)[35] (i.e. 1:10 dilution consistent with volumes administered in vivo). The dispersion was stirred using a magnetic stirrer (Teflon coated stirrer bar, 10 x 6 mm) and samples (200 µL) collected after 30 min.

For experiments using ex-vivo gastric fluids, male Sprague Dawley (SD) rats (300-400 g) were fasted for 12 h prior to surgery with free access to drinking water. Animals were anesthetised with isoflurane (5% v/v) and a ligature tied around the oesophageal and duodenal apertures of the stomach. The stomach was excised and rinsed with 900 µL MilliQ (the volume of fluid dosed with the formulations in the oral bioavailability experiments). The rinsing fluid ('ex vivo stomach fluid') was collected and utilised in low volume dispersion experiments as described above.

### *In vitro digestion of SEDDS formulations*

The impact of digestion on the solubilisation properties of SEDDS formulations containing danazol was examined using a range of protocols that employed different sources and quantities of digestive enzyme in an attempt to most effectively mirror conditions in the rat in vivo. The different conditions are summarised in Table 2 and described below.

## Dog digestion model – High dilution / High enzyme activity

***In vitro digestion model (high dilution) using 100% porcine pancreatin extract (1000 TBU/mL)***

This is the in vitro model used in previous in vitro studies,[11] and that was designed to estimate the impact of digestion on SEDDS formulations in larger species such as dogs or humans.[12] In these studies, 1 g of formulation was dispersed in 36 mL of digestion media in a thermostatically controlled (37°C) vessel and digestion initiated by addition of porcine pancreatin extract (4 mL).

## Rat digestion model - High dilution / Low enzyme activity

***In vitro rat digestion model (high dilution) using ex-vivo rat pancreatic/biliary fluid***

To better mimic conditions in the rat intestine, digestions were carried out using collected (ex vivo) rat pancreatic/biliary secretions (collection method described below). Due to the relatively low rate of secretion of these fluids in the rat (1-1.5 mL/h), initial studies were conducted in 10 mL in vitro digests. The ratio of formulation to digestion medium was kept constant relative to previous studies[36] and experiments conducted using 250 mg formulation in 9 mL digestion medium with 1 mL of ex vivo rat pancreatic/biliary secretions to stimulate digestion. Since rat pancreatic/bile secretions contain bile, experiments were conducted in digestion buffer without added bile salt and phospholipid but were otherwise conducted as described previously.[36] 1 mL samples of digestion medium were collected following 30 min dispersion and at 10, 20, 30 and 60 min post initiation of digestion.

***In vitro rat digestion model (low dilution) using 1.7% porcine pancreatin extract***

In vitro digests were also performed using the 'rat' digestion protocol described above (high dilution/low enzyme) but using a quantity (17 µL) of porcine enzyme that provided a similar enzyme activity to that of 1 mL ex-vivo rat pancreatic fluid. The activity of the ex-vivo rat pancreatic/biliary fluid and the quantity of porcine pancreatic fluid required to mimic this activity was evaluated using a tributyrin assay as described below.

Rat digestion model – Low dilution / Low enzyme activity

***In vitro rat digestion model (low dilution) using ex-vivo rat pancreatic/biliary fluid***

In conducting the surgical procedures required to collect bile in the rats, it became apparent that the volume of fluid in the rat intestine was low (certainly much lower than the dilution factor of 40 used in the initial in vitro rat digestion experiments), and that under fasted state conditions the major source of fluid flow into the intestine was from the bile. As such, a second series of experiments was conducted using a lower volume of digestion media (and therefore lower dilution). Low dilution digestions were performed using a 1:1 (w/w) mixture of dispersed formulation and rat bile/pancreatic secretion. The dispersed formulation contained 20% w/v formulation in MilliQ water reflecting the ratio of formulation to dispersion fluid employed in the in vivo studies (i.e. 100 mg formulation to 400 µL MilliQ). Additionally, 250 µL MilliQ water was added to simulate the low levels of basal intestinal fluids that might be present in the GI tract during in vivo studies. Studies were performed in a 10 mL glass vial and media was stirred magnetically (Teflon coated stirrer bar, 10 x 6 mm). Samples of the digest (300 µL) were collected after 30 min. It was not possible to employ a pH stat for maintenance of pH during the digestions due to the low volume employed, however, following digestion the pH of the digestion medium was 7.9 and 5.8 for SEDDS-IV and SEDDS-III, respectively.

***In vitro rat digestion model (low dilution) using 1.7% porcine pancreatin extract***

In vitro digestion experiments were also conducted as described above, but with ex vivo rat bile/pancreatic fluid replaced with 17 µL porcine pancreatic extract.

## *Determination of pancreatic lipase activity in rat pancreatic/biliary fluid*

The lipolytic activity of enzymes in ex vivo rat bile/pancreatic fluid was determined in tributyrin units (TBUs) as previously described,[37] where 1 TBU is defined as quantity of enzyme releasing 1 µmol of titratable butyric acid per minute.[38] Briefly, 6 g of tributyrin was dispersed in 9 g of digestion buffer (50 mM TRIS maleate, 150 mM NaCl, 5 mM $CaCl_2 \cdot 2H_2O$, pH 7.5). Experiments were performed at 37°C. Digestion was initiated by addition of 1 mL of collected ex vivo pancreatic fluids following 2 min dispersion and pH was maintained at 7.5 using a pH-stat titration unit (Radiometer, Copenhagen, Denmark). Digestion was followed for 30 min and lipase activity in TBU (µmol titratable

butyric acid per minute) calculated from the initial rate of digestion (i.e. via the slope of the titration profile (i.e. fatty acids (mmol) liberated per min) multiplied by the molar strength of titrant (0.5 M)).

To determine the relative lipase activity of ex-vivo rat pancreatic fluid and the porcine pancreatin extract used previously, the lipase activity of 1 mL of porcine pancreatin enzyme extract was also examined under the same conditions (although in this case the digestion buffer contained 5 mM NaTDC and 1.25 mM phosphatidycholine (PC) since the enzyme preparation did not contain bile). Porcine pancreatic lipase extract was prepared by dispersing 1000 mg of porcine pancreatin (8 x USP specification activity) in 5 mL digestion buffer. The mixture was stirred for 15 min and centrifuged for 10 min (2,880 x *g* at 5°C, Eppendorf 5804 R centrifuge, Eppendorf AG, Hamburg, Germany). The supernatant was separated and used for digestion studies on the day of preparation.

Further experiments were conducted using lower quantities of porcine pancreatic extract to provide a 'standard curve' of enzyme activity as a function of the volume/quantity of porcine pancreatic enzyme added. This identified the quantity of porcine pancreatic extract needed to match the activity of 1 mL of ex-vivo rat pancreatic/biliary fluid. In this case, experiments were conducted using volumes of porcine pancreatic extract of less than 1 ml and additional buffer was added to maintain a total volume of 1 mL added to the digestion vessel.

### *Pancreatic and biliary fluid collection from the rat*

Ex vivo pancreatic/biliary fluid from the rat (containing bile and pancreatic enzymes) was collected via bile duct cannulation. In the rat the bile duct also serves as the main duct for the transfer of pancreatic secretions to the GIT and to obtain an accurate secretion ratio between biliary and pancreatic fluids secreted in vivo, fluids were collected together. Anaesthesia and surgical procedures in rats were performed as previously described.[39-41] A ligature was then tied around the bile duct at the point of entry into the duodenum and an incision was made in the duct above the ligature and a polyethylene cannula (0.61 x 0.28 mm, o.d. x i.d.) inserted. Bile and pancreatic fluids were collected continuously for a 2 h period (achieving approximately 1.5 mL/h) and used immediately after collection. Rats were re-hydrated via saline infusion (1.5 mL/h) into a cannula inserted into the right jugular vein during the collection period.

**Table 2** Conditions for in vitro digestion experiments employing ex vivo rat bile/pancreatic fluids and porcine pancreatic extract

| **Digestion model (dilution/enzyme activity)** | **Dog (High / High)** | **Rat (High / Low)** | | **Rat (Low / Low)** | |
|---|---|---|---|---|---|
| Lipase source | Porcine pancreatin extract [a] | Porcine pancreatin extract [a] | Ex vivo rat pancreatic / biliary fluid | Porcine pancreatin extract [a] | Ex vivo rat pancreatic / biliary fluid |
| Formulation [mg] | 1000 | 250 | 250 | 200 | 200 |
| Digestion medium [b] | micelles | micelles | buffer | micelles | buffer |
| Pancreatic enzymes | 4 mL | 0.017 mL [c] | 1 mL | 0.017 mL [c] | 1 mL |
| Total volume | 40 mL | 10 mL | 10 mL | 2.05 mL | 2.05 mL |
| Formulation dilution | 40 (High) | 40 (High) | 40 (High) | 10 (Low) | 10 (Low) |
| Enzyme activity | High | Low | Low | Low | Low |

[a] Source of porcine pancreatic extract as previously published[37]

[b] Ex vivo rat pancreatic/biliary fluid contains bile. Experiments were therefore carried out in digestion buffer without added bile salt. In vitro digestions using porcine pancreatic extract were performed in digestion medium containing bile salt micelles (5 mM NaTDC and 1.25 mM phosphatidycholine (PC))

[c] The total volume of pancreatic enzyme added was kept constant (1 ml). The 1 ml volume was made up of 17 µL enzyme extract and 983 µL digestion medium. The quantity of enzyme extract is listed here to emphasise comparison with other groups. The additional volume of digestion medium is included in the total volume of media employed

### *Sample work up for in vitro dispersion/digestion experiments*

The lipase inhibitor 4-bromophenylboronic acid[42] (4-BPB; 9 µL of a 0.5 M solution in methanol per mL of digestion medium) was added to all in vitro digestion samples to prevent further digestion of the lipid components during phase separation. Dispersion samples were centrifuged for 10 min and digestion samples for 30 min at 21,100 x *g* (Heraeus Fresco 21 microcentrifuge, Thermo Scientific, Germany) to form a pellet phase and a solubilised aqueous phase (AP). Samples obtained from each phase were diluted (aqueous phase: 50 µL to 950 µL in ACN; precipitate (pellet) and oil phase: initially in 50 µL chloroform/methanol (2:1 v/v) and this further diluted with 950 µL MeOH) and then analysed by HPLC as below.

### *Quantification of Danazol in in vitro Experiments*

Danazol concentrations in samples taken from in vitro dispersion and digestion experiments were measured by HPLC as described previously.[11] The results are presented as the % drug distribution in the aqueous phase (AP), ie the proportion of the drug initially dissolved in the formulation that was recovered in the aqueous phase post centrifugation of the dispersion or digestion media.

## RESULTS

## In vivo evaluation

### *Intravenous pharmacokinetics of danazol*

The mean plasma concentration versus time profile for danazol following intravenous administration of a 15% HP-β-CD solution containing danazol at 1.2 mg/mL to rats is shown in Figure 1 with corresponding mean pharmacokinetic parameters summarised in Table 3. Plasma concentrations declined bi-exponentially. The total clearance and volume of distribution were high (87 mL $min^{-1}$ $kg^{-1}$ and 8.9 L $kg^{-1}$ respectively) and the terminal half-life relatively short (1.2 h).

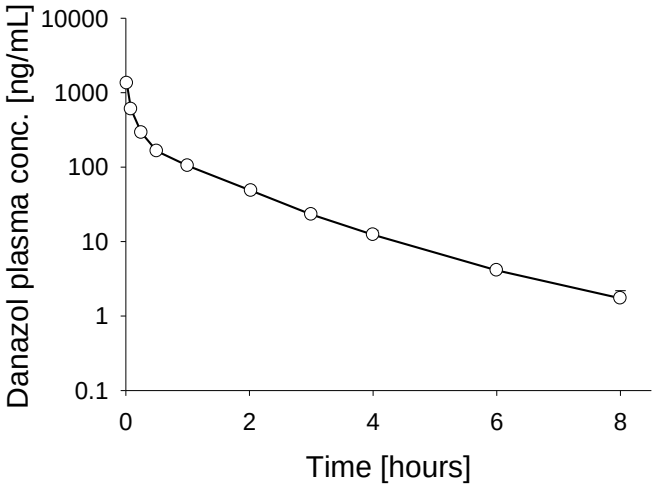



**Figure 1** Danazol plasma profile after IV administration of 15% HP-β-CD solution containing danazol at 1.2 mg/mL (total dose 2.3 mg $kg^{-1}$) to rats [mean ± SEM (n = 5)].

**Table 3** Pharmacokinetic parameters after intravenous administration of a 15% HP-β-CD solution containing 1.2 mg/mL danazol [mean ± SEM (n = 5)]

| Dose | $AUC_{0-\infty}$ | Cl | $Vd_{\beta}$ | $t_{1/2}$ |
|---|---|---|---|---|
| [mg $kg^{-1}$] | [ng h $mL^{-1}$] | [mL $min^{-1}$ $kg^{-1}$] | [L $kg^{-1}$] | [h] |
| 2.3 | 434 ± 18 | 87 ± 3.3 | 8.9 ± 0.5 | 1.2 ± 0.1 |

## *Bioavailability of danazol after oral administration of SEDDS*

The mean plasma concentration versus time profiles for danazol following oral administration of a LFCS type III lipid-containing SEDDS formulation (SEDDS-III) and a LBCS Type IV, lipid-free surfactant/cosolvent formulation (SEDDS-IV) at low drug loading are shown in Figure 2**.** Data comparing exposure from the SEDDS-III formulation at high and low drug loads ($SEDDS_H$-III vs. $SEDDS_L$-III) is also shown. The corresponding mean pharmacokinetic parameters, including absolute bioavailability compared to the intravenous formulation are summarised in Table 4 and dose normalized plasma profiles are presented in S4.

The higher solubility of danazol in SEDDS-IV compared to SEDDS-III resulted in the administration of a higher absolute dose when both formulations were administered at 40% of saturated solubility in the formulation. This resulted in increased plasma exposure after administration of SEDDS-IV compared to SEDDS-III. However, even when accounting for the difference in dose the absolute bioavailability of danazol after administration SEDDS-IV was higher than that following administration of SEDDS-III. As expected, administration of $SEDDS_H$-III (with a 2-fold higher drug dose) resulted in an increase in danazol plasma exposure when compared to $SEDDS_L$-III. However, the increase in exposure was non-linear with dose and a ~2-fold increase in absolute danazol bioavailability was evident at the higher dose. In all cases, absolute bioavailability was low (< 5%) (Table 4).

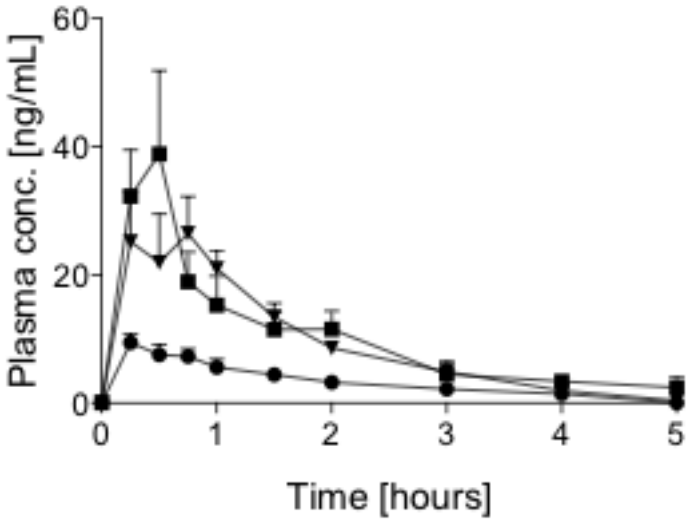



**Figure 2** Danazol plasma concentration profiles following oral administration to rats of SEDDS-III at low ($SEDDS_L$-III, circle), and high ($SEDDS_H$-III,square) dose and SEDDS-IV (triangle). All plasma concentration profiles are presented as mean ± SEM (n = 4) and the administered doses are tabulated in Table 4.

**Table 4** Pharmacokinetic parameters for danazol after oral administration to rats [mean ± SEM (n = 4)]

| Treatment | Dose | $AUC_{0\text{-}tz}$ | $AUC_{0\text{-}\infty}$ | $C_{max}$ | $T_{max}$ | *F* [a] |
|---|---|---|---|---|---|---|
| | [mg $kg^{-1}$] | [ng h $mL^{-1}$] | [ng h $mL^{-1}$] | [ng $mL^{-1}$] | [h] | [%] |
| $SEDDS_L$-III | 4.9 | 16 ± 3 | 19 ± 3 | 10 ± 1 | 0.4 ± 0.1 | 2.0 ± 0.4 |
| $SEDDS_H$-III | 8.6 | 52 ± 12 | 62 ± 13 | 43 ± 12 | 0.4 ± 0.1 | 3.7 ± 0.8 |
| SEDDS-IV | 5.8 | 47 ± 7 | 49 ± 7 | 32 ± 7 | 0.7 ± 0.2 | 4.4 ± 0.6 [b] |

[a] *F:* % Absolute bioavailability relative to IV data (refer Table 3)

[b] Statistically significant different compared to $SEDDS_L$-III ($P < 0.05$)

### *Bioavailability of danazol after intraduodenal administration of SEDDS*

The mean plasma concentration versus time profiles for $SEDDS_L$-III, $SEDDS_H$-III and SEDDS-IV following oral and ID administration are presented in Figure 3 A, B and C, respectively. The corresponding mean pharmacokinetic parameters following ID administration are summarised in Table 5 and dose normalized plasma profiles are presented in S5.

For all formulations, the bioavailability of danazol increased after ID administration when compared to oral administration of the equivalent formulation, and this was reflected in both $C_{max}$ and bioavailability (Table 5). Across the formulations, trends in relative performance were similar to that observed after oral administration and danazol bioavailability was highest from $SEDDS_H$-III ~ SEDDS-IV > $SEDDS_L$-III.

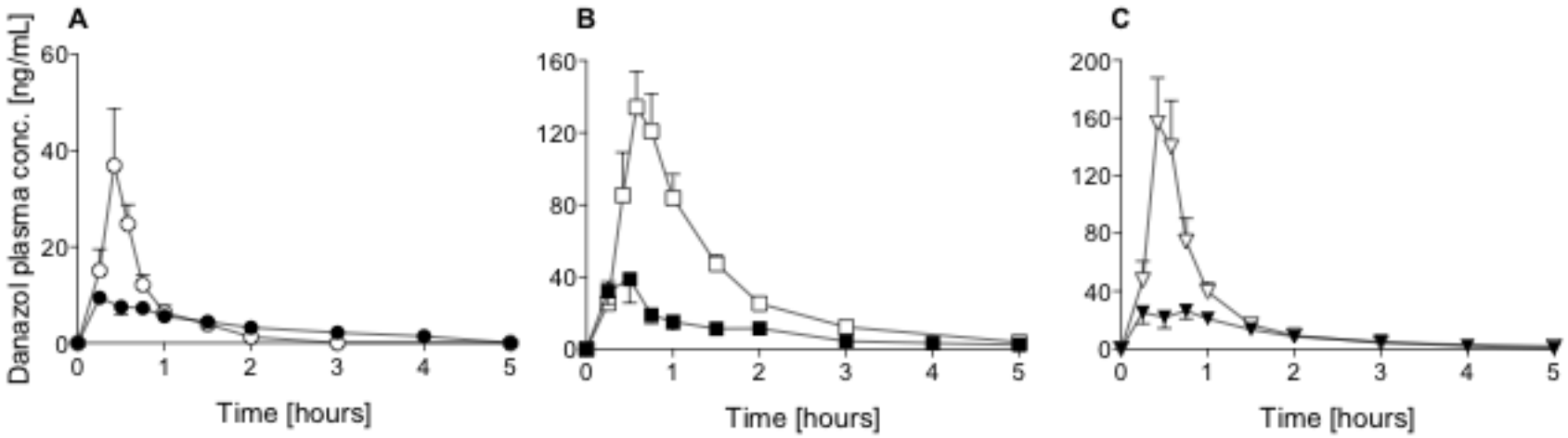



**Figure 3** Danazol plasma concentration profiles comparing oral (filled) versus intraduodenal (ID)(open) administration of A: SEDDS$_L$-III (circle), B: SEDDS$_H$-III (square), C: SEDDS-IV (triangle). All plasma concentration profiles are illustrated as mean ± SEM (n = 4) and the administrated doses are tabulated in Table 5.

**Table 5** Pharmacokinetic parameters for danazol after ID administration to rats [mean ± SEM (n = 4)]

| Treatment | Dose | $AUC_{0-tz}$ | $AUC_{0-\infty}$ | $C_{max}$ | $T_{max}$ | *F* [a] |
|---|---|---|---|---|---|---|
| | [mg $kg^{-1}$] | [ng h $mL^{-1}$] | [ng h $mL^{-1}$] | [ng $mL^{-1}$] | [h] | [%] |
| $SEDDS_L$-III | 4.0 | 25 ± 7 | 27 ± 8 | 39 ± 12 | 0.5 ± 0.0 | 3.6 ± 1.0 |
| $SEDDS_H$-III | 6.3 | 151 ± 18 | 156 ± 19 | 145 ± 21 | 0.6 ± 0.0 | 12.8 ± 1.5 [bc] |
| SEDDS-IV | 5.3 | 113 ± 23 | 116 ± 23 | 161 ± 29 | 0.5 ± 0.0 | 11.3 ± 2.3 [bc] |

[a] *F:* % Absolute bioavailability relative to IV data (refer Table 3)

[b] Statistically significant different compared to $SEDDS_L$-III ($P < 0.05$)

[c] Statistically significant different compared to oral administration of equivalent formulation ($P < 0.050$) (see Figure 2 and Table 4)

### *Impact of first pass metabolism on danazol bioavailability from SEDDS*

The impact of first pass metabolism was explored by administration of a non-specific cytochrome P450 inhibitor, 1-aminobenzotriazole (ABT), prior to danazol administration. The influence of ABT on danazol elimination was evaluated following IV administration of a 15% HP-β-CD solution containing danazol at 1.2 mg/mL in ABT pre-treated rats (a comparison of the mean plasma concentration versus time profiles for danazol in the presence and absence of ABT (S1) and the corresponding tabulated mean pharmacokinetic parameters (S2) can be found in supporting information).

ABT pre-treatment resulted in a significant reduction in danazol clearance (50 mL $min^{-1}$ $kg^{-1}$ compared to 87 mL $min^{-1}$ $kg^{-1}$ in the absence of ABT) with a corresponding increase in elimination half-life (1.8 h versus 1.2 h in the absence of ABT). No significant difference in volume of distribution was observed (7.6 L $kg^{-1}$ compared to 8.9 L $kg^{-1}$ in the absence of ABT).

The mean plasma concentration versus time profiles for danazol after oral and ID administration to ABT pre-treated rats are shown in Figure 4 with mean pharmacokinetic parameters summarised in Table 6 (dose normalized plasma profiles are presented in S6).

Panel A in Figure 4 shows the plasma profiles following oral administration of SEDDS-III at low and high drug load and SEDDS-IV in ABT pre-treated animals. Inhibition of CYP-metabolism resulted in significant increases in AUC compared to non ABT-treated animals (Figure 2). When compared with danazol exposure after IV administration in the presence and absence of ABT, oral bioavailability was much higher (up to 30 fold) suggesting the presence of a significant first pass effect for danazol in rats.

Oral administration of SEDDS-IV in the presence of ABT resulted in 45.4% danazol bioavailability compared to only 4.4% in the ABT untreated group. Increasing the lipid content in the formulation (and decreasing the surfactant content) by administering $SEDDS_L$-III, resulted in a small increase in bioavailability (to 59.7%) compared to SEDDS-IV, but again a very large increase relative to administration of the same formulation in the absence of ABT (~30-fold). When $SEDDS_H$-III was administered orally to ABT pre-treated rats (Figure 4A), increasing the drug loading in SEDDS-III led to a linear increase in $C_{max}$ and AUC and the oral bioavailability of SEDDS-III was unaffected.

Panel B in Figure 4 compares plasma profiles following ID and oral administration of $SEDDS_L$-III to ABT-treated rats. The oral bioavailability of danazol following ID administration in the presence of ABT was essentially complete (111.0%), and significantly higher than bioavailability after oral administration of the same formulation, also in the presence of ABT.

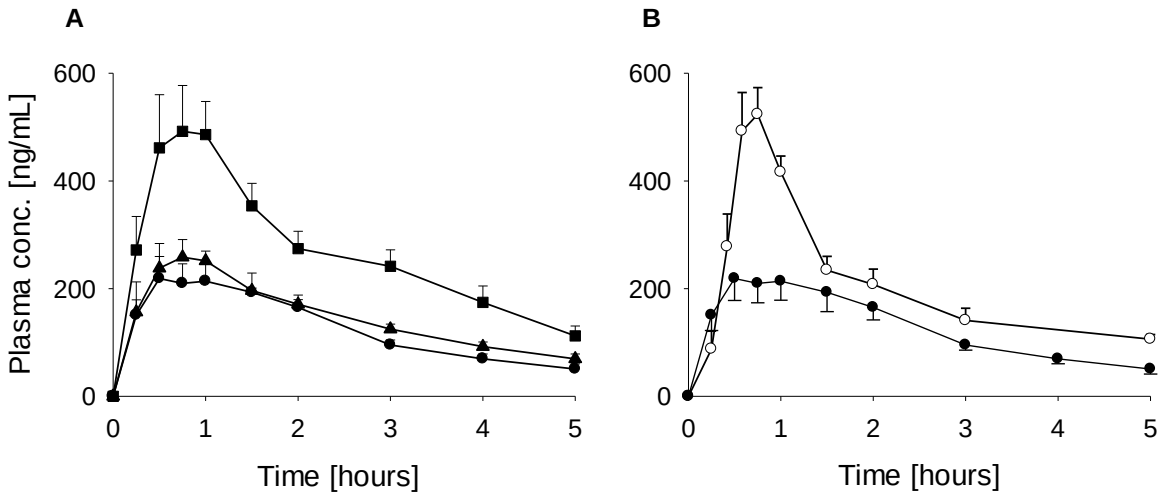


**Figure 4** Danazol plasma concentration profile from ABT pre-treated rats following A: oral administration of $SEDDS_L$-III (filled circle), $SEDDS_H$-III (filled square), SEDDS-IV (filled triangle); B: Intraduodenal (open) versus oral (filled) administration of $SEDDS_L$-III. All plasma concentration profiles are illustrated as mean ± SEM (n = 4).

**Table 6** Pharmacokinetic parameters for danazol after oral and intraduodenal administration to ABT pre-treated rats [mean ± SEM (n = 4)].

| Treatment | Dose | $AUC_{0-tz}$ | $AUC_{0-\infty}$ | $C_{max}$ | $t_{1/2}$ | $T_{max}$ | *F* [a] |
|---|---|---|---|---|---|---|---|
| | [mg $kg^{-1}$] | [ng h $mL^{-1}$] | [ng h $mL^{-1}$] | [ng $mL^{-1}$] | [h] | [h] | [%] |
| ABT-SEDDS-IV (O) [b] | 6.4 | 737 ± 21 | 975 ± 58 | 284 ± 36 | 2.3 ± 0.3 | 0.7 ± 0.2 | 45.4 ± 3 |
| ABT-$SEDDS_L$-III (O) | 3.9 | 636 ± 71 | 781 ± 103 | 258 ± 26 | 1.9 ± 0.3 | 1.3 ± 0.3 | 59.7 ± 8 |
| ABT- $SEDDS_H$-III (O) | 9.5 | 1344 ± 127 | 1643 ± 152 | 528 ± 72 | 1.8 ± 0.1 | 0.9 ± 0.1 | 51.6 ± 5 |
| ABT-$SEDDS_L$-III (I.D) [c] | 3.4 | 996 ± 102 | 1265 ± 141 | 541 ± 40 | 1.9 ± 0.2 | 0.8 ± 0.1 | 111.0 ± 12 [d] |

[a] *F:* % Absolute bioavailability calculated relative to IV data in ABT pre-treated rats (data in supplementary information Fig S2)

[b] Oral administration (O),

[c] Intraduodenal administration (I.D.)

[d] Statistically significant different compared to oral administration of $SEDDS_L$-III ($P < 0.050$)

## IN VITRO EVALUATION

### *Impact of gastric dispersion and ex vivo gastric fluid on drug precipitation from SEDDS*

The impact of formulation processing under gastric conditions on the in vitro performance of the investigated formulations (SEDDS-IV / $SEDDS_L$-III) was evaluated in a series of dispersion studies (Figure 5).

Dispersion of SEDDS-IV under high dilution conditions at pH 1.2 led to rapid drug precipitation and only 40% of the initial drug load was retained in a solubilized state after 30 min. In comparison, no drug precipitation was observed on dispersion of $SEDDS_L$-III under similar conditions (Figure 5A). Experiments were also conducted under lower dilution conditions (1 in 10) and at pH 5.5 (Figure 5B). Decreasing the volume of dispersion medium did not affect drug solubilisation patterns when compared to high volume conditions for either $SEDDS_L$-III or SEDDS-IV. Increasing the drug load in SEDDS-III, however, resulted in ~20% drug precipitation for $SEDDS_H$-III (Figure 5B).

Solubilisation/precipitation patterns following formulation dispersion in ex vivo gastric fluids from rats are shown in Figure 5C. The dispersion of $SEDDS_L$-III and SEDDS-IV in ex vivo gastric fluids resulted in similar drug solubilisation patterns to that observed using the simpler in vitro conditions. The mean pH of the collected ex vivo gastric fluid was 4.8, which is in agreement with previously published studies[35] and similar to that used in the low dilution simulated rat gastric fluid buffer (pH 5.5)

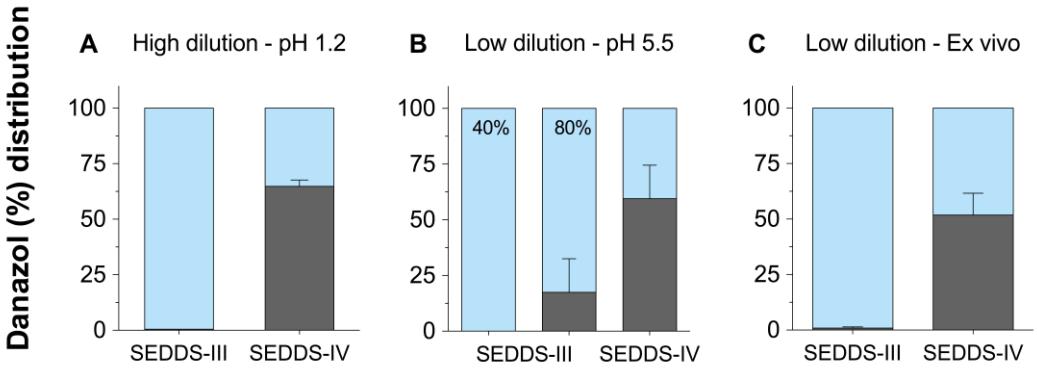



**Figure 5** The extent of danazol precipitation after 30 min dispersion of SEDDS-IV and $SEDDS_L$-III (drug loaded at 40% saturated solubility) under gastric condition [mean ± SD (n = 3)] in A: 40 mL 0.1 N HCL pH 1.2; B: 0.9 mL buffer, pH 5.5; C: 0.9 mL ex vivo rat gastric fluid pH 4.8. In addition, panel B also shows $SEDDS_H$-III (80% saturated solubility) and therefore the effect of drug loading on drug precipitation after 30 min of dispersion. Differences in drug loading in panel B are indicated by 40% and 80% labels within the bars. Bars represent danazol in aqueous phase (light blue) and precipitate (dark grey) [mean ± SD (n = 3)].

### *Impact of intestinal digestion on in vitro performance of danazol SEDDS*

Development of a rat in vitro digestion model

***Rat digestion model – high dilution / low enzyme activity***

To evaluate potential differences in formulation processing between species, in vitro digestions were initially conducted using ex vivo rat pancreatic enzymes and biliary fluids (Table 2 - Rat digestion model (high/Low)) rather than the porcine pancreatin extract previously employed.[11] Figures 6 A and B show the precipitation profiles for danazol over 60 min during in vitro digestion of SEDDS$_L$-III using the previous in vitro lipolysis model (dog digestion model (high dilution/high enzyme activity)) and the initial rat digestion model (Table 2 - Rat digestion model (high dilution/low activity)). Compared with the previous in vitro digestion model, the use of ex vivo rat pancreatic fluid to stimulate digestion resulted in much lower drug precipitation during the initial 30 min of digestion. Drug precipitation became more apparent after 60 min suggesting that the extent of precipitation may not vary significantly, but that there is a significant delay when using ex vivo rat pancreatic fluid rather than porcine pancreatic extract.

The much lower effect of ex vivo rat pancreatic enzymes on danazol solubilisation during digestion of SEDDS$_L$-III subsequently stimulated an evaluation of the relative enzyme activity of the ex vivo rat pancreatic enzyme when compared with the porcine pancreatic enzyme used previously.

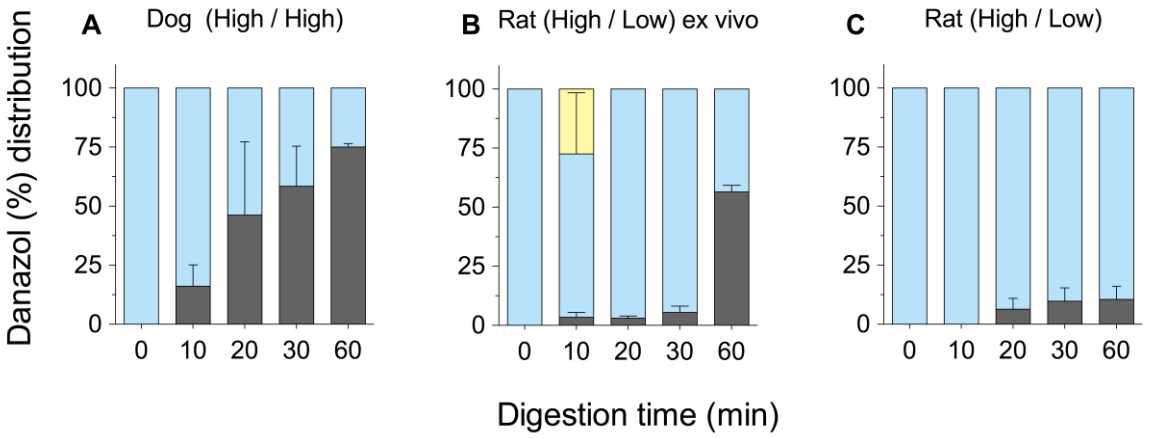



**Figure 6** Danazol solubilisation during digestion of SEDDS$_L$-III under three conditions; A: In vitro digestion utilizing the previous dog digestion model (high dilution/high enzyme activity) using 4 mL porcine pancreatin extract (data reproduced from Anby et al[11]), B: a rat digestion model (high dilution/low enzyme activity) using 1 mL rat pancreatic/biliary fluid and C: a rat digestion model (high dilution/low enzyme activity) using 17 µL of porcine

pancreatin extract to match the activity of ex vivo rat pancreatic/biliary fluid. Bars represent danazol in aqueous colloidal (light blue), oil phase below colloidal phase (light yellow) and precipitate (dark grey) [mean ± SD (n = 3)].

## Evaluation of ex vivo lipase activity

The activity of ex vivo rat pancreatic fluids was quantified using a tributyrin assay and compared to a 'standard curve' of the activity of known volumes of porcine pancreatin extract (data presented in Figure 6 and tabulated in S3). In the standardised tributyrin lipolysis test, 1 mL porcine pancreatin resulted in an effective lipase activity of 1097 TBU. Decreasing the volume of porcine pancreatin added resulted in a non-linear decrease in liberated butyric acid and a decrease in enzyme activity (measured in TBU). Using the same model, addition of 1 mL of ex vivo rat pancreatic/biliary fluid resulted in much lower lipolytic activity (194 TBU). The lipolytic activity of 1 mL of ex vivo pancreatic/biliary fluid was therefore approximately 5 times lower than that of 1 mL of the porcine pancreatin extract. By virtue of the markedly non-linear relationship between enzyme activity and mass of porcine pancreatic extract, 17 µL (1.7%) of porcine pancreatic extract gave the same activity (194 TBU) as that provided by 1 mL of ex vivo rat pancreatic fluid (Figure 7).

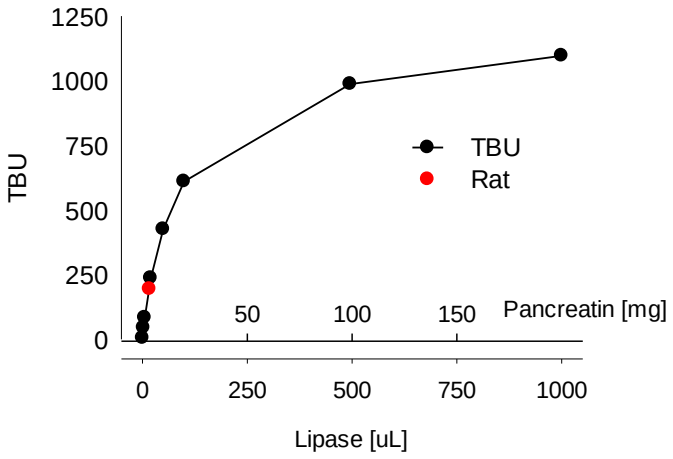



**Figure 7** Volume of porcine pancreatic extract (and the corresponding quantity of pancreatin powder) added to 10 mL digestions versus the correlating enzyme activity expressed in TBUs. The lipase activity in ex vivo rat pancreatic/biliary

fluid is illustrated by the red circle (194 ± 34 TBU). Similar activity is expected from 17 µL of porcine pancreatic extract (~ 1.7% of the volume utilized previously).

Drug distribution patterns following addition of 17 µL of porcine pancreatic extract rather than 1 mL of ex vivo rat bile are shown in Figure 6C. Similar data were seen when compared to the ex vivo rat pancreatic/biliary fluid up to 30 min digestion, although the increase in digestion at 60 min was not evident.

***Rat digestion model – low dilution / low enzyme activity***

In an attempt to better reproduce conditions in the GI tract of the rat with volumes likely to be less than 10 mL, a lower volume (low dilution) rat digestion model (Table 2 - Rat digestion model (low dilution/low activity)) was also evaluated using the same quantities/sources of enzyme used in the higher volume rat model (i.e. 1 mL of ex vivo rat pancreatic fluid or 1 mL of 1.7% porcine pancreatic extract). In this case, however, the total volume of digestion media (buffer plus enzyme) was reduced to 2.05 mL.

Under these conditions, formulation processing and danazol solubilisation profiles were markedly different when compared to patterns obtained using the much higher volume digests (Figure 8). The most notable change was the generation of a dense lipid-rich phase for the SEDDS-III formulation, which migrated to the bottom of the tube on centrifugation. In contrast, digestion and phase separation under higher enzyme loads and higher dilution led to a pellet phase containing precipitated drug and a highly dispersed micellar aqueous phase. In the low enzyme activity/ low volume model over 99% of the drug from the $SEDDS_L$-III formulation was recovered in the dense oil phase located below the colloidal aqueous phase (Figure 8A). Similar data were obtained using either ex vivo pancreatic fluid or 1.7% of the standard porcine pancreatic extract (Figure 8A).

The impact of drug load was also evaluated using the low volume digestion model (Figure 8B). For the $SEDDS_H$-III formulation, addition of ex vivo lipase led to a similar high-density oil phase that contained the majority of the drug. Limited drug precipitation was observed after 30 min as shown in Figure 8B, however following 60 min digestion some drug precipitation was observed, albeit at a relatively low level (< 20%), presumably reflecting the higher drug load.

The use of porcine pancreatic extract resulted in a similar profile (Figure 8B), but in this case precipitation was did not occur at later time points.

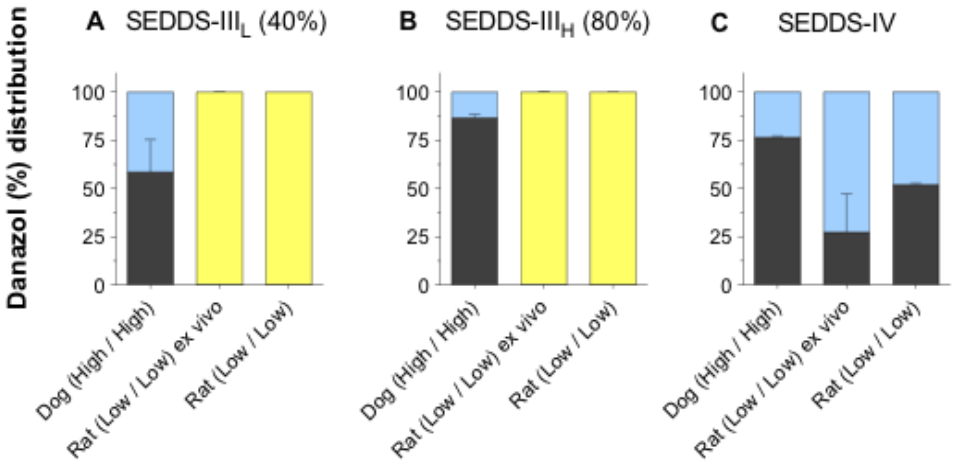



**Figure 8** Danazol distribution following 30 min digestion of A: SEDDS$_L$-III, B: SEDDS$_H$-III and C: SEDDS-IV under three conditions; 1: In vitro dog digestion model (high/high) using 1 mL of porcine pancreatin extract (reproduced from Anby et al[11]), 2: In vitro rat digestion model (low/low) using 1 mL of ex vivo rat pancreatic/biliary fluid, 3: In vitro rat digestion model (low/low) using 1 mL of 1.7% porcine lipase to reflect the activity in ex vivo rat pancreatic/biliary fluid. Bars represent danazol in aqueous colloidal (light blue), oil phase below colloidal phase (light yellow) and precipitate (dark grey) [mean ± SD (n = 3)].

Low dilution rat digestion experiments conducted with formulation SEDDS-IV resulted in high drug precipitation despite the low volume digestion model (Figure 8C). In this case, the ex vivo rat pancreatic fluid led to lower drug precipitation during digestion (albeit with more variability) when compared to the porcine pancreatic extract. However, regardless of the source of pancreatic enzymes, only 40-50% of the initial drug loading was recovered in the aqueous phase following digestion. In contrast to the SEDDS-III formulation no high-density oil phase was generated on digestion of formulation SEDDS-IV, consistent with the lack of classical oils in the formulation.

## DISCUSSION

Previous studies of danazol absorption from SEDDS revealed non-linear increases in exposure (bioavailability) with increasing drug dose in dogs.[19] This was suggested to reflect either saturation of first pass metabolism or an increase in thermodynamic activity at higher doses. Interestingly, the dose-dependent increase in bioavailability was evident in only one cohort of (older) animals, and not in another (younger) group. The uncertainty generated by these previous studies stimulated a more detailed examination of danazol absorption from SEDDS formulations in the current study. Here, the rat model was employed to allow more facile examination of the role of first pass metabolism and to examine whether the trends seen previously in dogs were replicated in another species (and therefore to rule out species-specific anomalies). The use of rats also prompted a re-evaluation of the utility of previous in vitro digestion protocols (that were originally established to reflect events in the dog) to mirror events in the rat where GI volumes and enzyme activities were expected to be lower.

### *Danazol bioavailability in rats after administration of SEDDS-III and SEDDS-IV*

The plasma profiles in Figure 2 and the pharmacokinetic parameters presented in Table 4 reveal moderate differences in in vivo exposure of danazol in rats following oral administration of two markedly different lipid based formulations (SEDDS-IV and SEDDS-III). In both cases, bioavailability was low, in agreement with previous studies in rats using similar lipid-based formulations where the absolute bioavailability of danazol was < 20%.[43, 44] When danazol was incorporated into SEDDS-III at a 2-fold higher dose ($SEDDS_H$-III), bioavailability increased when compared to the lower dose formulation ($SEDDS_L$-III). The dose effect data for SEDDS-III was consistent with previous studies in beagle dogs, at least in older animals.[19]

Danazol bioavailability after administration of SEDDS-IV, however, was significantly higher (~2-fold) than that observed after administration of $SEDDS_L$-III, in contrast to previous studies in beagle dogs where little difference in oral bioavailability was seen across the two formulations.[11] The very low absolute bioavailability of danazol in the rat prompted a more detailed evaluation of the potential causes of the limited exposure. In the first instance, intraduodenal (ID) administration was explored as a means of circumventing potential gastric processing of lipidic formulations, on the basis that drug precipitation in the stomach may reduce drug absorption. The plasma profiles in

Figure 3 and the pharmacokinetic parameters presented in Table 5 are consistent with this suggestion and ID administration of both the high cosolvent/surfactant formulation (SEDDS-IV), and the more lipid-rich formulations ($SEDDS_L$-III and $SEDDS_H$-III) led to increased danazol exposure (1.8-3.5 fold) when compared to oral administration (although the increase was not statistically significant for $SEDDS_L$-III). Interestingly, the non-linearity in danazol absorption with increasing dose was not only retained after intraduodenal absorption but was enhanced. Thus, the bioavailability of danazol after intraduodenal infusion of $SEDDS_H$-III was 12.8%v versus 3.6% for $SEDDS_L$-III (a 3.6-fold increase) whereas after oral administration danazol bioavailability from the same formulations was 3.6% versus 2% (a 1.8-fold increase).

The increase in exposure evident after ID administration may reflect avoidance of precipitation events in the stomach after oral administration. However, in light of the larger increase in intraduodenal bioavailability for the higher dose formulation ($SEDDS_H$-III) (where precipitation might be expected to be more prevalent, not less), it is also possible that direct introduction of high concentrations of drug directly into the intestine, may more effectively saturate first pass enterocyte-based metabolism than is the case after oral administration (where gastric emptying is expected to delay and dilute entry into the small intestine).

### *First pass metabolism is the major limitation to danazol oral bioavailability from SEDDS formulations in the rat*

Previous studies have shown that after oral administration of ($^{14}C$) labelled danazol to rats, biliary excretion of danazol metabolites is significant (~70%) and that extensive enterohepatic re-cycling occurs.[20] In vitro studies have further shown that danazol is primarily a substrate for CYP3A4 (86%) and to a lesser extent CYP2D6 (11%) and CYP2J2 (3%).[45] The role of CYP-mediated danazol metabolism in the rat was explored here using the non-specific cytochrome P450 inhibitor, 1-aminobenzotriazole (ABT). ABT has been used extensively in animal studies to probe the role of CYP3A and CYP2D in drug clearance and first pass metabolism.[30] In rats, pre-treatment with 100 mg/kg ABT 2 hours prior to the administration of a test compound (antipyrine) substantially inhibits CYP450 enzymes, and a single dose of ABT is sufficient to inhibit metabolism for over 24 h.[30, 31]

Comparison of oral and IV plasma AUCs obtained after ABT pre-treatment provides an indication of 'apparent' danazol bioavailability in the absence of CYP450-mediated metabolism, and therefore gives a clearer indication of the likely fraction of dose absorbed, unencumbered by first pass CYP-metabolism. Under these conditions, oral administration of $SEDDS_L$-III to ABT pre-treated rats resulted in a sharp increase in apparent bioavailability to 59.7% compared to true bioavailability of 2% in non-ABT pretreated animals (Figure 4 and Figure 2, respectively). First pass metabolism is therefore a very significant limitation to danazol oral bioavailability in rats under these conditions and at these doses.

Furthermore, in contrast to the data in non-ABT pretreated rats, increasing the drug load in the $SEDDS_H$-III formulation resulted in similar danazol exposure (51.6%) to that obtained after administration of $SEDDS_L$-III The lack of difference in danazol bioavailability after administration of the two drug doses in the presence of ABT suggests that the non-linear increase in danazol bioavailability observed in the absence of ABT stems from saturation of first pass metabolism rather than differences in thermodynamic activity and fraction of dose absorbed.

After pre-administration of ABT, danazol bioavailability was slightly higher after administration of $SEDDS_L$-III when compared to the cosolvent/surfactant-based formulation (SEDDS-IV), however, these differences were not significant (Figure 4). This is in contrast to the data obtained in the absence of ABT where danazol bioavailability from SEDDS-IV was higher than that from SEDDS-III. The enhanced bioavailability of danazol after administration of the SEDDS-IV formulation in the absence of ABT is consistent with higher danazol solubility in SEDDS-IV (17.9 mg/g vs. 12.1 mg/g in $SEDDS_L$-III) (Table 1) and therefore administration of a slightly higher dose at 40% of the saturation solubility. Realizing the role of first pass metabolism in dictating danazol bioavailability in the rat, it is likely that the higher dose may have led to greater saturation of first pass metabolism in the absence of ABT. Previous studies have also suggested that Cremophor EL (present at high concentrations in SEDDS IV) is able to inhibit CYP3A in human and rat liver microsomes.[46, 47] The results obtained here in the absence of ABT may therefore indicate differences in the ability of the two formulations to inhibit first pass metabolism. More specifically, the higher quantity of Cremophor in SEDDS-IV [65% (w/w)] compared to SEDDS-III [(30% (w/w)] may help to reduce first pass metabolism and promote bioavailability. In contrast, in the presence of ABT, metabolic limitations are circumvented and solubilisation events dominate. Under these circumstances, differences between danazol bioavailability from SEDDS-III and SEDDS IV were not significant (consistent with the previous data in dogs), although exposure was slightly higher from SEDDS-III.

Bearing in mind the increase in exposure in the absence of ABT after ID administration (compared to oral administration) $SEDDS_L$-III was also administered intraduodenally to ABT pre-treated rats to see whether the same trends were apparent. In this case, bioavailability increased to 111.0% after ID administration when compared to 59.7% after oral administration. Absorption of danazol was therefore essentially complete after intraduodenal administration of $SEDDS_L$-III. The increase in bioavailability observed after ID rather than oral administration of $SEDDS_L$-III to ABT pretreated animals (1.9-fold) was also consistent with the increases seen in non-ABT pretreated animals (1.8-fold) suggesting that the drivers of enhanced absorption after ID rather than oral administration, in the presence of ABT, were similar to the drivers of enhanced bioavailability in the absence of ABT. Alignment between increases in bioavailability in the presence and absence of ABT suggests that in this case the differences between ID and oral administration may have been mediated by changes to solubilisation rather than first pass metabolism.

In contrast, much greater increases in bioavailability were evident after intraduodenal versus oral administration of $SEDDS_H$-III to non ABT pre-treated animals (~3.5 fold) when compared to intraduodenal versus oral administration of $SEDDS_L$-III (1.8-fold). Since bioavailability of danazol after oral administration of $SEDDS_H$-III to ABT pre-treated animals was as high as ~52% (and therefore the fraction absorbed must have been at least 55%), the 3.5-fold increase in bioavailability seen after intraduodenal administration in the absence of ABT could not have stemmed (completely) from increases in absorption. The current data therefore indicate that at the higher drug load (i.e. after administration of $SEDDS_H$-III) intraduodenal administration was able to more effectively saturate first pass metabolism than was the case at lower drug doses. This trend was also replicated for SEDDS-IV where increases in ID versus oral bioavailability were slightly higher (2.6-fold) than were evident for $SEDDS\text{-}III_L$ at the lower danazol dose. Intraduodenal delivery therefore seems more able to take advantage of direct delivery to the absorptive site and to subsequently inhibit first pass metabolism when combined with formulations containing higher doses of danazol. Formulation strategies that deliver high concentration of danazol rapidly to the upper small intestine therefore seem most likely to benefit from increases in bioavailability, at least in the rat.

In summary, the in vivo data suggest that the primary limitation to danazol bioavailability in the rat is first pass metabolism, that increasing drug dose leads to increases in bioavailability via saturation of first pass metabolism, that intraduodenal administration results in increases in bioavailability probably as a result of both increases in absorption

and reductions in first pass metabolism, and that based on the data obtained in the presence of ABT, danazol absorption from the SEDDS formulations examined here is generally good (in contrast to bioavailability). Indeed after ID administration danazol absorption from $SEDDS_L$-III was almost complete. This is surprising based on previous in vitro dispersion/digestion data[11] that show significant drug precipitation after initiation of digestion for both SEDDS-IV and SEDDS-III. Interspecies differences in GI tract conditions may, however, influence formulation processing, and the efficiency of digestion (and subsequent drug precipitation) may be different in the rat when compared to larger species, such as the dog. The solubilisation behaviour of the SEDDS formulations was therefore also evaluated under in vitro conditions more reflective of the GI tract in the rat, when compared to GI conditions in the dog.

### *The effect of gastric dispersion on SEDDS performance in the rat*

To provide a comparative assessment of possible behaviour in rats and dogs, the impact of gastric dispersion on formulation performance was initially evaluated using experimental protocols designed to mimic conditions in the dog (high formulation dilution in simulated gastric fluid, pH 1.2). Dispersion of SEDDS-IV in pH 1.2, high dilution gastric media resulted in significant drug precipitation and increased drug precipitation compared to dispersion data conducted under simulated intestinal conditions (Figure 5A)**.** To better reflect the conditions expected in the GI tract of the rat, dispersion volume and pH were subsequently altered to 900 µL and pH 5.5, respectively, however, significant precipitation of danazol from SEDDS-IV was still evident. In contrast, drug solubilisation during dispersion of the formulation containing greater quantities of lipid (and lower quantities of surfactant and cosolvent), $SEDDS_L$-III, was not affected by pH or dispersion volume (Figure 5 A, B) and drug precipitation from $SEDDS_L$-III was limited under both conditions.

The interaction of lingual lipase with medium-chain triglycerides results in the liberation of fatty acid,[48] and lingual lipase activity is reportedly[49] high in rodents. Subsequent experiments were therefore conducted to explore the potential additional impact of ex vivo gastric fluids (containing any available lingual lipase) on danazol precipitation from SEDDS formulation. These data suggest limited effects of lingual lipase on danazol solubilisation in SEDDS in the rat stomach (Figure 5C)**.** However, pre-processing of lipids in the stomach may affect subsequent events in the

duodenum (i.e., secretion of digestive enzymes and the rate and extent of lipid digestion)[50-52] and as such, gastric digestion by lingual lipase may indirectly affect drug absorption in the small intestine.

### *Development of a modified in vitro digestion model for SEDDS evaluation in the rat*

To explore the potential impact of intestinal digestion on SEDDS performance, an in vitro lipolysis model previously used to examine digestion events in the dog was modified here to better reflect the conditions in the GI tract of the rat. Experiments were initially undertaken using the same formulation dilution factor as that previously used in the 'dog' in vitro digestion model, but where the source of digestive enzyme was replaced with ex vivo rat pancreatic fluid. This model is described in the methods as the rat high dilution / low enzyme activity model. Ex vivo pancreatic fluids were collected from rats by cannulation of the common bile duct resulting in collection of mixed bile and pancreatic fluids. Analysis of the in vitro activity of recovered rat pancreatic enzyme (using a standardized tributyrin activity test) revealed activities (~200 TBU/mL pancreatic/bile fluid) much below the values commonly reported in vivo in humans and dogs, and therefore far below the levels commonly used in in vitro digestion experiments modelled on those conditions.

Recently, Tønsberg et al[53] examined lipase activity in luminal intestinal samples from rats and reported lower activity (153 U/mL), consistent with the levels utilised in the rat digestion models employed here and consistent with dilution of pancreatic fluids with bile prior to entry into the intestine. It seems likely therefore that the pancreatic enzyme levels recorded here, whilst low, reflect lower lipase activity in the rat GI tract when compared to the dog or human.

Formulation digestion was evaluated in a series of in vitro experiments utilising different formulation dilutions, enzyme activities and enzyme sources. As expected, the conduct of studies using the high dilution/low enzyme activity model and employing 1 mL of ex vivo rat pancreatic/biliary fluid resulted in much lower lipid digestion and correspondingly lower levels of drug precipitation when compared to the previously employed dog in vitro conditions (Figure 6). Based on in vitro analysis of the activity of the ex vivo pancreatic/biliary fluids, subsequent studies were conducted using a quantity of porcine pancreatic enzyme that was equally active in the TBU test to 1 mL of ex vivo enzyme fluid (Figure 7). Similarly reduced levels of digestion and precipitation were evident, suggesting that substitution of low levels of porcine pancreatic enzyme may be sufficient to broadly mimic the lipolytic activity of ex vivo rat pancreatic fluids. For

different digestible substrates, however, different pancreatic enzymes may be required, and a more detailed series of studies would be required to fully characterise the similarity of rat pancreatic/biliary fluids to porcine pancreatic extract.

A digestion model was also evaluated using much lower dilution conditions (the rat low dilution/low activity model). This was designed to better mimic the lower fluid volumes expected in the rat GI tract where the volume of fluid administrated with the formulation was ~1 mL and the flow of fluid from the bile duct is ~1.5 mL/h. Under these conditions, the formulations behaved quite differently, and SEDDS-III formed a dense lipid phase that phase separated below the aqueous phase. This lipid-rich phase contained 80-99% of the incorporated drug. In contrast, no oil phase was generated on digestion of SEDDS-IV, the lipid-free formulation, suggesting that the high density oil phase generated by digestion of SEDDS-III consisted of fatty acids and mono/diglycerides generated via digestion of the lipids present in SEDDS-III.

Conduct of these experiments at low volume precluded the use of the pH stat titrator and as such, the pH in the digest was not constrained. Fatty acid liberation therefore resulted in a limited drop in pH during digestion of SEDDS-III (pH 5.8 following 60 min digestion). Nonetheless, pancreatic enzyme activity was expected to be retained at this pH [54]. In contrast, the terminal pH of digestion of SEDDS-IV was higher (pH 7.9) suggesting limited fatty acid liberation, consistent with the lack of lipid substrate in this formulation.

The presence of an oil phase that floats on centrifugation is common during lipid digestion and typically represents poorly digested tri- and diglycerides, and less readily solubilised monoglycerides and protonated fatty acids. In contrast, in the current low dilution/low activity rat digests, the oil phase that was generated on digestion, was a viscous isotropic phase that was more dense than the solubilised aqueous phase and sedimented when left unstirred, consistent with previous observations.[55, 56] The current data suggest that in the rat, under conditions of lower dilution in vitro (and potentially in vivo), less readily dispersed lipid phases are formed that are less dense than water. This may also be exacerbated by the lower pH, resulting in greater quantities of less polar unionised fatty acid. Where isotropic, partially digested lipid phases are formed under conditions of low enzyme activity and low dilution, the likelihood of drug precipitation appears to be diminished. Whether drug absorption is possible from these phases directly or whether further dispersion into e.g. bile salt micelles is required is unknown at this point. Continued

dilution is likely to occur and the possibility of transition through different phases, which may not be captured with the low dilution conditions employed here, is likely. This is supported by the difference between oral and ID administration in the presence and absence of ABT suggesting that differences in phase generation and how the drug is presented at the absorption site is important, and that drug precipitation from SEDDS-III may occur in spite of the low dilution/low enzyme in vitro model.

The current studies therefore suggest that comparison of in vivo drug absorption patterns in the rat with in vitro digestion data obtained using lipid digestion models that simulate dog/human conditions may lead to overestimation of drug precipitation and underestimation of absorption. Grove et al[57, 58] previously also suggested that the quantity of GI fluid present in the rat may be low, and that administration of self-emulsifying drug delivery systems under these conditions may lead to the formation of a more viscous, bicontinuous phase when compared to an emulsion system. The current in vitro data using the low volume low enzyme activity rat model are consistent with this contention.

### *Impact of animal model on danazol bioavailability from SEDDS*

In the current studies the primary limitation to danazol oral bioavailability in the rat was first pass metabolism, and this was reduced (and bioavailability enhanced) by administration of higher doses or by direct infusion of the dose into the duodenum. These data are broadly consistent with previous danazol dose-escalation studies in older beagle dogs where administration of higher doses also resulted in increased drug exposure.[19] However, in beagle dogs the absolute oral bioavailability of danazol was higher (10-26%)[19] than that seen here in rats, and much higher oral bioavailability of danazol in beagle dogs has previously been reported (64%, 82% and 107%).[21, 59] Collectively, the data suggest that where solubilisation is ensured, danazol bioavailability in the dog may be more than an order of magnitude higher than that observed here in the rat (< 5% after oral administration), and therefore that first pass limitations to bioavailability are likely to be lower. Whilst the rat data presented here indicate a very large first pass effect; under conditions where first pass metabolism was inhibited, absolute bioavailability was high suggesting that the fraction absorbed was also high. This was not expected based on previous in vitro digestion data showing considerable drug precipitation under simulated dog GI environments,[11, 22] which was seemingly reflected in previous bioavailability data in the dog[22, 60] showing significant formulation effects on bioavailability. However, much improved

absorption in the rat is consistent with the lower extent of precipitation obtained in the in vitro tests conducted in the current studies under the lower dilution conditions and lower digestive enzyme levels expected in the rat. In contrast, under conditions of lower metabolic effects, higher intestinal dilution and higher digestive enzyme load in the dog, bioavailability appears to be higher and more dependent on continued solubilisation. One significant lack of congruence between the current rat studies and previous beagle studies is the differential behaviour of the SEDDS-III and SEDDS-IV formulations. Here, in rats, SEDDS-IV outperformed SEDDS-III when first pass metabolism was the primary limitation to bioavailability leading to the suggestion that the higher absolute dose in the SEDDS-IV formulation, or the presence of higher quantities of Cremophor EL, may have enhanced bioavailability via a reduction in first pass metabolism. In contrast, in beagle dogs, danazol exposure was essentially the same after administration of SEDDS-III and SEDDS-IV.[11] This may be explained by lower first pass metabolism in the dog and therefore less impact of formulation effects that are mediated via differences in first pass rather than differences in solubilisation. Interestingly, in the presence of ABT (i.e. in the absence of first pass effects) danazol absorption from SEDDS-III and SEDDS-IV was similar in the rat and more consistent with the previous dog data.

This, however, also suggests that in spite of the somewhat higher prevalence of precipitation from SEDDS-IV when compared to SEDDS-III, in both dog and rat digestion conditions (although much lower for ex vivo rat conditions), this may not significantly impact absorption and in vivo exposure. For these formulations, in vitro digestion therefore seems to overestimate the extent, or impact, of precipitation in vivo in some cases. Similar conclusions have recently been drawn for correlations between the absorption of the model weakly basic drug substance AZD0865,[61] and the basic BSC class II drug mebendazole,[62] and simple in vitro models of drug precipitation where the in vitro tests also appeared to over-predict precipitation in vivo.

The current data also raise the question as to whether formulation or dose effects on first pass metabolism may have obscured data interpretation in previous dog studies, In particular, in studies by Cuine et al[22] danazol bioavailability was previously shown to correlate well with differences in solubilisation during in vitro digestion experiments and in particular to decrease with the inclusion of increasing proportions of surfactant (cremophor EL) and cosolvent in SEDDS formulations. In these studies, however, the formulations with the highest quantities of Cremophor EL (and therefore those that might be expected to more significantly inhibit first pass metabolism) resulted in the lowest in

vivo danazol exposure. Similarly, drugs were dosed at a fixed proportion of drug solubility in the formulation, and since danazol is more soluble in surfactant and cosolvent than in lipids, the absolute dose in the surfactant and cosolvent rich formulations was also higher. This in turn might be expected to lead to increased saturation of first pass metabolism. In contrast, the reverse was true and the formulations containing the highest absolute danazol doses led to the lowest exposure. These previous data are therefore consistent with the suggestion that whilst first pass metabolic limitation may dominate danazol bioavailability in the rat, this may not be the case in the dog where correlations with in vitro solubilisation profiles appear to provide good rank order indicators of in vivo exposure.

## CONCLUSION

SEDDS formulations have been widely employed to enhance the oral bioavailability of poorly water-soluble drugs. In the majority of cases, the ability of SEDDS to improve bioavailability has been ascribed to increases in apparent GI solubility or the ability to circumvent traditional dissolution process. SEDDS are therefore commonly used to enhance the oral bioavailability of poorly water-soluble drugs. Many poorly water-soluble drugs are also highly metabolised, and in some cases, first pass metabolism may be an additional limitation to oral bioavailability. Danazol is employed here as a poorly water-soluble and highly metabolised drug, to better understand the potential role of formulation, drug dose and first pass metabolism on drug bioavailability from SEDDS formulations. In the current studies the oral bioavailability of danazol in the rat, after administration of either a LFCS class III or class IV lipid based formulation, was extremely low (<5%). In contrast, data obtained in the presence of the metabolic inhibitor ABT revealed that the fraction absorbed was high from all formulations (45-60%). Since ABT is not expected to affect the fraction absorbed, it is likely that danazol absorption was relatively high after administration of all SEDDS formulations and that the major limitation to oral bioavailability was first pass metabolism. Interestingly, this was not consistent with data obtained during in vitro lipolysis studies, at least with models that reflected intestinal conditions in the dog or human, since these suggested that significant drug precipitation was expected on formulation processing in the GI tract. Efforts were therefore made to modify the lipolysis test to better reflect the intestinal conditions in the rat. When test conditions were modified to better reflect the much lower digestion challenge in the rat intestine and lower levels of dilution (i.e. the rat (low/low) digestion model), the degree of drug precipitation on formulation digestion was

reduced, providing data that was more consistent with the in vivo data (where danazol absorption was seemingly robust from both of the studied SEDDS formulations). The rat (low/low) in vitro digestion model may therefore provide a better indication of the utility of SEDDS formulations to promote drug absorption in the rat. Comparison of formulation performance across in vitro models reflecting species differences in dilution and enzyme activity may also provide an indication of differences in drug exposure in different species, however, significantly more data is required to support this suggestion.

Even when using the rat (low/low) digestion model, however, the class IV formulation outperforms the in vitro profile and resulted in good exposure in spite of significant in vitro drug precipitation, Recent studies suggest that in vitro GI models may have a tendency to overestimate drug precipitation in vivo, potentially due to non-sink conditions. Thus, formulations providing for transiently highly supersaturated conditions (i.e. the type IV formulations) may lead to good absorption in spite of significant in vitro drug precipitation. However a more detailed investigation of time dependent drug absorption, including the impact of gastric emptying, is required.

In the absence of ABT, danazol bioavailability was higher after administration of the class IV formulation when compared to class III formulations, whereas in the presence of ABT this difference was ameliorated. This suggests that the Cremophor rich type IV formulation may reduce first pass metabolism. However, the absolute drug dose was slightly higher in this formulation (which in turn may have led to saturation of first pass metabolism), the effect was only moderate and first pass metabolism was still highly significant. The ability of the SEDDS formulations studied here, to reduce first pass metabolism, was therefore limited, at least for danazol.

In contrast to the data obtained here in the rat (where digestive enzyme activity was low, metabolic activity high and effects on first pass metabolism critical), previous studies suggests that danazol bioavailability in the dog is less dependent on first pass metabolism, more dependent on continued solubilisation and therefore more sensitive to differential formulation processing by digestion.

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## ILLUSTRATIONS EMBEDDED IN THE MANUSCRIPT

## SUPPORTING INFORMATION

Supporting information **S1** illustrates the danazol plasma profile after IV administration of 15% HP-β-CD solution containing danazol at 1.2 mg/mL to control (open) [mean ± SD (n = 5)] and pre-treated (filled) ABT rats [mean ± SD (n = 4)]. **S2** shows the tabulated pharmacokinetic parameter after intravenous administration of a 15% HP-β-CD solution containing 1.2 mg/mL danazol to control [mean ± SEM (n = 5)] and ABT pre-treated rats [mean ± SEM (n = 4)]. **S3** shows the tabulated activity of ex vivo rat bile and lipase fluids in comparison to the activity of porcine pancreatin extract. This material is available free of charge via the internet at http://pubs.asc.org.

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