Understanding the determinants of drug absorption following oral administration of lipid-based drug delivery systems

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"An expert is a person who has found out by his own painful experience all the mistakes that one can make in a very narrow field"

Niels Bohr (1885-1963)

Nobel Prize in Physics (1922)

"When in the end, the day came on which I was going away, I learned the strange learning that things can happen which we ourselves cannot possibly imagine, either beforehand, or at the time when they are taking place, or afterwards when we look back on them"

Karen Blixen (1885-1963)

Out of Africa (1938)

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Abstract

The current project has explored the determinants of drug absorption following oral administration of lipid-based drug delivery systems (LBDDS) and the role of intestinal digestive processes on formulation performance. Particular focus has been directed to the role of formulation excipients and drug loading on the generation and maintenance of drug supersaturation during LBDDS processing and the subsequent impact on drug bioavailability.

The data show that initiation of digestion by pancreatic enzymes functions as an effective supersaturation trigger and that addition of polymeric precipitation inhibitors (PPI) may be utilised to stabilise supersaturation for longer periods and therefore to enhance absorption. Formulation performance was highly correlated with the maximum degree of supersaturation that the formulation generated on dispersion and digestion. In vitro, increasing drug dose initially increased drug thermodynamic activity in the aqueous colloidal phases formed by formulation digestion. Above a critical drug loading, however, supersaturation 'pressure' increased to a point above which nucleation and crystal growth dominated, resulting in drug precipitation. The utilisation of lower drug loads, higher surfactant levels, reduced cosolvent and the addition of PPI all enhanced formulation performance in vitro (i.e. supported ongoing solubilisation), however, subsequent studies showed that only in some cases was the addition of PPI able to support enhanced absorption in vivo.

Consistent with the potential for increases in thermodynamic activity with increase in drug dose, non-linear increases in bioavailability were evident after administration of a series of LBDDS containing increasing quantities of drug to beagle dogs. In further alignment with the in vitro data, non-linear increases in bioavailability were also only evident up to a critical point, beyond which further increases in drug dose resulted in a reduction in bioavailability. The initial in vivo studies were therefore highly consistent with the in vitro supersaturation data. Replication of the in vivo

study in a younger cohort of animals, however, was not able to reproduce the same trends and linear increases in exposure with dose were apparent in this animal cohort. Further studies failed to show a significant difference in hepatic function across the two cohorts, and instead suggested that age-related changes in GI solubilisation, potentially through increased bile salt secretion in the older cohort, may have led to better stabilisation of supersaturation and therefore increases in danazol absorption. Increases in the quantity of drug absorbed at higher doses in the older cohort may have also magnified differences in exposure due to greater saturation of first pass metabolism.

The latter data led to a more detailed evaluation of the role of drug dose on the bioavailability of danazol from LBDDS. These studies were conducted in rats to allow more direct exploration of the role of first pass metabolism, and gastric and intestinal processing on danazol bioavailability. Surprisingly, danazol exposure in the rat following oral administration of danazol formulated in similar LBDDS as those used in the dog studies was low (< 12%), and incorporation of PPI had limited effect. In contrast, co-administration of an inhibitor of CYP450 enzymes resulted in a large increase in bioavailability suggesting that the major limitation to oral bioavailability was first pass metabolism. The applicability of previous in vitro models of lipid digestion to events in the rat was also examined, and a number of modifications to the model suggested. The data obtained indicate that in the rat, lipid digestion may be less efficient than it is in the dog (or human), and therefore that digestion-mediated reductions in solubilisation capacity are less important, that danazol absorption from LBDDS formulations is high (> 50%) and that the principle limitation to danazol bioavailability in the rat is first pass metabolism.

In summary, this thesis contributes to a better understanding of the mechanisms by which LBDDS promote drug solubilisation and absorption and specifically to the influence of drug dose, animal model and the inclusion of polymeric precipitation inhibitors (PPI) on supersaturation generation and stabilisation.

Monash University
Monash Research Graduate School

Declaration for thesis based or partially based on conjointly published or unpublished work

General Declaration

In accordance with Monash University Doctorate Regulation 17/ Doctor of Philosophy and Master of Philosophy (MPhil) regulations the following declarations are made:

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes one original paper published in peer reviewed journals and two submitted publications. The core theme of the thesis is an understanding of the determinants of drug absorption following oral administration of lipid-based drug delivery systems. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the Drug Delivery, Disposition and Dynamics Theme of the Monash Institute of Pharmaceutical Sciences under the supervision of Professor Christopher J. H. Porter and co-supervisor Professor Colin W. Pouton.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

In the case of chapter three, four and five, my contribution to the work included the majority of the experimental work, data analysis and interpretation, the concept and design of all studies, the preparation of initial drafts of all manuscripts and the subsequent revision and formulation of conclusions and hypotheses resulting from the relevant studies.

Thesis chapter	Publication title	Publication status	Nature and extent of candidate's contribution
3	Lipid digestion as a trigger for supersaturation: evaluation of the impact of supersaturation stabilisation on the in vitro and in vivo performance of self-emulsifying drug delivery systems	Accepted / published online	Planning and conducting experimental work, data evaluation and drafting and revision of manuscript
4	Non-linear increases in danazol exposure with dose in older vs. younger beagle dogs: evaluation of the potential role of intestinal bile salt concentration, thermodynamic activity and formulation digestion	In submission	Planning and conducting experimental work, data evaluation and drafting and revision of manuscript
5	Evaluation of danazol bioavailability from lipid-based drug delivery systems in the rat: the role of formulation dispersion/digestion and first pass metabolism	In submission	Planning and conducting experimental work, data evaluation and drafting and revision of manuscript

I have not renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Signed:…… Date:……

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Publications

This thesis is a compilation of the following manuscripts:

Chapter 3:

Anby MU, Williams HD, McIntosh M, Benameur H, Edwards GA, Pouton CW & Porter CJH. Lipid digestion as a trigger for supersaturation: evaluation of the impact of supersaturation stabilisation on the in vitro and in vivo performance of self-emulsifying drug delivery systems. *Molecular Pharmaceutics*; 2012: 9(7), 2063-79. (Appended at the end of this thesis).

Chapter 4:

Anby MU, Williams HD, Benameur H, Edwards GA, Pouton CW & Porter CJH. Non-linear increases in danazol exposure with dose in older vs. younger beagle dogs: evaluation of the potential role of intestinal bile salt concentration, thermodynamic activity and formulation digestion (*Manuscript in submission).*

Chapter 5:

Anby MU, Nguyen TH, Yeap YY, Williams HD, Benameur H, Pouton CW & Porter CJH. Evaluation of danazol bioavailability from lipid-based drug delivery systems in the rat: the role of formulation dispersion/digestion and first pass metabolism (*Manuscript in submission*).

Contributions to publications during candidature

During the conduct of my PhD studies I made significant, but more moderate, contributions to the following manuscripts. Whilst these studies are also in the area of lipid-based formulations they are not included in this thesis as they are out of scope of the major aim of the studies described herein. These manuscripts are appended at the end of this thesis.

Warren DB, Anby MU, Hawley A & Boyd BJ. Real time evolution of liquid crystalline nanostructure during the digestion of formulation lipids using synchrotron small-angle X-ray scattering. *Langmuir;* 2011: 27(15), 9528-34.

Porter CJH, Anby MU, Warren DB, Williams HD, Benameur H & Pouton CW. Lipid-based formulations: Exploring the link between *in vitro* Supersaturation and *in vivo e*xposure. *Bulletin Technique Gattefossé;* 2011: 104, 61-69.

Williams HD, Anby MU, Sassene P, Kleberg K, Bakala-N'Goma J-C, Calderone M, et al. Toward the Establishment of Standardized in Vitro Tests for Lipid-Based Formulations. 2. The Effect of Bile Salt Concentration and Drug Loading on the Performance of Type I, II, IIIA, IIIB, and IV Formulations during in Vitro Digestion. *Molecular Pharmaceutics;* 2012: 9(11), 3286-3000.

Van Speybroeck M, Williams HD, Nguyen T, Anby MU, Porter CJH & Augustijns P. Incomplete desorption of liquid excipients reduces the in vitro and in vivo performance of self-emulsifying drug delivery systems solidified by adsorption onto an inorganic mesoporous carrier. *Molecular Pharmaceutics;* 2012: 9(9), 2750-2760.

Communications

Anby MU, Warren DB, Benameur H, Pouton CW & Porter CJH. Excipient effects on the generation and maintenance of supersaturation during in vitro digestion of SMEDDS. *The AAPS Journal* (2011): W4203.

Warren DB, Anby MU, Benameur H, Porter CJH & Pouton CW. Kinetic stabilisation of supersaturated poorly water soluble drug solutions using polymeric precipitation inhibitors. *The AAPS Journal* (2011): T2209.

Boyd B, Warren D & Anby MU. Lipid-based formulation nanostructures during digestion monitored using synchrotron small angle x-ray scattering. *The AAPS Journal* (2011): T3033.

Anby MU, Warren DB, Benameur H, Pouton CW & Porter CJH. Thermodynamic activity versus solubility as a driver of drug absorption from lipid-based drug delivery systems. *The AAPS Journal* (2010) 12 (S2): R6005.

Warren DB, Anby MU, Benameur H, Pouton CW & Porter CJH. Identification of key properties of polymeric precipitation inhibitors for use with poorly water soluble drugs to improve bioavailability. *The AAPS Journal* (2010) 12 (S2): T3079.

Anby MU, Warren DB, Benameur H, Pouton CW & Porter CJH. Using polymers to enhance the utility of lipid-based delivery systems (II). *The 37th Annual Meeting & Exposition of the Controlled Release Society (CRS)*, July 10-14 (2010) Abstract 559. Portland, OR, USA.

Anby MU, Warren DB, Benameur H, Pouton CW & Porter CJH. Using polymers to enhance the utility of lipid-based delivery systems (I). *Australasian Pharmaceutical Science Association (APSA) National Conference & CRS-AUS*, Dec. 9-11 (2009) Abstract 901. Hobart, Tasmania, AUS ([*]Award winning).

List of abbreviations

4-BPB	bromophenyl boronic acid
ABT	1-aminobenzodiazole
ACN	acetonitrile
An	absorption number
AUC	area under the curve
BA	bioavailability
BCS	biopharmaceutics classification system
BS	bile salt
C_{max}	peak plasma concentration
CrEL	Cremophor EL
CYP	cytochrome P450
DA	danazol
DG	diglycerides
Dn	dissolution number
Do	dose number
EtOH	ethanol
FA	fatty acids
g	grams
x *g*	relative centrifugation force
GI	gastrointestinal
HPLC	high performance liquid chromatography
h	hour
IS	internal standard
kg	kilogram

L	litre
LBDDS	lipid-based drug delivery system
LCMS	liquid chromatography mass spectrometry
LCT	long-chain triglycerides
LFCS	lipid formulation classification system
LLQ	lower limit of quantification
LogD	logarithm of the octanol/water partition coefficient at a set pH
LogP	logarithm of the octanol/water partition coefficient
MCT	medium-chain triglycerides
mg	milligrams
MG	monoglycerides
min	minutes
mL	millilitres
mM	millimolar
µg	micrograms
µL	microlitre
NaTDC	sodium taurodeoxycholate
ng	nanograms
nm	nanometer
PC	phosphatidylcholine
P-gp	p-glycoprotein
PL	Phospholipid
PPI	polymeric precipitation inhibitors
PWSD	poorly water-soluble drugs
s	seconds

SD	standard deviation
SEDDS	self-emulsifying drug delivery system
SEM	standard error of the mean
SMEDDS	self-microemulsifying drug delivery system
TBU	tributyrin units
TG	triglycerides
T_{max}	time of occurrence of peak plasma concentration
w/w	weight in weight

CHAPTER 1

GENERAL INTRODUCTION

1 General introduction

1.1 STATEMENT OF THE PROBLEM

To facilitate effective absorption following oral administration, drug candidates must be both stable and soluble in the gastrointestinal (GI) fluids and possess reasonable gastrointestinal permeability.[1] Approximately 40% of new drug candidates, however, are poorly water-soluble, and the low luminal solubility and slow dissolution rate of these compounds often limits their development as useful medicines.[2,3]

Lipid-based drug delivery systems (LBDDS) are one of several approaches that have been explored to improve the oral bioavailability of poorly water-soluble drugs (PWSD).[4,5] LBDDS range from simple solutions of drug in oil through to emulsions and self-microemulsifying systems containing lipids, surfactants and co-solvents.[6] LBDDS are typically encapsulated into hard or soft gelatin capsules to allow for oral administration. Historically, attention has focused on the composition and self-emulsification properties of LBDDS as the critical determinants of utility.[7-12] However, formulations which provide for efficient self-emulsification after dispersion in the GI tract, do not necessary provide the best foundation for drug absorption, as the properties of the formulation can change markedly on passing through the GI tract. In particular, a loss in solubilisation capacity of the formulation upon enzymatic lipid digestion has been shown, in many cases, to lead to supersaturation, drug precipitation and a decrease in bioavailability.[13-16]

Recently, interest has increased in the use of polymeric additives as a means of inhibiting drug precipitation in Pharmaceutical formulations. Polymeric precipitation inhibitors (PPIs), including methylcellulose (MC), hypromellose (HPMC), and povidone (PVP), are well recognised for their ability to suppress crystal nucleation in a range of circumstances, including the supersaturated drug reservoirs employed to promote flux in transdermal drug delivery systems and the

supersaturated solutions that are typically formed after dissolution of solid dispersion formulations.[17-23] To this point, however, relatively little has been published describing the utility of PPI in LBDDS.[24-29] The overarching hypothesis that underpins the current research project is therefore that the inclusion of PPIs in LBDDS can be utilised to stabilise the supersaturated solubilised drug concentrations that result after dispersion and/or digestion of LBDDS and in doing so, prevent drug precipitation and enhance drug absorption. In seeking to address this hypothesis, a broader range of variables became evident as key determinants of bioavailability from the LBDDS employed here, including drug dose, and the prospect of changes to both supersaturation and first pass metabolism on increasing drug dose. These aspects have been explored in more detail in the latter part of this thesis.

In general, therefore, this project aims to develop a more complete understanding of the mechanisms by which lipids, surfactants and polymers interact with the gastrointestinal content to support the solubilisation of poorly water-soluble drugs and promote bioavailability following oral administration.

1.2 FACTORS AFFECTING ORAL BIOAVAILABILITY OF DRUGS

The oral route is the most accepted and convenient means of drug administration. In order for drug to reach the systemic circulation after oral administration, drug molecules must first be absorbed from the gastrointestinal tract and subsequently avoid pre-systemic metabolism on 'first-pass' through the enterocyte and the liver. This relationship is illustrated by equation 1, where the bioavailable fraction is F, the fraction of the dose absorbed from the intestinal lumen is presented by fa and the effect of first pass is illustrated by F_G, (the fraction metabolised on passage across the gut wall) and F_H (the fraction metabolised on first pass through the liver (i.e. hepatic metabolism)).[30,31]

$$F = \frac{D_{IV} \times AUC_{po}}{D_{po} \times AUC_{IV}} = f_a \times F_G \times F_H \qquad \text{(Equation 1.1)}$$

1.2.1 Factors affecting the fraction of dose absorbed

For drugs with adequate chemical and metabolic stability, drug solubility and dissolution in the GI tract and drug permeability across the gastrointestinal membrane are the major barriers to oral drug absorption. The solubility and permeability properties of the drug are, in turn, dictated by the physicochemical properties of the molecule.[30,32] These include melting point, lipophilicity (normally expressed as the logarithm of the octanol/water partition coefficient (log*P*) or the effective partition coefficient at a given pH (log*D*)), the number of hydrogen bond donors and acceptors, the number of rotatable bonds, the polar surface area, the molecular weight and the potential for the drug to ionize (pK_a).[1,33]

1.2.1.1 Drug solubility in the gastrointestinal tract

Together with the volume of available intestinal fluid, the aqueous solubility of the drug provides an estimate of the maximum quantity of drug that may be solubilised in the GI tract prior to absorption.[34] The dissolution rate of the drug is also critical since the time available for drug to pass into solution is finite (and determined by the GI transit profile).[30,34,35] The Noyes Whitney equation dictates that the rate of dissolution is directly proportional to drug solubility.[1] As such, low solubility often also dictates slow dissolution, and together low solubility and slow dissolution provide a significant barrier to effective absorption.[35,36]

Aqueous solubility is determined by the strength of the intermolecular forces in the solid state, since these must be broken before drug can pass into solution; and the strength of attractive

forces between solute (drug) and solvent (water), since these dictate the affinity of the drug for the solvent. In turn these properties are typically predicated by differences in melting point and lipophilicity. Thus high melting, high log P compounds are poorly water-soluble, whereas low melting point, low (or negative) log P compounds are typically water-soluble. Solubility is also indicated by changes in molecular weight and planarity, since both have an impact on crystal packing (and therefore melting point). As described below, however, lipophilicity is a significant determinant of both solubility and permeability[37] and in general, drugs must be hydrophilic enough to be soluble in the aqueous environment of the GI tract, and at the same time possess sufficient lipophilic character to partition into and ultimately cross, the GI membrane.[32]

1.2.1.2 Drug transport across the gastrointestinal membrane

Drug transport across the intestinal epithelial layer can occur via passive diffusion or active transport.[37] In addition, macromolecules or particulates may be taken up by constitutive endocytotic pathways, although these typically have very low capacity.[38] Active or carrier mediated transport (i.e. transport against an electrical or chemical gradient) is a common form of transport for nutrients such as amino acids, lipids and peptides, and may be utilised by drugs that share common structures.[39] Most drugs, however, are absorbed by passive diffusion, a process largely dependent on the physicochemical properties of the drug.[1,37] In this regard, lipid-soluble molecules typically penetrate through the intestinal membrane more effectively than more polar compounds.[1] Smaller molecules also diffuse more rapidly than larger molecules, and drug molecules with reduced numbers of rotational bonds, fewer hydrogen bond donors and acceptors, and lower polar surface areas, also penetrate lipidic environments more effectively leading to enhanced permeability.[1,37]

An understanding of the physicochemical characteristics of drug candidates can therefore be used to identify factors that may limit drug absorption and bioavailability in the early stages of drug

discovery and development.[30,32,40] An example is Lipinski's rule of 5, perhaps the best known of the physicochemical 'gateway' tools that are used as a guideline to predict the likelihood of poor oral drug absorption.[32] Lipinski's rule of 5 states that for passively absorbed drugs, poor absorption is more likely when a drug molecule has a molecular weight over 500, a Log*P* > 5, more than 5 hydrogen bond donors or more than 10 hydrogen bond acceptors.[32]

The stability of the drug in the GI tract, and on transport across the enterocyte and the liver, are also key determinants of oral bioavailability. Drug stability may be affected by chemical degradation, for example as a result of exposure to the acidic pH within the stomach, by luminal instability such as breakdown caused by digestive enzymes, or by more classical first pass metabolic events mediated by enzymes present in the enterocyte or liver.[41]

1.2.2 Pre-systemic drug metabolism

Metabolism is a major elimination pathway for many drugs and in particular, lipophilic drugs where enzymatic transformation to more hydrophilic metabolites promotes renal or biliary excretion. Various metabolic pathways are involved in drug metabolism, and these are subdivided into Phase I and Phase II metabolic conversions ('Phase III' metabolism has been used to describe metabolic modification of the products of phase II metabolism, but this terminology is not widely used).[42,43]

Phase I reactions include four major pathways: oxidation, reduction, hydrolysis and hydration, where oxidation is the most important.[44] Phase I metabolism generally introduces a functional group to the drug molecule leading to changes in the physicochemical properties. Cytochrome P450 (CYP) enzymes play an important role in oxidative metabolic reactions and the CYP3A subfamily is the most prevalent phase I metabolic enzyme family.[45] CYP3A is strategically located within enterocytes in the intestine and in hepatocytes within the liver. CYP3A enzymes are involved in the oxidation of endogenous substrates, including steroids and bile acids and

contribute to approximately 35% of all phase I reactions[45] and approximately 50% of phase I reactions for marketed drugs.[46] CYP3A enzymes have wide substrate specificity[31], leading to significant overlap and the potential for drug-drug metabolic interactions. Differences in CYP activity across a population also has the potential to lead to inter-individual differences in CYP3A-mediated metabolism and drug exposure.[47,48]

Phase II metabolism can take place simultaneously with, or sequential to, phase I reactions, and leads to conjugation of drug functional groups (or functional groups inserted during phase 1 transformations). Functional groups include hydroxyl, amino, carboxyl, epoxide or halogen groups and these are subsequently conjugated with hydrophilic endogenous substrates such as sugars, amino acids, glutathione (GSH) and sulfates. UDP-glucuronosyltransferase (UGT) enzymes are localized in the endoplasmic reticulum of metabolic cells in both the intestine and liver[49] and contribute to approximately 40% of all phase II reactions.[45]

The fraction of an orally administered dose that reaches the systemic circulation can be reduced by both extra-hepatic (intestinal) and hepatic first pass metabolism.[50] However, orally administered drugs encounter intestinal drug-metabolising enzymes before reaching the liver[31,51] and as such, the intestinal enterocyte sees the highest concentrations of drug (and formulation excipients). Saturation of enterocyte based first pass metabolism is therefore a significant contributor to non-linear changes to drug exposure stemming from changes in first pas metabolism.[52-55]

Many CYP3A substrates are also substrates of the membrane efflux pump, P-glycoprotein (P-gp). P-gp efflux provide a potential limitation to drug absorption directly and can further influence the pre-systemic metabolism, and subsequent fraction of dose absorbed, by promoting the recycling of drugs between enterocytes and the gut lumen, thereby increasing drug exposure to intestinal CYP enzymes.[31,56]

Hepatic metabolism is limited by the rate of drug delivered to the liver (i.e. blood flow, Q), enzyme activity (usually captured by intrinsic clearance, Cl_{int}), and drug binding in blood (usually quantified by fu, the fraction unbound), where:

$$Cl = \frac{Q_H \times (fu \times Cl_{int})}{Q_H + (fu \times Cl_{int})} \qquad \text{(Equation 1.2)}$$

Some 'size-based' physiological factors including blood flow scale allometrically across species.[57,58] Others, including enzyme activity do not. Differences in drug metabolism across species are, therefore, to be expected, and extrapolation of metabolism data from one species to another is complex.[59-63] The impact of first pass metabolism on oral bioavailability and the influence of the animal model used in PK studies, is further evaluated in Chapter 4 and 5.

1.2.3 Additional considerations

Following absorption into and across the enterocyte, most drugs are transported to the systemic circulation via the portal vein and liver.

For a limited number of highly lipophilic drugs however, transport to the systemic circulation may also occur via the lymphatic system, which in turn may impact on oral bioavailability via avoidance of first pass metabolism.[64] Nonetheless, lymphatic transport to the systemic circulation is typically regarded as a post-absorptive event, and is therefore not expected to impact on the overall fraction absorbed (unless lymphatic transport provides an improved transport sink away from the intestine).[65]

It is apparent, therefore, that there are many potential determinants of low oral bioavailability, and the inclusion or exclusion of different excipients in a formulated product has the potential to impact on oral bioavailability via several mechanisms.

The focus of the current project, however, is the potential influence of intestinal processing of lipid-based formulations on drug absorption, and in particular, the impact of excipients, including PPIs, on drug solubilisation and subsequent exposure. The potential impact of changes to permeability and lymphatic transport, and the impact of formulation components on these processes, is not examined in detail and is therefore not expanded on here. The model compound employed in these studies, danazol, also does not have physicochemical properties consistent with lymphatic transport, and is expected to have high passive membrane permeability, limiting the likelihood that changes to permeability or lymphatic transport will impact the data obtained. Further details of lipid digestion, absorption and lymphatic transport are available in the following reviews.[64,66-69]

1.3 IN VITRO - IN VIVO CORRELATIONS FOR POORLY WATER-SOLUBLE DRUGS

As described above, the most important determinants of oral drug absorption include; drug dose, solubility, the rate of dissolution and intestinal permeability. In 1995, the biopharmaceutical classification system (BCS) was developed as an approach to give greater clarity to the drug absorption process, and to provide a basis for determining the conditions under which in vitro-in vivo correlation might be expected.[30] In the BCS, three dimensionless terms, the dose number (*Do*), dissolution number (*Dn*) and absorption number (*An*) were suggested as a means to capture and quantify the contribution of drug dose, dissolution and membrane permeation, to absorption. In particular the BCS was developed to quickly identify compounds where absorption was likely to be formulation independent and therefore compounds where regulators might reasonably waive

the need to run multiple bioavailability studies to prove bioequivalence for essentially similar formulations (a 'bio-waiver').

The BCS classifies drugs and drug candidates into 4 classes based on aqueous solubility and intestinal permeability.[30] Class I includes high solubility and high permeability drugs, class II are drugs with high permeability but low solubility, class III includes low permeability and high solubility drugs and when both solubility and permeability are low, the drug is categorised as class IV (**Figure 1**).

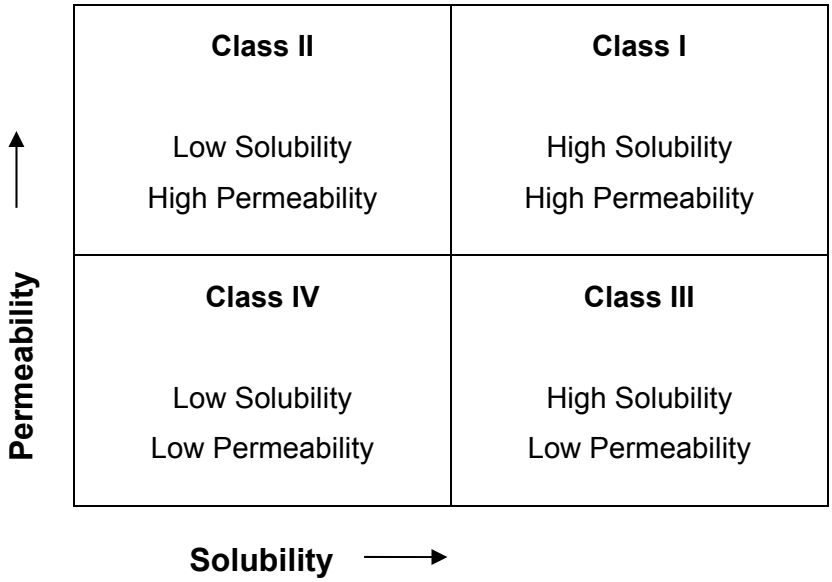

Figure 1.1 The biopharmaceutical classification system with in vitro-in vivo correlation expectations for immediate release products.[30,34]

A drug is considered highly soluble in the BCS when the highest strength dose is soluble in 250 mL or less of aqueous media over a pH range of 1.0 - 7.5 and where *in vitro* dissolution is fast (at least 85% of the drug dissolved in 30 min *in vitro*). The permeability classification is based on the extent of absorption as determined by pharmacokinetic studies in humans (such as mass balance studies

or absolute bioavailability studies), or is determined directly via intestinal permeability methods. These include in vivo intestinal perfusion studies in humans or animal models or *in vitro* permeation studies with human or animal intestinal tissue or cultured epithelial cells. The classification of 'highly permeable' applies when the absolute bioavailability is 90% or higher or when the *in vitro* permeability is high relative to a panel of drugs with known permeability.[70] Where drugs are highly soluble and demonstrate rapid *in vitro* dissolution, the in vivo dissolution rate is expected to be high, and unlikely to limit in vivo absorption. Under these circumstances, an in vitro-in vivo correlation between *in vitro* dissolution and in vivo bioavailability might be expected for class II drugs (where permeability does not limit), and for class I drugs assuming dissolution rate is slower than gastric emptying. In comparison, when the rate of dissolution is high relative to gastric emptying, the rate of gastric emptying is likely to dictate the timescales of absorption.[1,30,70] Class I drugs, where neither solubility nor permeability limit absorption, were suggested to be eligible for bio-waiver since differences in formulation were unlikely to alter in vivo absorption. Drugs in class III and IV are limited by gastrointestinal permeability and therefore, limited or no in vitro-in vivo correlation is expected with dissolution rate.[30] Class II drugs, where solubility is the major limitation to absorption, are those that most readily benefit from solubility enhancing formulation approaches, such as the lipid based formulations examined here.

1.3.1 Enhancement of oral drug bioavailability

Enhancement of oral drug bioavailability may be achieved by a number of different approaches. Improvements in permeability, however, are difficult and drug candidates where absorption is limited by permeability (i.e. BCS class III and IV) are ideally returned to lead optimisation/medicinal chemistry programs in order to design-in better permeability characteristics.[71] Improvement in bioavailability may be gained by changes to the administration route, for example, via changes from oral delivery to parenteral or pulmonary delivery.

Formulation modifications, such as the addition of permeability enhancers, may also be attempted however, in most cases, this is not sufficient to provide significant advances in bioavailability. Realising, that flux across a membrane is the product of permeability and concentration, i.e.:

$$\text{Flux} = \text{P} \times \text{C} \qquad \text{(Equation 1.3)}$$

It is also apparent that decreases in permeability can, to some extent, be off-set by increases in solubility, although for compounds with very low permeability increases in solubility are unlikely to provide for increases in flux sufficient to markedly improve bioavailability.

For BCS class II compounds, where membrane permeability is not limiting, the potential rate limiting steps to absorption include solubility and dissolution. However, the BCS classification for solubility is binary i.e. high or 'not high', and class II compounds therefore comprise a wide range of molecules with varying physicochemical properties, and widely varying degrees of formulation dependence on absorption.[72] Certainly, not all class II drugs show low absorption after oral delivery. This is particularly apparent for many weak acids that are classified as 'not-highly soluble' since the dose cannot be dissolved in 250 mL of water at pH 2, but where solubility at intestinal pH is often quite high.

Nonetheless, many BCS class II compounds do have intrinsically low solubility at both gastric and intestinal pHs and are poorly absorbed after oral administration in traditional solid dose forms (suspensions, tablets etc). These compounds, and the approaches that can be taken to enhance their absorption, are the main focus of this project.

1.3.1.1 Approaches to enhance the oral bioavailability of PWSD

Several approaches have been taken to enhance the solubility and dissolution rate of BCS class II drugs. These include the isolation of more soluble salts or polymorphs, complexation with cyclodextrins, the addition of polymers, particle size reduction to form micro- or nanonised suspensions, the use of solid dispersions, co-solvents and surfactants, and the application of lipid-based delivery systems such as emulsions and microemulsions.[4,5,71,73-77]

The current studies have focussed on the use of lipid-based drug delivery systems to enhance bioavailability for poor water-soluble drugs. Lipid-based delivery systems encompass a wide range of systems comprising different mixtures of oils, surfactants and co-solvents. Simplistically, these formulations enhance drug absorption by promoting drug incorporation into highly efficient endogenous lipid absorption pathways. These processes are discussed in more detail below.

1.4 ORAL ADMINISTRATION OF LIPID-BASED DRUG DELIVERY SYSTEMS

1.4.1 Gastric dispersion and digestion

Following oral administration, lipid-based formulations are initially diluted and, depending on included formulation excipients, often self-emulsify as the formulation is dispersed in the gastric fluids. Digestion of formulation-derived lipids and surfactants also starts in the stomach, where gastric lipase is secreted by the gastric mucosa and lingual lipase is present in saliva (**Figure 2**).[78] These 'acid' lipases (so called since they are stable in acidic environments) hydrolyse triglycerides (TG) to free fatty acids (FA) and diglycerides (DG) and are responsible for 10-30% of the hydrolysis of TG in food.[79] The acid lipases have a greater affinity for medium-chain triglycerides (MCT) when compared to long-chain triglycerides (LCT) and do not hydrolyse phospholipids and cholesterol esters.[67,68]

1.4.2 Enzymatic digestion and solubilisation of lipids in the small intestine

The presence of lipids and FA in the GI tract stimulate the secretion of bile salt (BS), phospholipids (PL) and cholesterol (in an approximate molar ratio of 16:4:1) from the gall bladder[64,80] and the release of pancreatic fluids including pancreatic lipase/co-lipase from the pancreas.[81]

Pancreatic fluids initiate lipolysis in the small intestine at the oil droplet-water interface, yielding two FA and a single 2-monoglyceride (MG) from each TG molecule through a two-step reaction.[66-68,82,83] 2-MG can subsequently undergo non-enzymatic isomerisation to 1-monoglyceride and subsequent enzymatic release of FA.[68] The relative composition of TG hydrolysis products in vivo is 22% glycerol, 72% 2-MG and 6% 1-MG.[84] Pancreatic lipase/co-lipase appears to be the dominant enzyme complex in the digestion of lipids,[85] however, recent studies suggest that other enzymes, including gastric lipase and carboxyl ester hydrolase, may also be responsible for the digestion of some formulation components.[86,87]

Lipid digestion products from exogenous (i.e. ingested) lipid sources are subsequently incorporated into a range of unsaturated mixed colloidal species. The generation of a mixed micellar phase containing bile salts, fatty acids and monoglycerides in the human intestinal fluid was first shown in 1964 by Hofmann and Borgström.[88] Later, a phase equilibrium between liquid crystalline species (unilamellar and multilamellar vesicles) and micellar phases (including endogenous solubilizers such as BS, PL and cholesterol) was reported[89-93], which was proposed to have a significant impact on lipid absorption.[91] Incorporation of dietary or formulation-derived lipids into these mixed colloidal species provides a means of solubilisation and also a means of solubilisation of co-administered PWSDs.

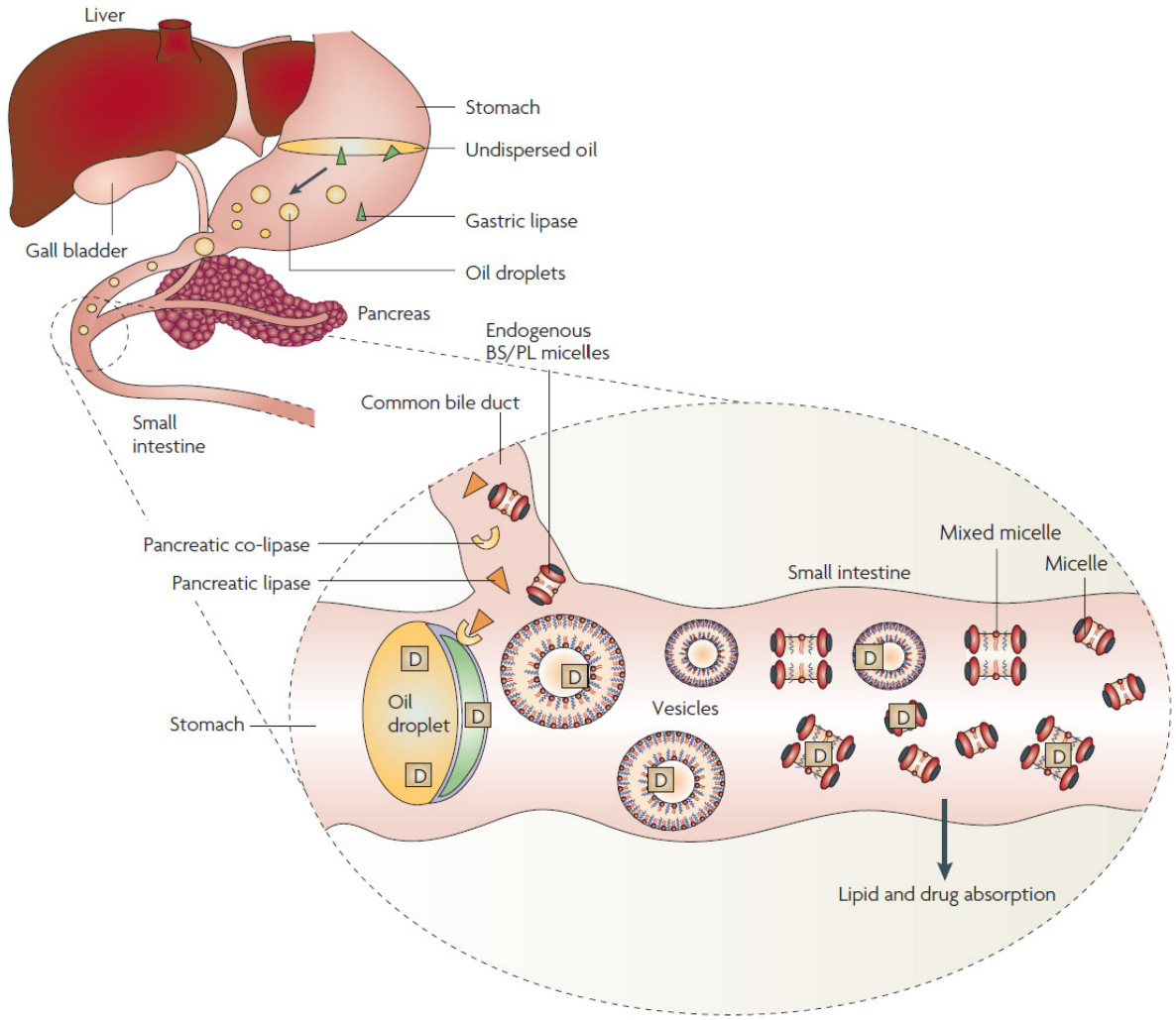

Figure 1.2 Lipid digestion and drug solubilisation in the stomach and small intestine. Lipid-based formulations typically disperse in the stomach, where lipid digestion commences upon gastric lipase release. When the formulation passes from the stomach to the duodenum after gastric emptying the formulation emulsifies further, and within the small intestine, pancreatic lipase/co-lipase completes the breakdown of TG to DG, MG and FA. The gall bladder releases biliary lipids including bile salts, lecithin and cholesterol, which together with lipid digestion products produces a colloidal range of structures which support drug solubilisation (Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Drug Discovery (Porter et al[64]). Copyright (2007).

1.4.3 Advantages of lipid-based drug delivery systems following oral administration

In almost all cases, drug molecules must be in solution in the GI fluids in order for absorption to occur. Where drugs are administered as a solid dose form, this is achieved by dissolution (Figure 1.3A). For poorly soluble class II drugs, dissolution is typically low and commonly the rate limiting step to absorption. When formulated in a lipid-based formulation, however, PWSD are typically administered in solution in the formulation (albeit a non-aqueous solution), effectively circumventing the need for dissolution (Figure 1.3B).[71] Drugs may be formulated simply as a solution in a lipid or a blend of lipids, or may be dissolved in a combination of lipids, surfactants and/or co-solvents that disperse or self-emulsify on contact with the GI fluids to form a fine (commonly submicron) emulsion. In all cases, however, drug is typically molecularly dispersed in the formulation (lipid suspensions are occasionally employed when the required dose is high, but in general these are less effective than solution formulations).

This non-aqueous solution of drug in lipid is subsequently dispersed and integrated into lipid digestion pathways prior to drug absorption. The dispersed colloidal species formed by integration of the formulation into endogenous lipid processing pathways, ultimately functions as a reservoir in which the co-formulated PWSD is solubilised (Figure 1.3B).

The current prevailing view, is that drug absorption from lipid based formulations occurs via the free drug concentration that exists in equilibrium with the solubilised reservoir, and that drug absorption subsequently displaces the equilibrium between solubilised and free drug, resulting in 'release' of further drug from the solubilised reservoir. The rate of replacement of the free fraction is expected to be rapid, and much more rapid than the analogous dissolution process for drug from a typical solid dose form. The principle advantage of lipid-based formulations is therefore the ability to maintain a high-capacity solubilised reservoir of molecularly dispersed

drug which exists in rapid equilibrium with drug in free solution. This circumvents the inherent problems of low drug solubility and significantly increases the rate of drug dissolution.

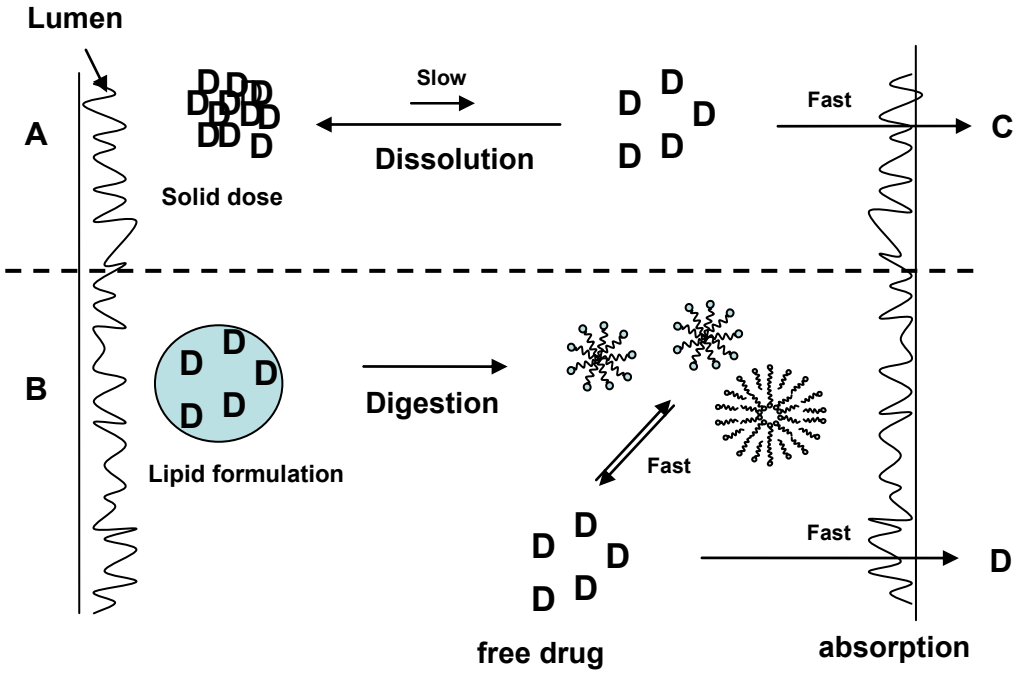

Figure 1.3 Illustration comparing traditional dissolution from a solid dose to drug in solution (A) and dispersion and digestion of a lipid-based formulation (B) for a PWSD. Digestion products of formulation derived lipids and endogenous BS mixed micelles combine to produce colloidal particles that increase the drug solubilisation capacity in the GI tract. The colloidal system functions as a drug reservoir where the co-formulated PWSD is in rapid equilibrium with drug in free solution. The theoretical maximum concentration of drug in free solution in the gastrointestinal tract is the same for both situations (and reflects the equilibrium solubility of drug in the intermicellar fluids, which in turn approximates the aqueous solubility). Drug permeability is a molecular property and as such is also expected to be the same (i.e. C and D). The principle difference between the two approaches is the rapid equilibrium that is established between drug solubilised in the colloidal species that are formed by lipid digestion and drug in free solution, when compared to the much slower rate of drug dissolution for a solid.

1.5 DEVELOPMENT OF LIPID-BASED DRUG DELIVERY SYSTEMS

The performance of a lipid-based drug delivery system is highly dependent on the nature of the excipients utilised in the formulation since drug solubilisation is dependent on the colloidal species formed by intercalation of lipid-digestion products into mixed micelles. The integrity of drug solubilisation must be maintained during formulation dispersion, emulsification and digestion, since precipitation re-capitulates the situation obtained after oral administration of a suspension formulation, where dissolution subsequently becomes the major rate limitation to absorption.

To help identify the critical performance characteristics of lipid-based formulations, Pouton proposed a classification system; the Lipid formulation classification system (LFCS) (Table 1.1).[94] The LFCS classifies formulations on the basis of the properties of the polar and amphiphilic components included in the formulation and the likely impact of lipid digestion on formulation performance.[71,94]

1.5.1 The lipid formulation classification system (LFCS)

The LFCS was introduced in 2000 as a tool to classify formulations according to the properties of included formulation components and to assist in the interpretation of in vivo studies.[94] The system was updated in 2006 to include an extra class of formulation (see Table 1.1).[6,71,94]

Type I lipid formulations consist of simple lipid solutions of drugs (usually glyceride mixtures). The low solvent capacity of mixed glycerides, and in particular triglycerides, may limit the application of this approach, however, oil solutions can be a useful formulation approach for highly lipid soluble drugs. The solvent capacity of type I formulations for less lipophilic drugs can be improved by adding more polar lipids, such as mono- and diglycerides.[94]

Table 1.1 Lipid formulation classification system (LFCS)[71,94] showing typical compositions and properties of lipid-based formulations.[a]

	Increasing hydrophilic content ⟶				
	Type I	Type II	Type IIIA	Type IIIB	Type IV
Typical composition (%)					
Triglycerides or mixed glycerides	100	40 - 80	40 - 80	< 20	0
Water-insoluble surfactants (HBL < 12)	-	20 - 60	-	-	0 - 20
Water-soluble surfactants (HBL > 12)	-	-	20 - 40	20 - 50	30 - 80
Hydrophilic co-solvents	-	-	0 - 40	20 - 50	0 - 50
Particle size of dispersion (nm)	Coarse	100 - 250	100 - 250	50 - 100	< 50
Significance of aqueous dilution	Limited importance	Solvent capacity unaffected	Some loss of solvent capacity	Significant phase changes / potential loss of solvent capacity	Significant phase changes / potential loss of solvent capacity
Significance of digestibility	Crucial requirement	Not crucial but likely to occur	Not crucial but may be inhibited	Not required	Not required

[a] Adapted from Pouton[71,94] and Porter & Charman[34]

Simple lipid solutions disperse poorly in aqueous media and therefore require digestion by pancreatic lipase/co-lipase in the GI tract to liberate more amphiphilic digestion products that are in turn better able to promote droplet dispersion. Digestion also supports transfer of drug and lipid digestion products into the dispersed colloidal environment produced by the combination of endogenous solubilisers and exogenous digestion products in the GI tract.[64]

The inclusion of lipophilic surfactants in type I formulations increases the solvent capacity for moderately lipophilic drugs and enhances dispersion properties. The latter results in a reduction in interfacial tension between the lipid and water phases.[1] The choice of surfactant can lead to formulations which disperse readily in aqueous environment under limited agitation. These formulations are classified as type II or self-emulsifying drug delivery systems (SEDDS).[6] Self-emulsification provides for improved dispersion and increases the available surface area of

interaction with the gastrointestinal milieu. Self-emulsification usually requires the inclusion of > 25% (w/w) surfactant.[94]

To further improve solvent capacity and dispersability of the formulation, water-soluble excipients, such as higher HLB (hydrophile/lipophile balance) surfactants and co-solvents can be added to the formulation. Formulations with surfactant levels ≥ 40% w/w typically produce very small droplets (< 1 μm) on dispersion and are commonly referred to as type III formulations (or self-microemulsifying (SMEDDS) or self nanoemulsifying (SNEDDS) drug delivery systems). Type III formulations can be further divided into class A and B depending on the proportion of included hydrophilic components, where type IIIB systems usually contain a greater quantity of water-soluble components.[6]

Type IV formulations are lipid-free and contains hydrophilic surfactants and co-solvents. Type IV formulations disperse to form micellar solutions in the GI tract and commonly exhibit higher drug loading capacities than type II and type III formulations.[74]

Dispersion and digestion of lipids and other excipients included in lipid based delivery systems (such as surfactants) may have a significant impact on the performances of lipid based delivery systems. This has led to the development of *in vitro* models that may be used to assess the impact of formulation processing on drug solubilisation under simulated GI conditions.[74,95,96]

1.5.2 In vitro assessment of the performance of lipid-based drug delivery systems

In vitro tests are widely used for the assessment of drug formulations, although no standard pharmacopoeia method has to this point been defined for the evaluation of lipid-based drug delivery systems. Nonetheless, several in vitro dispersion and digestion methods for lipid-based delivery systems have been described in recent years and provide a reasonable indication of the relative utility of different formulation approaches.[97-104]

1.5.2.1 In vitro dispersion of lipid-based drug delivery systems

A modification of the USP dissolution test may be used to give a preliminary indication of behaviour of lipid-based delivery systems on initial dispersion in GI fluids. The focus of the modified test is the potential for drug precipitation on formulation dispersion in the GI tract rather than classical drug dissolution, since true dissolution from the solid state does not occur.[71,103] Measurements of particle size on dispersion may also be included as a quality control indicator of consistent dispersion properties, although, several studies have shown that there is little correlation between particle size on dispersion and drug bioavailability from lipid-based delivery systems.[71,104,105]

Importantly, the performance of lipid-based delivery systems appears to be more highly influenced by the change in formulation properties that occurs as lipid digestion proceeds, rather than the composition of the initial formulation or the efficiency of dispersion. As such, methods to examine the fate of the drug following digestion are essential.[13,16,65,96-99,101,103-107] These are described below.

1.5.2.2 In vitro digestion of lipid-based drug delivery systems

Several laboratories have published methods for the evaluation of LBDDS using in vitro digestion tests. In all cases, a pH stat titration unit has been used to maintain pH during lipolysis, porcine pancreatin employed as source of digestive enzymes and BS-PL mixed micelles used as a simple model for simulated intestinal fluid.[97-102,104] In these tests, formulations are dispersed in simulated intestinal fluids in a temperature controlled vessel and digestion initiated by the addition of pancreatin extract. Digestion subsequently leads to the liberation of FA from glyceride lipids or FA ester surfactants. Release of FA results in a transient decrease in pH. The decrease in pH is detected by the pH stat, resulting in the titration of an equimolar quantity of base (usually NaOH) to maintain pH. During lipolysis, the formation of free fatty acid (FA) can be used as a measure of

the digestion kinetics and is quantified by the number of moles of base required to titrate the release FA.[97,108]

There are four primary experimental variables that appear to affect the rate of in vivo digestion; the enzyme activity, the concentration of BS, the concentration of Ca^{2+} and the quantity of formulation.[97] The activity of pancreatic lipase/co-lipase is normally expressed in tributyrin units (TBUs), where 1 TBU is the quantity of enzyme that can liberate 1 µmole of FA per min when the substrate (tributyrin) is in excess.[108,109] To determine whether the substrate is in excess, enzyme activity is ideally measured over a range of substrate/enzyme ratios. Plotting the initial rate of lipolysis as a function of the mass of substrate (at fixed enzyme), provides a curve that ultimately plateaus once the quantity of substrate is in excess. This indicates the (maximum) specific activity of the enzyme.[97]

Lipid digestion products (largely MG and FA) produced during lipolysis accumulate at the interface between oil droplets in the dispersed formulation and aqueous intestinal fluid. This eventually slows or prevents lipolysis if removal of the digestion products from the surface does not occur.[92,97,108,110] The principle method of removal of digestion products from the oil/water interface is by micellar solubilisation, where BS mixed micelles secreted in bile facilitate the solubilisation of the digestion products into the aqueous sub-phase.[111] This can only occur, however, when unsaturated BS micelles are present.[97] Consequently, the initial hydrolysis rate is influenced by the total concentration of solubilizing species (endogenous biliary lipids as well as exogenous surfactant) present in the digest (in vitro) or in the intestine (in vivo).[97] As such, there is an initial rapid rate of lipolysis in vitro, where the digestion products are solubilised in the micellar species that are present in the digest. Once the micellar solubilisation capacity is exceeded, however, the rate levels off and on-going digestion becomes dependent on processes that remove lipid from the solubilised phase and reinstate the presence of an unsaturated micellar 'sink'. This may be achieved by the addition of additional solubilisers or by lipid

complexation with, for example, Ca^{2+} to form insoluble fatty acid soaps.[100] In vivo, absorption of digestion products from the micellar phase maintains sink conditions and allows lipolysis to continue.

The formation of calcium salts between Ca^{2+} and FA [$CaFA_2$] can remove FA (but not MG) from the oil/water interface (via the formation of an insoluble soap) and therefore Ca^{2+} addition typically enhances the rate of in vitro lipolysis of MCT and LCT.[100,112] Calcium has also been suggested to enhance lipolysis directly via the formation of ternary complex with pancreatic lipase and BS mixed micelles which appears to be the catalytically most effective enzyme complex.[108] Interestingly, calcium has little influence on the rate of tributyrin lipolysis, likely reflecting the relatively high solubility of tributyrin digestion products (butyric acid) and therefore reduced reliance on the complexation of digestion products to shuttle digestion products away from the interface in order to promote lipolysis.[97,108] Finally, the quantity of formulation employed is also a determinant of the efficiency of digestion, where larger quantities of formulation result in the formation of larger quantities of lipid digestion products and therefore require higher solubilisation capacity in the aqueous phase to allow digestion product solubilisation. The degree of formulation dilution during lipid digestion may therefore impact on formulation performance. This is further evaluated in Chapter 5.

Given the number of parameters affecting the performance of a lipid-based formulation, a standard model for assessment would be of great value. Efforts to this end are continuing via a recently established consortium of academic and industrial groups (the LFCS consortium), such that results from different laboratories and research groups can be compared.[104] To this point, however, lipolysis models are evident with varying specifications in respect to bile salt, calcium concentration and enzyme concentration (Summary of lipid digestion model conditions used in the literature can be found in Porter et al[34]). In this thesis, a method has been employed that has previously been used widely by our group.[97,109] This system contains 5 mM Ca^{2+}, 5 mM BS and 1.25

mM phosphatidycholine (PC) to reflect fasted state intestinal conditions and a lipase activity of 10,000 TBU per mL pancreatin extract (1000 TBU/mL digest).

1.6 DRUG SOLUBILISATION FOLLOWING ORAL ADMINISTRATION OF LIPID-BASED DRUG DELIVERY SYSTEMS

1.6.1 Impact of excipients on solubilisation capacity and formulation performance

The ability of lipid-based formulations to maintain solubilisation of a co-formulated PWSD on dispersion in the aqueous media of the stomach is highly dependent on the composition of the formulation and in particular, the proportion of water-soluble components that are included or generated through digestion. Thus, type IIIB and type IV formulations (Table 1.1) more commonly lead to drug precipitation on formulation dispersion as water-soluble excipients separate from the more lipophilic excipients (such as lipids, low HLB surfactants) during dispersion and dissolve in the aqueous phase resulting in a loss of solvent capacity (Figure 1.4A).[6,105,113] In comparison, type II and type IIIA formulations (Table 1.1) have a greater potential for maintaining solubilisation capacity following dispersion in the aqueous medium of the stomach since they contain fewer-water miscible components (Figure 1.4B).[113]

Following initiation of digestion, changes to the chemical and physicochemical properties of many included excipients may also lead to decreases in solubilisation capacity.[65,67,96,114] In particular, digestion of formulations containing high quantities of digestible non-ionic surfactants or medium-chain lipids commonly results in drug precipitation since the digestion products formed are significantly more polar and less able to swell the solubilisation capacity of bile salt/phospholipid micelles than their undigested comparators (Figure 1.4C).[13,16,98,106]

The choice of included excipients is therefore dictated by the capacity to promote drug solvent capacity in the formulation and by the ability to enhance drug solubilisation during formulation digestion as opposed to simply improving in vitro dispersion properties.[64]

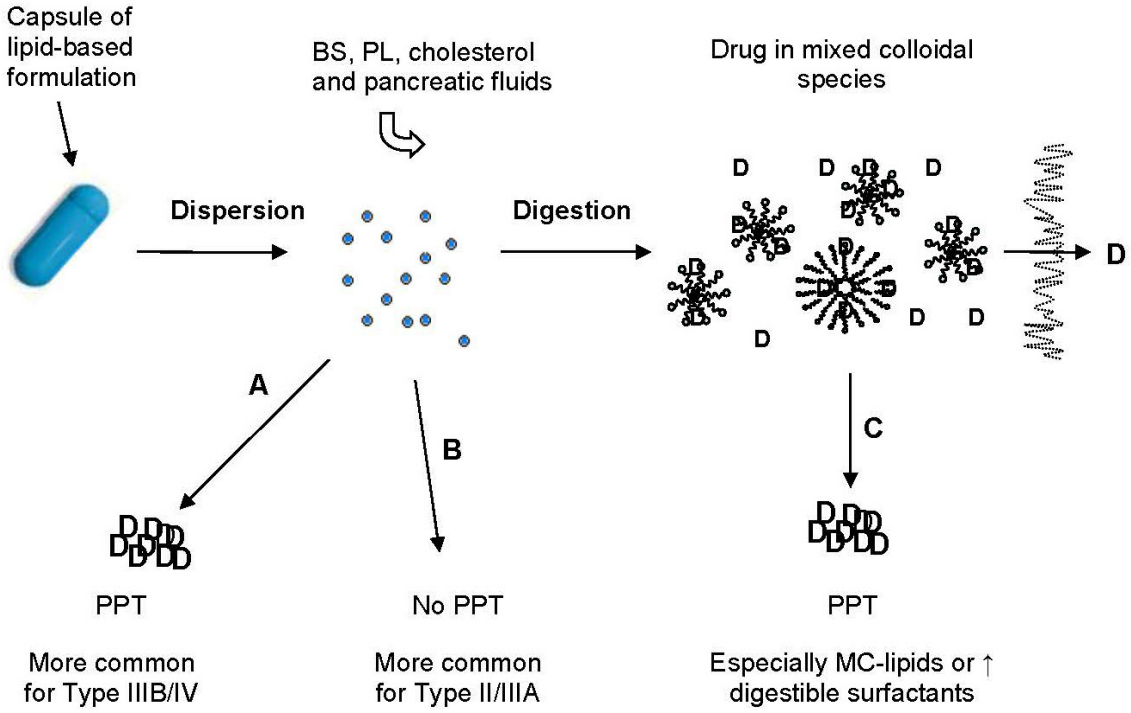

Figure 1.4 Illustrates the potential for drug precipitation during the dispersion and digestion of lipid based formulations. On dispersion, the solvent capacity of the formulation may decrease due to miscibility of water-soluble excipients in the aqueous media of the GI tract (A). However, for formulations with few water-soluble excipients, drug precipitation is less likely (B). Initiation of lipolysis may further change the solubilisation capacity of the aqueous media due to the production of more polar lipid digestion products that are less able to swell the solubilisation capacity of endogenous bile salt/phospholipid micelles (C). In general, drug precipitation leads to decrease in the quantity of drug molecules available for absorption (D) across the GI tract, regardless of whether this occurs during dispersion (A) or digestion (C).

1.6.1.1 Impact of choice of surfactant

A wide range of surfactants are available as potential excipients,[4,115] however, in general non-ionic surfactants are preferred due to lower toxicity and irritancy. When lipid-based formulations are dispersed in aqueous media, surfactants accumulate at the interface of the aqueous and lipophilic phase, lowering the interface tension and promoting dispersion.[1,6] For example, Cuine et al showed that the inclusion of Cremophor EL (CrEL) and ethanol facilitated emulsification and contributed to the production of very fine dispersions in vitro. However, they did not appear to enhance the solubilisation capacity of the formulation post digestion, as they were less capable of maintaining drug solubilisation than LC lipids.[13] Indeed, a reduction in the relative lipid content in the lipid-based delivery system and an increase in surfactant and cosolvent resulted in a significant loss of solubilisation capacity on in vitro digestion and a reduction in in vivo drug exposure.[13]

Cuine et al also showed that some non-ionic surfactants may be subject to pancreatic enzyme-mediated digestion, which may explain the loss in solubilisation capacity post in vitro digestion.[106] This problem, however, may be circumvented by substituting a digestible surfactant (i.e. CrEL) with a less digestible surfactant (i.e. Cremophor RH40 (CrRH40)).[106] This finding is consistent with an earlier study by Sek et al[105] where substitution of (digestible) CrEL with a series of (non-digestible) Pluronic surfactants improved the extent of atovaquone solubilisation during in vitro digestion. Christiansen et al[116] later confirmed that solubilisation capacity was influenced by the susceptibility of non-ionic surfactants to pancreatic lipase digestion and to their potential inhibitory effect on triglyceride (olive oil) digestion. Christiansen et al[116] also suggested that non-ionic surfactants, including polysorbate 80, tocophenyl polyethylene glycol succinate, CrRH40, CrEL and sucrose laurate, could interact with CYP450 enzymes and potentially function as inhibitors of CYP450-mediated metabolism, thereby affecting first pass metabolism. Non-ionic

surfactants have also been shown to inhibit efflux pumps and to act as permeation enhancers. These properties are well reviewed in Williams et al.[5]

1.6.1.2 Impact of choice of lipid

The nature and quantity of the lipids used in lipid-based formulations are also important determinants of formulation performance.[13,16,74,84] Formulations with increased lipid content may provide for higher drug solubilisation capacities after dispersion and digestion, however, this is highly dependent on the nature of the lipids.[14,74,96,98,114] For example, PWSDs are usually more soluble in medium chain (MC) triglycerides when compared to long chain (LC) triglycerides since drug solubility in triglycerides appears to depend on the molar concentration of ester bonds in the lipids and the formation of hydrogen-bonded complexes between drug and lipid.[117] As such, MC lipids provide benefits in terms of drug loading potential. However, the rate and extent of MC-lipid digestion is higher than that of LC-lipid formulation[15,90,98,112,118], and the aqueous solubility of MC-digestion products is significantly higher than that of LC-lipids. MC lipids therefore have a reduced ability to incorporate into BS micellar structures when compared to LC digestion products and this typically decreases the solubilisation capacity of the colloidal structures formed on digestion of MC lipid-based formulation.[15,16,68,74,90]

As such, whilst MC lipids improve the solvent capacity of the initial formulation, drug precipitation on digestion of the formulation is also common. In contrast, whilst long-chain lipids contained in LBDDS are also digestible, long-chain digestion products appear better able to retain solubilisation capacity when compared to their medium-chain equivalents. In support of this suggestion, Han et al[119] previously showed that self-emulsifying delivery systems containing LC-lipids appears to more effectively support solubilisation of the poorly water-soluble drug, anetholtrithione when compared to similar MC- and SC-lipid formulations. Porter et al[16] showed similar trends for danazol following in vitro digestion of MC-lipid formulations (36% Captex355, 18% Capmul MCM,

36% CrEL and 10% ethanol)and LC-lipid formulations (30% soybean oil, 30% Maisine 35-I, 30% CrEL and 10% ethanol). In contrast, Dahan et el[120] showed that the solubility of griseofulvin in the aqueous phase formed after in vitro digestion of MC and LC lipid-based formulation was higher after digestion of the MC formulation.[120] This is consistent with studies by Christensen et al[112] and Kossena et al[90], that suggest that the 'preferred' lipid chain length in order to generate ideal solubilisation capacity following digestion is dependent on the physicochemical characteristics (i.e. log*P*) of the model drugs evaluated. Thus, lower log*P* drugs are more soluble in micellar systems containing medium chain lipid digestion products, whereas increasingly lipophilic drugs require colloids containing LC lipids to provide enhanced solubilisation.

1.6.2 Characterisation of the colloidal phases generated by lipid digestion

Trends in drug solubilisation following in vitro lipid-digestion have therefore been widely utilised to predict the performance of lipid-based drug delivery systems. More recently, the test has been expanded to incorporate advanced microscopy and x-ray scattering to further characterise changes to the colloidal species produced during formulation digestion.

In 2007, Fatouros and co-workers[99] evaluated the colloidal phases present in bio-relevant media and the colloid species generated following in vitro digestion of a long-chain SEDDS formulation (30% sesame oil, 30% Maisine 35-I, 30% Cremophor RH40 (CrRH40) and 10% ethanol) using cryogenic transmission electron microscopy (cryo-TEM) and small angle x-ray scattering (SAXS). The SAXS experiments showed the generation of a lamellar phase on initiation of digestion. The nature of the colloidal species was also shown to change as hydrolysis of the digestible excipients continued and after 60 min a hexagonal phase was formed.[121] Cryo-TEM imaging revealed that the bio-relevant digestion media contained only micelles and that initiation of digestion of the LC formulation resulted in the formation of a colloidal phase containing oil droplets, unilamellar and bilamellar vesicles and micelles, although, after 30 min digestion, no bilamellar vesicles

remained.[99] The effect of increased bile salt (BS) and phosphatidylcholine (PC) concentrations in the digestion medium on the colloidal phases generated was also evaluated and the authors suggested that the PC levels can have an important impact on the colloidal structures generated on digestion with increased concentrations leading to preferential formation of multilamellar structures.[122]

The use of the in vitro digestion model combined with synchrotron SAXS has recently been applied by Warren et al[123] to study real time evolution of nanostructures formed during in vitro digestion of a LC SEDDS (30% sesame oil/30% Maisine 35-I, 30% CrRH40 and 10% ethanol). The group showed that the phase generation was affected by the composition of formulation, and that increasing the quantity of Maisine 35-I in the formulation increased the generation of hexagonal phase compared to lamellar phase. In addition, the studies showed that the surfactant, CrRH40, had little impact on the structures formed on digestion. The authors concluded that rapid digestion led to the fast appearance of a hexagonal phase and that the formation of hexagonal phase was therefore related to the production of fatty acids.[123]

Most recently, the Mullertz group evaluated the morphology of colloidal phases in ex vivo human intestinal fluid following ingestion of a lipid-rich meal (62.5 g olive oil, 1.25 g eggs, 25 g sucrose, 2.7 g sodium chloride and water to give a total volume of 500 mL).[124] The colloidal phases present in the intestinal fluid was characterised using atomic force microscopy (AFM) and cryo-TEM following collection of ex vivo fluid after 30 min and 60 min.[124] The study showed the generation of spherical micelles in equilibrium with plate-like structures, oil droplets and unilamellar vesicles (and a few cases of bi- and mulitlamellar vesicles). The study supported the phase generation model previously reported by cryo-TEM and SAXS following in vitro digestion of a long-chain formulation[99,121-123], and concluded that species (i.e. multivesicular, faceted vesicles and bilayer fragments) formed by incorporation of digestion products into the micellar phase was generated

when the ratio of digestion product/bile salt was ≥ 1.9, which is in agreement with the in vitro studies of Rigler et al.[93]

1.7 USING IN VITRO DRUG SOLUBILISATION PATTERNS TO PREDICT FORMULATION PERFORMANCE AND BIOAVAILABILITY IN VIVO

Formulation performance in vivo is usually evaluated through bioavailability studies conducted in dogs, mini-pigs or rats. In order to develop a more complete understanding of the in vivo processing and solubilisation capacity of lipid-based formulations, several groups have in the last decade utilised in intro digestion models to evaluate the influence of formulation characteristics and excipients on formulation performance in vivo and to estimate potential drug bioavailability. The in vitro digestion model is most often used to predict a rank order of formulation performance based on in vitro drug solubilisation compared to the AUC of the plasma profile in the animal model rather than obtaining a true in vitro-in vivo correlation (IVIVC) as defined by FDA.[125]

In the Porter et al[16] study, the group showed that LC-SEDDS appears to more effectively promote the oral bioavailability of danazol when compared to MC-SEDDS, which was supported by the in vitro danazol solubilisation following in vitro digestion demonstrating the efficacy of in vitro digestion model to assess in vivo performance of lipid-based formulations in beagle dogs.[16]. This was later confirmed in the study by Han et al[119], where the in vivo performance was evaluated following oral administration to rats. The group showed a clear trend between lipid chain length and in vitro and in vivo performance using lipid solutions (short chain (SC), medium chain (MC) and long chain (LC), and evaluation of the efficacy of simple oil formulation compared to microemulsions (addition of surfactant to oil solutions i.e. glycerol and Labrasol® to LC and LC microemulsions and CrEL to the SC microemulsion) illustrated that oil formulations generally performing better than microemulsions.[119]

Dahan and Hoffman[126] also studied the effect of lipid chain-length on in vivo bioavailability of progesterone and vitamin D3. For progesterone, a correlation between in vitro solubilisation and in vivo performance was evident for different lipid chain length formulations and more effective absorption was seen after administration of MC (Captex 355) > LC (peanut oil) > SC (triacetin). In contrast, the in vivo exposure of vitamin D3 was greater when administered in LC when compared to MC lipids, but this did not correlate with solubilisation capacity in vitro. The group suggested that lymphatic transport of high log*P* drugs such as vitamin D3, may lead to differences between in vitro and in vivo performance due to avoidance of first pass metabolism in vivo. This is consistent with the studies of Caliph et al who examined the effect of lipid chain length on the absorption and intestinal lymphatic transport of halofantrine and showed that lymphatic transport is typically enhanced by LCT > MCT > SCT.[127] The group subsequently evaluated patterns of in vitro and in vivo performance of lipid-based formulations using a lower log*P* drug, griseofulvin, where lymphatic transport was unlikely to play a role in in vivo disposition. Under these circumstances the correlation between formulation performance in vitro and in vivo performance in rats was much stronger.[120] As such, in vitro results may therefore occasionally lead to bias results due to lack of sick conditions in the in vitro model and incomplete lipid digestion, in particular, when using LC lipids and therefore generation of oil phase under these conditions. This may lead to false interpretation of the solubilisation capacity if only evaluating the aqueous phase, since drug concentrations often are superior in the remaining oil phase. The interpretation of in vitro data may therefore significantly impact on the IVIVC and evaluation of formulation performance.

In the study by Sek et al[105] evaluating the influence of the nature of the surfactant included in LC lipid-based formulations on formulation digestion, the in vivo performance following oral administration of the formulations containing the poorly water soluble drug, atovaquone to beagle dogs was also explored. Formulations comprised 30% soybean oil, 30% maisine 35-I, 30%

surfactant (CrEL or a series of Pluronic surfactants) and 10% ethanol. Significant differences in aqueous phase drug concentrations were obtained following in vitro digestion of formulations comprising different surfactants, however, surprisingly, no difference in atovaquone bioavailability was observed in vivo after administration of a control formulation (no surfactant) and formulations comprising 30% CrEL or Pluronic 121.[105]

Fatouros et al[128] later showed that complete removal of the surfactant component from a LC formulation (sesame oil, Maisine 35-I, CrRH40, ethanol) decreased the bioavailability of probucol following oral administration to male mini-pigs (fed) consistent with in vitro studies where aqueous phase concentration of probucol were also lower following digestion experiments in the absence of surfactant.

1.7.1.1 Impact of quantity of lipid

In another Porter et al study, the group examined the impact of the quantity of lipid included in a lipid based formulation on the in vivo bioavailability of halofantrine following oral administration of MCT, LCT or blended MCT/LCT solutions.[65] The study showed good correlation between in vivo exposure (where halofantrine exposure was greatest from LCT > LCT/MCT blend > MCT) and in vitro drug solubilisation following in vitro digestion, but only when lower quantities of lipid (5 mg lipid/mL digest) were examined in vitro. In contrast, when higher quantities of lipid were employed (25 mg/mL) the MC formulation out-performed the LC formulation in vitro (but not in vivo). The authors suggested that the seemingly higher than expected drug solubilisation patterns seen on digestion of the higher quantities of lipid in vitro reflected the formation of a vesicular phase, with high solubilisation capacity, that was not formed under greater dilution in vivo. In the same study, the effect of the dose of halofantrine per gram of LCT lipid was also evaluated and interestingly greater in vivo exposure was obtained using a suspension of 100 mg halofantrine per gram lipid when compared to a lipid solution containing 50 mg halofantrine per gram lipid. The

data obtained with the lipid suspension were, as expected, more variable than the lipid solution formulation but the increase in exposure at the higher drug load was ascribed to potentially beneficial effects of the increase in thermodynamic activity that might be expected with the suspension formulation.[65] In contrast, Larsen et al[129] explored the impact of increasing drug dose using a series of formulations based on Labrafil®2125CS (comprising mono-, di- and triglycerides and LC mono- and di-fatty esters of polyethylene glycol 300) and containing increasing concentrations of danazol. In this case increases in danazol quantity per mL Labrafil led to decreased bioavailability in rats.[129]

The impact of drug load has been extended further still in a recent study from Thomas et al where supersaturated solutions of halofantrine in SEDDS formulations were administered to beagle dogs and compared to administration of identical formulations but containing sub-saturated quantities of drug.[130] The SEDDS formulations comprised 55% lipid (either LC (soybean:Maisine 35-I), or MC (Captex 300:capmul MCM)) 35% CrRH40 and 10% ethanol. The study showed that doubling the drug load of halofantrine from 75% to 150% of the saturated solubility of drug in the SEDDS formulation led to increased exposure and that slightly greater than linear increases in exposure with dose were apparent in vivo (although increase in absolute bioavailability were not statistically insignificant). In contrast, the solubilised concentration of halofantrine following in vitro digestion was similar regardless of formulation drug load. In addition, the study also showed that in vitro solubilisation was greater when the quantity of formulation was increased 2-fold, however, in vivo studies showed a tendency towards increased bioavailability when the quantity of formulation was doubled while maintaining the dose of halofantrine constant.[130]

Evidently, many factors influence the in vivo performance of lipid based formulations and predicting in vivo performance is complex. Historical attention has focussed on the composition and self-emulsification properties of LBDDS as important parameters in formulation performance, with possible drug loading and continued drug solubilisation during digestion as key indicators of

success. However, it is increasingly apparent that a more detailed understanding of the kinetic events that evolve on administration of LBDDS is required to better understand formulation performance and in particular the potential for supersaturation generation as the formulation is digested. These concepts are explored further in the following sections, in particular, the physiological impact of lipid dose and the ratio to drug loading in the formulation administered.

1.8 GENERATION OF SUPERSATURATION DURING PROCESSING OF LIPID-BASED FORMULATIONS IN THE GI TRACT

As described in section 1.6.1, formulation dispersion (**Figure 3**A) or digestion (**Figure 3**B and C), may decrease drug solubilisation capacity. Regardless of the mechanism by which the solubilisation capacity is reduced, however, the process of drug precipitation may not be immediate. Under these circumstances, metastable, supersaturated conditions are generated such that drug concentrations solubilised in the aqueous colloidal phase are greater than the equilibrium solubility of drug in the same phases.

Supersaturation is potentially a key mechanism by which drug absorption may be promoted, and is the only means by which the concentration of drug in free solution, in equilibrium with the solubilised reservoir, can be increased above the saturated solubility of drug in the intermicellar phase. Thus, for a drug that is solubilised in a colloidal particle, such as a micelle, the concentration of drug in free solution (D_{free}) increases as the total concentration of solubilised drug increases. Assuming the properties of the micelle do not change (i.e. above the critical micelle concentration), the ratio of C_{free} to C_{TotaL} is unchanged and is defined by the micellar partition coefficient (K_M)[131-133] where:

$$K_M = \frac{D_{micelle}}{D_{Total}} \qquad \text{(Equation 1.4)}$$

The maximum D_{free} concentration is reached when the saturated solubility of drug in the micellar system is reached. At that point, the drug concentration in free solution is essentially the same as the aqueous solubility of the drug. Under normal circumstances, therefore, micellar solubilisation cannot result in drug concentrations in free solution that are higher than that obtained by simply dissolving drug in aqueous solution in the absence of micelles (although the rate of dissolution is typically markedly higher). However, where solubilised drug concentrations are supersaturated, free drug concentration also increase, usually in proportion (since K_M is unchanged). Simplistically therefore, a solubilised system where drug is present at twice the equilibrium solubility of drug in the colloidal particles (often referred to as a supersaturation ratio of 2), results in a doubling of D_{free}.

An alternative means of description of the same phenomena is to describe in terms of the thermodynamic activity of drug in the solubilised system. The thermodynamic activity of drug in solution (a) is a measure of the effective concentration of drug that is available to exert an effect, be that to diffuse, to bind to a receptor, or in this case, to be absorbed.[1] Activity is therefore related to the concentration (mole fraction) of drug in solution (χ) by the activity coefficient (γ) where:

$$a = \gamma \times \chi \qquad \text{(Equation 1.5)}$$

In dilute solution, where intermolecular forces between drug molecules are limited $\gamma = 1$ and activities and concentration may be used interchangeably. Thus, all drug molecules in solution are 'available'. In contrast, where circumstances conspire to limit the availability of drug molecules in solution, the activity coefficient < 1 and the activity is less than the concentration.[1] This may occur under a range of circumstances, but is well exemplified by micellar solubilisation where the effective concentration of drug is significantly reduced by sequestration into the core of the micelle.[133] Assuming a simple phase separation model for micellar solubilisation, the available

concentration (or a) is D_{free} and γ is effectively the free fraction. Under these circumstances, it is apparent that supersaturation can also be viewed in terms of thermodynamic activity, such that increasing degrees of supersaturation result in parallel increases in thermodynamic activity. Increases in thermodynamic activity in the aqueous colloidal phase are therefore expected to drive improvements in absorption, in the same way that increases in C_{free} might be expected to drive increases in absorption.[132]

In the case of a LBDDS, increases in drug dose in the formulation are expected to increase the thermodynamic activity of drug in the colloids produced by formulation digestion and intercalation into endogenous bile salt/phospholipid micelles. This is expected regardless of whether digestion or dispersion triggers supersaturation, and increasing drug dose is expected to lead to greater degrees of saturation or supersaturation. As the degree of supersaturation increases, however, the likelihood of drug nucleation and crystallisation increase and ultimately drug precipitation is expected, returning the system to equilibrium (i.e. to equilibrium solubility). Simplistically therefore, increases in drug dose are expected to increase thermodynamic activity to a fixed point, above which further increases in dose will likely lead to precipitation and a reduction the thermodynamic activity. These two opposing drivers for absorption (increases in thermodynamic activity versus precipitation) must be balanced and the capacity of formulation excipients (or their digestion products) to maintain supersaturation and to limit drug precipitation subsequently dictates formulation performance.

In order to take advantage of the metastable supersaturated state, supersaturated drug concentrations need to be maintained for long enough to allow for drug absorption to occur. In recent years, significant attention has focussed on the potential for various formulation excipients to inhibit precipitation and to maintain supersaturation, although relatively few of these studies have applied these concepts to supersaturation generation during the processing of lipid based formulations. As such, one of the aims of the current research project is to evaluate the

conditions under which metastable supersaturated conditions can be generated and the potential for formulation excipients to stabilise this condition. These issues are addressed explicitly in Chapter 3.

1.9 PRECIPITATION INHIBITION TO STABILISE SUPERSATURATION

1.9.1 Drug crystallisation and precipitation

All supersaturated systems are thermodynamically unstable, and ultimately, drug precipitation will occur until the solubilised drug concentration reaches the equilibrium solubility of drug in the system and supersaturation is lost. Although the free energy of supersaturated drug is higher than that of crystalline drug (hence the drive towards precipitation)[134-136], for drug molecules to precipitate, two distinct processes must occur; firstly, the formation of a crystal nucleus of critical size and secondly, crystal growth.[134,137] Both of these processes are influenced by the physical conditions of the solution and the environment.[136,137]

The nucleation process involves the diffusion of molecules through the bulk of the solution, molecular collision and ultimately, the formation of nuclei of a critical size. Although drug precipitation is thermodynamic favoured (see Figure 3), initiation of nucleation requires the activation energy for nucleation to be overcome.[134,136,137] Where this activation energy is high, the stability of the supersaturated solution can be enhanced.

The period of time from when supersaturation is initiated to the point of nucleation is referred to as the induction time.[134,136-138] This period is reduced (i.e. the stability of the supersaturated solution is reduced) with increasing degrees of supersaturation. The critical supersaturation point for nucleation is the maximum degree of supersaturation that a metastable phase can tolerate

prior to initiation of nucleation; this has also been referred to as the metastable zone width.[135,137,139]

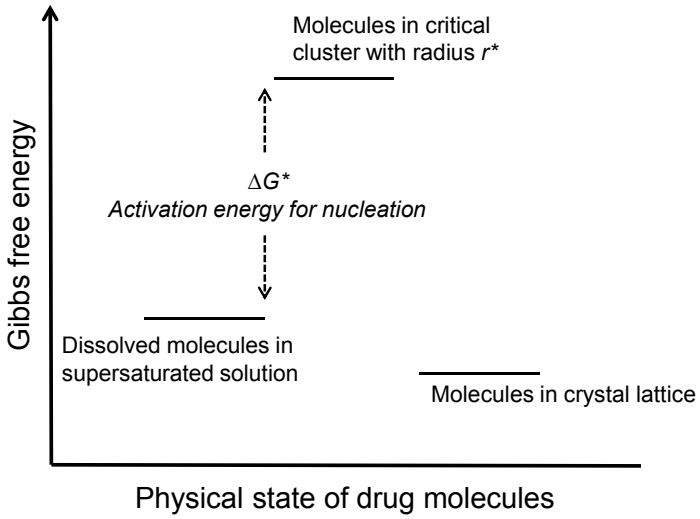

Figure 1.5 Schematic representation of the Gibbs free energy of molecules present in a supersaturated solution. Adapted from Brouwers et al[136]

Once a nucleus of critical size is reached, crystal growth takes place via a two-step process, transport of drug molecules to the crystal surface and subsequent arrangement in an ordered fashion in the lattice. For more detailed reviews on nucleation and crystal growth see Warren et al[138] and Brouwers et al[136].

1.9.2 Stabilising supersaturation using polymeric precipitation inhibitors

Polymers, for example, hydroxypropyl methylcellulose (HPMC), and povidone (PVP), are well recognised for their ability to suppress crystal nucleation in a range of different formulations.[17-23,138,140] For example, Raghavan et al showed that the addition of HPMC to a supersaturated solution of hydrocortisone acetate (HA) in propylene glycol (PG) and water delayed the onset of drug crystallisation, and the lag-time prior to drug crystallisation was increased as the polymer

concentration increased.[23] DiNunzio et al also showed that solid dispersions containing polymers including CAP (cellulose acetate phthalate) and PVAP (polyvinyl acetate phthalate) provided for a greater extent of supersaturation on dissolution and a reduction in the extent of drug precipitation due to the stabilising effect of the polymer.[141]

Raghavan et al[17], also examined the flux of hydrocortisone acetate (HA) across a silicone membrane to evaluate the potential for different polymers to stabilise a supersaturated cosolvent solution. The studies showed that the utility of the added polymer reached a maximum effect at polymer concentrations that varied depending on polymer type (1% for HPMC, 1% for HPC and 10% for PVP), and that further increases in polymer concentrations reduced drug flux.[17] This study suggested that the maximum enhancement in supersaturation was polymer dependent and that the degree of supersaturation was increased with an increase in polymer concentration, but only to a certain point.[17]

Polymers have been shown to reduce drug precipitation via interference with crystal nucleation and suppression of crystal growth. Inhibition of nucleation can be mediated via direct intermolecular interactions, such as hydrogen bonding, between drug and a co-formulated precipitation inhibitor, reducing the likelihood of the drug-drug interactions required to initiate nucleation.[17-23] Taylor and Zografi [142], for example, used IR spectroscopy to show the existence of hydrogen bonds between indomethacin and PVP in polymeric solid dispersion formulations and suggested that this may be responsible for the enhanced stability of indomethacin in a higher energy crystal form. Raghavan et al[17] also showed the presence of hydrogen bonds between hydrocortisone acetate (HA) and PVP in a solid dispersion formulation and suggested that the strength of the drug-polymer interaction determined the time required for nucleation to take place.

Once nucleation has occurred, precipitation inhibitors may further influence the precipitation process by decreasing crystal growth, in this case by reducing the rate of drug diffusion to the

crystal surface.[141] Raghavan et al,[22] for example, demonstrated that the presence of a 0.5% aqueous solution of HPMC, significantly reduced the particle size of drug crystals formed on dilution of a concentrated solution of triclosan in propylene glycol into an aqueous solution. The latter method of supersaturation generation (the solvent shift method) is a common experimental means of supersaturation generation. Retardation of crystal growth was explained by polymer adsorption to the crystal surface and the presence of a hydrodynamic boundary layer surrounding the crystal.[22,23] The capacity of many polymeric materials to form hydrogen-bonds with drug molecules dictates that many have the potential to interact with the surface of developing drug crystals and to suppress crystal growth.[19,23] Non-absorbed polymer molecules may also accumulate at the crystal interface providing a diffusion barrier for drug transfer to the crystal, effectively reducing crystal growth.[23]

The ability of polymers to maintain supersaturation in the GI tract has been well studied using itraconazole (ITZ).[21] ITZ is a weak base (pKa 3.7) with greater solubility in the acidic environment of the stomach than in the small intestine. Dissolution of ITZ in the stomach is therefore favoured, but gastric emptying typically leads to drug precipitation in the small intestine.[21] Solid dispersion formulations that include polymeric material, however, facilitate rapid dissolution in the stomach and subsequently, provide a stabilising effect against ITZ precipitation following transit from acidic to neutral pH. For example, after dissolution under acidic (gastric) pH and subsequent pH-shift to neutral pH, approximately 40% of the dose of ITZ from a formulation containing HPMC was still in solution compared to only 0-5% for alternative polymeric carriers including PVP, EUDRAGIT L100-55 (poly(methyacrylic acid,ethyl acrylate)1:1) and HPMC-P (hypromellose phthalate). This finding was explained by the different capabilities of the polymers to form hydrogen bonds with ITZ. Thus, PVP does not contain any hydrogen bond donors whereas HPMC may provide for up to 6 free hydroxyl groups as possible donors.[21] The authors concluded that strong intermolecular interactions as well as high local viscosity in solution were the underlying causes for

supersaturation stabilisation seen on polymer addition.[21] Subsequently, an ITZ solid dispersion formulation containing HPMC was evaluated *in vivo* and the extent of ITZ supersaturation following acidic to neutral pH change in vitro was well correlated with the extent of *in vivo* drug absorption.[21]

The prevention of nucleation and crystal growth and therefore the utility of PPIs is highly dependent on the physicochemical properties of both the drug and the polymer.[140] Recently, screening studies utilising the solvent-shift methods described above have been employed to assess the effect of a range of polymers on supersaturation stabilisation for a range of PWSD, in order to better understand the physicochemical determinants of drug-polymer interaction and supersaturation stabilisation. In this regard, Vandecruys et al[140] evaluated the effect of different pharmaceutical polymers; hydroxypropyl cellulose (HPC), hydroxypropyl methylcellulose (HPMC), Polyox, polyvinylpyrrolidone (PVP) and PVP-co-vinyl acetate (PVP VA) on supersaturated stabilisation of solutions of 25 drug candidates. The authors reported that the polymers were able to increase the period of supersaturation however the extent of performance was dependent on polymer type and drug candidate. The in vivo performance of solid formulations of the drug candidates and polymers were following evaluated in beagle dogs and a good correlation between supersaturation stabilisation and in vivo exposure was obtained. Subsequently, Warren et al[138] evaluated a broader range of 53 polymeric materials in a similar testing protocol with the poorly water-soluble drug, danazol. These studies identified a group of PPIs that showed superior precipitation inhibition effects, the majority being cellulose-derivatives.

1.9.3 Polymeric precipitation inhibitors in lipid-based formulations

In the lipid formulation area, relatively little work has been performed to evaluate the potential utility of the addition of PPIs in suppressing drug precipitation during formulation dispersion and digestion. The exception is a series of studies by Gao and colleagues[24,25,27], recently supplemented

by Kakaoka et al[29] and Wei et al[28]. Gao et al[27], showed that when 5% (w/w) HPMC was added to a SEDDS formulation comprising EtOH (151.5 mg/g), PEG400 (151.5 mg/g), CrEL (400 mg/g) and glyceryl dioleate (190 mg/g), differences were apparent on formulation dispersion in simulated gastric fluid (SGF, pH 2.0 at 37°C).[27] Thus, the formulated drug candidate (Paclitaxel, 57 mg/g) remained solubilised in the system containing polymer for longer periods than the equivalent SEDDS formulation without HPMC. Furthermore, when the same formulation was examined *in vivo* in rats following oral administration of the pre-dispersed formulation in aqueous media, a 10 fold increase in bioavailability was observed for the SEDDS containing 5% (w/w) HPMC when compared with the equivalent polymer free formulation.[27] This is in agreement with Wei et al[28] where a similar approach was utilised to enhance the oral bioavailability of the poorly water-soluble drug, silybin.

In a subsequent study, Gao and co-workers attempted the same approach for the poorly soluble drug, PNU-91325 formulated in polyethyleneglycol (PEG) 400 (90 mg/g), water (80 mg/g), Cremophor EL (300 mg/g), dimethyl acetamide (50 mg/g), pluronic-L44 (180 mg/g) and glycerol monooleate and dioleate (8:2)(60 mg/g).[25] In vitro dispersion under similar gastric conditions (as described above) revealed a five-fold decrease in drug precipitation when the formulation was tested in the presence (S-SEDDS) and absence (SEDDS) of HPMC (grade E50LV) (20%). This was suggested to be due to polymer stabilisation of supersaturated conditions on dispersion of the S-SEDDS formulation in aqueous media. In vivo evaluation in beagle dogs following oral administration led to an absolute bioavailability of the S-SEDDS formulation of 76% compared to only 12% for a PEG 400 formulation. However, a control experiment using the SEDDS formulation without HPMC was not performed limiting the conclusions that could be drawn regarding the specific effect of the polymer, and when compared to a simple Tween 80 formulation only a small difference (68% versus 76% for Tween 80 versus S-SEDDS) was observed.[25] Interestingly, a subsequent study conducted in monkeys, where AMG 517 in an aqueous suspension (OralPlus

suspension vesicle containing 100 mg/g Pluronic F108) and a SEDDS formulation (56% PEG 400, 30% Tween 80, 9% Capmul MCM and 2% HPMC) were evaluated, showed no significant difference in absolute bioavailability between the two formulation types.[24] The impact of HPC and HPMC on the in vivo performance of SEDDS formulations has subsequently also been evaluated in Kataoka et al[29], and no significant effect of polymer addition was observed on the oral bioavailability of danazol in rats. However, the control SEDDS formulation (LFCS type II) was again different to the polymer-comprising SEDDS (LFCS type IIIA) and the performance therefore difficult to comparable.

As an alternative approach to the direct incorporation of HPMC into the formulation, SEDDS formulations have also been examined after filling into HPMC capsules (Quali-V capsules, Shionogi). In this case, similar results to that obtained by direct addition of HPMC to the SEDDS formulation were reported.[26] The utility of PPI therefore does not appear to be dependent on incorporation into the SEDDS formulation.

In contrast to much of the data obtained with HPMC, one study where the utility of PVP in SEDDS formulations containing the poorly water soluble drug AMG 517 was examined, failed to reveal differences in drug precipitation on dispersion reinforcing the specificity of the interactions between drug and polymer in dictating the utility of PPIs.[24] In an attempt to further evaluate the precipitation process of drug from the SEDDS formulations, the same authors[24] examined the nature of the precipitated solids obtained on dispersion of formulations containing AMG 517 by x-ray powder diffraction. Interestingly, the precipitated solids from SEDDS formulations lacking HPMC or those containing PVP were crystalline whereas the solids obtained from SEDDS formulation containing HPMC were amorphous. Gao et al[24] suggested this was due to differences in the hydrophilicity of the polymers (with PVP being the more hydrophilic) and that hydrophobic interactions between the drug and the polymer were important for polymer adsorption on to the crystal surface. Interestingly, the change in crystal form observed suggests that the increases in

drug solubilisation obtained for these systems post digestion, may be due to changes in polymorphic form, rather than supersaturation stabilisation. However, differences in supersaturation stabilisation between PVP and HPMC have also been suggested to reflect stronger intermolecular interactions between drug (in this case ITZ) and HPMC (due to high number of hydrogen bond donors in HPMC) in contrast to PVP (that is a hydrogen bond accepter).[21] In this case, increases in supersaturation stabilisation may be due to attractive intermolecular interactions in solution may lead to decreased enthalpy of the solution and therefore reduced thermodynamic activity, hence reduced drug precipitation.

The research conducted by Gao and co-workers[24-27] and others[28,29] suggest the potential utility of PPIs incorporation into SEDDS formulations to prevent drug precipitation. However, the reported data set is limited, and provides somewhat contradictory evidence of utility. The mechanisms responsible for the enhanced absorption that occurs in some cases on addition of PPIs are also still to be determined. A more detailed evaluation of the utility of PPIs in lipid-based SEDDS formulations including detailed in vitro dispersion and digestion experiments and correlation with in vivo performance is described in Chapter 3.

1.10 CHOICE OF MODEL POORLY WATER-SOLUBLE DRUG

Danazol (MW: 337.46 g/mol) is a synthetic androgen used in the treatment of endometriosis. It was chosen as a model poorly water-soluble drug to investigate the hypotheses stated above since it is highly lipophilic (log*P* value (4.53))[143], poorly water-soluble (< 1 µg/mL)[144] and has poor oral bioavailability. Previous work has shown that the bioavailability of danazol can be enhanced using lipid vehicles.[13,16,106]

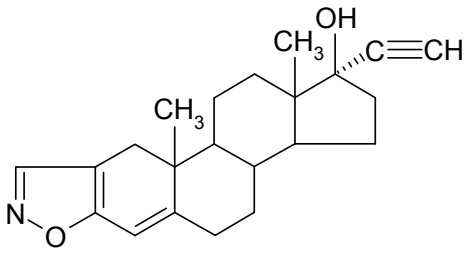

Figure 1.6 Chemical structure of danazol

1.11 AIMS OF RESEARCH

The overarching hypothesis that underpins the current research project is that the potential for supersaturation generation during the processing of lipid-based SEDDS formulations has a great impact on the formulation performance, and that inclusion of PPIs in SEDDS may be utilised to stabilise the supersaturated condition, prevent drug precipitation and enhance drug absorption.

This project therefore aims to develop a more complete understanding of the mechanisms by which lipids, surfactants and polymers interact with the gastrointestinal content to support the solubilisation of poorly water-soluble drugs, the potential for supersaturation generation during processing of SEDDS formulations and the ability of PPIs to enhance the performance of SEDDS and promote bioavailability after oral administration of drug candidates.

Additionally, the project endeavour to examine the influence of increasing drug dose on in vitro drug solubilisation and supersaturation, and to evaluate whether increases in thermodynamic activity (through increases in dose) in the aqueous colloidal phase (produced by formulation dispersion and digestion and the intercalation into endogenous bile salt/phospholipid micelles in the GI tract) drives improvements in absorption and therefore in vivo bioavailability. In this

regard, the impact of drug dose on first pass metabolism, and therefore the possibility of saturation of first pass metabolism following administration of higher drug doses, is also explored.

Finally, a more mechanistic evaluation of the impact of formulation processing on oral bioavailability after administration of SEDDS is attempted including in vitro and in vivo evaluation and species comparison studies in rats and beagle dogs to more effectively mimic events in the GI tract to obtain better in vitro - in vivo correlation.

1.12 THESIS STRUCTURE

This thesis is a compilation of 3 manuscripts. Chapter 3 is a manuscript recently published in Molecular Pharmaceutics, and Chapters 4 and 5 are manuscripts in submission. The format of the journals to which these manuscripts have been submitted has precluded the inclusion of detailed methods. Chapter 2 is therefore added as a general method section to allow a more detailed description of the methods that have been developed and validated throughout the thesis.

The thesis is concluded in Chapter 6 by a summary and perspectives chapter. This is followed by appendices that comprise my published manuscripts. These include the manuscript in Chapter 3 and four additional papers that are not a part of the main aspects of the thesis but where the contributed work has been carried out during my PhD candidature.

CHAPTER 2

GENERAL METHODS

2 General methods

2.1 FORMULATION PREPARATION

2.1.1 Preparation of self-emulsifying lipid-based formulations comprising danazol

All formulations were prepared by weighing individual excipients and the model compound, danazol, into 20 mL glass scintillation vials followed by vortexing for 60 seconds (s). The formulations were stored in a 37°C incubator overnight to allow for equilibration prior to experiments. Danazol was incorporated at various drug loads and the drug load expressed as the percentage of saturated solubility based on the equilibrium solubility in the formulation (at 37°C).

Drug solubility in each of the formulations was assessed by addition of excess solid danazol to excipients or formulations and samples taken over time. All experiments were performed in triplicate.[15] Equilibrium solubility was defined as the value attained when the concentration of danazol in at least three consecutive solubility samples varied by ≤ 5%. This was typically reached after equilibration times of between 48 and 72 hours (h).

Formulations with varying ratios of medium chain lipids (Captex 300:Capmul MCM (50:50)), surfactant (CrEL) and cosolvent (absolute ethanol) were used as model formulations. A ternary plot of the composition of the individual formulations employed is shown in Figure 2.1 and numeric values are tabulated in Table 2.1.

Formulations containing polymeric precipitation inhibitors (PPIs) were assembled using the formulations presented in Table 2.1 and subsequent addition of the PPIs at the stated concentrations (% w/w). After the addition of PPI, the formulations were vortexed for 30 s and equilibrated overnight at 37°C.

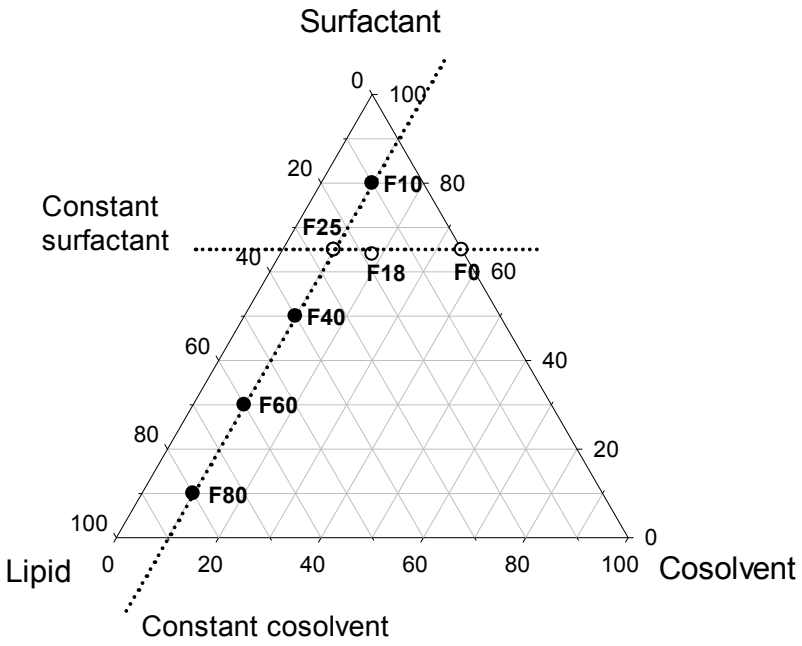

Figure 2.1 Ternary plot of formulation composition at constant surfactant (65%) or constant co-solvent (10%) comprising medium chain lipid (Captex 300: Capmul MCM 50:50), Surfactant (CrEL) and co-solvent (ethanol).

2.1.2 Preparation of intravenous formulation

An intravenous formulation of danazol (1.2 mg/mL) was prepared using 15% (w/v) hydroxypropyl-β-cyclodextrin (HP-β-CD). Danazol and HP-β-CD were firstly dissolved in 0.9% saline using a magnetic stirrer (Teflon coated stirrer bar, 10 x 6 mm) at ambient temperature. The formulation was filtered through a 0.22 µm filter (Millix®-GV) before use and the concentration of drug in the formulation checked after filtration.

Table 2.1 Composition (% w/w) of SEDDS and danazol solubility data in the formulation and in the aqueous phase (AP) formed during in vitro digestion

Formulation components	**F0** [a]	**F10**	**F18**	**F25**	**F40**	**F60** [a]	**F80**
Captex 300: Capmul MCM (1:1) (% w/w)	0	10	18	25	40	60	80
Cremophor EL (% w/w)	65	80	64	65	50	30	10
Ethanol (absolute) (% w/w)	35	10	18	10	10	10	10
LCFS classification type [b]	**IV**	**IV/IIIB**	**IV/IIIB**	**IIIB**	**IIIA**	**IIIA**	**IIIA**
Danazol solubility in formulation [mg/g]	45 ± 1.3	36 ± 0.6	39 ± 0.8	35 ± 2.3	32 ± 0.7	30 ± 0.7	25 ± 1.0
AP_{max} (@ 80% sat) [ug/mL] [c]	994	800	860	784	718	674	544
AP_{max} (@ 40% sat) [ug/mL] [c]	497	400	430	392	359	337	272
Apparent equilibrium solubility [ug/mL]:							
AP_{DISP} [d]	178 ± 6.8	259 ± 5.1	243 ± 2.5	282 ± 5.5	306 ± 3.7	301 ± 3.9	269 ± 15.9
AP_{DIGEST} (5 min) [e]	134 ± 5.0	155 ± 2.5	126 ± 1.0	132 ± 0.3	118 ± 2.9	106 ± 3.6	88 ± 0.6
AP_{DIGEST} (60 min) [e]	89 ± 2.8	102 ± 0.9	81 ± 0.8	91 ± 2.7	71 ± 3.2	56 ± 0.9	46 ± 1.0
AP_{DIGEST} + HPMC (60 min) [e]	82 ± 0.3	98 ± 3.3	80 ± 1.7	81 ± 2.5	67 ± 2.6	59 ± 0.3	38 ± 1.1

Captex 300 (MC triglycerides); **Capmul MCM** (MC monoglyceride/ diglyceride); **CrEL** (surfactant); **Ethanol** (cosolvent); **HPMC** (hydroxypropyl methylcellulose);

[a] Formulations re-named in Chapter 5 to SEDDS-IV (F0) and SEDDS-III (F60)

[b] Classification scheme taken from Pouton[71,94]

[c] Data represent 80% or 40% of the saturated solubility of danazol in the formulation

[d] Data represent equilibrium solubility of danazol in the AP produced by dispersion of blank (drug-free) formulation for 10 min

[e] Data represent equilibrium solubility of danazol in the AP produced by dispersion and digestion of blank (drug-free) formulation for either 5 or 60 min. Data at 60 min also generated for formulations containing HPMC

2.2 DRUG SOLUBILISATION DURING FORMULATION DISPERSION UNDER GASTRIC CONDITIONS

2.2.1 Dispersion of SEDDS in simulated gastric fluids

Dispersion experiments were carried out in 36 mL of 0.1 N HCL (pH 1.2) [simulated gastric fluid (SGF) (without pepsin)] in accordance to USP regulations. The experiments were performed by dispersing 1 g of lipid-formulation in 36 mL SGF in a 60 mL glass vessel at 37°C. The temperature was controlled by a thermostatically controlled water jacket and the dispersion was stirred magnetically (disc-shaped Teflon coated stirrer bar, 10 x 14 mm) and samples (200 µL) collected at t = 1, 15 and 30 min.

2.2.2 Dispersion of SEDDS under simulated low dilution gastric conditions

In some studies (Chapter 5), a low dilution/ intermediate pH model was utilised to better reflect gastric conditions in the rat. In these cases, 100 mg of lipid-based formulation was weighed into a flat bottom glass vial and 900 µL of buffer (pH 5.5) used as the dispersion medium (i.e. a 1 in 10 dilution). A pH of 5.5 was used based on previously reported values of rat gastric pH.[79] The dispersion was stirred using a magnetic stirrer (Teflon coated stirrer bar, 10 x 6 mm) and samples (200 µL) collected at t = 1, 15 and 30 min.

2.2.3 Dispersion of SEDDS in low dilution ex-vivo gastric fluid

Dispersion studies were also performed using ex vivo gastric fluid obtained from the rat (obtained as described in section 2.5.1). In this case dispersion experiments were performed using the low volume dispersion protocol described above (section 2.2.2).

2.2.4 Sample work up for in vitro gastric dispersion experiments

Dispersion samples were centrifuged for 10 min at 21,100 x *g* (Heraeus Fresco 21 microcentrifuge, Thermo Scientific, Germany) in order to pellet any drug that precipitated on dispersion. Samples obtained from each phase were diluted (aqueous phase: 50 µL to 950 µL ACN; Precipitate (pellet) and oil phase was dissolved in 50 µL chloroform/methanol (2:1 v/v) and 950 µL MeOH) and analysed by HPLC as described in section 2.4.

2.3 DRUG SOLUBILISATION DURING FORMULATION DISPERSION AND DIGESTION UNDER INTESTINAL CONDITIONS

In vitro digestion experiments were conducted using 3 different models, a dog-relevant digestion model with high dilution & high enzyme activity (section 2.3.2), a rat-relevant digestion model with high dilution & low enzyme activity (section 2.3.3) and a second rat digestion model with low dilution/low enzyme activity (section 2.3.4). A detailed comparison of in vitro model details is tabulated Table 13. The in vitro dog model was utilised as the standard lipid digestion model in Chapter 3 and 4, and the in vitro rat digestion models were utilised in the studies described in Chapter 5.

2.3.1 Simulated intestinal conditions

2.3.1.1 Preparation of digestion buffer

Digestion buffer was prepared in a volumetric flask by dissolving 50 mM TRIS maleate, 150 mM NaCl, 5 mM $CaCl_2 \cdot 2H_2O$ and 2.4 g NaOH pellets in MilliQ water and made up to 1000 mL. The pH was adjusted to 7.5 (Metrohm 632 pH-meter, Metrohm herisan Switzerland) by addition of 1 N HCL or NaOH. The digestion buffer was stored in the fridge until use for up to 30 days.

2.3.1.2 Preparation of simulated intestinal fluids (digestion medium)

The thin-film hydration procedure was used for the preparation of the micellar digestion medium. In the majority of experiments, simulated intestinal fluids contained 5 mM sodium taurodeoxycholate (NaTDC, 97%) and 1.25 mM phosphatidylcholine (PC, 99%).[145-151] To simulate high bile salt conditions in chapter 5, 20 mM NaTDC and 5 mM PC were also employed in specific studies. In both cases, the PC component was dissolved in 2 mL chloroform in a round-bottom flask. The chloroform was subsequently removed under vacuum using a rotary evaporator (Rotorvapour RE, Buchi, Switzerland) to produce a thin layer of lecithin on the sides of the round-bottom flask. When the flask was free from chloroform, the NaTDC was added to the flask along with digestion buffer (45 mL to give 50 mL micelles to allow for dilution effect on subsequent addition of enzyme extract to the digestion vessel) and the flask was wrapped in foil to minimise exposure to light. The flask was stirred magnetically overnight to allow equilibration and stored in the fridge for up to 7 days prior to use.

2.3.1.3 Lipid digestion apparatus

Experiments were performed at 37°C in a 60 mL glass vessel (Bartelt Instruments, Melbourne, Australia), attached to a water jacket. The temperature was controlled thermostatically (Thermomix 1440, D. Braun Melsungen AG, Germany) and the fluids contained in the vessel (i.e. digestion medium plus lipids) were stirred using a magnetic stirrer (SAM7 sample stand, Radiometer analytical, France) and a disc-shaped Teflon coated stirrer bar (10 x 14 mm, Kartell Plastilab, Italy).

Liberation of fatty acids (FA) were quantified using a pH stat titration unit containing a pH electrode (Ag/AgCL reference combined pH electrode, Radiometer Analytics, France) coupled to a pH-stat controller (PHM290 MeterLab, Radiometer Copenhagen, Denmark) and an autoburette (ABU901, Radiometer Copenhagen, Denmark). The pH of the digestion media was maintained

within 0.1 pH units of a set point (pH 7.5) by automatically titrating liberated FA by addition of equivalent molar quantities of NaOH. The cumulated volume of titrant added and the pH of the digestion medium was recorded at 5 s intervals acquired using the StatSheet software, version 1.3 (Radiometer Copenhagen, Denmark).

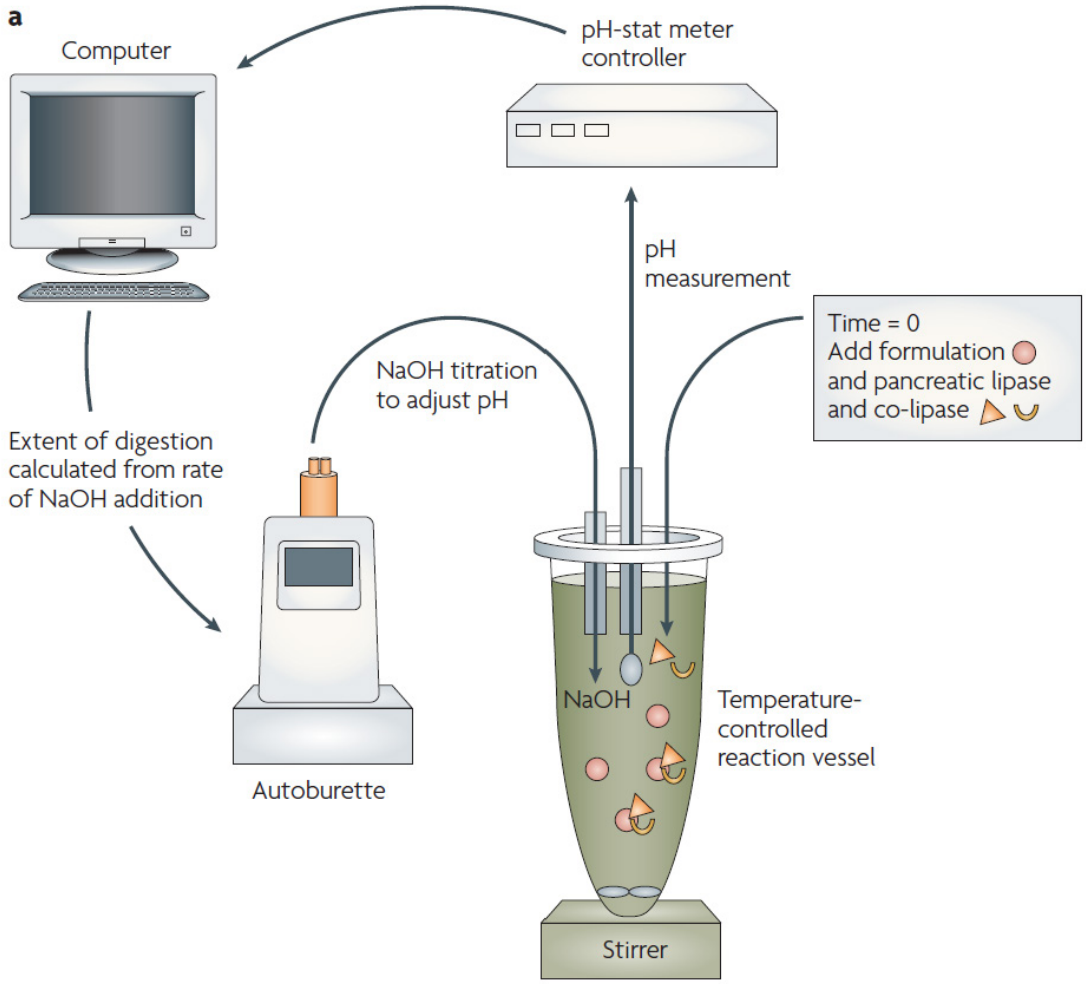

Figure 2.2 Schematic illustration of the lipid-digestion apparatus. Experiments were performed in a glass vessel where the fluids (digestion medium, formulation and lipase) were stirred magnetically using a magnetic stirrer. Liberated fatty acids (FA) were quantified using a pH stat titration unit containing a pH electrode coupled to a pH-stat controller and an autoburette. The pH of the digestion media was maintained by automatically titrating liberated FA by addition of equivalent molar quantities of NaOH. The cumulated volume of titrant added and the pH of the digestion medium was recorded by a computer (Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Drug Discovery (Porter et al[64]). Copyright (2007).

2.3.1.4 Preparation of the pancreatin extract

Pancreatic lipase/co-lipase (Sigma, P-7545) enzyme extract was prepared using 1000 mg of porcine pancreatin (8 x USP) in 5 mL digestion buffer (200 mg/mL). The mixture was stirred for 15 min and centrifuged (2880 x *g* at 5°C, Eppendorf 5804 R centrifuge, Eppendorf AG, Hamburg, Germany) for 10 min. The supernatant was separated and used for digestion studies on the same day it was prepared.

2.3.1.5 Validation of pancreatic enzyme activity

The activity of pancreatic lipase was determined using the in vitro lipolysis assay and tributyrin as a model substrate. This provides enzyme activity in tributyrin units (TBUs) where 1 TBU (lipase unit) is defined as the amount of enzyme that releases 1 μmol of titratable butyric acid per minute.[108] The lipase activity was determined as previously described[97], where 6 g of tributyrin was dispersed in 9 g of digestion medium (digestion media simulating typical fasted state) (see section 2.3.1.2). Experiments were performed using the digestion apparatus described in section 2.3.1.3. The fluids contained in the vessel (i.e. digestion medium plus tributyrin) were stirred magnetically (10 x 14 mm disc-shaped Teflon coated stirrer bar, Kartell Plastilab, Italy), and after 2 min of mixing, digestion was initiated by addition of a fixed volume (1 mL) of enzyme extract, containing different proportions of the porcine pancreatin enzyme extract such that the equivalent concentration of pancreatin extract ranged from 200 mg/mL (undiluted) to 0.056 mg/mL. This allowed the generation of a 'standard curve' of enzyme activity as a function of the volume/quantity of porcine pancreatic enzyme added. 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. Digestion was followed for 30 min (or until 10 mL NaOH) had been added using a pH-stat titration unit as described in section 2.3.1.3.

Lipase activity in TBU (μmol titratable butyric acid per minute) was calculated from the initial rate of digestion determined 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). The intrinsic activity of the enzyme was defined by the maximum rate obtained, i.e. when substrate was in excess and the generated 'standard curve' for porcine lipase activity is illustrated in Figure 2.3 as volume of porcine pancreatic extract versus the correlating enzyme activity expressed in TBUs.

2.3.1.6 Determination of pancreatic lipase activity in rat pancreatic/biliary fluid

The lipolytic activity of enzymes in ex vivo rat bile/pancreatic fluid (collected as described in section 2.5.2) was determined using an in vitro lipolysis assay and tributyrin as a model substrate as described above (section 2.3.1.5).

The relative lipase activity of the ex-vivo rat pancreatic fluid was determined based on the generated porcine pancreatin extract enzyme activity 'standard curve' in order to identify the quantity of porcine pancreatic extract needed to match the activity of 1 mL of ex-vivo rat pancreatic/biliary fluid. The lipase activity in ex vivo rat pancreatic/biliary fluid is illustrated by the red circle in Figure 2.3 with a similar activity expected from 17 μL of porcine pancreatic extract (~1.7% of the volume utilized previously). The data is further discussed in Chapter 5.

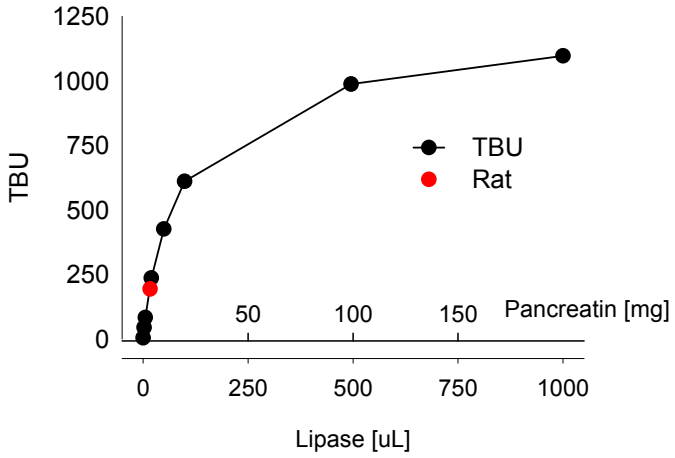

Figure 2.3 Volume of porcine pancreatic extract (and the corresponding quantity of pancreatin powder) added to 10 mL digestions versus the correlating enzyme activity expressed in TBUs. The lipase activity in ex vivo rat pancreatic/biliary fluid (collected as described in section 2.5.2) is illustrated by the red circle. Similar activity was expected from 17 µL of porcine pancreatic extract (~ 1.7% of the volume utilized previously).

2.3.2 In vitro dog digestion model - High dilution / High enzyme activity

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

In vitro experiments were conducted using a modification of a previously described lipid digestion model, although in this case a dispersion step was added prior to initiation of digestion to allow for evaluation of the potential for drug precipitation on formulation dispersion.

The lipid-based formulation (1 g) was dispersed in 36 g of digestion medium using the digestion apparatus described in section 2.3.1.3. The first experimental period (0-30 min) comprised the dispersion stage of the study, where the formulation was simply dispersed in the digestion medium by stirring. After 30 min, digestion was initiated by addition of 4 mL of pancreatin extract (see section 2.3.1.4 for preparation). Digestion was followed for 60 min and pH maintained at 7.5 via titration of liberated fatty acids with 0.6 M NaOH (see section 2.3.1.3). Aliquots (4.2 mL) were

taken from the dispersion/digestion media throughout the 90 min experimental period at t = 10, 30, 40, 50, 60 and 90 min. Samples were ultracentrifuged and treated as described in section 2.3.5 and analysed for danazol by HPLC (see section 2.4).

2.3.2.1 Evaluation of physical form of danazol by x-ray powder diffraction

The pellet phase obtained after digestion and ultracentrifugation was dried at room temperature and examined by x-ray powder diffraction (XRPD) to investigate the physical form in which danazol precipitated from LBDDS, and whether the inclusion of PPI (HPMC) in the formulation had any effect on this process. Samples of HPMC and crystalline danazol were measured as controls using a quartz sample plate. The pellet phase obtained post digestion of the F60 formulation (+/- 5% (w/w) HPMC E4M) (see section 2.1.1) was placed on a zero background silicone holder. XRPD was performed using a Philips 1140 vertical diffractometer (Philips, Holland) for scanning from 2° to 60°, with an angular increment of 0.02° and a speed of 2°/min. A copper (Cu) tube anode and Kα radiation (λ 1.54184 Å) was used. The voltage and current applied was 45 kV and 40 mA. The data were acquired and analysed using the Visual XRD and traces software package, version 6.6.1 (GBD Scientific Equipment).

2.3.2.2 Solubility in aqueous phase pre-digestion (dispersion phase) (AP_{DISP})

The solubility of danazol in dispersed emulsified blank (i.e. drug-free) formulation was evaluated. Triplicate samples of the aqueous phase were taken after dispersion of blank formulations for 10 min and excess danazol (10-20 mg) added and incubated at 37°C. The equilibrium solubility of danazol in the dispersed aqueous colloidal phase was defined as the mean solubility value obtained after 2, 4 and 6 h incubation. Solubility samples were centrifuged for 30 min at 21,100 x *g* (Heraeus Fresco 21 microcentrifuge, Thermo Scientific, Osterode, Germany) at 37°C and samples of the aqueous phase were subsequently taken. The aqueous phase samples were

diluted from 50 µL to 950 µL with ACN and analysed for danazol content by HPLC as described in section 2.4.

2.3.2.3 Solubility in aqueous phase post digestion (AP_{DIGEST})

The solubility of danazol in the aqueous colloidal phase generated by digestion of blank (i.e. drug-free) formulation was evaluated after 5 and 60 min digestion. Conditions for digestion of the blank formulation were as described above (see section 2.3.2) and digestion samples were ultracentrifuged (see section 2.3.5) and the aqueous phase separated prior to measurement of drug solubility. Triplicate samples comprising danazol in excess (10-20 mg) were incubated at 37°C and centrifuged for 30 min at 21,100 x *g* (Heraeus Fresco 21 microcentrifuge, Thermo Scientific, Osterode, Germany) at 37°C. As the solubility was found to decrease up to 20% over 24 h for some of the formulations, presumably due to instability of the colloids present, the apparent equilibrium solubility in the aqueous colloidal phase post digestion was defined as the mean solubility value obtained after 2, 4 and 6 h incubation following attainment of the maximum solubility.

The aqueous phase samples were diluted from 50 µL to 950 µL with ACN and analysed for danazol content by HPLC as described in section 2.4.

2.3.3 In vitro 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 also carried out using collected (ex vivo) rat pancreatic/biliary secretions (collection method described in section 2.5.2). Initial studies were conducted in 10 mL in vitro digests where the ratio of formulation: digestion medium was kept constant relative to the in vitro dog digestion model (section 2.3.2) and employed a

formulation dilution factor of 40, i.e. 250 mg formulation in 9 mL digestion medium with 1 mL of ex vivo rat pancreatic/biliary secretions employed to stimulate digestion (see Table 2.2). Since rat pancreatic/bile secretions contain bile, experiments were conducted in digestion buffer without added bile salt and phospholipid. Otherwise, in vitro digestion experiments were conducted as described above (section 2.3.2) and 1 mL samples of digestion medium were collected following 30 min dispersion and at 10, 20, 30 and 60 min post initiation of digestion. All samples were treated as described in section 2.3.5 and analysed for danazol by HPLC (see section 2.4).

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

In vitro digests were also performed using the rat digestion protocol (high dilution / low enzyme) but using a quantity of porcine enzyme that provided a similar enzyme activity to that of ex-vivo rat pancreatic fluid (17 µL pancreatic lipase extract, diluted to 1 mL with MilliQ water). The activity of the ex-vivo rat pancreatic/biliary fluid, and the quantity of porcine pancreatic fluid required to mimic this activity was assessed using the tributyrin assay described in section 2.3.1.6. All samples were treated as described in section 2.3.5 and analysed for danazol by HPLC (see section 2.4).

2.3.4 In vitro rat digestion model - Low dilution / Low enzyme activity

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

The apparent volume of fluid in the rat intestine is low, and under fasted state conditions the major source of fluid flow into the intestine appears to be bile and pancreatic secretions. 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 secretions (see section 2.5.2). 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)(described in section 2.8). This condition was intended to reflect the *in vivo* studies where administration of the dispersed formulation was expected to mix with endogenous rat bile/pancreatic secretions. An additional 250 µL MilliQ water was added to the vial to simulate the low levels of additional (basal) intestinal fluids that might be present in the GI tract during *in vivo* studies. Due to the low volume it was not possible to employ a pH stat for maintenance of pH during the digestions and studies were performed in a 10 mL glass vial with screw cap at room temperature and media was stirred magnetically (Teflon coated stirrer bar, 10 x 6 mm). Samples of the digest (300 µL) were collected at t = 10, 20, 30 and 60 min and at the end of the experiment and the pH of the digest was measured (PHM290 MeterLab, Radiometer Copenhagen, Denmark coupled to an Ag/AgCL reference combined pH electrode, Radiometer Analytics, France). All samples were treated as described in section 0 and analysed for danazol by HPLC (see section 2.4).

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

The low dilution rat model was also re-run using a quantity of porcine enzyme that provided a similar enzyme activity to that of ex-vivo rat pancreatic fluid (17 µL lipase extract and 983 µL MilliQ water to give a total of 1 mL as determined in section 2.3.1.6). All samples were treated as described in section 2.3.5 and analysed for danazol by HPLC (see section 2.4).

Table 2.2 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[97]

[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

[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

2.3.5 Sample work up for in vitro dispersion/digestion experiments under intestinal conditions

The lipase inhibitor 4-bromophenylboronic acid[152] (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) in order to pellet any drug that precipitated on dispersion/digestion. Digestion samples from the dog in vitro model were centrifuged more thoroughly via ultracentrifugation for 30 min at 37°C and 400,000 x *g* (Optima xL-100 K centrifuge, SW-60 rotor, Beckman, Palo Alto, CA, USA.

Aqueous phase samples obtained during dispersion experiments under intestinal conditions and after initiation of lipolysis were diluted 1:20 (v/v) with acetonitrile before HPLC analysis. Samples of danazol in the pellets and oil from the digestion studies were first dissolved in 1 mL chloroform/methanol (2:1 v/v) and subsequently diluted 1:20 (v/v) in methanol prior to analysis by HPLC as described in section 2.4.

2.4 DANAZOL QUANTIFICATION BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

2.4.1 Preparation of standards

A stock solution of 1 mg/mL danazol in acetonitrile: MilliQ (60:40 v/v) was prepared. Standard solutions of danazol (DA) in acetonitrile: MilliQ (60:40 v/v) were prepared from the stock solution

containing 0.25, 0.50, 1, 2.5, 5, 10, 25 and 50 μg/mL DA. All solutions were stored under refrigeration (< 5°C).

2.4.2 Sample preparation

See section 2.2.4 for in vitro gastric dispersion sample preparation and section 2.3.5 for in vitro intestinal dispersion/digestion sample preparation.

2.4.3 Instrument and chromatography

The danazol HPLC assay was adapted from a previous publication[13] with modification of the mobile phase composition. Chromatography was performed using a Waters 2690 separation Module and a Waters 486 tunable absorbance detector (Waters, MA, USA). A Waters symmetry C18 column (3.9 x 150 mm) (Waters, MA, USA) was maintained at 25°C. The mobile phase consisted of 75% (v/v) methanol and 25% (v/v) Milli-Q water. The flow rate was 1 mL/min. All samples were maintained at 10°C in the autosampler prior to injection of 50 μL. Detection of danazol was conducted by single wavelength monitoring at 286 nm.

2.4.4 Assay validation

The assay was validated by analysis of n = 5 replicate standards made up at three different concentrations (0.5, 5, 50 μg/mL). Intra-assay variability was accurate to 96.6, 101.1 and 99.7% and precise to ± 0.5, 0.3 and 3.8% of 0.5, 5 and 50 μg/mL. Inter-assay variability was assessed over three separate days and was accurate to 100.5, 99.3 and 100.3% and precise to ± 2.9, 0.2 and 0.1% at 0.5, 5 and 50 μg/mL. The recovery of danazol spiked into aqueous and pellet phases obtained from blank digests of soybean oil was 96.4 ± 0.3 % and 100.0 ± 0.8 % (n = 6), respectively.

Animal work

All surgical and experimental procedures in this thesis were approved by the Monash Institute of Pharmaceutical Sciences animal ethics committee and were conducted in accordance with the Australian and New Zealand Council for the Care of Animals in Research and Teaching guidelines.

2.5 SURGICAL AND EXPERIMENTAL PROCEDURES FOR COLLECTION OF EX VIVO RAT FLUIDS

2.5.1 Gastric fluid collection from the rat

To collect gastric fluids from the rat, male Sprague Dawley (SD) rats (300-400 g) were fasted in metabolic cages (to prevent coprophagy and ingestion of bedding material) for 12 h prior to surgery with free access to drinking water. Animals were anesthetised with isoflurane (5% v/v) and a ligature was tied around the oesophageal and duodenal apertures of the stomach. The stomach was excised and rinsed with 900 µL MilliQ (equivalent to the volume of fluid dosed with the formulations in the oral bioavailability experiments) using a 1 mL syringe with a 25G (0.50 x 16 mm) needle. The rinsing fluid ('ex vivo stomach fluid') was collected into an eppendorf tube and pH was measured (PHM290 MeterLab, Radiometer Copenhagen, Denmark coupled to a Ag/AgCL reference combined pH electrode, Radiometer Analytics, France). Dispersion experiments were conducted using the low dilution dispersion protocol described above (section 2.2.3.).

2.5.2 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.[153] There is no gall bladder in a rat and the bile duct therefore also serves as the main duct for the transfer of pancreatic secretions to the GI tract.

Male SD rats (300-400 g) were fasted overnight and anaesthetised by subcutaneous injection of 1.0 mL/kg of 'Cocktail I' (100 mg/mL ketamine (Parnell Labs, NSW, Australia), 100 mg/mL xylazine (Iliul xylazine-100, Troy Labs, NSW, Australia), 10 mg/mL acepromazine (A.C.P 10, Ceva Delvet, NSW, Australia). Anaesthesia was maintained throughout study with subcutaneous doses of 0.15 mL of 'Cocktail II' (100 mg/mL ketamine, 10 mg/mL acepromazine) when required.

The body temperature of the anesthetized rats was maintained at 37°C by placing them on a heated pad (Ratek Instruments, Subiaco, Australia). At the conclusion of the experiment, rats were euthanized via a lethal IP dose of 1 mL sodium pentobarbitone (100 mg/mL).

A midline incision was made in the abdomen 1-2 cm below the line of the ribcage, and the bile duct was located near the pyloric sphincter surrounded by pancreatic tissue. A ligature was tied around the bile duct where it enters the duodenum and catheterisation of the duct facilitated through a cut made near the duodenal end of the duct in the pancreatic region to allow exocrine pancreatic secretions. The common bile duct was cannulated with polyethylene tubing (0.5 x 0.8 mm (i.d. x o.d.); Dural Plastics, Huntingdale, Australia) and secured with a drop of superglue. Bile flow was obstructed with suture ligation, a site of cannulation made by piercing with a 25G needle, and the duct cannulated with a 30 cm piece of polyethylene tubing (0.61 x 0.28 mm, o.d. x i.d.) and secured with surgical suture. Bile and pancreatic secreted fluids were continuously collected for a 2 h period (achieving approximately 1.5 ml/h) and used immediately after collection. To prevent dehydration of the animal, the right jugular vein was cannulated with a 30 cm piece of polyethylene tubing (0.80 x 0.50 mm, o.d. x i.d.) to allow for intravenous (IV)

administration (1.5 mL/h, Harvard pump 11 plus, Harvard Apparatus, MA, USA) of saline during the collection period.

In vivo experiments

2.6 BIOAVAILABILITY STUDIES IN BEAGLE DOGS

This thesis contains data from four different in vivo beagle studies carried out in two beagle dog cohorts. The older cohort (n = 3) had a mean age of 8 ± 1.0 years (depending on study number) and weighed approximately 18-23 kg. The younger cohort (n = 4) was on average 9 months in study 2 and 15 months in study 4 with an average weight of 11-16 kg.

2.6.1 Study design for in vivo bioavailability studies in beagle dogs

Studies were conducted as four-way cross-over studies in male beagle dogs where each animal received four different treatments (see Table 2.3) with a minimum of a 7 day washout period between doses. Treatments were based on formulation types outlined in Table 2.1 and details of the individual studies are shown in Table 2.3.

2.6.2 Administration and Sampling

Treatments were prepared 16 h prior to dosing and drug load, formulation stability and dispersability properties of each batch were assessed prior to in vivo studies. Treatments were hand filled into air filled soft gelatine capsules (size 22 oblong) using a 1 mL syringe and a 23G needle. The capsules contained oil residues from production and were therefore rinsed with blank (i.e. drug free) formulation before filling of active formulation. Subsequently, the perforation point in the capsule was sealed with a hot spatula by melting the gelatine.

Table 2.3 Summary of in vivo experimental design matrix for beagle dog studies

Study	I [ab]	II [b]	III [b]	IV [a]
Beagle cohort	Old	Young	Old	Young
Formulation [c]	F60	F60	F60	F0
Treatment: [d]				
1	40% DA [e]	15% DA	15% DA	40% DA
2	40% DA + HPMC [f]	30% DA	30% DA	40% DA + HPMC
3	80% DA	60% DA	60% DA	-
4	80% DA + HPMC	90% DA	90% DA	-

[a] Data are reported in Chapter 3
[b] Data are reported in Chapter 4
[c] Formulation compositions are outlined in Table 2.1
[d] Treatments administered in two gelatine capsules containing 800 mg formulation each
[e] Refers to a danazol incorporation at 40% of the saturated solubility in the formulation (Table 1)
[f] Hydroxypropyl methylcellulose (USP grade E4M) was added at a concentration of 5% (w/w)

The dogs were fasted for at least 20 h prior to dosing. After dosing, animals remained fasted until 10 h post-dosing, after which they were fed on a daily basis. Water was available ad libitum during the study. Each treatment was administered in two soft gelatine capsules (2 x 800 mg) with approximately 50 mL water using a 50 mL syringe after the capsule was placed in the back of the mouth (pharynx) of the animal.

Blood samples of approximately 3 ml were collected before dosing and at 0, 15, 30, 45, 60, and 90 min, then at 2, 3, 4, 6, 8 and 10 h post-dosing. Samples were collected via an indwelling catheter inserted in the cephalic vein prior to dosing and 2 mL of saline with heparin (1 IU/mL) was injected after each blood sample to prevent clotting in the catheter. At 24, 32 and 48 h post-dose additional samples were obtained by individual venipuncture. Blood samples were collected into 4 mL tubes containing 5.4 mg dipotassium EDTA dissolved in MilliQ (pH 7.4). Plasma was separated within 2 h by centrifugation (1,598 x *g*, 10 min) in an 80-2 centrifuge (Shanghai Surgical

Instruments Factory, Shanghai, China) and stored at -80°C until samples could be analysed. Danazol plasma concentrations were quantified by LC-MS as described in section 2.9.1.

2.6.3 Pharmacokinetic Data analysis in beagle studies

The maximum plasma concentrations (C_{max}) and the time for their occurrence (T_{max}) were noted directly from the individual plasma concentration vs. time profiles. The area under the plasma concentration vs. time profiles (AUC_{0-10}) was calculated using the linear trapezoidal method. Danazol plasma concentrations were typically below the limit of quantification of the assay at 24, 32 and 48 h post-dose and accurate determination of the terminal elimination rate constant and ($AUC_{0-\infty}$) was not possible. Since the danazol plasma concentrations at 10 h were low, the extrapolated AUC ($AUC_{10-\infty}$) was expected to contribute only a minor proportion of the total AUC ($AUC_{0-\infty}$). Relative bioavailability comparisons were therefore performed using (AUC_{0_10}). 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.

2.7 COLLECTION OF SERUM BILE ACID SAMPLES IN BEAGLE DOGS

To assess the liver function of beagle dogs, serum bile acid levels were quantified pre- and post-prandially via a standard liver function test. Animals were fasted for at least 20 h with free access to drinking water prior to the test. Samples were collected via an indwelling catheter inserted in the cephalic vein and blood samples of approximately 2 ml were collected pre-prandially (just prior to feeding) and post-prandially (2 h after being fed a small meal (~ 85 g) of standard canned dog food. Blood samples were collected into 3 mL vacutainer tubes (no clotting agent) and serum separated by centrifugation (1,598 x *g*, 10 min, 80-2 centrifuge, Shanghai Surgical Instruments

Factory, Shanghai, China). Samples were analysed using an enzymatic, colorimetric test for total serum bile acids (Randox Laboratories Limited, Crumlin, UK).

2.8 BIOAVAILABILITY STUDIES IN RATS

Experiments were conducted as a series of one-way parallel studies in four male Sprague Dawley rats (250-320 g) per group. Danazol bioavailability was explored following oral administration in the presence and absence of HPMC (5% w/w) and also after intraduodenal (ID) administration to assess the impact of gastric processing on subsequent formulation performance. To allow calculation of absolute bioavailability, danazol was also administered intravenously (IV).

In some groups, a non-specific cytochrome P450 inhibitor (1-aminobenzotriazole, ABT) was administered orally to inhibit hepatic and intestinal CYP enzymes.

A total of 15 experimental groups were evaluated (9 oral, 4 intraduodenal and 2 intravenous) and a summary of in vivo experimental design matrix can be found in Table 2.4.

Table 2.4 Summary of in vivo experimental design matrix for studies in rats (n = 4 per study group)

Formulation [a]	IV (± ABT) [c]	Oral [b]	ID	Oral (+ ABT) [c]	ID (+ ABT) [c]
15% HP-β-CD solution	x	-	-	-	-
F60-40% ($SEDDS_L$-III)	-	x	x	x	x
F60-80% ($SEDDS_H$-III)	-	x	x	x	-
F0-40% (SEDDS-IV)	-	x	x	x	-

[a] Composition of individual formulations is described in section 2.1 and Table 2.1 (the nomenclature in the brackets refers to the formulation name used in Chapter 5)

[b] Studies were also conducted in the presence of 5 % (w/w) HPMC dispersed in the formulations

[c] Pre-treatment with oral dosing of 100 mg/kg ABT to inhibit CYP450 enzymes and first pass metabolism

2.8.1 Surgical and experimental procedures

Rats were anaesthetised using isoflurane inhalation (2.5% v/v) throughout the duration of the surgical procedures. The body temperature of the anesthetized rats was maintained at 37°C by placing them on a heated pad (Ratek Instruments, Subiaco, Australia). Surgical procedures were adapted from previously published methods.[154]

2.8.1.1 Cannulation of carotid artery for blood sampling

All animals had cannulas inserted into the carotid artery to facilitate blood sampling. Prior to incision, subcutaneous application of a local anaesthetic (bupivacaine 0.5%) at the site of incision was utilised to provide analgesia at the incision site. A 1-1.5 cm vertical incision was placed to the right of the central neck muscle revealing the carotid artery approximately 1 cm below the skin surface. The artery was freed from surrounding tissue and the vagus nerve, a suture was placed at the top and bottom of the isolated section of artery, and the top suture was tied off. The sutures were clamped to reduce blood pressure in the isolated section and a small incision made in the artery. A cannula (0.96 x 0.58 mm (o.d. x i.d.) polyethylene tubing) was inserted into the carotid artery and advanced 2.5 cm. The cannula was secured by suturing into place.

To maintain the patency of the cannula, the cannula was tunnelled subcutaneously to exit at the back of the neck of the animals. A midline incision (1-1.5 cm) was placed on the dorsal side of the shoulder blades, the skin was separated from the underlying tissue and the cannula was tunnelled over the left shoulder to exit at the neck. The incision was afterwards closed with sterile sutures.

2.8.1.2 Cannulation of jugular vein for intravenous (IV) dosing

In some groups, cannulas were also inserted into the jugular vein for IV administration. Using the same incision but prior to cannulation of the carotid artery, the jugular vein was localised close to

the skin surface. The vein was freed from surrounding tissue and the cannula (0.96 x 0.58 mm (o.d. x i.d.) polyethylene tubing) inserted in a similar manner to the cannulation of the carotid artery described above and subcutaneously tunnelled to exit at the back of the neck of the animal.

2.8.1.3 Cannulation of the intestine to allow for intraduodenal (ID) dosing

To circumvent formulation processing in the stomach, formulations were also infused directly into the duodenum. This required the placement of a cannula in the duodenum. To facilitate cannulation, a 4 cm incision was made in the abdomen, 1-2 cm below the ribcage. The duodenum was isolated (~2 cm below the pyloric sphincter) and a small puncture made with a 21 G needle. A small section (1.5 cm) at the end of a 30 cm polyethylene cannula (1.7 x 1.2 mm, o.d. x i.d.) was bent under heating to form a 'U' shaped hook. This was inserted into the duodenum and glued into place with superglue. The duodenum was then placed back into the abdominal cavity. The cannula was subcutaneously tunnelled to exit at the back of the neck of the animal as described above, the abdomen muscle layer closed with sterile sutures and the skin tissue sealed with superglue.

After surgery, animals were connected to a swivel/tether apparatus to avoid cannula fouling and placed into a wire bottomed metabolic cages. Animals were checked regularly until recovery from the anaesthetic, after which rats were allowed to recover for at least 12 h prior to dosing.

2.8.2 Formulation administration and sample collection

Rats were fasted for 12 h prior to dosing with free access to drinking water. Animals continued to be fasted until after the last blood sample was taken on the study day. SEDDS formulations (prepared as described in section 2.1.1) were pre-dispersed in water (100 mg formulation + 400 mg MilliQ water) immediately prior to oral or intraduodenal administration.

2.8.2.1 Intraduodenal (ID) administration

The pre-dispersed formulations were administered into the duodenum via an intraduodenal cannula at a constant infusion rate (1 mL/h) (Harvard apparatus pump 11 plus, Harvard Apparatus, MA, USA) followed by 0.5 mL of MilliQ for flushing of the cannula. Blood samples were taken at pre-dose, 15, 25, 35, 45, 60, 90 min and 2, 3, and 5 h after ID dosing.

2.8.2.2 Oral administration

Rats were lightly anaesthetised via inhalation of isoflurane (5% v/v), and the pre-dispersed SEDDS formulations were dosed via oral gavage followed by 0.5 mL MilliQ water. Blood samples were taken at pre-dose, 15, 30, 45, 60, 90 min and 2, 3, 4 and 5 h after oral dosing.

2.8.2.3 Intravenous (IV) administration

The intravenous formulation (0.5 mL of a danazol in 15% HP-β-CD solution prepared as described in section 2.1.2) was administered by infusion pump (1 mL/h)(Harvard apparatus pump 11 plus, Harvard Apparatus, MA, USA) via an indwelling jugular vein cannula. The cannula was subsequently flushed with 2 IU/mL sodium heparin saline. Blood samples (300 µL) were taken at pre-dose, 1, 5, 15, 30, 60, 120, 180, 240, 360 and 480 min after IV dosing.

2.8.2.4 Pre-treatment with ABT

In order to inhibit CYP450 metabolism, rats were pre-treated with 1-aminobenzotriazole (ABT). A similar dosing protocol has been shown previously to provide almost complete inhibition (93%) of antipyrine clearance.[155] Rats were lightly anaesthetised via inhalation of isoflurane (5% v/v), and a single bolus dose (100 mg/kg) of ABT in 0.9 % saline (Baxter, Australia) was administered via oral gavage post-surgery approximately 14 h prior to IV, oral or intraduodenal dosing of the formulations.[155-158]

2.8.2.5 Sample collection

Aliquots (300 µL) were taken at various time points depending on administration route. Saline with heparin (1 IU/mL) was injected after each blood sample to prevent clotting in the cannula. After collection of the final blood sample, rats were euthanised via a lethal IP dose of phenobarbitone (100 mg/kg).

Blood samples were collected into 1.5 mL eppendorf tubes containing 10 µL heparin (2 IU/mL). Samples were centrifuged (5 min at 6700 x *g*, Eppendorf minispin plus, Eppendorf AG, Hamburg, Germany) to separate plasma within 2 h of the time of collection and plasma was stored individually in eppendorf tubes at -80°C until analysis.

2.8.3 Pharmacokinetic data analysis in rat studies

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 area under the plasma concentration vs. time profiles ($AUC_{0\text{-}tz}$) was calculated using the linear trapezoidal method. In the absent of ABT treatment, plasma concentrations were low and the elimination phase difficult to quantify accurately. As such the terminal elimination rate constant obtained from the IV study was used to extrapolate the AUC from the last time point to infinity. In the presence of ABT, plasma concentrations were considerably higher and the extrapolated AUC was therefore based on individual plasma profiles. Clearance (Cl), volume of distribution (Vd_{β}) 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.

2.9 DEVELOPMENT AND VALIDATION OF AN LC-MS ASSAY FOR DANAZOL IN PLASMA

Several chromatographic methods have been developed for the quantification of danazol in biological matrixes such as high performance liquid chromatography (HPLC)[13,159], gas-chromatography (GC-MS)[160] and liquid chromatography-electrospray ionisation tandem mass spectrometry (LC-MS/MS)[161]. Although triple-quadrupole mass spectrometry is very sensitive and specific, an instrument was not available for the quantification of danazol in plasma at the time of the current project and the available HPLC-UV assay required large plasma sample volumes with an extensive plasma extraction procedure and a long (30 min) runtime. A single-quad LC-MS assay was therefore developed to simplify sample preparation and to provide higher sensitivity and a 2-fold faster run time than the previous HPLC-UV assay. The analysis of steroids by single-quad LC-MS is challenging due to their relatively low polarity and proton affinity[162] and no published chromatographic methods for danazol using LC-MS and ESI interface were apparent at the time of study initiation.

2.9.1 Quantification of danazol in beagle plasma

2.9.1.1 Preparation of standards

Standard solutions of danazol (DA) in acetonitrile were prepared containing 25, 62.5, 125, 250, 1250, 2500 and 6250 ng/ml DA. An internal standard solution (IS) of progesterone was prepared in acetonitrile at the concentration 1 mg/mL and diluted to 2000 ng/mL in ACN. All solutions were stored under refrigeration (< 5°C).

2.9.1.2 Preparation of spiked plasma standards for calibration curves

Calibration standards for danazol were prepared to provide extracted plasma concentrations in the range of 1-250 ng/ml danazol and IS (progesterone). Quality control (QC) samples were prepared in at concentrations 1, 10 and 100 ng/mL DA and were afterwards stored at -20°C until analysis.

2.9.1.3 Preparation of plasma samples and protein precipitation

Protein precipitation was carried out using the salting-out principle, where a decrease in solubility with increasing ionic strength of the solution (due to high salt concentrations, in this case ammonium sulphate) leads to protein precipitation.[163] Samples were prepared by spiking 250 µl aliquots of blank beagle plasma with 10 µl of a danazol standard solution and 10 µL of IS solution to each sample. The tubes were vortexed for 30 s, and 125 µl of aqueous ammonium sulphate (saturated at room temperature) added. After an additional 30 s of vortexing, 250 ml ACN was added. The tubes were vortexed again for 30 s, left to stand for 20 min at room temperature prior to centrifugation for 5 min at 20,800 x *g* (Eppendorf 5804 R centrifuge, Eppendorf AG, Hamburg, Germany). The supernatant (organic phase) was transferred into autosampler vials and 10 µl injected onto the LCMS.

2.9.1.4 Instrument and chromatography

LCMS was performed using a liquid chromatograph-mass spectrometer system (Shimadzu LCMS 2020, Kyoto, Japan) which included a LC-20AD binary pump, a SiL-20AC refrigerated autosampler, a mobile phase vacuum degassing unit (DGU-20A_3), and a temperature-controlled column compartment (CTO-20A), coupled to a single-quadrupole mass spectrometer (Shimadzu LCMS 2020) equipped with an electrospray ionization source. The autosampler was maintained at 4°C and the column at 40°C. A Phenomenex Gemini C6-phenyl column (50 × 2.0 mm, 3 µm) was used

for separation. Samples were eluted via gradient elution at a flow rate of 0.3 mL/min. The mobile phases consisted of a mixture of solvent A (95% v/v MilliQ water: 5% v/v MeOH) and solvent B (5% v/v MilliQ water: 95% v/v MeOH) both containing 1 mM ammonium formate and 0.1% formic acid. The initial percentage of solvent B was 60%. The proportion of solvent B was linearly increased to 100% over 6.5 min and was held at 100% for 7 min. After 14.25 min, the gradient was returned to 60% solvent B until the end of the 17 min run time to achieve re-equilibration. The MS conditions were as follows: drying gas flow, 20 L/min; nebulizing gas flow, 1.5 L/min; drying gas temperature, 200°C; interface voltage, 3.5 kV and detector voltage 1.0 kV. Selected-ion monitoring was accomplished at *m/z* 338.2 for danazol, and *m/z* 314.9 for the internal standard, progesterone. The chromatographic data were acquired and analysed using the LabSolution software package, 5.31.277 (Shimadzu, Kyoto, Japan).

2.9.1.5 Assay validation

For validation of the method, quality control samples containing 1, 10 and 100 ng/mL danazol were generated (n = 6) from a pool of drug-free beagle plasma. Calibration curves were in a concentration range of 1-250 ng/ml danazol and IS (progesterone). To determine recoveries, non-extracted samples were compared with extracted samples at 1, 10 and 100 ng/mL danazol.

2.9.1.6 Selectivity

Selectivity was assessed by comparing the chromatograms of six batches of blank plasma with the corresponding spiked plasma. Chromatograms of blank plasma are shown in Figure 2.4A and no interfering endogenous compound peaks were observed after injection of blank extracted plasma samples (n = 6). Chromatograms of spiked plasma samples containing danazol at 100 ng/mL and IS are showed in Figure 2.4B. The retention times of danazol and progesterone were 4.59 and 4.35 min, respectively.

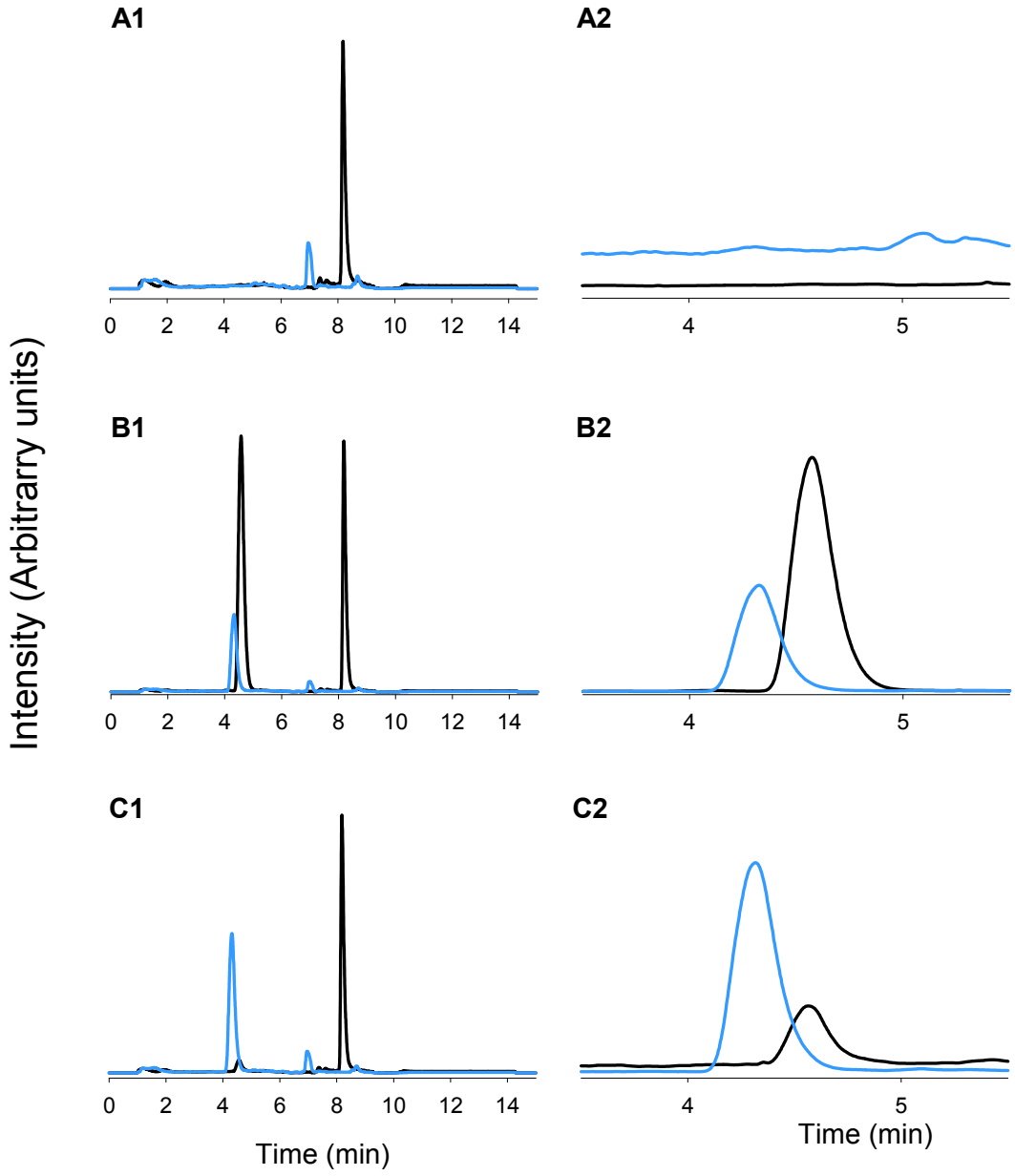

Figure 2.4 LC-MS chromatograms of danazol (black) and IS (blue) of A: blank beagle plasma, B: beagle plasma spiked with danazol standard (100 ng/mL DA + IS) and C: beagle plasma spiked with danazol standard (1 ng/mL DA + IS) illustrating LOQ. Panel 1 shows the total run time and panel 2 shows a section of the chromatogram illustrating the retention time of danazol and IS.

Unknown concentrations were determined by comparison of the unknown danazol:IS peak area ratio with a calibration curve of danazol:IS peak area ratio vs. danazol concentration constructed using the calibration standards. The danazol calibration curve and regression parameters were calculated by least-squares linear regression analysis using a weighting factor of 1/x. A typical calibration curve for danazol was $y = 0.0158x - 0.0028$ and $r^2 = 1$, where Y is the peak area ratio of danazol to IS and X the danazol concentration.

2.9.1.7 Extraction efficiency and matrix effect

Danazol was spiked into the supernatant after blank plasma extraction or to the blank plasma prior to extraction at concentrations 10 and 100 ng/mL in six replicates and the extraction efficiency of danazol was 98% and 94%, respectively.

The matrix effect of plasma was found to be approximately 15% by comparing the peak ratio of danazol concentrations 10 and 100 ng/mL and IS spiked into supernatant of blank plasma extraction and acetonitrile in six replicates.

Crossover interference between danazol and IS was evaluated by comparing each individual analyte in the presence of each other and no interferences were detected (Figure 2.5).

2.9.1.8 Precision and accuracy

The plasma assay was validated by analysis of n = 6 quality control samples containing 1, 10 and 100 ng/ml danazol in blank plasma. Intra-assay variability was accurate to 113.5, 103.8 and 111.8% and precise to ± 11.2, 9.2 and 4.4% of 1, 10 and 100 ng/ml, respectively and the inter-assay variability was accurate to 105.8, 96.1 and 100.0% and precise to 7.6, 9.5 and 4.7% of 1, 10 and 100 ng/ml, respectively.

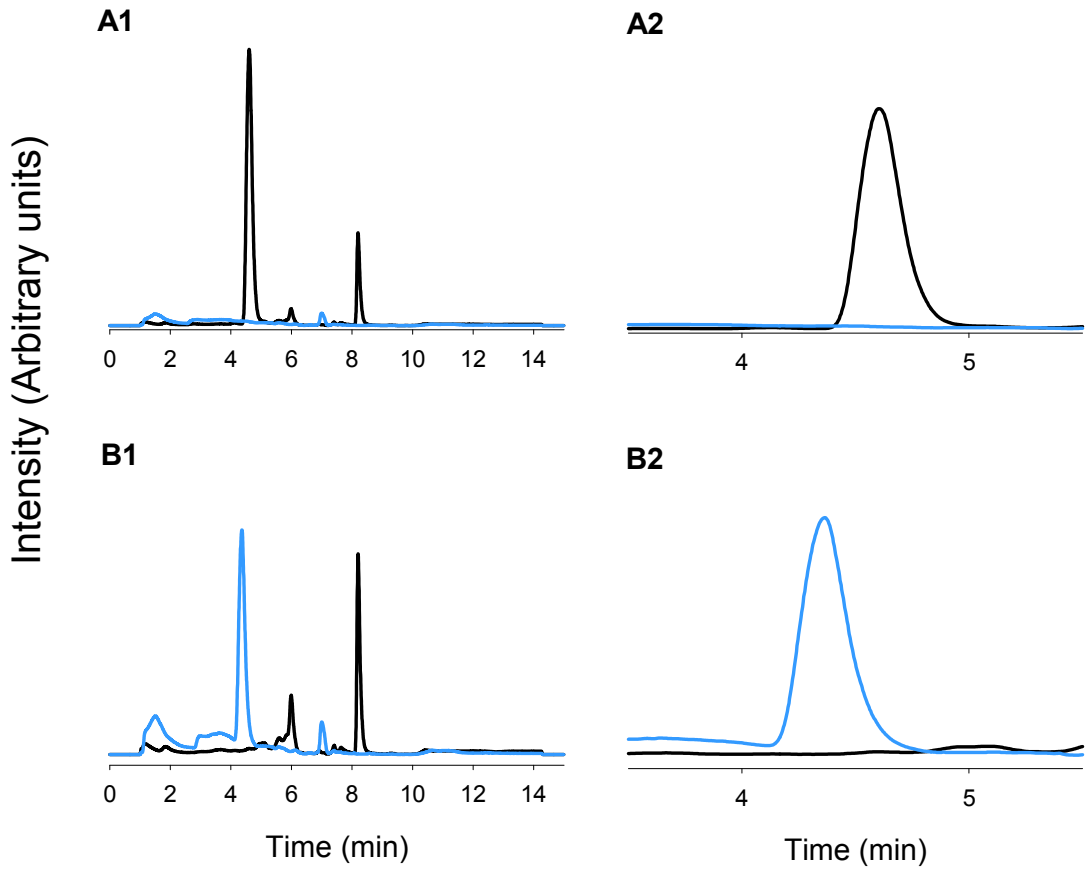

Figure 2.5 LC-MS chromatograms of danazol (black) and IS (blue) of A: beagle plasma spiked with DA and B: beagle plasma spiked with IS. Panel 1 shows the total run time and panel 2 shows a section of the chromatogram illustrating the retention time of danazol and IS.

2.9.2 Quantification of danazol in rat plasma

The LC-MS assay was also validated for rat plasma using a similar extraction approach but at lower quantities of plasma and danazol standard and IS as described below.

2.9.2.1 Preparation of calibration standards

Standard solutions of danazol (DA) and internal standard solution (IS) of progesterone was prepared as previously described in section 2.9.1.1.

2.9.2.2 Preparation of spiked plasma standards for calibration curves

Calibration standards for danazol were prepared by spiking 125 µl aliquots of blank rat plasma with 5 µl of an acetonitrile solution containing 25, 62.5, 125, 250, 1250, 2500 and 6250 ng/ml danazol. This provided spiked plasma concentrations in the range of 1-250 ng/ml danazol and 40 ng/mL IS (progesterone). Quality control (QC) samples were prepared at concentrations of 1, 10 and 100 ng/mL DA and were stored at -20°C until analysis.

2.9.2.3 Preparation of plasma samples and protein precipitation

Five microliters of an internal standard solution (2000 ng/ml of progesterone in acetonitrile) was added to each plasma sample or standard, the tubes were vortexed for 30 s, and 62.5 µl aqueous ammonium sulphate solutions (saturated at room temperature) added. After 30 s of vortexing 125 µl ACN was added. The tubes were vortexed again for 30 s and left to stand for 20 min at room temperature prior to centrifugation (Eppendorf 5804 R, 20,800 x *g*, 5 min, 20°C). The organic phase was transferred into autosampler vials and 10 µl injected onto the LC-MS.

Unknown concentrations were determined by comparison of the unknown danazol:IS peak area ratio with a calibration curve of danazol:IS peak area ratio vs. danazol concentration constructed using the calibration standards.

2.9.2.4 Instrument and chromatography

Danazol concentrations in rat plasma were performed using the LC-MS system described in section 2.9.1.4.

2.9.2.5 Assay validation

The assay was validated by replicate analyses of quality control samples (n = 6) containing 1, 10 and 100 ng/ml danazol in blank plasma. Intra-assay variability was accurate to 97.6, 117.5 and 118.1% and precise to 5.6, 9.8 and 2.6%. Inter-assay variability was accurate to 92.9, 104.8 and 113.9% and precise to 10.2, 7.1 and 6.0% for the quality control samples containing 1, 10 and 100 ng/ml, respectively. The lower limit of quantification for the plasma assay (1 ng/ml) was determined by replicate analyses (n = 6) of spiked plasma samples and defined as the lowest concentration at which appropriate accuracy and precision was obtained.

Monash University

Declaration for Thesis Chapter 3

Declaration by candidate

In the case of Chapter 3, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution
Planning and executing experimental work, data evaluation and drafting and revision of manuscripts	60%

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	Nature of contribution	Extent of contribution
C.J.H. Porter	Project supervisor, data and manuscript review	NA
C.W. Pouton	Project co-supervisor and manuscript review	NA
H.D. Williams	Manuscript review	NA
M. McIntosh	Supervision of analytical work with LC-MS	NA
H. Benameur	External supervision and manuscript review	NA
G.A. Edwards	Supervision and responsibility for large animal work	NA

Candidate's Signature		**Date:** 20/11/2012

Declaration by co-authors

The undersigned hereby certify that:

(1) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
(2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(4) there are no other authors of the publication according to these criteria;

(5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and

(6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)	**Monash Institute of Pharmaceutical Sciences, Parkville Campus**	
Signature 1	[redacted]	21/09/2012
Signature 2	[redacted]	11/10/2012
Signature 3	[redacted]	11/10/2012
Signature 4	[redacted]	21/11/2012
Signature 5	[redacted]	19/09/2012
Signature 6	[redacted]	13/09/2012

CHAPTER 3

LIPID DIGESTION AS A TRIGGER FOR SUPERSATURATION: EVALUATION OF THE IMPACT OF SUPERSATURATION STABILISATION ON THE IN VITRO AND IN VIVO PERFORMANCE OF SELF-EMULSIFYING DRUG DELIVERY SYSTEMS

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3 Lipid digestion as a trigger for supersaturation: evaluation of the impact of supersaturation stabilisation on the in vitro and in vivo performance of self-emulsifying drug delivery systems

3.1 ABSTRACT

The generation of supersaturation in the gastrointestinal (GI) tract is an increasingly popular means of promoting oral absorption for poorly water-soluble drugs. The current study examined the impact of changes to the quantities of medium-chain (MC) lipid (Captex 300: Capmul MCM), surfactant (Cremophor EL) and cosolvent (EtOH), and the addition of polymeric precipitation inhibitors (PPI), on supersaturation during the dispersion and digestion of MC self-emulsifying drug delivery systems (SEDDS) containing danazol. The data suggest that digestion acts as a 'trigger' for enhanced supersaturation and that solubilisation/precipitation behaviour is correlated with the degree of supersaturation on dispersion (S^M_{DISP}) or digestion (S^M_{DIGEST}). The ability of the formulation to maintain solubilisation in vitro decreased as the S^M of the formulation increased. PPI significantly increased supersaturation stabilisation and precipitation was inhibited where $S^M_{DISP} < 3.5$ and $S^M_{DIGEST} < 4$. In the presence of polymer, some degree of supersaturation was maintained up to S^M_{DIGEST} ~8. Differentiation in the ability of SEDDS to maintain drug solubilisation stems from the ability to stabilise supersaturation stabilisation and for MC SEDDS, utilisation of lower drug loads, higher surfactant levels (balanced against increases in S^M_{DISP}), lower cosolvent and the addition of PPI enhanced formulation performance. In vivo studies confirmed the ability of PPI to promote drug exposure at moderate drug loads (40% of saturated solubility in the formulation). At higher drug loads (80% saturation) and in lipid-free SEDDS, this effect was lost, suggesting the ability of PPIs to stabilise supersaturation in vitro may, under some circumstances, overestimate utility in vivo.

3.2 INTRODUCTION

The oral absorption of poorly water-soluble drugs (PWSD) is frequently compromised by low gastrointestinal (GI) solubility and slow dissolution. Limited absorption dictates a reduction in oral bioavailability and may eventually limit effective drug development. Self-emulsifying drug delivery systems (SEDDS) are one of several approaches that have been explored to address these issues and improve the oral bioavailability of PWSD.[4]

In contrast to traditional solid dose forms, the drug dose in SEDDS is usually dissolved in the formulation. SEDDS therefore present drug to the GI tract in solution, albeit in non-aqueous solution, and circumvent the conventional dissolution processes that commonly limit the absorption of PWSD. The key to the utility of SEDDS is maintenance of the integrity of drug solubilisation as the formulation is processed in the GI tract and delivery of drug, in a solubilised reservoir, to the absorptive surface (enterocyte). There is little evidence to suggest the potential for direct absorption of the solubilised construct (for example, a micelle or an emulsion droplet), and the prevailing view is that drug absorption is facilitated by drug equilibration between the solubilised reservoir and drug in free intermicellar solution followed by absorption of drug from the intermicellar phase. Drug absorption subsequently shifts the equilibrium between solubilised and free drug, maintaining sink conditions and perpetuating absorption via ongoing release from the solubilised reservoir. The nature of the solubilised reservoir is a critical aspect of the utility of SEDDS and is a composite function of formulation derived materials such as lipids and surfactants (and their digestion products), and endogenous solubilizers such as bile salts and phospholipids that are secreted in bile in the form of mixed micelles.[64,89,92,99,114,164]

Historically, the utility of lipid- and surfactant-based formulations such as SEDDS has been thought to lie in their ability to supplement the solubilisation capacity of the GI fluids. Solubilisation enhancement occurs via the provision of additional surfactants and the generation of lipid

digestion products that swell the solubilisation capacity of bile salt-phospholipid micelles.[88,89,92,97,99,114,121,124] It is increasingly apparent, however, that this explanation is incomplete and that a more detailed understanding of the kinetic events that evolve on administration of SEDDS is required to better understand formulation performance.

Figure 3.1 provides a diagrammatic representation of possible changes to PWSD solubilisation in the GI fluids after oral administration of a conventional immediate release (IR) solid oral dose form and a SEDDS formulation. For the IR formulation, dissolution is usually slow and ultimately limited by the solubility of the drug in the GI fluids. In contrast, administration of drug in solution in the SEDDS dictates that initial dispersion of the formulation results in the attainment of solubilised drug concentrations that are orders of magnitude higher than the equilibrium solubility of drug in the GI fluids alone (upper dotted line [C] in Figure 3.1). However, SEDDS usually contain digestible lipids[67,92,164,165] and surfactants[115], and the nature of these components is expected to change significantly as digestion occurs.[115] Indeed, in many cases, the solubilisation capacity of SEDDS formulations reduces on digestion leading to drug precipitation.[14,16,65,96,166] In a classical scenario, digestion reduces the solubilisation capacity of the colloidal aqueous phase and drug precipitates in line with the reduction in solubilisation capacity. Ultimately, this results in a drop in solubilised drug concentrations to values approximating the equilibrium solubility of drug in the 'composite' GI fluids containing bile salt and phospholipid from bile and lipid and surfactant digestion products from the formulation ([A] in Figure 3.1). However, as we have reported previously[167] and describe in some detail here, certain formulations resist precipitation, even though digestion leads to a decrease in solubilisation capacity. As a result, a transiently supersaturated condition is created ([B] in Figure 3.1).

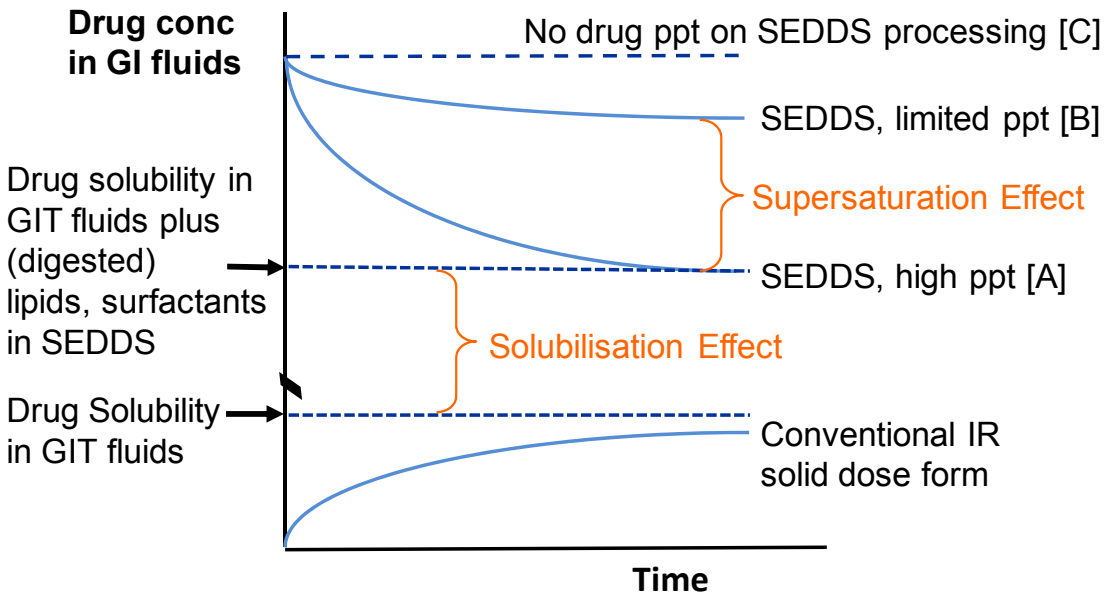


Figure 3.1 Schematic representation of potential changes to solubilised drug concentration in GI fluids after oral administration of a conventional immediate release (IR) solid oral dose and a SEDDS formulation. Dissolution of a typical solid dose form results in increasing concentrations of drug in the GI fluids that are ultimately limited by drug solubility. In contrast, SEDDS formulations do not undergo traditional dissolution and instead initially disperse to provide high solubilised drug concentrations where drug is present molecularly dispersed in the formulation [C]. However digestion of formulation components typically leads to a reduction in solubilisation capacity and drug precipitation (ppt) [A]. In some cases, precipitation to [A] is not immediate resulting in transient supersaturation [B] (the supersaturation effect). Even in the absence of supersaturation, the presence of lipid and surfactant digestion products results in increases in solubilised drug concentrations [A] when compared to drug solubility in the GI fluids alone (the solubilisation effect).

The overarching goal of the recent work conducted in our laboratories has been to explore the formulation dependence of supersaturation generation during the dispersion and digestion of lipid-based SEDDS formulations, and the studies reported here are the first to explore these trends in detail. In particular, we describe the impact of changes to the relative quantities of lipid, surfactant and cosolvent contained within a SEDDS formulation on supersaturation using a

formulation comprising medium-chain triglyceride and a blend of medium-chain mono-, di-, and tri-glycerides as a lipid source, Cremophor EL (CrEL) as a surfactant and ethanol as a cosolvent. In addition to the inherent potential of conventional SEDDS components such as lipids, surfactants and cosolvents to promote supersaturation on formulation dispersion or digestion, several authors have also shown that supersaturation, in both lipidic[24-27] and non-lipidic[17,18,20,73,168-170] formulations, can be stabilised by polymers. The additional effect of polymers on supersaturation in lipid-based formulations has therefore also been explored. The data suggest that much of the differentiation between solubilisation properties for SEDDS stems from differences in the ability to stabilise supersaturation and that although polymers can be very effective in stabilising supersaturation in vitro, these effects are not always apparent in vivo.

3.3 MATERIALS AND METHODS

3.3.1 Materials

Danazol (pregna-2,4-dien-20-yno[2,3-d]isoxazol-17-ol) was kindly supplied by Sterling Pharmaceuticals (Sydney, Australia) and progesterone was from Sigma-Aldrich (St Louis, MO, USA). Captex 300, a medium-chain triglyceride (MCT), and Capmul MCM, a blend of medium-chain mono-, di-, and tri-glycerides, were donated by Abitec Corporation (Janesville, WI, USA). Soybean oil (predominantly C18 triglycerides), Cremophor EL (polyoxyl 35 castor oil), sodium taurodeoxycholate 97% (NaTDC) and porcine pancreatin (8 x USP specification activity) 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), 4-bromophenylboronic acid (4-BPB) was obtained from Sigma Aldrich (St Louis, MO, USA) and 1 M sodium hydroxide, which was diluted to obtain 0.6 M NaOH titration solution, was purchased from Merck (Darmstadt, Germany). Water was obtained from a Milli-Q (Millipore, Bedford, MA,

USA) purification system. All other chemicals and solvents were of analytical purity or high performance liquid chromatography (HPLC) grade.

The following polymers were obtained from Sigma Aldrich Pty Ltd., Australia: Hydroxypropyl methylcellulose (HPMC) E4M, polyvinylpyrrolidone (PVP) 10 and PVP 360. Hercules Chemical Company Inc., c/o APS Healthcare, Nuplex Industries Pty Ltd Australia supplied hydroxyethylcellulose (HEC) 250GF, hydroxypropyl cellulose (HPC) HXF, methylcellulose (MC) A4C and HPMC E5. Eudragit E100 and L100 were supplied by Evonik Degussa Australia Pty Ltd, Aerosil 200 was supplied by Degussa AG, Frankfurt am Main and HPMC phthalate (HPMC-P) 55S, HPMC acetate succinate (HPMC-AS) LF and HF were supplied by ShinEtsu Chemical Co. Ltd.

3.3.2 Preparation of SEDDS formulations containing danazol

All formulations were prepared as previously described[13] and danazol, a synthetic steroid originally developed to treat endometriosis, was utilised as a model poorly water-soluble drug (aqueous solubility 0.59 ug/ml[144], log *P* = 4.53[143]). Danazol was incorporated into formulations at 40% or 80% of saturated solubility in the formulation (based on measured values at equilibrium at 37°C). Drug solubility in each of the formulations was assessed using standard methodologies and all experiments were performed in triplicate.[15] Equilibrium solubility was defined as the value attained when at least three consecutive solubility samples varied by ≤ 5%. This was typically reached after equilibration times of between 48 and 72 h.

Formulations were constructed to contain i) varying ratios of medium-chain (MC) lipid to surfactant at a constant cosolvent level (10% v/v) and ii) varying ratios of cosolvent to MC lipid at approximately constant surfactant levels (64-65% v/v). A ternary plot of the composition of the individual formulations is shown in Figure 3.2 and numeric values are given in Table 3.1.

Formulations containing polymeric precipitation inhibitors (PPI) were assembled using the formulations presented in Table 3.1 and subsequent addition of the PPIs at the stated concentrations (% w/w)**.** The formulations were vortexed for 30 sec and equilibrated overnight at 37°C. The following polymers were utilised: Aerosil 200, Eudragit E100, Eudragit L100, hydroxypropylmethyl cellulose (HPMC) (E4M), HPMC (E5), HPMC-AS (LF), HPMC-AS (HF), methylcellulose (MC) (A4C), hydroxypropylcellulose (HPC), hydroxyethylcellulose (HEC), HPMC-P (55S) and polyvinylpyrrolidone (PVP) 10 or PVP360.

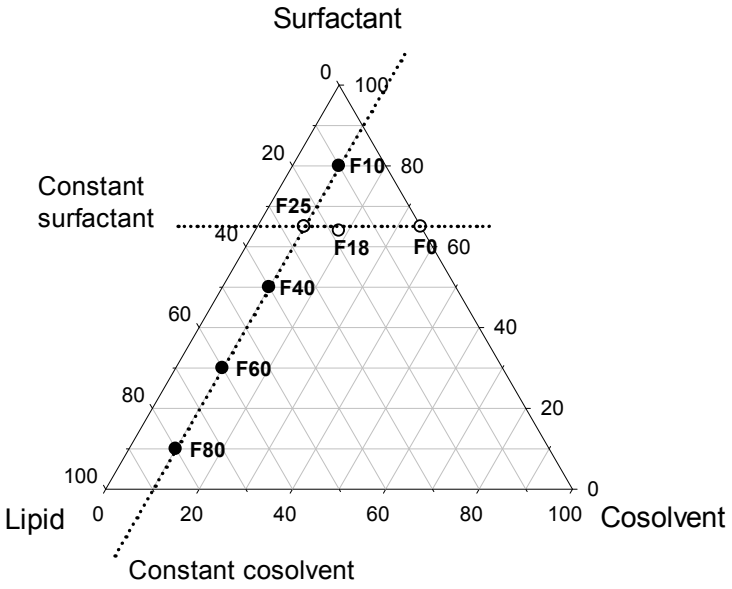


Figure 3.2 Ternary plot of formulation composition at approximately constant surfactant (64-65%) or constant cosolvent (10%) for formulations comprising medium-chain lipid (Captex 300: Capmul MCM 50:50), surfactant (Cremophor EL) and cosolvent (ethanol). Exact compositions are shown in Table 3.1.

Table 3.1 Composition (% w/w) of SEDDS and danazol solubility data in the formulation and in the aqueous phase (AP) formed during in vitro digestion

Formulation components	**F0** [a]	**F10**	**F18**	**F25**	**F40**	**F60** [a]	**F80**
Captex 300: Capmul MCM (1:1) (% w/w)	0	10	18	25	40	60	80
Cremophor EL (% w/w)	65	80	64	65	50	30	10
Ethanol (absolute) (% w/w)	35	10	18	10	10	10	10
LCFS classification type [b]	**IV**	**IV/IIIB**	**IV/IIIB**	**IIIB**	**IIIA**	**IIIA**	**IIIA**
Danazol solubility in formulation [mg/g]	45 ± 1.3	36 ± 0.6	39 ± 0.8	35 ± 2.3	32 ± 0.7	30 ± 0.7	25 ± 1.0
AP_{max} (@ 80% sat) [ug/mL] [c]	994	800	860	784	718	674	544
AP_{max} (@ 40% sat) [ug/mL] [c]	497	400	430	392	359	337	272
Apparent equilibrium solubility [ug/mL]:							
AP_{DISP} [d]	178 ± 6.8	259 ± 5.1	243 ± 2.5	282 ± 5.5	306 ± 3.7	301 ± 3.9	269 ± 15.9
AP_{DIGEST} (5 min) [e]	134 ± 5.0	155 ± 2.5	126 ± 1.0	132 ± 0.3	118 ± 2.9	106 ± 3.6	88 ± 0.6
AP_{DIGEST} (60 min) [e]	89 ± 2.8	102 ± 0.9	81 ± 0.8	91 ± 2.7	71 ± 3.2	56 ± 0.9	46 ± 1.0
AP_{DIGEST} + HPMC (60 min) [e]	82 ± 0.3	98 ± 3.3	80 ± 1.7	81 ± 2.5	67 ± 2.6	59 ± 0.3	38 ± 1.1

Captex 300 (MC triglycerides); **Capmul MCM** (MC monoglyceride/ diglyceride); **CrEL** (surfactant); **Ethanol** (cosolvent); **HPMC** (hydroxypropyl methylcellulose);

[a] Formulations re-named in Chapter 5 to SEDDS-IV (F0) and SEDDS-III (F60)

[b] Classification scheme taken from Pouton[71,94]

[c] Data represent 80% or 40% of the saturated solubility of danazol in the formulation

[d] Data represent equilibrium solubility of danazol in the AP produced by dispersion of blank (drug-free) formulation for 10 min

[e] Data represent equilibrium solubility of danazol in the AP produced by dispersion and digestion of blank (drug-free) formulation for either 5 or 60 min. Data at 60 min also generated for formulations containing HPMC

3.4 IN VITRO EVALUATION

3.4.1 Drug solubilisation during formulation dispersion and digestion

In vitro experiments were conducted using a modification of a previously described lipid digestion model, although in this case a dispersion step was added prior to initiation of digestion to allow for evaluation of the potential for drug precipitation on formulation dispersion.[13,109] To simplify evaluation, formulation dispersion properties were assessed under intestinal conditions. This allowed distinction between the contribution of dispersion versus digestion events as a driver of supersaturation or precipitation, but does not provide an indication of the potential impact of dispersion under gastric (acidic) conditions. Previous studies, however, suggest relatively moderate differences between formulation dispersion properties under intestinal or gastric conditions, especially for unionised drugs.[13] The impact of pH on the dispersion properties of danazol will be examined in more detail in subsequent publications.

Briefly, 1 g of lipid-based formulation was dispersed in 36 g of digestion buffer (50 mM TRIS maleate, 150 mM NaCl, 5 mM $CaCl_2 \cdot 2H_2O$, pH 7.5) containing 5 mM NaTDC and 1.25 mM phosphatidycholine (PC) to reflect typical fasted state intestinal conditions.[145-151] Experiments were performed at 37°C in a glass vessel, the temperature was controlled by a thermostatically controlled water jacket and the fluids contained in the vessel (i.e. digestion buffer plus formulation) were stirred magnetically. The first experimental period (0-30 min) comprised the dispersion stage of the study, where the formulation was simply dispersed in the digestion medium by stirring. After 30 min, digestion was initiated by addition of 4 mL of pancreatin extract containing 40,000 TBU (to provide approximately 10,000 TBU per mL of extract and approximately 1000 TBU per mL of digest) of pancreatic lipase. Digestion was followed for 60 min using a pH-stat titration unit (Radiometer, Copenhagen, Denmark) that maintained the pH at 7.5 via titration of

liberated fatty acids with 0.6 M NaOH. Aliquots (4.2 mL) were taken from the dispersion/digestion media throughout the 90 min experimental period at t = 10, 30, 40, 50, 60 and 90 min. Lipid digestion inhibitor (4-BPB, 9 µL of a 0.5 M solution in methanol per mL of dispersion/digestion medium) was added to each sample immediately after sampling to prevent further lipolysis.[89,171] Dispersion samples were centrifuged for 10 min at 21,100 x *g* (Heraeus Fresco 21 microcentrifuge, Thermo Scientific, Osterode, Germany) in order to pellet any drug that precipitated on dispersion. Digestion samples were centrifuged more thoroughly via ultracentrifugation for 30 min at 37°C and 400,000 x *g* (Optima xL-100 K centrifuge, SW-60 rotor, Beckman, Palo Alto, CA, USA) to separate the digestion sample into an aqueous phase and a pellet phase. Since all formulations dispersed to form microemulsified dispersions, an undispersed 'oil' phase was not evident on digestion. Samples obtained from each phase were assayed for danazol content by HPLC.

3.4.2 X-ray powder diffraction (XRPD)

The pellet phase obtained after digestion and ultracentrifugation was examined by x-ray powder diffraction to investigate the influence of HPMC on the physical form of precipitated danazol. Samples of HPMC and crystalline danazol were measured as controls using a quartz sample plate. The pellet phase obtained post digestion of the F60 formulation (+/- 5% (w/w) HPMC E4M) was placed on a zero background silicone holder. XRPD was performed using a Philips 1140 vertical diffractometer (Philips, Holland) for scanning from 2° to 60°, with an angular increment of 0.02° and a speed of 2°/min. A copper (Cu) tube anode and Kα radiation (λ 1.54184 Å) was used. The voltage and current applied was 45 kV and 40 mA. The data were acquired and analysed using the Visual XRD and traces software package, version 6.6.1 (GBD Scientific Equipment).

3.4.3 Solubility in aqueous phase pre-digestion (dispersion phase) (AP_{DISP})

The solubility of danazol in dispersed emulsified blank (i.e. drug-free) formulation was evaluated. Triplicate samples of the AP were taken after dispersion of drug-free (blank) formulations for 10 min and excess danazol added and incubated at 37°C. Solubility samples were subsequently taken over 6 h and centrifuged for 30 min at 21,100 x *g*, 37°C. The equilibrium solubility of danazol in the dispersed aqueous colloidal phase was defined as the mean solubility value obtained after 2, 4 and 6 h incubation.

3.4.4 Solubility in aqueous phase post digestion (AP_{DIGEST})

The solubility of danazol in the aqueous colloidal phase generated by digestion of blank (i.e. drug-free) formulation was evaluated after 5 and 60 min digestion. Conditions for digestion of the blank formulation were as described above and digestion samples were ultracentrifuged and the aqueous phase separated prior to measurement of drug solubility. Triplicate samples comprising danazol in excess were incubated at 37°C and centrifuged for 30 min at 21,100 x *g*, 37°C. As the solubility was found to decrease up to 20% over 24 h for some of the formulations, presumably due to instability of the colloids present, the apparent equilibrium solubility in the aqueous colloidal phase post digestion was defined as the mean solubility value obtained after 2, 4 and 6 h incubation following attainment of the maximum solubility.

3.4.5 Quantification of danazol in in vitro experiments by HPLC

Aqueous phase samples obtained during dispersion experiments and after initiation of digestion were diluted 1:10 (v/v) with acetonitrile before HPLC analysis. Samples of danazol in pellets were first dissolved in 5 mL of chloroform/methanol (2:1 v/v) and subsequently diluted 1:10 (v/v) in acetonitrile prior to analysis by HPLC.

Danazol chromatography was conducted using a Waters 2690 separation Module and a Waters 486 tunable absorbance detector (Waters, MA, USA). A Waters symmetry C18 column (3.9 x 150 mm) (Waters, MA, USA) was maintained at 25°C. The mobile phase consisted of 75% (v/v) methanol and 25% (v/v) Milli-Q water. The flow rate was 1 mL/min. All samples were maintained at 10°C in the autosampler prior to injection of 50 µL. Detection of danazol was conducted by single wavelength monitoring at 286 nm.

The assay was validated by analysis of n = 5 replicate standards made up at three different concentrations (0.5, 5, 50 µg/mL). Intra-assay variability was accurate to 96.6, 101.1 and 99.7% and precise to ± 0.5, 0.3 and 3.8% of 0.5, 5 and 50 µg/mL. Inter-assay variability was assessed over three separate days and was accurate to 100.5, 99.3 and 100.3% and precise to ± 2.9, 0.2 and 0.1% at 0.5, 5 and 50 µg/mL. The recovery of danazol spiked into aqueous and pellet phases obtained from blank digests of soybean oil was 96.4 ± 0.3% and 100.0 ± 0.8% (n = 6), respectively.

3.5 DATA ANALYSIS FOR IN VITRO EXPERIMENTS

3.5.1 Solubilisation profiles

The ability of excipients to maintain danazol in a metastable, supersaturated state during kinetic dispersion and digestion experiments was assessed using solubilisation/precipitation profiles. **Figure 3** shows a typical profile of a SEDDS during dispersion and digestion including the potential maximum concentration in the aqueous colloidal phase assuming no precipitation (AP_{MAX}) and the apparent equilibrium solubility in the aqueous colloidal phase post dispersion (AP_{DISP}) and digestion (AP_{DIGEST}). The maximum concentration that could be obtained in the aqueous colloidal phase was determined from the mass of drug contained in the 1 g of formulation added to the dispersion/digestion experiment, divided by the volume of the digest. Since the solubilised concentrations varied as a function of time, an indication of changes to solubilisation/

precipitation across the entire experiment were gained via the use of areas under the solubilisation curves (AUC).

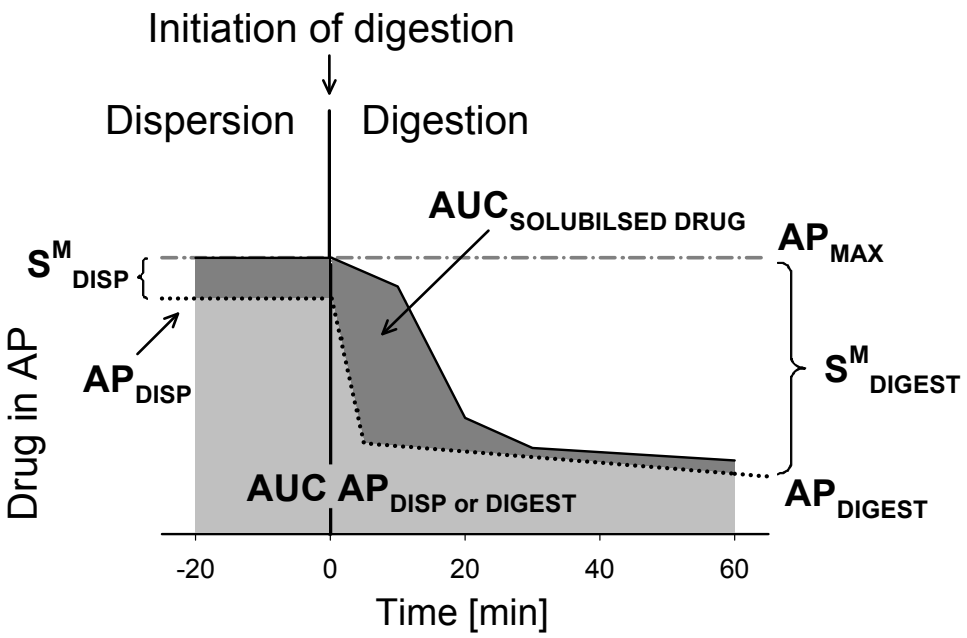


Figure 3.3 Supersaturation during dispersion and digestion of SEDDS formulations; The upper (dash) line indicates the potential maximum concentration in the aqueous colloidal phase in the absence of drug precipitation (AP_{MAX}). The lower dotted line indicates drug solubility in the colloidal phase produced by dispersion (AP_{DISP}) or digestion (AP_{DIGEST}) of drug-free SEDDS. S^{M}_{DISP} or S^{M}_{DIGEST} are the maximum supersaturation ratios for each formulation on dispersion or digestion, respectively (eq. 3.2).

The ability of the formulation to maintain supersaturation during dispersion and digestion was expressed as a supersaturation ratio, S (eq. 1). S was determined as the area under the solubilised drug concentration versus time profile divided by the AUC of the apparent equilibrium solubility of danazol in the AP during dispersion (AP_{DISP}) and digestion (AP_{DIGEST}). To allow representation of AP_{DIGEST} in the current plots, the change in solubility (during the course of digestion) was assumed to be linear between 5 min and 60 min post digestion (see Figure 3.1).

$$S = \frac{AUC_{Solubilised\ drug}}{AUC\ AP_{DISP\ or\ DIGEST}} \quad \text{(Equation 3.1)}$$

The ratio of the potential maximum drug levels in the aqueous colloidal phase in the absence of drug precipitation (AUC AP_{MAX}) and the equilibrium solubility in the AP obtained after dispersion/digestion of a drug-free formulation (AUC $AP_{DISP\ or\ DIGEST}$) provides a measure of the metastability of the dispersed or digested formulation; i.e. the potential maximum supersaturation ratio (S^M) (eq. 3.2) that the formulation may generate.

$$S^M = \frac{AUC\ AP_{max}}{AUC\ AP_{DISP\ or\ DIGEST}} \quad \text{(Equation 3.2)}$$

A measure of how effectively solubilisation was maintained was gained by comparing the AUC for the solubilised drug concentration as a function of time, divided by the area under the maximum drug concentration that may be attained in the absence of precipitation (eq. 3).

$$\%\ Solubilised = \frac{AUC_{Solubilised\ drug}}{AUC\ AP_{max}} \cdot 100\% \quad \text{(Equation 3.3)}$$

3.6 BIOAVAILABILITY STUDIES IN BEAGLE DOGS

3.6.1 Administration and Sampling

Based on the in vitro experiments, six formulations with varying drug load and polymer (HPMC E4M) concentration were evaluated *in vivo* to examine the impact of the addition of polymeric material and the potential role of drug saturation in the formulation on in vivo exposure.

All experiments were approved by the local animal ethics committee and were conducted in accordance with the Australian and New Zealand Council for the Care of Animals in Research and Teaching guidelines. Treatments were based on two formulation types, F0 and F60 (Table 3.1). F0, a lipid-free SEDDS contained danazol at nominally 40% of saturated solubility in the formulation. F60, a MC lipid-based SEDDS contained danazol at nominally 40% or 80% of saturated solubility in the formulation. All treatments were evaluated in the presence and absence of 5% (w/w) HPMC E4M.

The studies were conducted as a four-way crossover (7-days washout) in male beagle dogs (13-23 kg). Treatments were hand filled into gelatine capsules 16 h prior to dosing as previously described.[103] Each treatment was administered in two capsules (2 x 800 mg formulation) with approximately 50 mL water. The actual dose administered for F0 (nominally at 40% saturation in the formulation) was 26.3 mg (representing 37% saturation) in the absence of polymer and 23.6 mg (33% saturation) in the presence of polymer. For F60 (nominal 40%), the actual dose was 17.3 mg (36% saturation) and 17.2 mg (35% saturation) in the absence and presence of polymer. For F60 (nominal 80%), the actual dose was 34.2 mg (70%) and 34.1 mg (70%) in the absence and presence of polymer respectively. The dogs were fasted for at least 20 h prior to dosing. After dosing, animals remained fasted until 10 h post-dosing, after which they were fed on a daily basis. Water was available *ad libitum* during the study.

Blood samples of approximately 3 ml were collected before dosing and at 15, 30, 45, 60, and 90 min, then at 2, 3, 4, 6, 8 and 10 h post-dosing. Samples were collected via an indwelling catheter inserted in the cephalic vein prior to dosing and additional samples were obtained by individual venipuncture at 24, 32 and 48 h post-dose. Blood samples were collected into 4 mL tubes containing dipotassium EDTA. Plasma was separated within 2 h by centrifugation for 10 min at 1,328 x *g* (Eppendorf 5702 R/A-4-38 centrifuge, Eppendorf AG, Hamburg, Germany) and stored at -80°C until samples analysis.

3.6.2 Plasma sample preparation

Calibration standards for danazol were prepared by spiking 250 µl aliquots of blank beagle plasma with 10 µl of an acetonitrile (ACN) solution containing 25, 62.5, 125, 250, 1250, 2500 and 6250 ng/ml danazol. This provided spiked plasma concentrations in the range of 1-250 ng/ml danazol. Ten microliters of an internal standard (IS) solution (2000 ng/ml of progesterone in ACN) was also added to each plasma sample or standard, the tubes were vortexed for 30 s, and 125 µl aqueous ammonium sulphate solutions (saturated at room temperature) added. After an additional 30 s of vortexing, 250 µl ACN was added. The tubes were vortexed again for 30 s and left to equilibrate for 20 min at room temperature prior to centrifugation for 5 min at 20,800 x *g* (Eppendorf 5804 R centrifuge, Eppendorf AG, Hamburg, Germany). 100 µl of the organic phase was transferred into autosampler vials and 10 µl injected onto the LC-MS. Unknown concentrations were determined by comparison of the unknown danazol: IS peak area ratio with a calibration curve of danazol: IS peak area ratio vs. danazol concentration constructed using the calibration standards.

3.6.3 Quantification of danazol in plasma samples by LC-MS

Plasma analysis was conducted using a LCMS 2020 system (Shimadzu, Japan) which included a LC-20AD binary pump, a SiL-20AC refrigerated autosampler, a mobile phase vacuum degassing unit

(DGU-20A_3), and a temperature-controlled column compartment (CTO-20A), coupled with a single-quadrupole mass spectrometer (Shimadzu LCMS 2020) equipped with an electrospray ionization source. The autosampler was maintained at 4°C and the column at 40°C. A Phenomenex Gemini C6-phenyl column (50 × 2.0 mm, 3 µm) was used to allow separation. Samples were eluted via gradient elution at a flow rate of 0.3 mL/min. The mobile phases consisted of a mixture of solvent A (95% v/v Milli-Q water: 5% v/v MeOH) and solvent B (5% v/v Milli-Q water: 95% v/v MeOH) both containing 1 mM ammonium formate and 0.1% formic acid. The initial percentage of solvent B was 60%. The proportion of solvent B was linearly increased to 100% over 6.5 min and was held at 100% for 7 min. After 14.25 min, the gradient was returned to 60% solvent B until the end of the 17 min run time to achieve re-equilibration. Under the above-mentioned conditions, the retention times of danazol and progesterone were 4.59 and 4.35 min, respectively. The MS conditions were as follows: drying gas flow, 20 L/min; nebulizing gas flow, 1.5 L/min; drying gas temperature, 200°C; interface voltage, 3.5 kV and detector voltage 1.0 kV. Selected-ion monitoring was accomplished at *m/z* 338.2 for danazol, and *m/z* 314.9 for the internal standard, progesterone. The chromatographic data were acquired and analysed using the LabSolution software package, 5.31.277 (Shimadzu).

The plasma assay was validated by analysis of n = 6 quality control samples containing 1, 10 and 100 ng/ml danazol in blank plasma. Intra-assay variability was accurate to 113.5, 103.8 and 111.8% and precise to 11.2, 9.2 and 4.4% of 1, 10 and 100 ng/ml, respectively. The lower limit of quantification for the plasma assay (1 ng/ml) was determined by replicate analysis (n = 6) of spiked plasma samples and defined as the lowest concentration at which appropriate accuracy and precision was obtained. Calibration curves and regression parameters were calculated by least-squares linear regression analysis using a weighting factor of 1/x.

3.6.4 Pharmacokinetic data analysis

The maximum plasma concentrations (C_{max}) and the time for their occurrence (T_{max}) were noted directly from the individual plasma concentration vs. time profiles. The area under the plasma concentration vs. time profiles (AUC_{0-10}) was calculated using the linear trapezoidal method. Danazol plasma concentrations were typically below the limit of quantification of the assay at 24, 32 and 48 h post-dose and accurate determination of the terminal elimination rate constant and ($AUC_{0-\infty}$) was not possible. Since the danazol plasma concentrations at 10 h were low, the extrapolated AUC ($AUC_{10-\infty}$) was expected to contribute only a minor proportion of the total AUC ($AUC_{0-\infty}$). Relative bioavailability comparisons were therefore performed using (AUC_{0_10}). 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.

3.7 RESULTS

In vitro evaluation

3.7.1 Characterisation of SEDDS formulations containing danazol

Table 3.1 provides the exact composition of the model formulations shown in the ternary plot in Figure 3.2 and also danazol solubility data in the formulations and in the aqueous phases formed by in vitro dispersion or digestion of the blank formulations. The in vitro performance of the formulations was evaluated using formulations containing drug (danazol) loads equivalent to 40% and 80% of danazol saturated solubility in the formulation. The maximum solubilised dose that the formulations could provide at low (40% of saturated solubility) and high (80% of saturated solubility) drug loading in the formulation is also shown. As the lipid/surfactant ratio in the

formulation increased at constant cosolvent levels (i.e. F10 – F25 – F40 – F60 – F80), danazol solubility in the formulation decreased. Therefore, the potential maximum drug concentration that could be attained in the aqueous phase of the digest in the absence of drug precipitation (AP_{MAX}) also decreased at drug loads representing either 40% or 80% of danazol solubility in the formulations. The same trend was observed for formulations containing decreasing cosolvent/lipid ratios at constant surfactant levels (i.e. F0 – F18 – F25).

3.7.2 Polymeric inhibition of drug precipitation from SEDDS formulations

To evaluate the potential effect of polymeric precipitation inhibitors (PPI) on the performance of danazol-containing SEDDS formulations, formulation F60 was used as a typical formulation composition (60% lipid, 30% surfactant, 10% cosolvent) and a screening study was conducted to explore the effect of 13 different polymers on drug precipitation during formulation dispersion and digestion. In this screening exercise, danazol was included at 40% of saturated solubility in the formulation. Figure 3.4 shows the percentage of the original quantity of drug dissolved in the formulation that precipitated after dispersion and a period of 60 min digestion in the absence (control) and presence of the different polymers. Consistent with previous studies describing similar medium-chain SEDDS formulations of danazol,[16] digestion led to significant drug precipitation. After a 60 min digestion period, 75% of the initial drug load in the formulation had precipitated. The addition of polymer to the formulation, however, had in many cases a profound effect on drug precipitation. Data below the dotted line in Figure 3.4 show polymers that resulted in lower drug precipitation compared to the control formulation. Seven of the polymers examined (Eudragit E100, HPMC (E4M), HPMC (E5), HPMC-AS (LF), HPMC-AS (HF), MC (A4C) and HPC) reduced drug precipitation by more than 50% and the cellulose derivatives led to the greatest reduction in drug precipitation. In contrast, Eudragit L100, Aerosil 200, HEC, HPMC-P (55S) and PVP10 or PVP360 had little effect on drug precipitation (Aerosol was included in the current

comparison, even though it is not a polymer, since this high surface area material was seen as a prospective precipitation inhibitor). HPMC (E4M grade) was chosen as a candidate PPI for further evaluation as it more easily dispersed in the SEDDS formulation when compared to e.g. HPMC-AS, which resulted in formulations with much higher viscosity that were more difficult to manipulate.

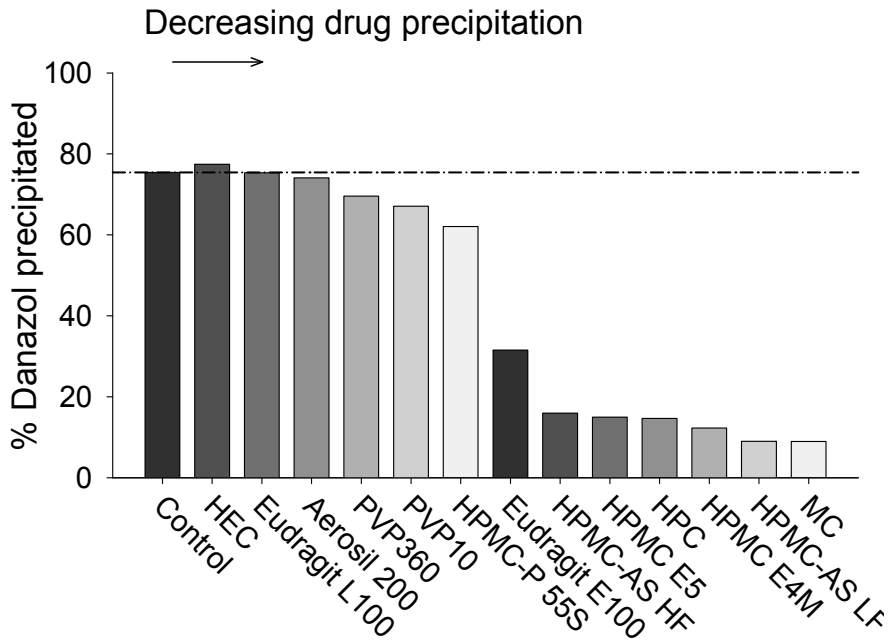


Figure 3.4 Screening of various polymers as precipitation inhibitors after 60 min in vitro digestion of a model SEDDS (F60) containing danazol. The dotted line represents the extent of drug precipitation from the formulation in the absence of polymer. Data below the line represent formulations where polymer addition leads to lower drug precipitation on formulation digestion.

3.7.3 Effect of polymer concentration on drug precipitation

To probe the impact of HPMC (E4M) concentration on drug precipitation, two SEDDS formulations were examined at different HPMC loads. F60 was again employed as a typical SEDDS formulation and formulation F0 was explored as an example of a formulation containing much higher

surfactant and cosolvent levels. Figure 3.5 shows the effect of polymer on drug precipitation from the formulations as the HPMC concentration in the formulations was altered. For F60 (Figure 3.5A), a greater than threefold decrease in drug precipitation was evident when HPMC was added to the formulation at levels of 0.5% w/w or higher. In contrast, the polymer effect on drug precipitation from the formulation containing high surfactant and cosolvent [F0] was more (polymer) concentration dependent, and larger quantities of polymer (5% w/w) were required to inhibit precipitation to a similar extent (Figure 3.5B). Subsequent studies included HPMC at 5% w/w in all formulations.

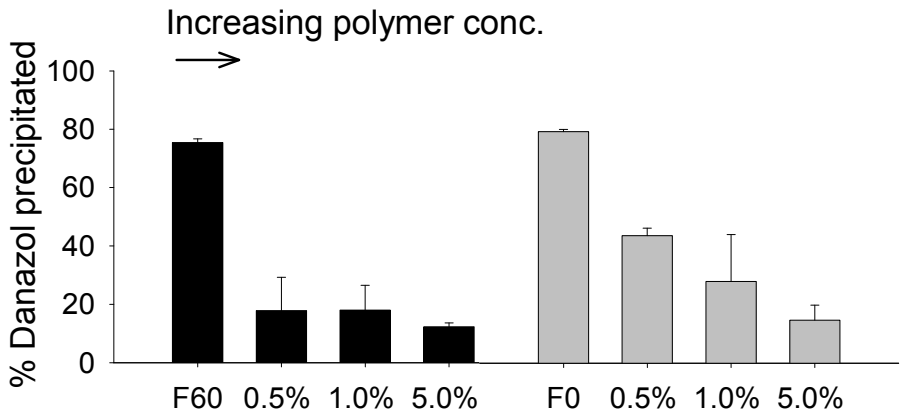


Figure 3.5 Effect of polymer concentration on danazol precipitation from F60 (black bars) and F0 (grey bars) after 60 min digestion. HPMC was incorporated at 0, 0.5, 1 and 5% (w/w) in F0 and F60 [mean ± SD (n = 3)].

3.7.4 Drug solubilisation during in vitro dispersion and digestion

After identification of the most appropriate PPI (HPMC E4M) and an appropriate polymer concentration (5% w/w) for the model drug and formulations investigated here, subsequent studies sought to explore the impact of systematic changes to the nature of the SEDDS

formulation (including the presence and absence of polymer) on drug precipitation during dispersion and digestion and in particular the potential for stabilisation of supersaturation. Experiments were conducted using formulations containing drug at two different loads - 40% and 80% of drug solubility in the formulation, since increasing drug load was expected to increase the likelihood of drug precipitation and decrease the potential for either excipients or polymer to maintain supersaturation for extended periods. Data exploring the impact of changes to the lipid/surfactant ratio (at fixed cosolvent) are presented in Figure 3.6. Similar data exploring the effect of changes to the ratio of cosolvent/lipid at fixed surfactant are shown in Figure 3.7.

3.7.4.1 Impact of lipid/surfactant ratio on performance of SEDDS ± HPMC

Low drug loading (40% saturation)

Drug precipitation profiles from formulations with varying lipid/surfactant ratios containing danazol at 40% of saturated solubility are shown in the left hand panels of Figure 3.6 in the presence and absence of 5% w/w HPMC. The potential maximum drug concentration that could be attained in the aqueous phase (in the absence of drug precipitation) (AP_{MAX}) is shown as the top horizontal dashed line in each plot. Consistent with the decrease in drug solubility in the formulations, increasing the ratio of lipid/surfactant led to lower AP_{MAX} values from 400 µg/mL for F10 to 272 µg/mL for F80.

To gain a better understanding of the drivers of drug precipitation during dispersion and digestion and to evaluate the possibility of supersaturation during formulation processing, the apparent equilibrium solubility of danazol in the AP generated by dispersion (AP_{DISP}) of blank (drug free) formulations and digestion (AP_{DIGEST}) of blank formulations (at 5 min and 60 min post digestion) was also measured. Only moderate decreases in drug solubility were evident over the 5 - 60 min digestion period, and the equilibrium drug solubility in the dispersed and digested AP is shown as the lower dotted line in Figure 3.6 and 3.7.

The ratio between AP_{MAX} and drug solubility in the aqueous phase (see Figure 3.3) provides a measure of the maximum degree of supersaturation that each formulation is able to generate and is described by the maximum supersaturation ratio (S^M) (see eq. 2). S^M quantifies the degree of thermodynamic instability generated by formulation dispersion or digestion (when S^M is > 1) and is expected to influence the degree of drug precipitation. The S^M values are annotated on Figure 3.6 and 3.7 for both dispersion (S^M_{DISP}) and digestion (S^M_{DIGEST}) phases.

All the formulations dispersed readily and no evidence of drug precipitation was apparent during the initial 30 min dispersion period at low drug loading despite the generated level of supersaturation. As such AP concentrations over the 30 min dispersion period were consistent with AP_{MAX} (dashed line).

Examination of the drug concentrations measured in the AP of the digest after initiation of digestion (filled symbols in Figure 3.6) reveals a very rapid drop in drug solubilisation on digestion initiation for all formulations and approach towards the equilibrium solubility of drug in the phases formed post digestion. A brief period of supersaturation was evident in all cases, but by 30 min digestion drug precipitation had lowered the AP concentration to a level consistent with drug solubility in the phase formed, effectively removing the driving force for further precipitation.

As digestion was initiated, the equilibrium solubilisation capacity of the system also rapidly decreased due to changes in the physical and chemical properties of the aqueous colloidal phase upon digestion. This is reflected in the data describing eq. solubility of drug in the AP after 5 min of digestion (AP_{DIGEST} 5 min in Table 3.1).

Addition of 5% (w/w) HPMC to all formulations (open circles in Figure 3.6) resulted in greatly reduced drug precipitation and maintenance of supersaturation for extended periods. This occurred regardless of lipid/surfactant ratio. To evaluate whether the enhanced drug solubilisation that was obtained in the presence of HPMC reflected stabilisation of a

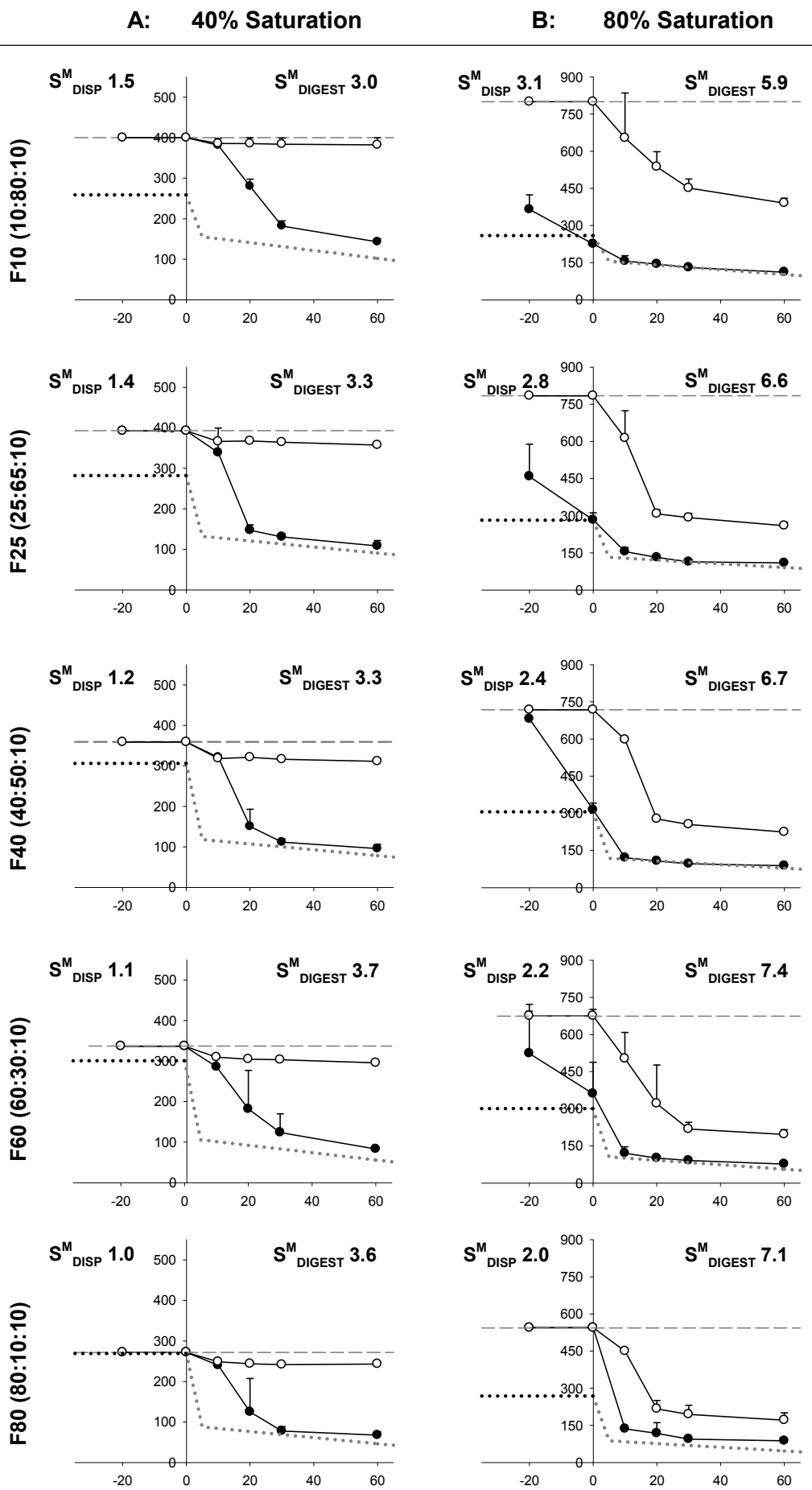
supersaturated state or changes to the inherent solubilisation capacity of the post digestion AP, the equilibrium solubility of danazol in blank (drug-free) AP was measured in the presence of polymer. Table 3.1 summarises the equilibrium solubility values obtained after 60 min digestion in the presence of 5% w/w HPMC, and no significant difference was evident between drug solubility in the presence and absence of HPMC. The impact of the polymer on drug solubilisation during digestion was therefore to stabilise a supersaturated state rather than to enhance intrinsic solubility.

High drug loading (80% saturation)

Panel B in Figure 3.6 shows the drug precipitation profiles of SEDDS with varying lipid/surfactant ratio at high drug loading (80%) in the presence and absence of 5% w/w HPMC during in vitro dispersion and digestion. Doubling the drug load in the formulations resulted in a corresponding 2-fold increase in S^M. In the absence of polymer, all formulations with S^M_{DISP} above 2 resulted in rapid drug precipitation during the initial 30 min dispersion period. Precipitation continued with initiation of digestion, and within 10 min, the drug concentration in the aqueous colloidal phase reduced to the level of the equilibrium solubility in the AP regardless of the lipid/surfactant ratio or $S^M_{DIGEST.}$

At the high drug load, addition of 5% (w/w) HPMC prevented drug precipitation on dispersion of all formulations, maintaining supersaturated conditions. However, initiation of digestion led to drug precipitation despite the presence of HPMC. The presence of the polymer slowed drug precipitation, resulting in profiles similar to that obtained at the lower drug loading in the absence of polymer. Interestingly, after 20-30 min digestion, drug concentrations appeared to plateau at a level above the equilibrium solubility in the AP.

Figure 3.6 Drug precipitation profiles during dispersion and digestion of SEDDS formulations (FX (X:Y:Z) where X is the % composition of lipid, Y is surfactant and Z is cosolvent) when decreasing lipid/surfactant ratio at constant cosolvent (10%) [mean ± SD (n = 3)]. Danazol was incorporated at 40% (panel A) and 80% (panel B) of the equilibrium solubility in the formulation and in the absence (closed symbols) and presence (open symbols) of 5% (w/w) HPMC. The upper dashed line indicates AP_{MAX} and the lower dotted line indicates the drug solubility in the aqueous colloidal phase produced by dispersion (AP_{DISP}) and digestion (AP_{DIGEST}) of drug-free SEDDS. S^{M}_{DISP} or S^{M}_{DIGEST} are the maximum supersaturation ratios for each formulation on dispersion or digestion respectively. Axis-labels are not shown on the plots to improve clarity; x-axis represents digestion time [min]; y-axis represents danazol concentration in AP [ug/mL].


A: 40% Saturation
B: 80% Saturation
F10 (10:80:10)
F25 (25:65:10)
F40 (40:50:10)
F60 (60:30:10)
F80 (80:10:10)
S^M DISP 1.5
S^M DIGEST 3.0
S^M DISP 3.1
S^M DIGEST 5.9
S^M DISP 1.4
S^M DIGEST 3.3
S^M DISP 2.8
S^M DIGEST 6.6
S^M DISP 1.2
S^M DIGEST 3.3
S^M DISP 2.4
S^M DIGEST 6.7
S^M DISP 1.1
S^M DIGEST 3.7
S^M DISP 2.2
S^M DIGEST 7.4
S^M DISP 1.0
S^M DIGEST 3.6
S^M DISP 2.0
S^M DIGEST 7.1

3.7.4.2 Impact of increasing cosolvent/lipid ratio in SEDDS ± HPMC

Low drug loading (40% saturation)

Panel A in Figure 3.7 shows the drug precipitation profiles of SEDDS with varying cosolvent/lipid ratio at low drug loading (40%) in the presence and absence of 5% w/w HPMC during in vitro dispersion and digestion. At constant surfactant levels, increasing the cosolvent/lipid ratio in the formulation increased drug solubility in the formulation and therefore AP_{MAX}. In contrast, increasing cosolvent resulted in little difference to the equilibrium solubility of drug in the post digestion AP (consistent with the decrease in solubilisation capacity for diluted cosolvents[172]). As a result, S^{M}_{DIGEST} increased as the cosolvent/lipid ratio in the formulation was increased.

In the absence of polymer, formulations containing low levels of cosolvent were able to prevent drug precipitation during dispersion and maintain supersaturation, however as cosolvent concentrations increased (> 18% in the formulation), precipitation was evident even during the dispersion phase. After initiation of digestion, rapid drug precipitation occurred for all formulations resulting in limited supersaturation and drug concentrations in the AP approaching eq. solubility after 10-20 min digestion. Addition of 5% (w/w) HPMC to all formulations containing danazol at low drug loads significantly reduced drug precipitation on digestion and stabilized supersaturation regardless of cosolvent levels and S^{M}_{DIGEST} at low drug loading (Figure 3.7A).

High drug loading (80% saturation)

As the drug loading in the formulations was increased, S^{M} increased, as did the degree of drug precipitation from the formulations. In the absence of polymer, significant precipitation was evident even on dispersion and drug concentrations in the post digestion aqueous phase approached equilibrium solubility more quickly than was evident in the lower dose profiles, regardless of the cosolvent/lipid ratio (Figure 3.7B).

Figure 3.7 Drug precipitation profiles during dispersion and digestion of SEDDS formulations (FX (X:Y:Z) where X is the % composition of lipid, Y is surfactant and Z is cosolvent) when increasing cosolvent/lipid ratio at constant surfactant [mean ± SD (n = 3)]. Danazol was incorporated at 40% (panel A) and 80% (panel B) of the equilibrium solubility in the formulation and in the absence (closed symbols) and presence (open symbols) of 5% (w/w) HPMC. Figure labels as described in legend for Figure 3.6.

Addition of 5% w/w HPMC to the formulations containing lower cosolvent (F25, F18) prevented precipitation on dispersion, however, the increased level of cosolvent in F0 resulted in rapid precipitation within 15 min of dispersion, even in the presence of polymer. In all formulations, initiation of digestion led to rapid drug precipitation, although the presence of polymer appeared to result in a plateau in the AP concentrations at a level slightly above the equilibrium solubility of drug in the post digestion AP, similar to the results presented in Figure 3.6.

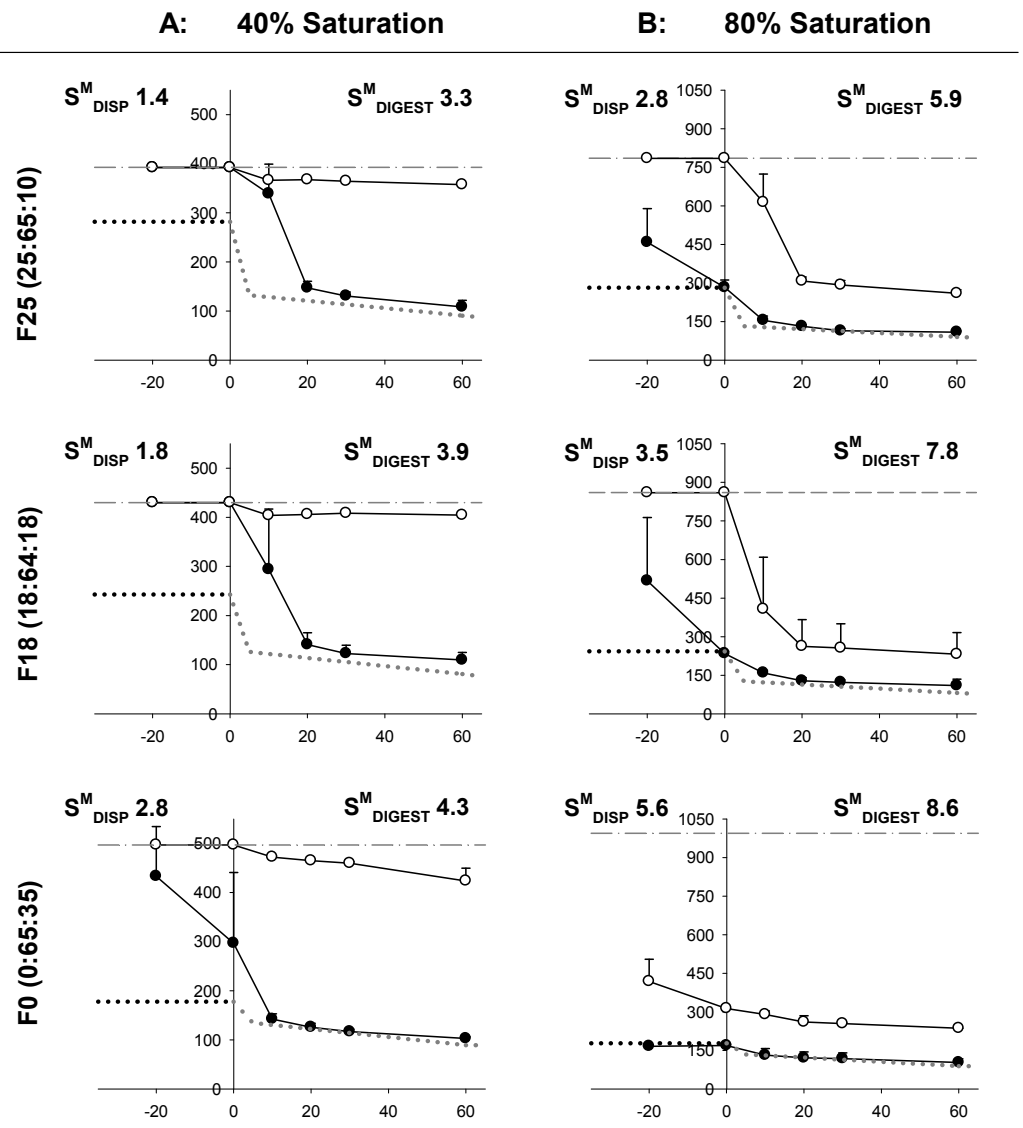


Figure 3.8 shows the XRPD diffractograms of the pellet phase obtained after digestion and ultracentrifugation of F60. Physicochemical examination of precipitated danazol in both the presence and absence of HPMC revealed similar XRPD signals to the original crystalline material suggesting that polymer addition did not affect the nature of the crystallised material that precipitated out of solution. The polymer effect was therefore more likely to be an effect of retardation of crystal nucleation and growth rather than the promotion of phase separation of amorphous drug in the precipitate.

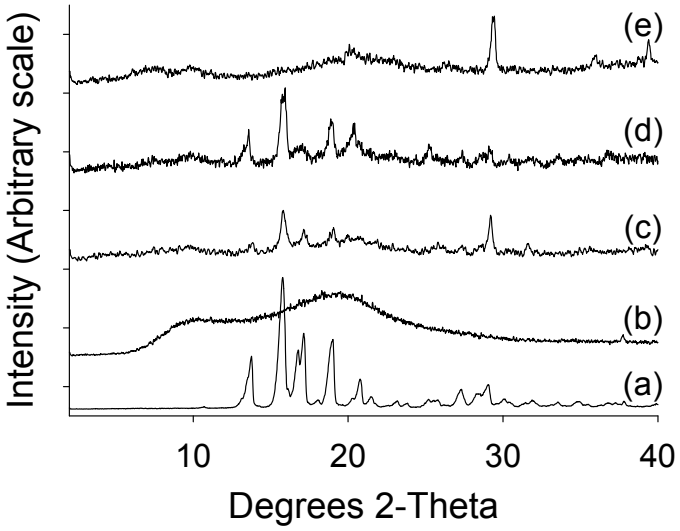


Figure 3.8 XRDP profiles of (a) crystalline danazol, (b) HPMC, (c) pellet after digestion of F60 comprising danazol and 5% HPMC, (d) pellet after digestion of F60 comprising danazol, (e) Blank pellet after digestion of drug-free F60.

3.8 BIOAVAILABILITY DATA

3.8.1 Effect of formulation composition and the presence of PPI in vivo

The mean plasma concentration versus time profiles for danazol following oral administration of a high cosolvent formulation (F0) and high lipid formulation (F60) in the presence and absence of 5% w/w HPMC (E4M grade) to fasted beagle dogs are shown in Figure 3.9 and the corresponding mean pharmacokinetic parameters are summarised in Table3.2. Danazol plasma concentrations at 24, 32 and 48 h were typically below the limit of quantification of the analytical assay, and data were therefore truncated to 10 h for pharmacokinetic evaluation.

Panel A in Figure 3.9 shows the administration of F0 containing danazol at 40% saturated solubility. In the absence of polymer, the dose normalised plasma profile and AUC of danazol after oral administration was consistent with a previous reported study using the same formulation providing confidence in reproducibility.[13] In contrast to the in vitro precipitation profiles, however, the addition of 5% w/w HPMC to the formulation failed to increase danazol bioavailability and resulted in a decrease in C_{max} and similar exposure as expressed by AUC.

The mean plasma concentration time profiles for F60 are shown in Figure 3.9 at low (panel B) and high (Panel C) drug loading, and in the presence and absence of HPMC. At low drug loads, addition of HPMC to F60 led to a 65% increase in danazol exposure. Increasing the drug loading from 40% to 80%, however, resulted in loss of the polymer effect and AUCs in the presence and absence of polymer were similar.

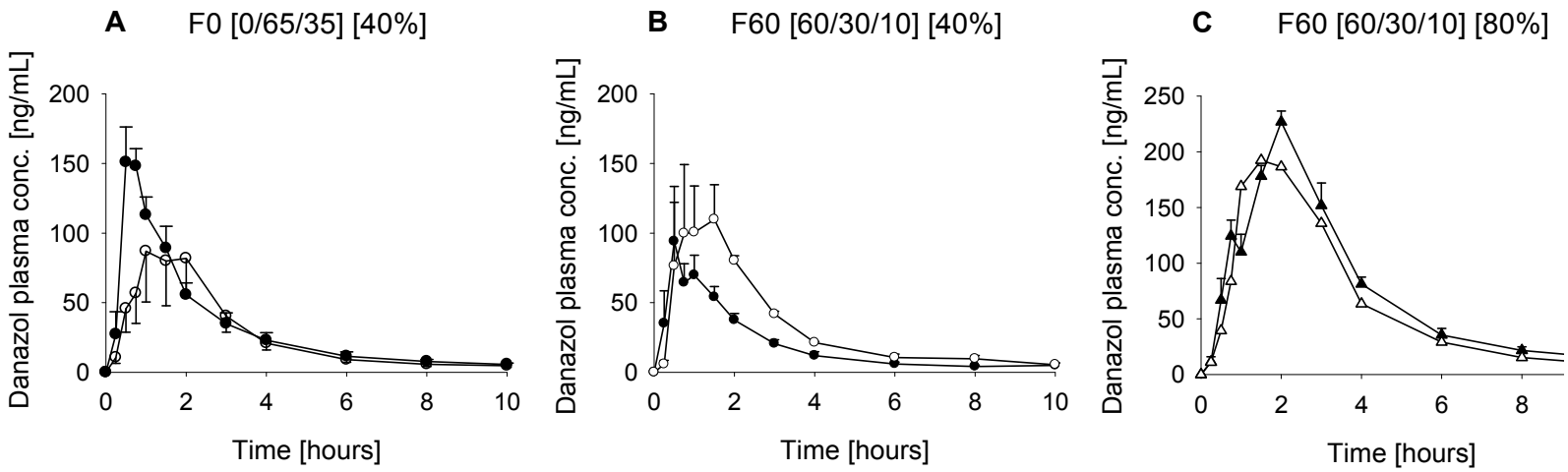


Figure 3.9 Mean plasma conc. vs time profiles for danazol after oral administration to fasted beagle dogs in the presence (open) and absence (filled) of 5% (w/w) HPMC. A: Formulations F0 [0/65/35; lipid/CrEL/ethanol], danazol incorporated at 40% saturated solubility [mean ± SEM (n = 4)], B: F60 [60/30/10], danazol at 40% saturated solubility [mean ± SEM (n = 3)], C: F60 [60/30/10] in the absence of polymer (filled), [mean ± SEM (n = 3)] and in the presence of polymer (open), [mean (n = 2)] with danazol incorporated at 80% saturated solubility.

To compare excipient effects on bioavailability across formulations and at different drug loads, the $AUC_{0\text{-}10h}$ was also normalised to a standardised 15 mg dose, and these data are compared in Figure 3.10. The administration of F0 [high cosolvent/lipid-free] and F60 [high lipid] resulted in similar drug exposure at the low drug load in the absence of polymer. In the presence of HPMC however, bioavailability from F60 was higher than that of F0 (although statistical significance was not reached), reflecting the effect of the polymer in increasing exposure for F60 but not F0.

At the higher drug load, the polymer had no effect on bioavailability for F60. Interestingly, however, bioavailability was increased when compared to the low drug dose formulation regardless of the presence of polymer.

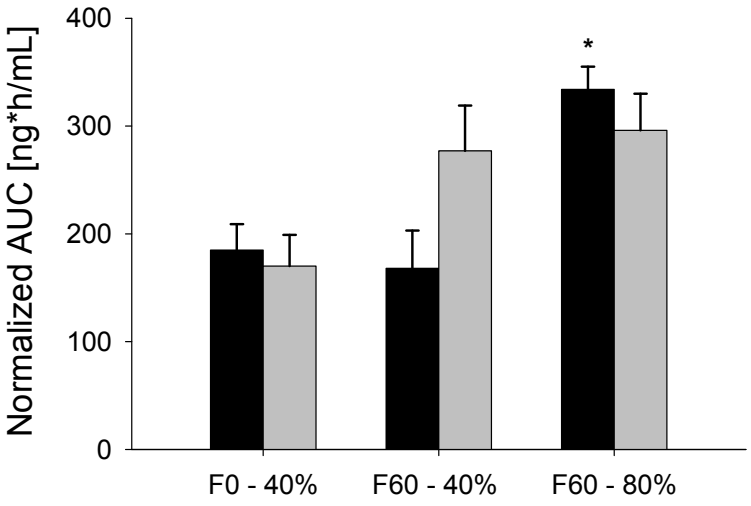


Figure 3.10 Normalised $AUC_{0\text{-}10h}$ [ng.h/mL] to 15 mg dose. Formulation F0 with danazol incorporated at 40% saturated solubility and F60 with danazol incorporated at 40% and 80% saturated solubility in the absence (black) and presence (grey) of 5% (w/w) HPMC. *Statistically significant different to F0 (40%) and F60 (40%) ($P < 0.050$).

Table 3.2 Pharmacokinetic parameters for danazol after oral administration of F0 [mean ± SEM (n = 4)] and F60 [mean ± SEM (n = 3)] to fasted beagle dogs.

	F0		F60			
Formulation	40%[a]	40% + PPI	40%	40% + PPI	80%	80% + PPi [c]
$AUC_{0\text{-}10h}$ (ng.h/mL)	325 ± 42	267 ± 45	194 ± 40	316 ± 48	762 ± 48	738.6: 628.9
$AUC_{0\text{-}10h}$ (normalised)[b]	185 ± 24	170 ± 29	168 ± 35	277 ± 42	334 ± 21	320.1: 272.6
C_{max} (ng/mL)	160 ± 22	127 ± 26	104 ± 30	124 ± 37	227 ± 10	196.7: 249.2
T_{max} (h)	0.7 ± 0.1	1.3 ± 0.2	0.8 ± 0.3	1.4 ± 0.4	2.0 ± 0.0	3.0; 1.5:
$t_{1/2}$ (h)	2.5 ± 0.1	2.1 ± 0.2	4.4 ± 0.7	2.9 ± 0.4	3.3 ± 0.2	2.4: 2.5

[a] Nominal % saturation shown here. Accurate doses and saturation level for each dose based on variation in fill concentrations and fill weights detailed in methods

[b] AUC normalised to 15 mg dose to compare relative exposure across different drug doses

[c] Dog 3 was excluded due to vomiting after dosing. Data presented for the individual dogs (n = 2)

3.9 DISCUSSION

Self-emulsifying drug delivery systems (SEDDS) show great potential for improvements in oral bioavailability for poorly water-soluble drugs. The ability of SEDDS to maintain drug in a solubilised state and to prevent drug precipitation is highly dependent on the nature of included formulation components, and this is further complicated by the realisation that the properties of lipid and surfactant based excipients can change markedly during dispersion and digestion in the GI tract.[13,16,74,84,120] In particular, digestion may lead to a loss in solubilisation capacity of the formulation and a decrease in bioavailability.[13-16,106]

The loss of solubilisation capacity that results from either dispersion or digestion of a SEDDS formulation, also results in the generation of transient supersaturation. Where precipitation can be delayed for a period sufficient to allow absorption, supersaturation increases thermodynamic activity and may promote absorption. In recent years, considerable efforts have therefore been

directed towards the design and development of drug formulations that promote the formation and stabilisation of supersaturation during GI formulation processing. Hydrophilic polymers have been shown to be effective in stabilising supersaturation in several applications – perhaps most notably the marketed itraconazole formulation (Sporanox®) and as such have also started to be explored for their potential to stabilise supersaturation during the processing of SEDDS formulations.[24,25,27,173]

The balance between on the one hand increasing supersaturation and thermodynamic activity and on the other avoiding precipitation is further complicated by differences in dose, where increasing dose will increase the potential for supersaturation but also the risk of precipitation.

The current studies were conducted to evaluate the potential for supersaturation generation during the processing of SEDDS formulations, to probe the ability of polymers to enhance the performance of SEDDS by stabilising supersaturation, and to evaluate the effect on solubilisation and supersaturation of increasing drug dose.

3.9.1 Polymers as drug precipitation inhibitors

The cellulose-based polymers (e.g. HPMC, HPC and MC) were the most effective in preventing drug precipitation during the dispersion and digestion of medium-chain SEDDS containing danazol (Figure 3.4). This is consistent with recent data from our laboratories that detailed the effect of polymers on drug precipitation for danazol using a solvent shift method[138] and also the work of others.[140,174] Data obtained using simple in vitro methods to screen for polymeric precipitation inhibitors (PPIs) therefore appears to translate into utility in more complex in vitro dispersion and digestion evaluation protocols for SEDDS formulations (at least for danazol).

Evaluation of the impact of increasing HPMC concentration on the ability of the polymer to maintain danazol in a solubilised state post digestion revealed differences in polymer

performance depending on the nature of the formulation. For the F60 formulation, addition of 0.5%, 1% or 5% (w/w) HPMC resulted in a significant decrease in drug precipitation however, no differences were seen as a function of polymer concentration, suggesting that only low quantities of polymer were sufficient to inhibit precipitation. In contrast, for formulation F0, a concentration dependency in polymer effect was evident, and maximum precipitation inhibition occurred only at the highest polymer concentration (5% w/w). These data are consistent with the findings of Raghavan et al[17], where the flux of hydrocortisone acetate across a silicone membrane was used to evaluate the potential for different polymers to stabilise a supersaturated cosolvent solution. In these studies, the utility of the added polymer reached a maximum effect at polymer concentrations that varied depending on polymer type (1% for HPMC, 1% for HPC and 10% for PVP), although in this case further increases in polymer concentrations were shown to reduce drug flux.[17]

3.9.2 Formulation sensitivity of polymer precipitation effect

Data describing the formulation dependency of the ability of 5% HPMC to prevent drug precipitation on dispersion and digestion of SEDDS formulations is shown in Figure 3.6 and 3.7. To facilitate easier cross-formulation comparison, the area under the AP drug concentration versus time profiles after initiation of digestion was utilised as a composite measure of drug solubilisation or total drug 'exposure' to the AP over time. Figure 3.11 shows the in vitro AP AUC as a function of formulation composition in the presence and absence of HPMC. In all cases, the addition of polymer significantly enhanced total drug solubilisation. The addition of HPMC to the formulations did not, however, affect drug solubility in the AP produced by digestion of blank formulations (Table 3.1). The effect of the polymer on drug solubilisation during digestion was therefore due to stabilisation of a transiently supersaturated state rather than an increase in solubilisation capacity of the aqueous colloidal phase. Polymer addition has been suggested to

prevent drug precipitation via a reduction in crystal nucleation and/or crystal growth, and intermolecular interactions (e.g. hydrogen bonding) between drug and polymer implicated in mediation or accentuation of the precipitation inhibition effect.[17-23] The utility of polymeric precipitation inhibitors is therefore highly dependent on the physicochemical properties of both drug molecule and polymer.[140]

Drug solubilisation patterns after digestion of SEDDS formulations have also been suggested to reflect changes in crystal form and the prospect of drug precipitation in the amorphous form after digestion of SEDDS formulation has been raised by Sassene et al.[175] The presence of polymer has also been suggested to change the crystal form, and Gao et al[24] previously showed that polymer addition to SEDDS formulations of the poorly water-soluble drug, AMG 517, did not prevent precipitation on formulation dispersion, but instead led to drug precipitation in the amorphous rather than the crystalline form, thereby enhancing the re-solubilisation potential by forming a more rapidly dissolving amorphous drug precipitate. In the current studies, danazol precipitated out of solution in a crystalline form on in vitro digestion both in the absence of HPMC (consistent with previous studies[166]) and in the presence of HPMC. In the case of danazol therefore, the data reported here suggest that polymer addition was more likely to be an effect of retardation of crystal nucleation and growth rather than the promotion of phase separation of amorphous danazol (Figure 3.8). More detailed discussion of the potential mechanism of action of PPIs is available in the following reviews.[136,138]

Increasing surfactant and decreasing lipid resulted in greater in vitro drug exposure at low drug loading (40% of saturated solubility in the formulation) in the absence and presence of polymer (Figure 3.11A), and at high drug loading in the presence of polymer (Figure 3.11C). At higher drug loads and in the absence of polymer, precipitation was rapid and drug solubilisation was low and invariant for all formulations (Figure 3.11C). A similar trend was observed when the cosolvent/lipid ratio in the formulation was increased (Figure 3.11B, D), consistent with a drop in

the solubilisation capacity of cosolvents on dilution. One exception was the data obtained at low drug loads and in the presence of polymer where a small increase in drug solubilisation was evident at higher cosolvent (Figure 3.11B).

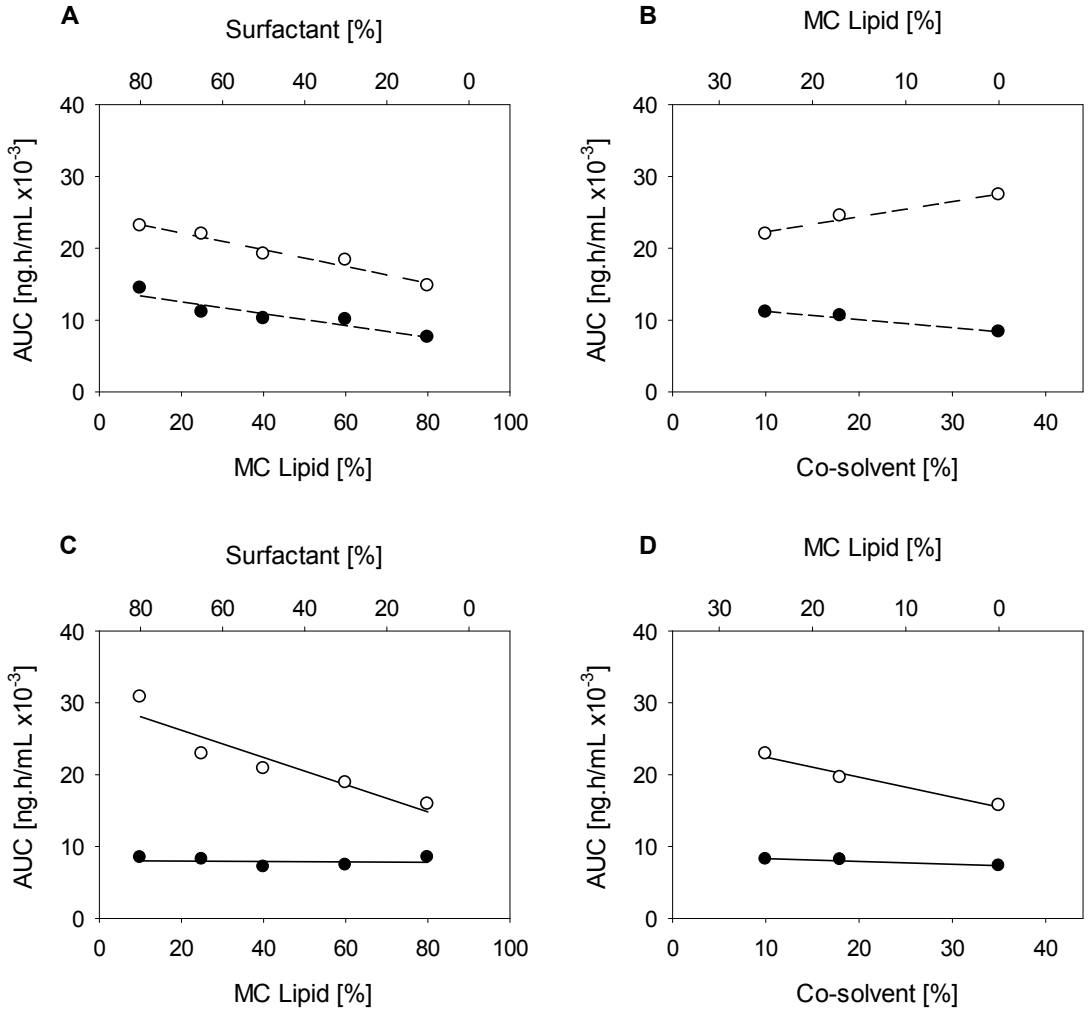


Figure 3.11 Area under the solubilisation profile after initiation of digestion (AUC) in the presence (open) and absence (filled) of 5% w/w HPMC versus A: increasing lipid/surfactant ratio and B: increasing cosolvent/lipid ratio in the formulation with danazol incorporated at 40% saturated solubility and C: increasing lipid/surfactant ratio and D: increasing cosolvent/lipid ratio in the formulation with danazol incorporated at 80% saturated solubility.

The trends in in vitro exposure are a function of both the quantity of drug initially dissolved in the formulation and the subsequent ability of the formulation to maintain solubilisation on GI processing. Thus, at the 40% drug load and in the presence of polymer, precipitation was almost entirely inhibited, and therefore the solubilised drug concentrations in the AP (in vitro 'exposure') reflected the initial drug load in the formulation. Increasing AP exposure was therefore evident on increasing surfactant and cosolvent levels in the formulation since these components result in greater drug solubility in the formulation. In contrast, at the 80% drug load and in the absence of polymer, precipitation was rapid, exposure was low and differences in formulation had little impact on solubilisation profiles.

Cuine et al[13] previously showed that the inclusion of Cremophor EL (CrEL) and ethanol in danazol SEDDS containing long-chain (rather than medium-chain) lipids increased drug solubility in the formulation and contributed to enhanced formulation dispersion. However, CrEL and ethanol did not enhance solubilisation post digestion, and instead a reduction in lipid content and an increase in surfactant and cosolvent composition resulted in a significant reduction in drug solubilisation post digestion and a reduction in *in vivo* exposure.[13] Comparison of the data across the two studies suggests that increasing surfactant may be beneficial in formulations comprising medium-chain lipids (where the lipids provide limited on-going solubilisation capacity post digestion), however, for long-chain lipid-containing formulations, the inclusion of high quantities of surfactants (or at least digestible surfactants such as CrEL) may be counterproductive.

In all cases evaluated here, differences in danazol equilibrium solubility in the AP formed on dispersion or digestion of the model formulations (lower dotted lines in Figure 3.6 and 3.7) were relatively small when compared across the different formulations, and differences in formulation performance were largely evident in the level of supersaturation seen after dispersion or initiation of formulation digestion. Consistent with the data reported here, analysis of danazol equilibrium solubility in the AP formed on digestion of similar long-chain SEDDS formulations has

also shown that differences in formulation performance are driven largely by differences in supersaturation post digestion, rather than differences in the intrinsic solubilisation capacity of the colloids formed.[167] Collectively, these data suggest the possibility that design specifications for lipid-based formulations might be reorientated such that emphasis is placed on the ability of excipients to maintain drug in a solubilised and supersaturated state, rather than focussed on changing the equilibrium solubilisation capacity of the colloids that are formed post digestion.

3.9.3 Impact of S^M on drug solubilisation and supersaturation

More detailed examination of the patterns of in vitro solubilisation of danazol during dispersion and digestion of medium-chain lipid-containing SEDDS formulations reveals the importance of the initial (maximum) supersaturation ratio generated by formulation dispersion or digestion, prior to drug precipitation (a property we have defined as S^M).

Figure 3.6 and 3.7 show that danazol precipitation on dispersion of the evaluated formulations was only evident when S^M_{DISP} values were higher than 2, and the presence of polymer was able to prevent drug precipitation on dispersion for formulations up to a S^M_{DISP} of 3.5. Indeed, drug precipitation on formulation dispersion in the presence of polymer was only evident for the F0 formulation that contained surfactant and cosolvent alone and had the highest S^M_{DISP} (5.6).

A notable observation from examination of Figure 3.6 and 3.7 is the significant increase in S^M that occurred on initiation of digestion. In these formulations, therefore, digestion acts as a very effective supersaturation 'trigger', and S^M increased by a factor of at least 2 and often 3-4 fold within 5 min of initiation of digestion. Generation of supersaturation subsequently predisposed the formulations to crystallisation and precipitation. Whether precipitation occurred and how rapidly precipitation occurred was highly formulation dependent, however, similar trends were evident to those seen on formulation dispersion, and increased precipitation was associated with formulations that led to higher S^M_{DIGEST} values (Figure 3.6 and 3.7). For the formulations

examined, increasing surfactant resulted in higher S^{M}_{DISP} and lower S^{M}_{DIGEST}, trends reflecting greater loss in solubilisation capacity on dispersion of formulations containing greater quantities of surfactant rather than lipid, whereas on digestion, the loss in solubilisation capacity was greater for MC lipid-rich formulations rather than surfactant-rich systems.

In an attempt to illustrate the impact of the degree of supersaturation on the potential for drug precipitation, Figure 3.12 provides a correlation between the S^{M} for the different formulations and a measure of the ability of the formulation to resist precipitation on dispersion and digestion (% solubilised, eq. 3). The data show that the ability to maintain in vitro exposure decreases as the S^{M} of the formulation increases (Figure 3.12) consistent with the realisation that at higher S^{M}, the concentration of supersaturated drug is higher, and therefore provides the greatest driving force for nucleation and drug precipitation.[134] This relationship appears to hold equally true in either the dispersion (Figure 3.12A) or digestion (Figure 3.12B) period. It is also apparent from Figure 3.12C, that the presence of polymer significantly increases the ability of the formulation to maintain supersaturation close to maximum levels. Indeed, up to S^{M} of approximately 4 (consistent with conditions encountered at drug loadings of 40% of saturated solubility in the formulation), precipitation was largely suppressed. Above S^{M} of 6, however (conditions in line with the higher drug loading), increases in S^{M} led to significant decreases in solubilisation.

Comparison with previous data for danazol obtained for similar long-chain formulations suggests the trends revealed here may be applicable beyond medium-chain formulations.[13] Thus, previous studies have shown increases in oral bioavailability for poorly water-soluble drugs when administered in lipid-based formulations comprising long-chain rather than medium-chain lipids.[16,176] In the past, this has been ascribed to the presence of long-chain lipid digestion products more effectively swelling the solubilisation capacity of intestinal mixed micelles. We recently reported, however, that retrospective analysis of at least one of these studies[13], including generation of equilibrium solubility data for danazol in the colloids formed by digestion of the

long-chain SEDDS employed, suggests that much of the difference in formulation performance was also driven by differences in supersaturation, rather than equilibrium solubility.[167] Further evaluation of these data and applying a similar analysis to that described here, suggests that the AP_{MAX} values for the long-chain formulations are typically lower than that for medium-chain formulations (reflecting lower drug solubility in long-chain rather than medium-chain lipids) and that AP_{DIGEST} values are somewhat higher than that observed here for analogous medium-chain SEDDS (presumably due to the improved micelle swelling properties of long-chain digestion products). As such, the combination of lower AP_{MAX} and higher AP_{DIGEST} values on digestion of the long-chain SEDDS formulations results in lower S^M - i.e. a lower degree of supersaturation on digestion. This is entirely consistent with the trends observed here. The common observation of improved performance of long-chain lipid-based SEDDS formulations when compared to analogous medium-chain formulations[16,176] may therefore reflect an inherent tendency towards lower S^M on dispersion and digestion, reducing the drive towards precipitation and increasing in vivo exposure.

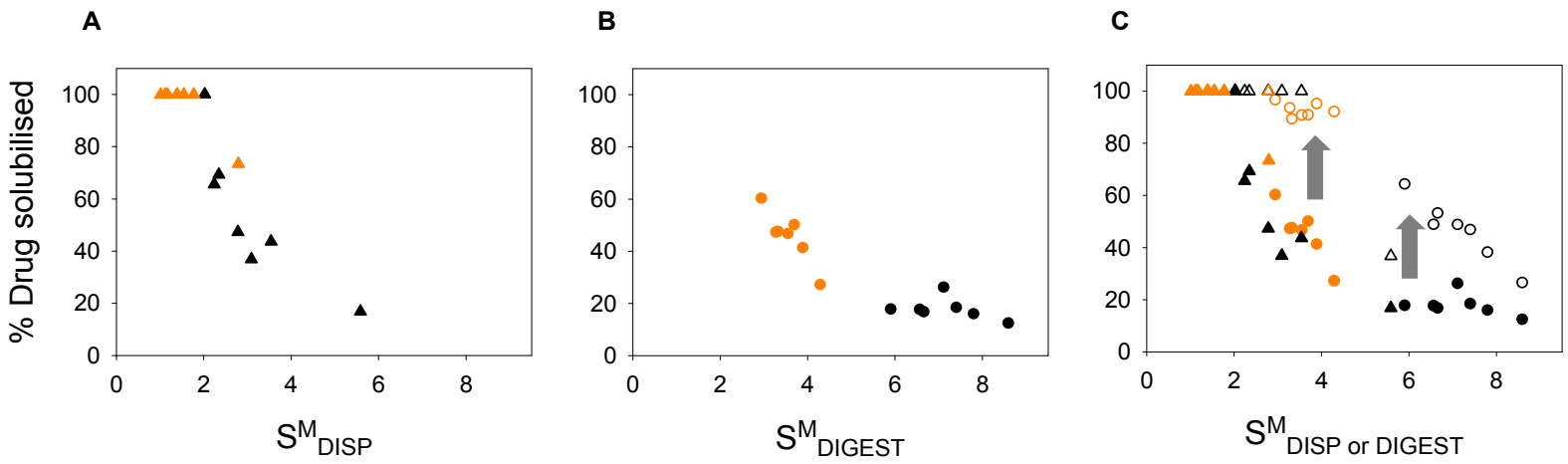


Figure 3.12 Percentage drug solubilised versus S^M at 40% (orange) and 80% (black) on A: dispersion and B: digestion. Panel C shows percent drug solubilised versus $S^M_{DISP\ \&\ DIGEST}$ at 40% (orange) and 80% (black) in the presence (open) and absence (closed) of PPI. Arrow illustrates the effect of PPI on percentage of solubilised drug at constant $S^M_{DISP\ \&\ DIGEST}$.

3.9.4 Effect of formulation composition on in vivo performance

The plasma profiles in Figure 3.9 and the pharmacokinetic parameters presented in Table 3.2 reveal limited differences in *in vivo* exposure of danazol after oral administration of formulations F0 and F60. In spite of the considerable difference in formulation composition between the two formulations, the in vitro digestion profiles share similarities with S^{M}_{DIGEST} values of 4.3 and 3.7 for F0 (Figure 3.7) and F60 (Figure 3.6), and in vitro solubilisation AUCs of 8.3 and 11.2 (Figure 3.11). The similarity in *in vivo* exposure is therefore largely consistent with the in vitro data. Addition of polymer to F60 also resulted in a moderate increase in *in vivo* exposure, again consistent with the in vitro data, however, for F0, no increase in *in vivo* exposure was seen, in spite of significant increases in in vitro solubilisation in the presence of polymer. Indeed, the addition of polymer appeared to reduce C_{max} *in vivo*. The lack of in vivo benefit provided by the addition of polymer to formulation F0 was consistent with the much greater dependency on polymer concentration for precipitation inhibition when compared to F60 (Figure 3.5) and a higher S^{M}_{DISP}, perhaps pointing to a greater inclination towards precipitation. For F0 in the presence of polymer, therefore, the in vitro digestion experiments appear to overestimate in vivo solubilisation. It is also possible that drug precipitation from a supersaturated state is more likely in the less well controlled, highly kinetic environment of the GI tract when compared with a relatively 'clean' in vitro digestion experiment, and therefore that larger amounts of polymer are required to prevent precipitation. A complete explanation for the lack of correlation between in vitro solubilisation and in vivo exposure for F0 is not apparent at this time. However, this observation is consistent with previous studies by Gao and co-workers[24-26], where the incorporation of PPIs into SEDDS led to increased drug exposure only in selected studies and where the polymer effect was dependent on the formulation, drug and animal species used.

In an attempt to further evaluate the effect of drug loading (and therefore S^M) on SEDDS performance, the in vivo performance of F60 was also evaluated in the presence and absence of polymer at higher drug loads. In contrast to the F60 data obtained at lower drug load, the addition of polymer at the higher drug load had little effect on bioavailability. The in vitro data suggest an increase in supersaturation stabilisation when assessed by in vitro AUC (Figure 3.11), however, even in the presence of polymer the extent of danazol precipitation was high and the ability to stabilise the supersaturated state was far less efficient than was the case at the lower dose. Consistent with the data obtained with F0, it seems likely that polymeric stabilisation of danazol supersaturation on digestion of F60 is more readily achieved in vitro than in vivo, and therefore that care should be taken when looking to extrapolate from in vitro to in vivo data – especially where the extent of stabilisation is relatively low (e.g. F60 high dose and F0).

Evaluation of the dose normalised AUC data for the high dose and low dose data obtained for F60 also revealed a significant non-linear increase in bioavailability at the higher dose (Figure 3.10). This non-linear increase in bioavailability may be due to increased initial supersaturation driving increases in thermodynamic activity in the aqueous colloidal phase at the higher drug load. However, the in vitro data suggest rapid precipitation and whether the higher thermodynamic activity is maintained in vivo for long enough to enhance bioavailability is not known. Alternatively, danazol has previously been shown to be subject to extensive hepatic metabolism[177] and the increase in bioavailability with increasing dose may also reflect saturation of first pass metabolism. Studies are ongoing to explore these possibilities.

3.10 CONCLUSION

The current studies have explored in detail the impact of supersaturation on the performance of self-emulsifying drug delivery systems. The data suggest that knowledge of the extent to which excipients generate supersaturation and the stability of the metastable state as the formulation is

processed in the GI tract may be more significant determinants of formulation performance than the equilibrium solubilisation capacity of the colloids that are formed post digestion. The current data also illustrate that digestion can act as an effective supersaturation 'trigger' and that solubilisation/precipitation behaviour is highly correlated with the maximum supersaturation ratio on dispersion and digestion (as defined by S^{M}_{DISP} and S^{M}_{DIGEST}). Thus, reducing the degree of generated supersaturation protects against precipitation, at least in vitro. To this end, utilisation of lower drug loads, higher surfactant levels, reduced cosolvent and the addition of polymer all enhanced performance in vitro. Correlation between in vitro solubilisation and in vivo exposure was evident at moderate drug loads and the inclusion of HPMC in the formulation enhanced drug exposure. In contrast, at higher drug loads, in vivo exposure increased in both the presence and absence of polymer, and the stabilising effects of polymer on supersaturation in vitro were not replicated in vivo. An explanation for the increase in exposure at higher dose is not clear at this time but may reflect changes to first pass metabolism or thermodynamic activity.

3.11 ACKNOWLEDGEMENT

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Monash University

Declaration for Thesis Chapter 4

Declaration by candidate

In the case of Chapter 4, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution
Planning and executing experimental work, data evaluation and drafting and revision of manuscripts	65%

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	Nature of contribution	Extent of contribution
C.J.H. Porter	Project supervisor, data and manuscript review	NA
C.W. Pouton	Project co-supervisor and manuscript review	NA
H.D. Williams	Manuscript review	NA
H. Benameur	External supervision and manuscript review	NA
G.A. Edwards	Supervision and responsibility for large animal work	NA

Candidate's Signature		**Date:** 20/11/2012

Declaration by co-authors

The undersigned hereby certify that:

(1) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
(2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(4) there are no other authors of the publication according to these criteria;
(5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and

(6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)	**Monash Institute of Pharmaceutical Sciences, Parkville Campus**	
Signature 1	[redacted]	21/9/2012
Signature 2	[redacted]	11/10/2012
Signature 3	[redacted]	11/10/2012
Signature 4	[redacted]	19/9/2012
Signature 5	[redacted]	13/9/2012

CHAPTER 4

NON-LINEAR INCREASES IN DANAZOL EXPOSURE WITH DOSE IN OLDER VS. YOUNGER BEAGLE DOGS: THE POTENTIAL ROLE OF DIFFERENCES IN BILE SALT CONCENTRATION, THERMODYNAMIC ACTIVITY AND FORMULATION DIGESTION

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Manuscript in submission

4 Non-linear increases in danazol exposure with dose in older versus younger beagle dogs: the potential role of differences in bile salt concentration, thermodynamic activity and formulation digestion

4.1 ABSTRACT

Previous studies have shown that increasing the drug load in lipid-based formulations of danazol results in increased bioavailability following oral administration to beagle dogs. The current study has explored this relationship in more detail, including the possibility that age-related changes in physiology may result in differences in bioavailability across older and younger dog cohorts. Danazol absorption from SEDDS formulations containing increasing drug loads was examined in two dog cohorts, one younger (8 months) and one older (8 years). Hepatic function in both cohorts was assessed via monitoring changes in serum bile acid concentrations, and in vitro lipolysis experiments were performed to evaluate the impact of potential differences in lipolytic enzyme activity and intestinal bile salt concentration on drug solubilisation.

Linear increases in drug exposure with increases in drug dose were evident in the younger animals. In contrast, in the older cohort, bioavailability increased with increasing dose up to a tipping point (21 mg/g in the formulation), beyond which bioavailability reduced and returned to values more consistent with that observed in the younger cohort. No differences in hepatic function were apparent across the two animal cohorts, as assessed using the serum bile acid assay. Similarly, in vitro studies showed that decreasing the quantity of enzyme extract employed in the lipolysis studies had little impact on drug precipitation, and was therefore unlikely to explain the differences in exposure observed in the two groups of animals. In contrast, enzymatic

digestion studies showed that supersaturation at higher drug loads was more readily supported at higher bile salt concentrations, consistent with the in vivo data in the older animals.

The studies illustrate the complexity of interpretation of dose-linearity studies from solubilised formulations and suggest that differences in animal cohort can have a significant impact on absorption. At least in the case of danazol, bioavailability appears to be enhanced, under some circumstances, in older animals. In vitro experiments suggest that this may be explained by an increase in luminal [BS/PL] in these animals, resulting in more robust solubilisation, increased supersaturation and elevated exposure.

4.2 INTRODUCTION

After oral administration, the absorption of drugs with intrinsically poor water-solubility is often low, reflecting slow drug dissolution and incomplete solubilisation in the gastrointestinal (GI) fluids.[72] Several formulation approaches have been explored as a means to overcome these limitations[5], and lipid-based drug delivery systems (LBDDS) represent one method that has proven highly effective in enhancing the oral bioavailability of poorly water-soluble, lipophilic drugs.[7,74,78,178]

In most cases, LBDDS present drug to the GI tract in a molecularly dispersed, solubilised state thereby circumventing traditional dissolution (the exception being lipid suspension formulations). LBDDS also enhance drug solubilisation via stimulation of bile salt secretion and the formation of mixed colloidal species in the GI tract comprising exogenous (i.e. formulation derived) and endogenous (bile salts, phospholipids) lipids.[64,89,92,164] The majority of LBDDS formulations are also digested after oral administration, and digestion of the lipids and surfactants present in the formulation commonly results in a reduction in solubilisation capacity.[67,92,115,164,165] Where digestion results in a loss in solubilisation capacity, supersaturation usually eventuates. The

degree of induced supersaturation is dose-dependent and expected to increase with increasing drug load in the formulation.[179] Supersaturation may promote absorption via an increase in the thermodynamic activity of solubilised drug, however, the metastable supersaturated state also increases the likelihood of drug precipitation, which may reduce drug absorption (by re-introducing the need for dissolution from precipitated drug particles). The potential for a reduction in drug absorption on precipitation may be attenuated in situations where drug phase-separates in the amorphous form, but in most cases, the performance of LBDDS is expected to be dictated by the solubilisation capacity, the degree of supersaturation and the ability of the formulation to maintain supersaturation for sufficient time to allow for drug absorption.[107,179]

In a previous study[179], we examined the impact of the addition of a polymeric precipitation inhibitor (PPI), hydroxypropyl methylcellulose (HPMC), to a danazol-containing self-emulsifying LBDDS formulation, as a means of stabilising supersaturation, reducing precipitation and promoting absorption. The PPI had a marked impact on supersaturation stabilisation in vitro, however, in vivo effects were more moderate. As part of the same study, it was also observed that increasing the dose of drug in the formulation resulted in an increase in bioavailability on oral administration. Interestingly, this occurred in spite of the traditional paradigm of drug absorption that suggest that increasing the dose of a poorly water-soluble drug is expected to reduce absorption (since the mass of drug that must be dissolved increases).[179]

The current study was therefore initiated to explore in more detail the mechanism(s) by which increasing drug loading resulted in increased danazol bioavailability following oral administration to beagle dogs. The working hypothesis that underpinned these investigations was that the increase in danazol bioavailability on increasing drug dose was a result of either an increase in thermodynamic activity in the formulation (and ultimately in the colloidal species formed by formulation digestion), or a decrease in first pass metabolism (following saturation of first pass events). As part of this investigation, it became evident that the non-linearity in danazol

bioavailability observed previously[179], was dependent on the animal cohort in which the study was conducted and was only apparent after administration of higher doses to an older group of animals. The current studies therefore also explored the possibility that age-related changes in physiology may result in differences in bioavailability across the two dog cohorts. In particular, the potential for differences in hepatic function, lipolytic enzyme activity and intestinal bile salt concentration have been addressed in an attempt to explain the in vivo data obtained.

4.3 MATERIALS AND METHODS

4.3.1 Materials

Danazol (pregna-2,4-dien-20-yno[2,3-d]isoxazol-17-ol) was supplied by Sterling Pharmaceuticals (Sydney, Australia) and Coral drugs PVT (New Delhi, India) and progesterone was from Sigma-Aldrich (St Louis, MO, USA). Captex 300, a medium-chain triglyceride (MCT), and Capmul MCM, a blend of medium-chain mono-, di-, and tri-glycerides, were donated by Abitec Corporation (Janesville, WI, USA). Cremophor EL (polyoxyl 35 castor oil), sodium taurodeoxycholate 97% (NaTDC) and porcine pancreatin (8 x USP specification activity) were from Sigma Aldrich (St Louis, MO, USA). Lipoid E PC S, (Lecithin from egg, approximately 99% pure phosphatidylcholine) was from Lipoid GmbH (Ludwigshafen, Germany), 4-bromophenylboronic acid (4-BPB) was obtained from Sigma Aldrich (St Louis, MO, USA) and 1 M sodium hydroxide, which was diluted to obtain 0.6 M NaOH titration solution, was purchased from Merck (Darmstadt, Germany). Water was obtained from a Milli-Q (Millipore, Bedford, MA, USA) purification system. All other chemicals and solvents were of analytical purity or high performance liquid chromatography (HPLC) grade.

4.3.2 Preparation of SEDDS formulations containing danazol

A type IIIA SEDDS (SEDDS-III) was used in all studies. The formulation was based on medium-chain (MC) lipids and comprised 30% (w/w) Captex 300, 30% Capmul MCM, 30% Cremophor EL (as surfactant) and 10% ethanol (as cosolvent). All formulations were prepared as previously described[13] and contained danazol as a model poorly water-soluble drug. Danazol is a synthetic steroid originally developed to treat endometriosis and has an aqueous solubility of 0.59 ug/ml[144] and a log *P* of 4.53[143]. The drug was incorporated into the SEDDS-III at various drug loadings (mg/g) (tabulated in Table 4.1)**,** representing different proportions of saturated solubility in the formulation (based on the measured equilibrium solubility determined at 37°C) as previously described[179].

Table 4.1 Danazol solubility in SEDDS-III and the corresponding drug load and treatment doses utilized in in vivo and in vitro studies.

Saturation level [%]	15	30	40	60	80	90	100 [a]
Danazol [mg/g][b]	4.5	9.1	12.1	18.2	24.3	27.3	30.3
Treatment dose [mg][c]	7.3	14.5	19.4	29.1	38.8	43.6	-

[a] Saturated solubility of danazol in the MC SEDDS-III formulation comprising 60% lipid, 30% Cremophor EL and 10% ethanol Anby et al[179]

[b] Quantity of danazol dissolved in the SEDDS-III formulation

[c] Total dose administered in two gelatine capsules containing 800 mg SEDDS-III formulation each

4.4 BIOAVAILABILITY STUDIES IN BEAGLE DOGS

4.4.1 Study Design

A previously published study[179] (referred to here as the 'pre-study' for clarity) showed evidence of non-linear increases in danazol exposure after oral administration to beagle dogs of a MC SEDDS

formulation comprising 30% (w/w) Captex 300, 30% Capmul MCM, 30% Cremophor EL and 10% ethanol and increasing quantities of danazol (SEEDDS-III). In this pre-study, danazol was administered in two capsules each containing 800 mg of the SEDDS-III formulation containing danazol dissolved at either 40% or 80% of danazol saturated solubility in the formulation (equivalent to 12 mg/g and 24 mg/g, respectively).[179] Bearing in mind the non-linearity in exposure in this pre-study, but realising that data was only obtained at two dose levels, the current study sought to initially expand the previous study and to evaluate the potential for dose linearity/non-linearity across a wider dose range. In the first dog study described herein (study I), animals were therefore administered the same MC SEDDS-III formulation that was used in the pre-study, but with danazol incorporated (nominally) at 5 mg/g, 9 mg/g, 12 mg/g, 18 mg/g, 24 mg/g and 27 mg/g (equivalent to 15%, 30%, 60% and 80% of the saturated solubility in the formulation, respectively). Surprisingly, however, in this repeat study no evidence of non-linearity with dose was apparent. Since study I was conducted in a separate (and younger) cohort of animals than that employed in the pre-study[179], a second study (study II) was conducted in the original (older) beagle cohort to explore whether the non-linearity observed in the pre-study was specific to the older dog cohort. In study II, the older animal cohort was therefore administered the same MC SEDDS-III formulation, with danazol dissolved (nominally) at 5 mg/g, 9 mg/g, 18 mg/g and 27 mg/g (equivalent to 15%, 30%, 60% and 90% saturation, respectively).

4.4.2 Administration, Sampling and Analysis

Both studies (Study I and Study II) were conducted as four-way crossovers in male beagle dogs (13-23 kg) (with a 7-days washout period). Treatments were hand filled into gelatine capsules 2 h prior to dosing as previously described.[103] Each treatment was administered in two capsules (2 x 800 mg formulation) with approximately 50 mL water. Treatments were based on a SEDDS-III as described above and danazol was incorporated at various drug loadings with specific dosing data

tabulated in Table 4.9 and Table 4.10. Dogs were fasted for at least 20 h prior to dosing and remained fasted until 10 h post-dosing after which they were fed on a daily basis. Water was available *ad libitum* during the study.

Blood samples of approximately 3 ml were collected before dosing and at 0, 15, 30, 45, 60, and 90 min, then at 2, 3, 4, 6, 8 and 10 h post-dosing. Samples were collected via an indwelling catheter inserted in the cephalic vein prior to dosing and additional samples were obtained by individual venipuncture at 24, 32 and 48 h post-dose. Blood samples were collected into 4 mL tubes containing dipotassium EDTA. Plasma was separated within 2 h by centrifugation for 10 min at 1,328 x *g* (Eppendorf 5702 R/A-4-38 centrifuge, Eppendorf AG, Hamburg, Germany) and stored at -80°C until samples analysis. Danazol concentrations in plasma were quantified by LC-MS as described previously.[179]

4.5 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 area under the plasma concentration vs. time profiles (AUC_{0-10}) was calculated using the linear trapezoidal method. Because the danazol plasma concentrations were typically below the limit of quantification of the assay at 24, 32 and 48 h post-dose, accurate determination of the terminal elimination rate constant and ($AUC_{0-\infty}$) was not possible. However, the danazol plasma concentrations at 10 h were low and the extrapolated AUC ($AUC_{10-\infty}$) was therefore expected to contribute only a minor proportion of the total AUC ($AUC_{0-\infty}$). Relative bioavailability comparisons were therefore performed using (AUC_{0-10}). 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.

4.6 LIVER FUNCTION ASSESSMENT IN BEAGLE DOGS

To assess whether the differences in dose linearity in the two animal cohorts was a result of differences in liver function that in turn resulted in a difference in hepatic clearance, a measure of liver function was obtained via quantification of serum bile acid levels pre- and post-prandially. Serum bile acid levels are routinely utilised as a liver function test in dogs.[180-182] The test is based on the understanding that the presence of food in the GI tract stimulates the release of bile salts from the gall bladder into the intestine and that these bile salts are then absorbed (largely via active transport) in the lower small intestine. This stimulates a transient increases in serum bile acid levels.[13,180-183] Where liver function is normal, however, serum bile salt levels remain relatively constant post-prandially due to rapid and efficient bile salt uptake into the liver. In contrast, in animals with reduced hepatic function, hepatic uptake of serum bile acids is reduced leading to increased serum bile acid levels.[184-186]

To test liver function, animals in both cohorts were fasted for at least 20 h with free access to drinking water prior to the test. Blood samples were collected via an indwelling catheter inserted in the cephalic vein pre-prandially (just prior to feeding) and post-prandially (2 h after being fed a small meal (~85 g) of standard canned dog food). Blood samples (approximately 2 ml) were collected into 3 mL vacutainer tubes (no clotting agent). Serum was analysed using an enzymatic, colorimetric test for total serum bile acids (Randox Laboratories Limited, Crumlin, UK).

4.7 IN VITRO EVALUATION

4.7.1 Drug solubilisation during formulation dispersion and digestion

To explore the impact of increased drug loading on the potential for drug supersaturation and/or precipitation during processing of the formulations in the GI tract, drug solubilisation patterns in

vitro were assessed using a previously described in vitro model of lipid digestion.[179] Formulations containing danazol at differing levels of saturated solubility in the formulation were utilised as described in Table 4.1, and the in vitro solubilisation/precipitation behaviour was examined as described previously.[179] Briefly, 1 g of each formulation was first dispersed in 36 g of digestion medium (50 mM TRIS maleate, 150 mM NaCl, 5 mM $CaCl_2.2H_2O$, pH 7.5) containing bile salt (BS) and phospholipids (PL) (as phosphatidylcholine) at either low (BS: 5 mM NaTDC, PL: 1.25 mM) or high (BS: 20 mM NaTDC, PL: 5 mM) concentrations ([BS/PL]), at 37°C for 30 min. After 30 min, digestion was initiated by addition of 4 mL of pancreatin extract containing 40,000 TBU (to provide approximately 10,000 TBU per mL of extract and approximately 1000 TBU per mL of digest) of pancreatic lipase. Digestion was followed for a subsequent 60 min period using a pH-stat titration unit (Radiometer, Copenhagen, Denmark) that maintained the pH at 7.5 via titration of liberated fatty acids with 0.6 M NaOH. Aliquots (4.2 mL) were taken from the dispersion/digestion media throughout the 90 min experimental period at t = 10, 30, 40, 50, 60 and 90 min. Lipid digestion inhibitor (4-BPB, 9 µL of a 0.5 M solution in methanol per mL of dispersion/digestion medium) was added to each sample immediately after sampling to prevent further lipolysis.[89,171] Samples were centrifuged and phase separated as previously described[179] and each phase was assayed for danazol content by HPLC.

4.7.2 Solubility in aqueous phase pre-digestion (dispersion phase) (AP_{DISP})

The solubility of danazol in dispersed emulsified blank (i.e. drug-free) formulation was evaluated as described previously.[179]

4.7.3 Solubility in aqueous phase during digestion (AP_{DIGEST})

The solubility of danazol in the aqueous colloidal phase generated by digestion of blank (i.e. drug-free) formulation was evaluated after 5 and 60 min digestion as described previously.[179]

Conditions for digestion of the blank formulation were as described above and digestion samples were ultracentrifuged and the aqueous phase separated prior to measurement of drug solubility.

Quantification of danazol in in vitro experiments by HPLC

Aqueous phase samples obtained during dispersion experiments and after initiation of digestion were diluted 1:10 (v/v) with acetonitrile before HPLC analysis. Samples of danazol in the pellets (precipitate) from the digestion studies were first dissolved in 5 mL of chloroform/methanol (2:1 v/v) and subsequently diluted 1:10 (v/v) in acetonitrile prior to analysis by HPLC as described previously.[179]

4.8 DATA ANALYSIS FOR IN VITRO EXPERIMENTS

4.8.1 Solubilisation and supersaturation during in vitro experiments

The impact of drug loading on the ability of the formulation to maintain danazol in a metastable, supersaturated state during dispersion and digestion experiments was assessed using solubilisation/precipitation profiles as described previously.[179]

The ability of the formulation to maintain supersaturation during dispersion and digestion was expressed as a supersaturation ratio, S (eq. 4.1). S was determined as the solubilised drug concentration in the AP during dispersion (AP_{DISP}) and digestion (AP_{DIGEST}) divided by the solubility of danazol in AP_{DISP} and AP_{DIGEST}.

$$S = \frac{\text{Solubilised drug conc. in } AP_{DISP \text{ or } DIGEST}}{\text{Drug solubility in } AP_{DISP \text{ or } DIGEST}} \qquad \text{(Equation 4.1)}$$

Note that in the previous publication[179], AP_{DISP} or AP_{DIGEST} was used to describe the solubility in the aqueous phase during dispersion or digestion. We have since progressed to use this term more broadly to simply indicate the aqueous phase in general. Thus 'AP_{DISP}' as used in Anby et al[179] becomes 'drug solubility in AP_{DISP}' here. 'Solubilised drug conc. in AP_{DISP}' is used to make a distinction between the drug concentration measured kinetically during the dispersion and the equilibrium solubility of drug in the equivalent phase ('drug solubility in AP_{DISP}'). Analogous changes have been made to the terminology used to describe drug concentrations measured in the AP during digestion and the equivalent solubility measurements.

To allow representation of drug solubility in the AP_{DIGEST} in the current plots, the change in solubility (during the course of digestion) was assumed to be linear between 5 min and 60 min post digestion.

4.9 RESULTS

In vivo evaluation

4.9.1 Effect of increasing dose/thermodynamic activity on danazol exposure in vivo

The mean plasma concentration versus time profiles for danazol following oral administration of the MC lipid SEDDS formulation (SEDDS-III) containing danazol at 5-27 mg/g (15-90% of saturation in the formulation) administered to a relatively young (mean age; 9 months) cohort of fasted beagles (Study I) is shown in Figure 4.1A. The corresponding mean pharmacokinetic parameters are summarized in Table 4.2 and show a dose-proportional increase in maximum danazol concentration (C_{max}). The mean T_{max} was 1.2 ± 0.2 h and the danazol half-life was 5.9 ± 1.2 h. The relationship between exposure (AUC) and administered dose is presented in Figure 4.2. The non-

linearity with dose observed previously[179] was not evident in this younger cohort (Study I), and the study was therefore repeated in the original cohort used in the pre-study.[179] This group of animals was considerably older (mean age 8 ± 1.0 yrs.) The mean plasma concentration versus time profiles for danazol following oral administration to this older cohort (Study II) is shown in Figure 4.1B (along with the data obtained in the previously published study). The corresponding mean pharmacokinetic parameters are summarized in Table 4.3 showing non-linear increases in C_{max}, a mean T_{max} of 1.6 ± 0.3 h and a danazol half-life of 3.8 ± 0.5 h across doses.

A comparison of dose-response relationship is shown in Figure 4.2A where the area under the plasma concentration versus time curves in Figure 4.1A and B are plotted against dose administered. For the younger cohort (closed symbols), a linear relationship was obtained indicative of dose independent pharmacokinetics, and is consistent with a previous study[187] in humans where danazol was administered in a lipid emulsion formulation in the dose range 50-200 mg (equivalent to ~0.7-3 mg/kg). The dose-response curve for the older cohort, however, displayed marked deviations from linearity where drug was incorporated in the formulation at concentrations higher than 11 mg/g (equivalent to 40% of the saturated solubility in the formulation).

Figure 4.2B shows the relative bioavailability for the two cohorts based on the lowest absolute dose in the older beagle cohort. In the young cohort, the relative bioavailability was constant across all doses. In contrast, in the old cohort the relative bioavailability increased with increasing dose leading to a more than 2-fold increase in the relative bioavailability at a 21 mg/g dose (70% saturation in the formulation). Above this dose, however, exposure reduced significantly and approached that obtained in the younger cohort at the same dose. This trend in exposure was found to be consistent across the older beagle cohort (the relative bioavailability in individual dogs (older cohort) compared to the younger cohort following administration of increasing danazol doses can be found in the supporting information, Figure 4.9).

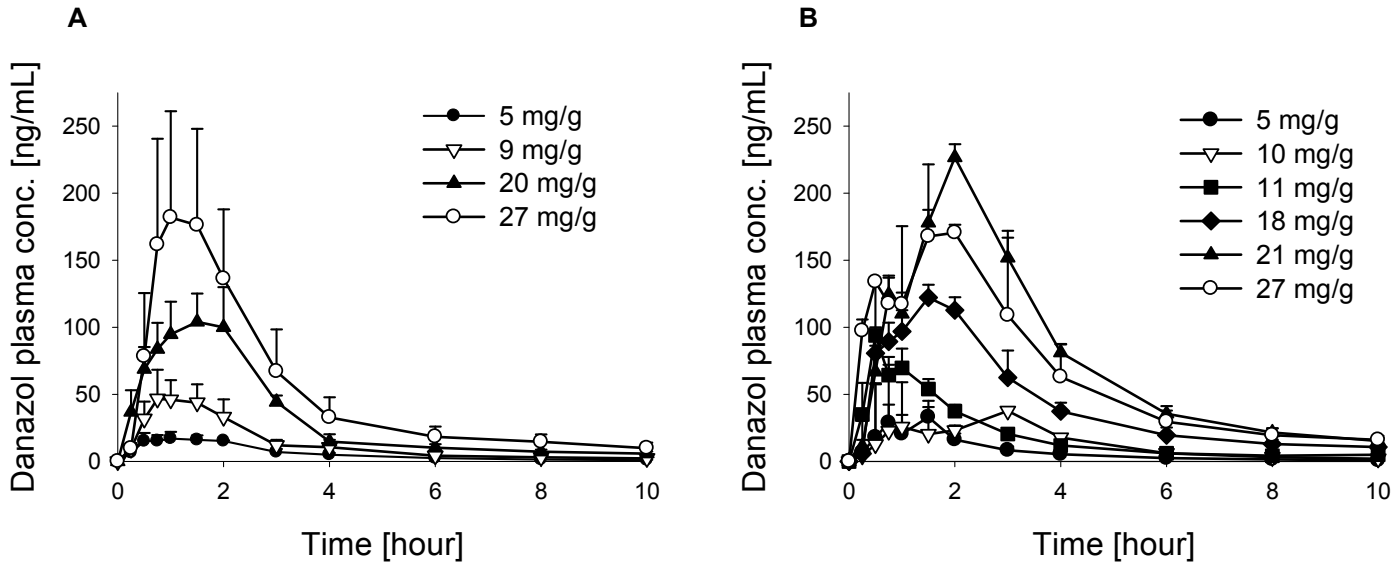


Figure 4.1 Mean plasma conc. vs time profiles for danazol after oral administration of a SEDDS-III containing [60/30/10% w/w] of [lipid/CrEL/ethanol]. SEDDS-III administered to A: a young beagle cohort (study I) with danazol loading of 5 mg/g (●), 9 mg/g (▽), 20 mg/g (▲) and 27 mg/g (○) (doses and equivalent saturation levels are tabulated in Table 4.2 [mean ± SEM (n = 4)], and B: to an older cohort (pre-study and study II) with danazol doses of 5 mg/g (●), 10 mg/g (▽), 11 mg/g (■)[a], 18 mg/g (◆), 21 mg/g (▲)[a] and 27 mg/g (○), respectively (doses and equivalent saturation levels are tabulated in Table 4.3 [mean ± SEM (n = 3)]. [a] Data reproduced from Anby et al[179].

Table 4.2 Summary pharmacokinetic parameters for danazol after administration in the SEDDS-III formulation comprising increasing drug loadings to the young beagle cohort (study I) [mean ± SEM (n = 4)] (Corresponding plasma profiles are presented in Figure 4.1A).

	SEDDS-III formulation treatments in younger beagle dogs			
Danazol loading [mg/g]	5	9	20	27
Treatment dose [mg] [a]	8.5	14.5	32.1	43.2
$AUC_{0\text{-}10h}$ (ng.h/mL)	59 ± 12	132 ± 46	321 ± 69	454 ± 128
Relative BA (%) [b]	70 ± 14	97 ± 36	102 ± 22	106 ± 21
C_{max} (ng/mL)	24 ± 4	53 ± 20	107 ± 21	168 ± 33
T_{max} (h)	1.3 ± 0.3	1.1 ± 0.2	1.4 ± 0.1	1.1 ± 0.2
$t_{1/2}$ (h)	5.4 ± 1.8	4.9 ± 0.5	6.8 ± 0.8	6.6 ± 1.7

[a] Each treatment was administered in two capsules (2 x 800 mg formulation)

[b] Relative BA was the relative bioavailability (%) expressed in comparison to the danazol $AUC_{0\text{-}10}$ obtained after oral administration of SEDDS-III at the lowest absolute dose (5 mg/g in the older beagle cohort) as determined by the ratio of the dose-normalized $AUC_{0\text{-}10}$

Table 4.3 Summary pharmacokinetic parameters for danazol after administration in the SEDDS-III formulation comprising increasing drug loadings to the older beagle cohort (pre-study and study II) [mean ± SEM (n = 3)] (Corresponding plasma profiles are presented in Figure 4.1B).

	SEDDS-III formulation treatments in older beagle dogs					
Danazol loading [mg/g]	5	10	11 [a]	18	21 [a]	27
Treatment dose [mg] [b]	7.6	15.6	17.3	28.1	34.2	43.4
$AUC_{0\text{-}10h}$ (ng.h/mL)	75 ± 9	133 ± 12	194 ± 41	421 ± 51	762 ± 46	617 ± 15
Relative BA (%) [c]	100 ± 12	86 ± 8	114 ± 24	152 ± 18	226 ± 14	144 ± 4
C_{max} (ng/mL)	45 ± 13	42 ± 3	104 ± 30	150 ± 38	227 ± 10	211 ± 23
T_{max} (h)	1.1 ± 0.2	2.5 ± 0.5	0.8 ± 0.3	1.5 ± 0.5	2.0 ± 0.0	1.5 ± 0.5
$t_{1/2}$ (h)	2.7 ± 0.4	2.9 ± 0.7	4.4 ± 0.7	4.7 ± 0.5	3.3 ± 0.2	4.8 ± 0.4

[a] Data reproduced from Anby et al[179]

[b] Each treatment was administered in two capsules (2 x 800 mg formulation)

[c] Relative BA was the relative bioavailability (%) expressed in comparison to the danazol $AUC_{0\text{-}10}$ obtained after oral administration of SEDDS-III at the lowest absolute dose (5 mg/g in the older beagle cohort) as determined by the ratio of the dose-normalized $AUC_{0\text{-}10}$

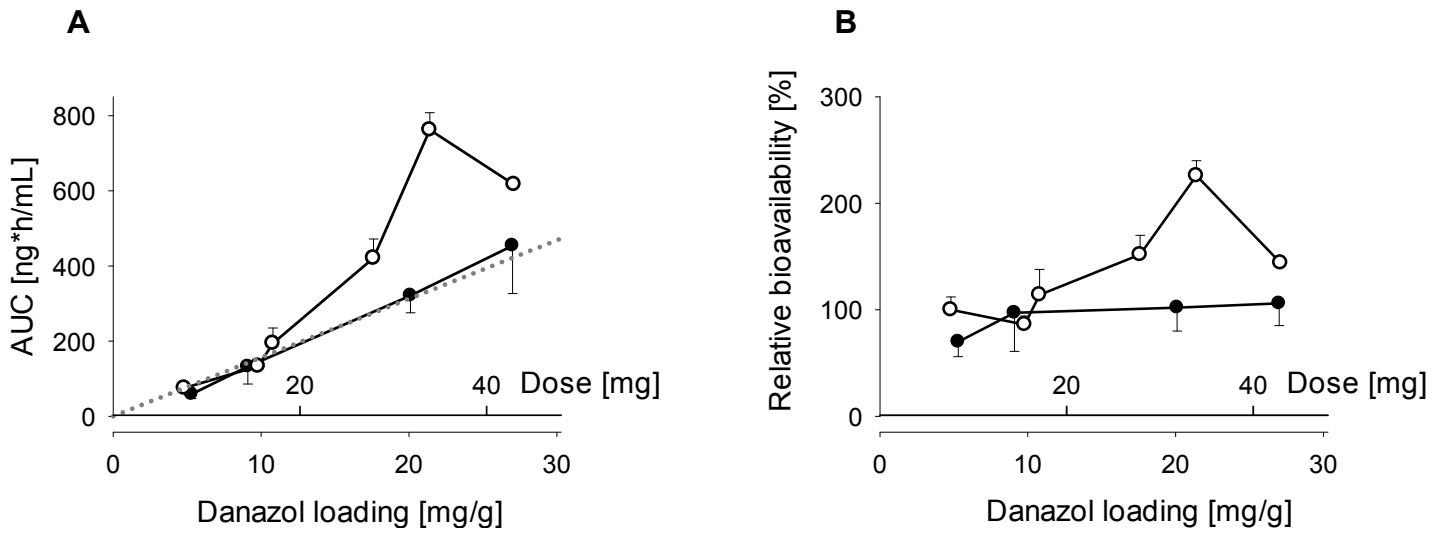


Figure 4.2 Dose linearity of (A) danazol exposure (AUC) and (B) Relative bioavailability after oral administration of increasing danazol doses (expressed both as absolute dose (mg) and drug concentration in the formulation (mg/g) in a type III MC SEDDS formulation to both a younger beagle cohort (●) [mean ± SEM (n = 4)] and an older beagle cohort (○)[mean ± SEM (n = 3)].

The appearance of dose dependency in danazol bioavailability after oral administration in the older beagle cohort, but not the younger cohort, prompted a closer examination of the possible explanations for these effects, and in particular, the potential for cohort (or age) dependent drivers of non-linearity. Two working hypotheses were suggested to explain these potential trends. Firstly, that the increases in exposure with increasing dose resulted from saturation of first pass metabolism (and that differences in metabolic capabilities were evident in the two cohorts). Secondly, that the increase in exposure at higher dose resulted from increases in thermodynamic activity in the colloids formed during digestion of formulations containing higher drug loads (and that more robust solubilising conditions were prevalent in the intestinal tract of the older animals, allowing ongoing solubilisation of the supersaturated solutions).

4.9.2 Evaluation of liver function in young versus old beagle cohorts

To provide an indication of potential differences in metabolic function in the two cohorts, liver function tests were performed in both sets of animals. To assess liver function, the serum bile acid (SBA) test was conducted under pre-prandial and post-prandial conditions. Figure 4.3 illustrates the SBA levels for the two cohorts. Pre-prandial SBA levels of 4.9 ± 0.9 µmol/L and 7.5 ± 4.2 µmol/L and post-prandial levels of 4.8 ± 2.8 µmol/L and 10.3 ± 4.5 µmol/L were obtained for the young and old cohort, respectively. The data are consistent with previously reported SBA values for dogs under similar conditions.[181,186,188] No significant differences in liver function were evident between the two cohorts, although SBA levels were, in general, insignificantly higher in the older dogs and showed higher variability than the equivalent data for the younger animals.

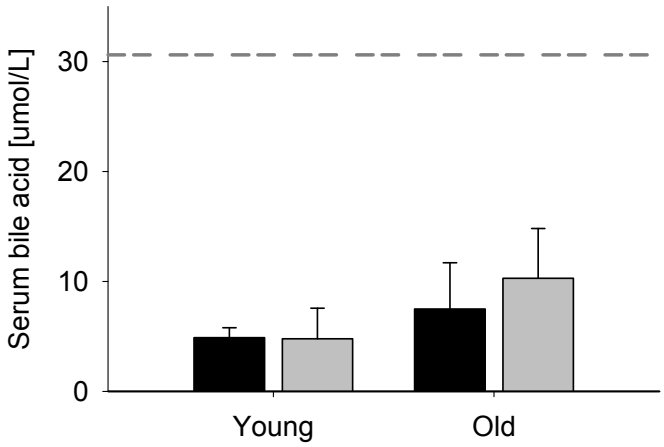


Figure 4.3 Serum bile acid (SBA) concentrations measured pre-prandially (black bars) and post-prandially (2 h) (grey bars) in two beagle dog cohorts. Mean SBA levels are shown for the young [mean ± SD (n = 4])] and old cohort [mean ± SD (n = 3]). The upper dashed line represents the reference value for normal liver function post-prandial conditions. No statistical significant difference was evident between old and young beagle cohort.

Unfortunately, the advanced age of the older animals dictated that ethics approval to conduct further studies to explore this in more detail could not be obtained. As such, experiments were conducted in vitro to explore possible differences in drug solubilisation/precipitation (and accompanying differences in supersaturation and thermodynamic activity) as a function of (i) drug dose, and (ii) differing intestinal conditions (that might be expected to vary in differing cohorts) such as differences in endogenous bile salt concentrations or lipase levels in the GI tract (the latter might be expected to impact solubilisation capacity due to differential hydrolysis of digestible excipients).

4.10 IN VITRO EVALUATION

4.10.1 Impact of increasing drug loading on solubilisation during in vitro dispersion and digestion at low and high bile salt levels

To evaluate the impact of drug saturation in the SEDDS-III formulation on danazol solubilisation patterns following dispersion and digestion of the formulation, a series of studies were conducted using a previous described in vitro lipid digestion model.[179] These experiments sought to explore the impact of increasing drug load in the formulation on drug precipitation during dispersion and digestion and in particular, the potential for supersaturation stabilization. Experiments were conducted in biorelevant digestion medium containing low [BS/PL] (i.e. 5 mM BS: 1.25 mM PC) and high [BS/PL] (i.e. 20 mM BS: 5 mM PC) to explore the potential for increased bile salt levels in either dog cohort to explain the in vivo data obtained. Data were obtained using SEDDS-III formulations at a danazol loading of 5-27 mg/g (equivalent to 15% - 90% of danazol saturation in the formulation).

To gain a better understanding of the drivers of drug precipitation during dispersion and digestion and to quantify the degree of supersaturation during formulation processing, the apparent solubility of danazol in the colloidal aqueous phase (AP) generated by dispersion (AP_{DISP}) and after 5 min and 60 min digestion (AP_{DIGEST}) of a blank (drug free) formulation was measured. The data are tabulated in Table 4.4 and show that initiation of formulation digestion led to a significant decrease in solubilisation capacity, regardless of BS/PL concentration in the digestion medium. Within 5 min of digestion initiation, danazol solubility in the AP_{DIGEST} decreased > 60% when compared to solubility in AP_{DISP}. As digestion continued to 60 min, danazol solubility in AP_{DIGEST} under low [BS/PL] decreased a further 2-fold compared to the solubility at 5 min. In contrast, under high [BS/PL], continued digestion (to 60 min) only led to a further ~10% decrease in

danazol solubility in AP_{DIGEST}. Significant differences in danazol solubility in the AP at low and high [BS/PL] were evident at each time point, but were most obvious at 60 min post digestion.

The impact of drug loading in the SEDDS-III formulation on kinetic changes to danazol solubilisation during dispersion and digestion (rather than solubility in the phases formed) under low [BS/PL] are presented in Figure 4.4A with the dotted line illustrating the solubility in the AP over time.

Table 4.4 Danazol solubility in the aqueous colloidal phase post dispersion or during digestion of the drug-free SEDDS-III formulation [mean ± SD (n = 3)].

	Low bile salt [5 mM][a]	High bile salt [20 mM]
Solubility in AP_{DISP} [ug/mL] [b]	301 ± 3.9	331 ± 6.0 [f]
Solubility in AP_{DIGEST} (5 min) [ug/mL] [c]	106 ± 3.6 [d]	120 ± 3.2 [df]
Solubility in AP_{DIGEST} (60 min) [ug/mL] [c]	56 ± 0.9 [de]	109 ± 7.9 [df]

[a] Data reproduced from Anby et al[179]
[b] The solubility of danazol in the AP formed by dispersion of blank (drug-free) formulation for 10 min
[c] The solubility of danazol in the AP formed by digestion of blank (drug-free) formulation for 5 or 60 min
[d] Statistically significant difference compared to AP_{DISP} ($P < 0.050$)
[e] Statistically significant difference compared to AP_{DIGEST} (5 min) ($P < 0.050$)
[f] Statistically significant difference compared to low bile salt [5 mM] ($P < 0.050$)

Dispersion of SEDDS-III formulations containing danazol at 5 mg/g and 9 mg/g resulted in AP concentrations below the danazol solubility limit in AP_{DISP} (time - 20 to 0 min in Figure 4.4A) and therefore, no drug precipitation was evident on dispersion. Initiation of digestion led to rapid changes to the nature of the colloids present in the aqueous phase as illustrated by the decrease in danazol solubility in AP_{DIGEST} (Table 4.4). However, the moderate drug load of 5-9 mg/g in the SEDDS-III formulations resulted in relatively low drug concentrations in AP_{DIGEST}, and therefore limited precipitation on digestion. In the case of SEDDS-III containing 5 mg/g, the concentrations attained in the digest were approximately equivalent to the danazol solubility (hence the alignment of the dotted line and the black line in Figure 4.4A. The concentrations attained in the AP_{DIGEST} for SEDDS-III at 9 mg/g were approximately 2-fold higher than the danazol solubility in

AP_{DIGEST}, however, supersaturation was well maintained over the 60 min digestion period and precipitation was limited. Increasing the drug loading to 12 mg/g and 18 mg/g in SEDDS-III initially led to danazol concentrations above solubility in $AP_{DISP,}$ but the degree of supersaturation generated did not lead to drug precipitation. On digestion, however, the drop in solubilisation capacity of the AP (Table 4) resulted in more significant initial supersaturation and more rapid drug precipitation. This was faster at the higher drug loading (18 mg/g) compared to 12 mg/g and as a result, supersaturation was maintained for a shorter time period. Increasing the drug loading to 80% and 90% of solubility in the formulation resulted in greater drug precipitation on dispersion and limited supersaturation was evident on initiation of digestion.

Increasing the [BS/PL] in the digestion media (Figure 4.4B), resulted in moderate (but significant) differences in danazol solubility in the aqueous colloidal phase on dispersion and digestion (dotted line in Figure 4.4, Table 4.4). The increase in [BS/PL], therefore, promoted maintenance of supersaturation and reduced the extent of precipitation. Thus, at raised [BS/PL], no drug precipitation was evident on dispersion regardless of drug loading in SEDDS-III in contrast to the data at low [BS/PL]. In addition, no drug precipitation was evident and supersaturation was maintained during the 60 min digestion period for SEDDS-III containing 12 mg/g and precipitation was reduced and delayed for SEDDS-III containing danazol at up to 18 mg/g. The significant decrease in solubilisation capacity on initiation of digestion did lead to rapid precipitation for SEDDS-III when drug loading was high (24-27 mg/g), although some degree of supersaturation was maintained up to and beyond 20 min post digestion. In general, therefore, the significantly higher danazol solubility in the colloids formed under high [BS/PL] (Table 4.4), reduced the extent of saturation and resulted in the maintenance of higher danazol concentrations in AP_{DIGEST} when compared to low [BS/PL].

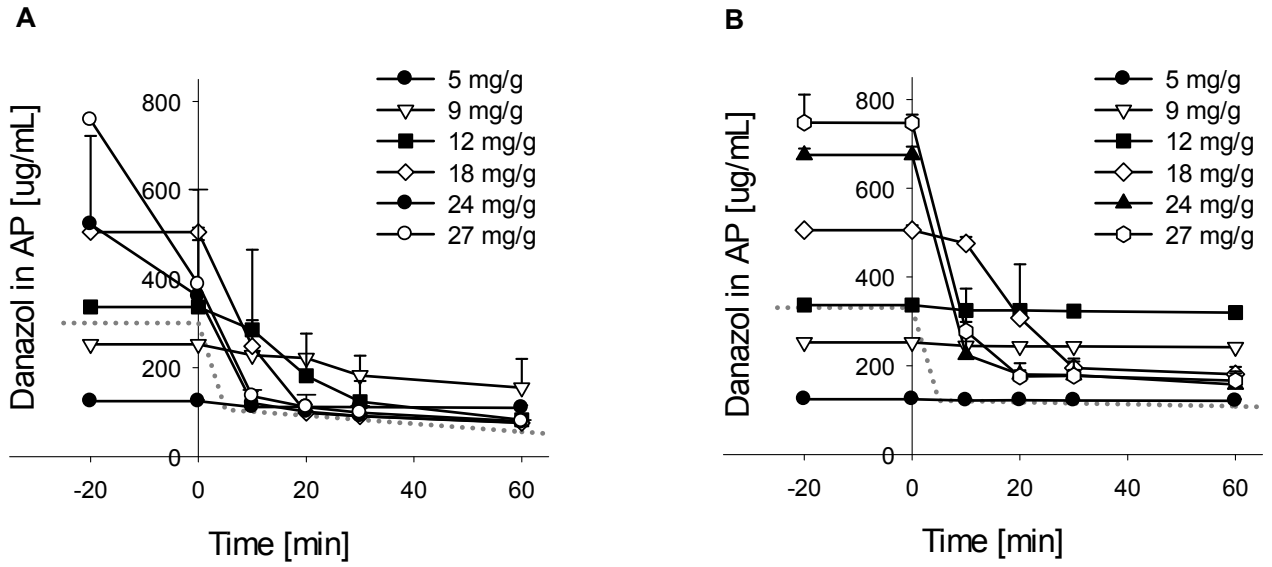


Figure 4.4 Drug solubilisation profiles during dispersion (-20 to 0 min) and digestion (0 to 60 min) of SEDDS formulations with danazol loading of; 5 mg/g (●), 9 mg/g (▽), 12 mg/g (■)[a], 18 mg/g (◇), 24 mg/g (▲)[a] and 27 mg/g (○) under A: low [BS/PL] (i.e. 5 mM BS: 1.25 mM PL), B: high [BS/PL] conditions (20 mM BS: 5 mM PL). The dotted line indicates the drug solubility in the aqueous colloidal phase produced on dispersion (AP_{DISP}) and digestion (AP_{DIGEST}) of drug-free SEDDS-III. All data presented as mean ± SD (n = 3). [a] Data reproduced from Anby et al[179].

4.10.2 Impact of pancreatic lipase activity on drug solubilisation during in vitro digestion

To simulate the potential for differences in intestinal lipid digestion (as a result of differences in pancreatic lipase secretion) in the two dog cohorts to affect drug precipitation from the SEDDS formulation, the impact of changes to the volume of pancreatic lipase added to the digestion vessel was explored. A SEDDS-III formulation containing 12 mg/g danazol was utilized during in vitro digestion experiments and the impact of decreasing pancreatic lipase extract concentrations on danazol solubilisation profiles is shown in Figure 4.5.

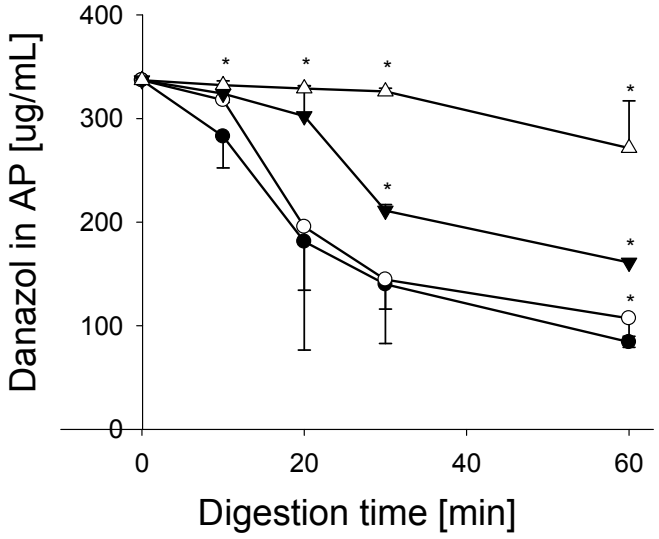


Figure 4.5 Drug solubilisation during 60 min in vitro digestion of a SEDDS-III formulations as a function of pancreatic enzyme concentration [mean ± SD (n = 3)]. Danazol loading was 12 mg/g (equivalent to 40% of the equilibrium solubility in SEDDS-III). The quantities of enzyme employed were 4000 µL (●)[a], 1000 µL (○), 400 µL (▼) and 100 µL (△) of pancreatic lipase extract, where 4 mL is the volume added under normal conditions. [a] Data reproduced from Anby et al.[179], [*]Statistically significant different compared to data obtained using 4000 µL lipase extract ($P < 0.050$).

Dispersion of SEDDS-III containing danazol at 12 mg/g did not result in drug precipitation as shown in Figure 4.4A, however, initiation of digestion with 4 mL of pancreatic enzyme extract led to significant precipitation and only transient supersaturation.

Figure 4.5 shows that decreasing the volume of pancreatic lipase extract added to the digest reduced drug precipitation during digestion, but that significant changes were only evident when the volume of lipase extract was reduced considerably (from 4000 µL to 100 µL). After 60 min digestion, drug precipitation was still evident after addition of only 100 µL lipase extract (equivalent to 10% of the original quantity), however, the degree of precipitation was much lower than that seen at higher enzyme levels.

4.11 DISCUSSION

Following oral administration, the properties of the colloidal species that are formed on dispersion of SEDDS formulations typically change as digestive enzymes hydrolyse included glyceride lipids and fatty acid ester surfactants. In most cases, these chemical changes lead to decreases in solubilisation capacity for co-formulated poorly water-soluble drugs.[65,67,96,114] Depending on the drug load in the formulation, this may result in the generation of transiently supersaturated conditions in the GI tract.[179] Supersaturation has the potential to enhance absorption via increases in thermodynamic activity, but may also reduce absorption by increasing the likelihood of drug precipitation. SEDDS performance under digesting conditions is therefore a balance between an increase in supersaturation (and thermodynamic activity) and absorption promotion, and the potential for highly metastable conditions to increase drug precipitation and reduce absorption. This is further complicated where drugs are substrates for first pass metabolism.

Increases in drug dose have an inherent impact on these processes since an increase in drug dose is expected to increase the degree of supersaturation generated on formulation digestion and also to increase the likelihood of inhibition of first pass metabolism. In this regard, previous studies of danazol absorption from a LFCS type III SEDDS (termed SEDDS-III here) comprising 60% MC lipid, 30% Cremophor EL and 10% ethanol revealed non-linear increases in exposure (bioavailability) with increasing drug dose in beagle dogs.[179] This prompted the current, more detailed, investigation of the impact of drug dose on danazol absorption using the same formulation. Initial studies revealed that these effects were highly dependent on the dog cohort, and the age difference in the two groups of animals employed suggested the potential for age-related differences in absorption or bioavailability. As such further studies were performed to probe the potential for age-related changes to physiology to lead to differences in danazol exposure.

4.11.1 Effect of drug dose on in vivo exposure in older vs. younger beagles

The plasma profiles in Figure 4.1 and the pharmacokinetic parameters presented in Table 4.2 and 4.3 reveal lower exposure and a dose-dependent relationship with AUC for the younger beagle cohort with constant relative bioavailability evident across a range of drug doses (Figure 4.2). This is in contrast to the previous studies conducted using an identical formulation in an older group of animals where increases in bioavailability were seen with a two-fold increase in dose [179]. This prompted an extended evaluation of the dose-response in the first (older) cohort, in an attempt to better understand the non-linearity observed (Figure 4.2). In the older cohort, deviations from linearity in dose versus exposure relationships were apparent after administration of formulations containing danazol at concentrations above 12 mg/g in the formulation (the mass of SEDDS-III formulation was constant across all studies so increases in saturation level in the formulation resulted in increases in drug dose) (Figure 4.2A). Relative bioavailability increased with dose until

a 'tipping point' was reached at doses equivalent to concentrations of 21 mg/g danazol (equivalent to 71 % of drug solubility in the formulation) in the SEDDS-III formulation. Above this, relative exposure dropped significantly and approached that obtained in the younger cohort (Figure 4.2B).

The variation in exposure as a function of dose and animal cohort, and the fact that the increase in exposure in the older cohort, was evident only to a critical point suggests the potential for multiple controlling factors. Increasing age is known to alter various physiological parameters and may change absorption and bioavailability. In particular, gastrointestinal changes are evident in older animals including reduced gastric acidity (although this might not occur in dogs[189] and gastric pH is often higher in dogs when compared to humans[190]), gastric emptying[191] and intestinal motility.[192,193] Changes in permeability of the intestinal tissue[194] and a reduction in renal and/or hepatic drug elimination have also been described with increases in age.[195,196]

Previous studies have also shown that oral administration of (^{14}C) labelled danazol to rats leads to significant (~70%) biliary excretion of danazol metabolites and extensive enterohepatic recycling.[177] In vitro studies utilizing pooled human liver microsomes and cytochrome P450 inhibitors further suggest that danazol is a substrate for CYP-enzymes, in particular CYP3A4.[197] A significant role for CYP-mediated metabolism in danazol clearance in dogs is therefore likely and is consistent with the potential for increases in danazol exposure with dose to result from saturation of first pass metabolism. It is less clear, however, why this might only be evident in the older animals (unless there are significant differences in hepatic function across the two cohorts), or why these effects are only evident up to a certain dose in the older animals.

An alternate explanation for the increase in absorption lies in the potential for increases in dose to increase thermodynamic activity in the colloidal species produced post digestion of the SEDDS-III formulation (through induction of supersaturation). The degree of supersaturation generated by dose escalation might also be expected to ultimately lead to spontaneous nucleation in the

supersaturated solution resulting in precipitation and reduced absorption (and bioavailability) at the highest doses. This suggestion is consistent with the trends in absorption with dose observed in vivo in the older cohort.

In this latter scenario, the differences in exposure in the two beagle cohorts might be explained by differences in intestinal conditions, such as differences in digestive enzyme levels or bile salt secretion, that lead to differences in solubilisation capacity and/or an altered capability to support supersaturation. In an attempt to clarify these possibilities, a series of in vitro and in vivo studies were therefore undertaken to explore the potential for differences in metabolism or intestinal solubilisation to explain the differences in exposure observed in the dog studies.

4.11.2 Differences in serum bile acid levels as an indicator of age-related changes to danazol metabolism

The potential for changes in liver function to lead to different degrees of pre-systemic hepatic metabolism in the two beagle cohorts was assessed via comparison of pre- and postprandial serum bile acid (SBA) levels. Many aspects of the hepatobiliary function are involved in bile acid metabolism, including bile acid synthesis by cytochrome P450 hydroxylation of cholesterol in the pericentral hepatocytes, bile acid conjugation with e.g. amino acids (i.e. glycine or taurine), and ultimately active secretion into bile.[183,198,199] Due to the structural similarities between danazol and the endogenous steroids involved in bile acid metabolism, differences in serum bile acid levels were utilised to provide an indicator of potential differences in microsomal hydroxylation and conjugation of danazol across the two cohorts.

The SBA test showed no significant differences in SBA levels pre- and post-prandially for either cohort, and SBA levels were within the range of previously published reference values.[181,186,188] Comparison between cohorts revealed an insignificant increase in SBA with age, and in particular identified a single dog in the older cohort that had a higher SBA level (albeit within normal levels)

compared to the other animals. However, this difference was not replicated across other animals, and although differences in serum bile acid levels have been reported in older versus younger rats, this was previously suggested to reflect differences in bacterial growth in the GI tract (and therefore changes to enterohepatic recycling) rather than differences in bile acid secretion.[200,201] Differences in GI bacterial growth have also been reported in aged dogs[202], however, it is unclear whether this has any impact on systemic danazol levels via differences in enterohepatic recycling, and little evidence of recycling was apparent in the oral profiles obtained here (Figure 4.2). Note that double peaks were seen in the early time periods of the mean plasma profiles in the older animals (Figure 4.2B), however, this reflected differences in T_{max} in individual dogs rather than the occurrence of double peaks in individual animals, thus unrelated to enterohepatic recycling.

The SBA data therefore suggest limited differences in liver function across the two cohorts and do not support the suggestion that the differences in dose linearity and bioavailability across the groups reflect differences in hepatic first pass metabolism. This is also consistent with the fact that danazol bioavailability at the highest dose was similar in both cohorts, a situation that would seem at odds with significant differences in first pass metabolic function. Differences in pre-hepatic, pre-systemic, metabolism may also play a role in differences in danazol disposition however, the lack of readily accessible biomarkers for enterocyte-based metabolic function precluded further assessment here.

4.11.3 Age-related changes to drug absorption from the gastrointestinal tract

Several studies have addressed the potential for age related physiological changes to alter drug absorption. These are well reviewed in Cusack[203], Fahey et al[204] and McLean & Le Couteur[205]. Physiological changes include changes to body weight, intestinal permeability, gastric pH, gastric emptying and gastric motility. Increases in body fat, for example, can lead to changes in volume of

distribution due to accumulation in the adipose tissue, thereby prolonging elimination half-life and exposure for lipid-soluble drugs.[203,205] In the current study, the older animal cohort was 5-10 kg heavier than the younger cohort and might therefore be expected to carry a higher proportion of body fat. The apparent half-life of danazol was, however, not significantly different in either group suggesting that differences in body weight were unlikely to explain the observed exposure differences.

Intestinal permeability has also been reported to increase for some drugs in older rats, due to age-related changes to membrane permeability in the enterocyte.[194] However, for a typical class II BCS drug like danazol, intestinal permeability is expected to be relatively high and for most substrates, passive intestinal permeability appears to be unchanged with age.[206,207] Increases in gastric pH, delays in gastric emptying and decreases in gastrointestinal motility are also commonly associated with increased age.[195] These changes might all be expected to alter absorption, and potentially increase absorption but are rarely clinically significant[203] and seem unlikely to explain the increase in bioavailability seen here at higher doses when compared to lower doses.

For poorly water-soluble drugs such as danazol, drug solubility in the GI fluids is expected to be the most significant determinant of drug absorption. Changes in *in situ* solubilisation are therefore likely to have the most significant impact on absorption. Indeed recent studies have reported age-related increases in danazol solubility in human GI fluid and coincident increases in intestinal bile salt concentrations (although the changes observed were not statistically significant due to high variability within groups[194]). In light of the importance of intestinal solubility in danazol absorption and the potential for increasing solubilisation properties and bile salt concentrations with age, a more detailed evaluation of the potential impact of changes to GI solubilisation capacity on danazol solubilisation during the digestion of SEDDS was undertaken. These studies focussed on the impact of potential changes to lipolysis and bile salt secretion in older animals since both are critical to patterns of drug solubilisation during the intestinal processing of a lipid based SEDDS.

4.11.4 Impact of lipase activity on drug solubilisation during SEDDS digestion in the GI tract

Following oral administration of SEDDS, the solubilisation capacity of the formulation (or the colloidal species generated by intercalation of the formulation components into the lipid digestion cascade) typically decreases as a result of lipid digestion. The degree of digestion (and potentially, therefore, the extent of solubilisation) is expected to be dependent on the quantity and activity of secreted pancreatic lipase (the main lipase responsible for lipid digestion in the GI tract).[85] To evaluate the impact of pancreatic lipase levels on digestion and subsequent drug solubilisation, the effect of changes to the quantity of pancreatic enzyme employed in an in vitro digestion model of SEDDS processing in the GI tract, was therefore explored. The data are summarized in Figure 4.5.

Under normal conditions, the specific activity of pancreatic lipase in the dog is high[208] and similar to that in humans.[145] In the current in vitro experimental protocol, replication of the lipase activities expected in the human (or canine) GI tract resulted in significant formulation digestion and danazol precipitation. In order to probe the potential impact of physiological changes in enzyme activity or in the quantity of enzyme secreted in the different animal cohorts, these studies were also conducted using decreasing quantities of enzyme extract. Decreasing the quantity of enzyme extract employed, however, had little impact on digestion or drug precipitation and very large decreases were required before appreciable differences in drug precipitation were evident. Lower lipase secretion in the older cohort, resulting in reduced lipid digestion and increased solubilisation capacity, is therefore unlikely to explain the higher danazol absorption seen in the older cohort. Indeed, the digestibility of protein and fat in beagle dogs has previously been reported to be significantly higher in older dogs when compared to weanlings.[209]

4.11.5 Impact of bile salt concentration on drug solubilisation in the GI tract

The influence of [BS/PL] on drug solubilisation and the kinetics of drug precipitation during formulation dispersion and digestion are shown in Figure 4.4. Two BS/PL conditions (low (5 mM BS: 1.25 mM PL) and high (20 mM BS: 5 mM PL)) were employed to provide a proof-of-concept indication of whether changes in intestinal bile salt concentrations (should they be evident across the two animal cohorts) were likely to explain the patterns of absorption seen in the in vivo studies.

After dispersion and initiation of digestion, the patterns of drug solubilisation varied significantly over time revealing simultaneous effects of both drug loading in the formulation and the [BS/PL] in the digestion medium. To facilitate better comparison of the results over time, the solubilised drug concentration as a function of the quantity of drug in the formulation under high and low [BS/PL] at different digestion times are presented in Figure 4.6. The figure also illustrates the impact of digestion time on drug solubilisation in an attempt to present relative in vitro 'exposure' at different time points.

Following 10 min dispersion (Figure 4.6A), linear increases in in vitro solubilisation/exposure were apparent with increases in drug concentration in the formulation under most conditions. However after 30 min of dispersion, some precipitation was evident at low [BS/PL] and higher drug loads (Figure 4.6B). Initiation of digestion resulted in a rapid drop in the solubilisation capacity of mixed colloidal species present in the digest (Figure 4.4). For example, at low [BS/PL], drug solubility dropped from 301 µg/mL in the dispersed formulation to 106 µg/mL after only 5 min of digestion (Table 4.4). However, at low drug loads (5 mg/g) the quantity of drug in the formulation was sufficiently low that it remained below the solubility limit throughout the digestion period and no precipitation occurred at either [BS/PL]. At drug loads of 9 mg/g, precipitation was evident, but minimal, and only observed under low [BS/PL] after 30 min digestion.

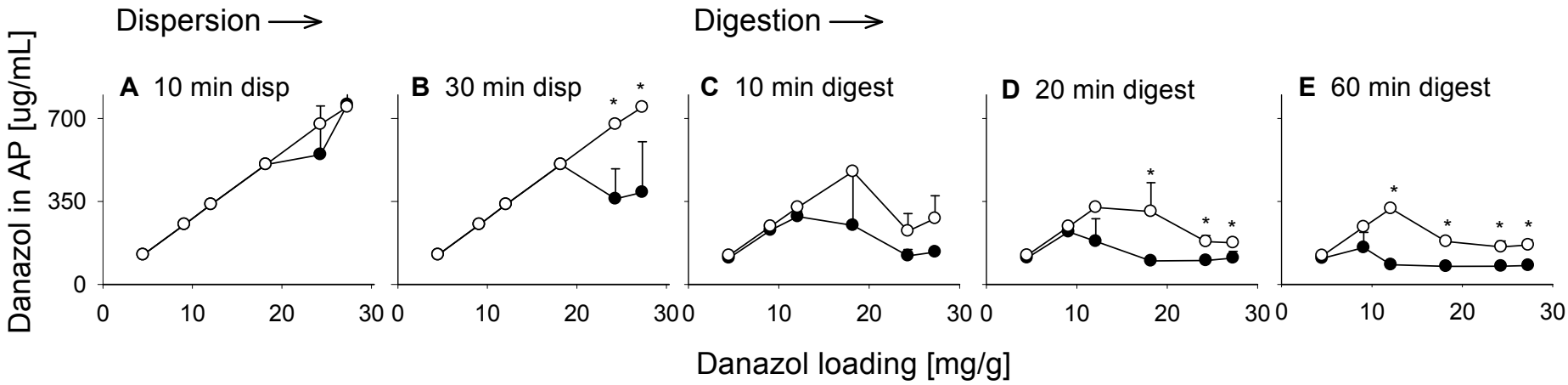


Figure 4.6 Drug solubilisation versus initial danazol saturation level in the formulation during in vitro digestion at low [BS/PL] (●) and high [BS/PL] (○) [mean ± SD (n = 3)]. Individual figures represent time points in Figure 4.4; A: early dispersion (-20 min), B: the end of the dispersion period (time zero); C: 10 min post digestion initiation (10 min), D: 20 min post digestion initiation (20 min) and E: 60 min post digestion initiation (60 min). *Statistically significant different compared to danazol solubilisation under low [BS/PL] ($P < 0.050$).

As such, linear increases in in vitro exposure with increases in drug dose up to 9 mg/g were evident on digestion in almost all cases (Figure 4.6). Higher drug loading, however, did result in precipitation, and this was more dependent on [BS/PL] concentration. Thus, at low [BS/PL], linear increases in in vitro 'exposure' were evident only up to drug loads of 12 mg/g (representing ~40% of drug solubility in the formulation) and then only up to 10 min post digestion (Figure 6C). At later time points and at higher drug loads, precipitation was more significant and further increases in drug load led to little additional solubilisation benefit (Figure 4.6C-E, filled symbols). In contrast, at the higher [BS/PL] (Figure 4.6, open symbols), solubilisation increases with dose were more apparent and total quantities of solubilised drug were significantly greater, especially at later time points (Figure 4.6C-E, open symbols).

The data in Figure 4.6 therefore provide a possible explanation for more robust absorption in the older animal cohort in the event that intestinal BS concentrations were elevated as described previously.[194] However, the in vivo data shows that bioavailability (not just exposure) increased with increasing dose in the older animals. This suggests the presence of a mechanism by which increasing dose increases bioavailability rather than simply maintaining linear increases in exposure. Closer examination of Figure 4.4 provides some indication of a possible means by which bioavailability increases with dose may occur, and also a rationale for how this might be more prevalent under increased [BS/PL]. Thus, initiation of digestion resulted in a very significant reduction in equilibrium solubility of drug in the digested formulation (dotted line in Figure 4.4, Table 4.4) and yet drug precipitation, was either avoided or delayed in many cases. This results in supersaturation[179], an increase in thermodynamic activity, and therefore a rationale for increases in membrane flux and (potentially) bioavailability. Importantly, the degree of supersaturation produced was highly dependent on dose and also sensitive to [BS/PL]. This is well illustrated in Figure 4.7 where the data have been presented as the supersaturation ratio (i.e. the ratio of the solubilised drug concentration in the digest to the solubility of drug in the same colloids).

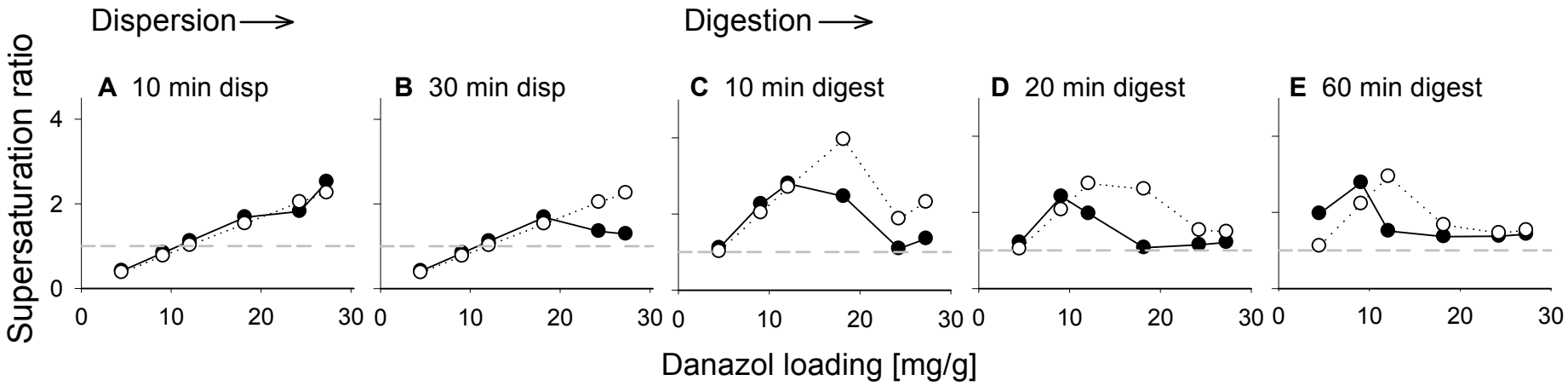


Figure 4.7 Supersaturation ratios versus danazol load in the formulation during in vitro digestion at 5 mM (●) and 20 mM (○) bile salt concentration [mean ± SD (n = 3)]. Individual figures represent time points in Figure 4.4; A: early dispersion (-20 min), B: the end of the dispersion period (time zero); C: 10 min post digestion initiation (10 min), D: 20 min post digestion initiation (20 min) and E: 60 min post digestion initiation (60 min). The dashed grey line illustrates the supersaturation ratio of 1 with ratios above this line indicating supersaturated conditions.

A supersaturation ratio of 1 suggests no increase in thermodynamic activity and in the absence of solubility limitations might be expected to translate into linear increases in exposure with dose. In contrast, supersaturation ratios above 1 dictate an increase in thermodynamic activity and the potential for non-linear increases in exposure or increases in bioavailability. On dispersion, it is apparent that increasing dose resulted in an increase in supersaturation ratio under most conditions. After initiation of digestion, however, the degree of supersaturation increased at lower drug loads and decreased at higher drug loads (due to precipitation), resulting in a parabolic relationship between drug load and supersaturation (similar to the parabolic relationship between bioavailability and dose seen in the older cohort (Figure 4.2)). This was most marked under higher [BS/PL], consistent with the hypothesis that elevated bile salt levels in the older cohort may provide an explanation for non-linear increases in bioavailability at moderate drug loads, whereas at the highest drug loads, increases in precipitation ultimately limit bioavailability enhancement.

4.12 CONCLUSION

In current studies, danazol absorption from SEDDS formulations containing drug at increasing drug loads was examined in two cohorts of beagle dogs, one younger (8 months) and one older (8 years). In the younger animals, linear increases in exposure were evident and bioavailability remained constant, even after administration of formulations where drug was dissolved in the formulation at up to 27 mg/g (90% of saturated solubility in the formulation). This occurred in spite of in vitro studies that suggested significant drug precipitation as the formulations were digested in the GI tract. Even more surprisingly, in the older cohort, not only was bioavailability maintained with increasing dose, bioavailability increased with increasing dose up to a tipping point (drug dissolved at 21 mg/g in the formulation and equivalent to 71% saturation in the

formulation), beyond which bioavailability returned back towards that observed in the younger cohort.

Unfortunately, definitive data to explain the results obtained remain elusive. Danazol has been reported to be a substrate of CYP3A[197] and therefore saturation of pre-systemic metabolism at increasing dose might be expected to provide an explanation for increases in exposure with dose. Similarly, increases in thermodynamic activity with increases in dose might also promote exposure. In contrast, increases in drug dose are also expected to encourage drug precipitation from the digesting formulation and to therefore reduce exposure with increasing dose. The net effect of these three competing forces is likely to dictate the ultimate profile of exposure with dose. This is illustrated in Figure 4.8, where the potential relationship between dose and exposure for solubility-limited exposure (dotted line) and first pass/thermodynamic activity enhanced exposure are shown (dashed line).

In the current studies, it seems likely that in the younger cohort as dose increased, negative effects on exposure mediated by increased precipitation with increasing dose were attenuated by positive effects on exposure mediated by saturation of first pass metabolism or increases in thermodynamic activity. The net result was therefore the observed linear relationship between dose and exposure (Figure 4.2).

In contrast, in the older animals, the increase in bioavailability observed up to a critical drug load (21 mg/g) was consistent with the maintenance of more robust solubilisation conditions in the GI tract such that the benefits obtained by an increase in thermodynamic activity or saturation of first pass metabolism outweighed the decreases in exposure due to precipitation. Under these conditions exposure is expected to increase to a critical point or critical supersaturation ratio, beyond which precipitation pressures start to outweigh solubilisation and supersaturation capacity and exposure is reduced.

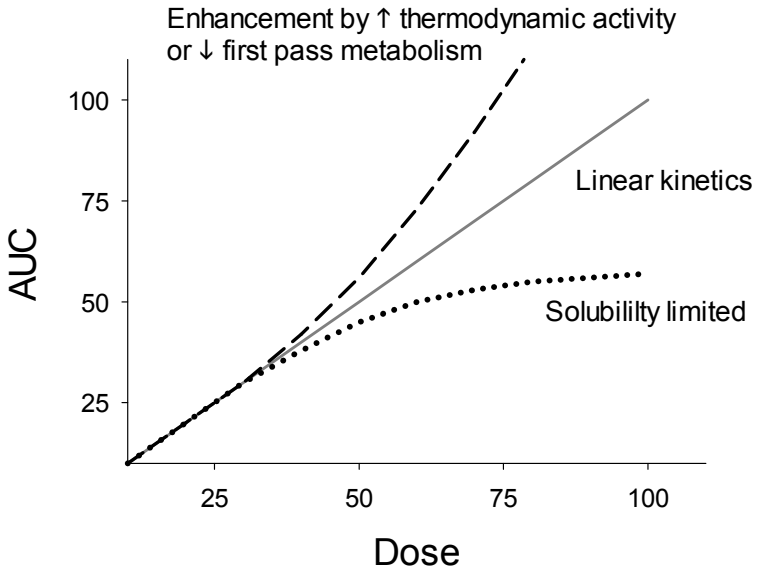


Figure 4.8 Theoretical relationship between exposure and dose following oral administration in a solubilized formulation of a poorly water-soluble drug that undergoes first pass metabolism. The plot illustrates the exposure following linear kinetics (solid line) and the potential beneficial effects of saturation of first pass metabolism or promotion of supersaturation and thermodynamic activity (dashed line) and the unfavourable effect of solubility-limited absorption conditions (dotted line).

These suggestions are consistent with the available in vivo data, but require a physiological change in the older animal cohort to drive the differences seen. One explanation would be a notable difference in metabolic behaviour in the two cohorts such that first pass metabolism is more readily saturated in the older animals. However, the serum bile salt assay data suggest limited differences in hepatic function between the two animal cohorts (at least in steroid processing), and the literature provides little evidence to support significant reductions in hepatic enzymatic activity (as an indicator of pre-systemic (hepatic) metabolic activity) with age.[203,205,210-212] The latter suggestion, however, should be viewed with the caveat that hepatic function is

notoriously difficult to capture with a single biomarker, and that differences in enterocyte-based metabolism may also be responsible for differences in metabolic activity.

Alternatively, differences in first pass metabolism may be limited and instead differences across the two cohorts may be driven by differences in intestinal conditions and therefore absorption. This is consistent with recent reports describing increases in danazol luminal solubility in the elderly.[194] To explore the potential impact of changes in intestinal conditions on exposure, we examined the impact of changes to drug solubilisation with increasing drug dose under differing [BS/PL] and these data provide conditions and data consistent with the scenario described above. Thus, in vitro digestion data obtained at higher [BS/PL] resulted in more sustained drug solubilisation and greater supersaturation than was evident at lower [BS/PL]. Increased luminal [BS/PL], resulting in prolonged solubilisation and increased thermodynamic activity, is therefore a possible explanation for the non-linear increases in bioavailability observed in the older versus younger animals. The increase in absorption stemming from an increase in thermodynamic activity might also be expected to lead to more effective saturation of first pass metabolism, leading to even greater increases in exposure.

In summary, the current studies illustrate the complexity of interpretation of dose-linearity studies from solubilised formulations, especially where first pass metabolism provides a limitation to bioavailability. The data also show that differences in animal cohorts can have a significant impact on absorption, and at least in the case of danazol, that under some circumstances bioavailability appears to be enhanced in older animals. In vitro experiments suggest that this could be explained by an increase in luminal [BS/PL] in these animals resulting in more robust solubilisation, increased supersaturation and enhanced exposure. These trends are likely to be amplified in the event of significant first pass metabolism, although the latter has not been directly studied here.

4.13 ACKNOWLEDGEMENT

Funding support from the Australian Research Council (ARC) and Capsugel is gratefully acknowledged. We also thank Anya Carlson and Gail Squires for technical assistance with the studies in beagle dogs.

4.14 SUPPORTING INFORMATION

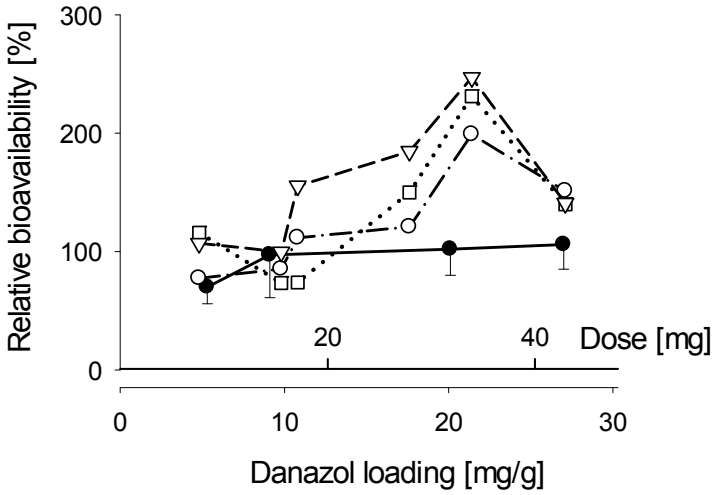


Figure 4.9 Dose linearity of the relative bioavailability following oral administration of increasing danazol doses (expressed both as absolute dose (mg) and drug concentration in the formulation (mg/g) in a type III MC SEDDS formulation compared to both the younger beagle cohort (●) [mean ± SEM (n = 4)] and the older beagle cohort represented as individual dogs (open symbols).

Monash University

Declaration for Thesis Chapter 5

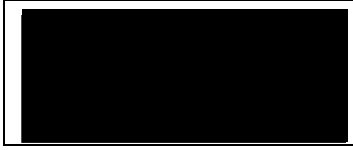
Declaration by candidate

In the case of Chapter 5, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution
Planning and executing experimental work, data evaluation and drafting and revision of manuscripts	50%

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	Nature of contribution	Extent of contribution
C.J.H. Porter	Project supervisor, data and manuscript review	NA
C.W. Pouton	Project co-supervisor and manuscript review	NA
T-H. Nguyen	Performing surgical procedures, formulation dosing, responsible for animal care	NA
YY. Yeap	Performing surgical procedures, responsible for animal care	5%
H.D. Williams	Manuscript review	NA
H. Benameur	External supervision and manuscript review	NA

Candidate's Signature		**Date:** 20/11/2012

Declaration by co-authors

The undersigned hereby certify that:

(1) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
(2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(4) there are no other authors of the publication according to these criteria;

(5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and

(6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)	**Monash Institute of Pharmaceutical Sciences, Parkville Campus**

Signature 1	[redacted]	21/09/2012
Signature 2	[redacted]	11/10/2012
Signature 3	[redacted]	20/11/2012
Signature 4	[redacted]	13/08/2012
Signature 5	[redacted]	11/10/2012
Signature 6	[redacted]	19/09/2012

CHAPTER 5

EVALUATION OF DANAZOL BIOAVAILABILITY FROM LIPID-BASED DRUG DELIVERY SYSTEMS IN THE RAT: THE ROLE OF FORMULATION DISPERSION/DIGESTION AND FIRST PASS METABOLISM

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Manuscript in submission

5 Evaluation of danazol bioavailability from lipid-based drug delivery systems in the rat: the role of formulation dispersion/digestion and first pass metabolism

5.1 ABSTRACT

Purpose: To evaluate the impact of gastrointestinal (GI) processing and first pass metabolism on danazol oral bioavailability (BA) after administration of self-emulsifying drug delivery systems (SEDDS) in the rat.

Methods: 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 in vivo processing of SEDDS in the rat.

Results: 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.

Conclusion: 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.

5.2 INTRODUCTION

Self-emulsifying drug delivery systems (SEDDS) provide a means to enhance the absorption of poorly water-soluble drugs (PWSD),[7,74,78,178] and their utility has been exemplified with a range of drugs including cyclosporine, halofantrine and danazol.[16,65,103,213-215] 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.[167,179] 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.[14,34,65,96,100,101,104,107,166]

Where supersaturation is maintained for extended periods of time, drug absorption continues and may be enhanced by virtue of an increase in thermodynamic activity. Several studies have therefore focused on the potentially positive role of supersaturation in drug absorption from SEDDS.[24-28,107,130,167,179] 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.[135,137,139] The degree of supersaturation is typically captured via the calculation of a supersaturation ratio that reflects the concentration of drug in the GI fluids relative to the equilibrium solubility of drug in the same system.[134,136]

Formulations that promote the stabilization of metastable drug concentrations at high supersaturation ratios are expected to more robustly facilitate drug absorption, and recent studies suggest that the addition of polymeric precipitation inhibitors (PPIs) to SEDDS may promote stabilization of supersaturation in vitro and under some circumstances in vivo.[24-29,179]

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/supersaturation resulting in an increase in thermodynamic activity (and therefore absorption promotion) versus increases in precipitation (and therefore a reduction in absorption). Interestingly, the data obtained varied as a function of the animal cohort. 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 pressure). 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, evaluation of the effects of dose on bioavailability is complicated by the possibility of changes to first pass metabolism. Thus the increase to bioavailability observed with

increases in dose (at least in the older beagle cohort), might also reflect saturation of first pass.[216] The prospect of first pass metabolism of danazol has been suggested previously[177], however, subsequent studies have also described oral bioavailabilities as high as 100% in beagle dogs[217] in seeming contradiction of the possibility of significant pre-systemic metabolic limitations to bioavailability.

The lack of consistency in the published literature with respect to the effects of dose on danazol bioavailability from SEDDS and the lack of consensus on the potential impact of first pass metabolism stimulated the current studies. Here we sought to further explore the role of drug dose on the bioavailability of danazol from SEDDS and in this case, studies have been conducted in rats to allow more mechanistic comparison of the impact of first pass metabolism versus gastric and intestinal processing on danazol bioavailability. The ability of PPI to enhance drug absorption from SEDDS formulations was also explored, to provide a species comparison of effects relative to previous studies in dogs. As part of the current studies, the applicability of previous in vitro models of lipid digestion to events in the rat has also been examined, and a number of modifications to the model have been suggested to more effectively mimic 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 human), that danazol absorption from SEDDS formulations is high (> 50%) and that the principle limitation to danazol bioavailability is first pass metabolism.

5.3 MATERIALS AND METHODS

5.3.1 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, a medium-chain triglyceride (MCT), and Capmul MCM EP, a blend of

medium-chain mono-, di-, and tri-glycerides, were donated by Abitec Corporation (Janesville, WI, USA). Soybean oil (predominantly C18 triglycerides), 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). The anaesthetics; Parnell ketamine (100 mg/ml) from Parnell Labs, NSW, Australia, Iliul 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 purchased from Merck (Darmstadt, Germany) and water was obtained from a Milli-Q (Millipore, Bedford, MA, USA) purification system. All other chemicals and solvents were of analytical purity or high performance liquid chromatography (HPLC) grade.

5.3.2 Formulation preparation

5.3.2.1 SEDDS formulations for oral/intraduodenal administration

All formulations were prepared as previously described[13] and danazol (aqueous solubility 0.59 µg/ml[144], log *P* of 4.53[143]) 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 of the formulations was assessed using standard methodologies and all experiments were performed in triplicate.[15] Equilibrium solubility was defined as the value attained when at least three consecutive solubility samples varied by ≤ 5%. This was typically reached after equilibration times of between 48 and 72 h.

In an attempt to stabilise potential supersaturation generated on formulation processing, the effect of the addition of a polymeric precipitation inhibitor (PPI), (5% w/w HPMC) to the formulation was also evaluated. The formulations are summarised in Table 5.1**.** The formulations were vortexed for 30 s and equilibrated overnight at 37°C.

Table 5.1 Composition (% w/w) of self-emulsifying lipid-based formulations containing danazol

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

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

[a] Hydroxypropyl methylcellulose (HPMC) was also added at 5% w/w to a series of formulations generated using the proportions described in the table

[b] Lipid Formulation Classification System (LFCS) applied from Pouton et al[71,94]

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

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

5.3.3 Intravenous formulation

An intravenous formulation of danazol (1.2 mg/mL) was prepared using 15% (w/v) hydroxypropyl-β-cyclodextrin (HP-β-CD). 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.

5.4 BIOAVAILABILITY STUDIES IN RATS

SEDDS formulations were evaluated in vivo in the presence and absence of HPMC to examine the impact of the addition of PPI in the formulation on drug absorption. The formulations included a LFCS class III formulation containing medium-chain lipids, Cremophor EL and ethanol (SEDDS-III), and a LFCS class IV formulation (i.e. lipid free) containing Cremophor EL and ethanol alone (SEDDS-IV) (Table 5.1). In the latter case, classification as a 'self-emulsifying' formulation has been retained for simplicity and to reflect the fact that the formulation disperses readily on contact with the GI fluids to form a solubilised formulation (even though the resulting dispersion is a micellar solution rather than an emulsion, and have previously been referred to as F60 and F0, respectively.[179] For the SEDDS-III formulation, the effect of drug dose was also evaluated via drug loading at both 40% ($SEDDS_L$-III) and at 80% of solubility in the formulation ($SEDDS_H$-III). SEDDS-IV contained drug at 40% solubility in the formulation, i.e. at the same thermodynamic activity as $SEDDS_L$-III. To allow calculation of absolute bioavailability, danazol was also administered intravenously (IV).

In some test groups, a non-specific cytochrome P450 inhibitor (1-aminobenzotriazole, ABT) was administered orally to inhibit hepatic and intestinal CYP enzymes. Comparison of data obtained after administration of danazol in the presence (pre-treated ABT rats) and absence of ABT provides an indication of the impact of first pass metabolism on danazol bioavailability.

Danazol bioavailability was explored following oral and intraduodenal (ID) administration to assess the impact of gastric processing on subsequent formulation performance. A total of 15 experimental groups were evaluated (9 oral, 4 intraduodenal and 2 intravenous).

5.4.1 Surgical and experimental procedures

All surgical and experiment procedures were approved by the Monash Institute of Pharmaceutical Sciences animal ethics committee and were conducted in accordance with 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) per group (n = 4). Surgical and recovery procedures were as described previously.[218,219] Briefly, polyethylene tubing (0.96 x 0.58) was surgically cannulated 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).

5.4.2 Formulation administration and sample collection

Rats were fasted for 12 h prior to dosing and remained fasted till the conclusion of the study, however, drinking water was provided *ad libitum*.

In groups pre-treated with ABT, a single bolus dose of ABT (100 mg/kg) was administered via oral gavage post surgery, approximately 14 h prior to IV, oral or ID dosing.[155-158] A similar dosing protocol has been shown previously to provide almost complete inhibition (93%) of antipyrine clearance.[155]

The intravenous formulation (0.5 mL of a 1.2 mg/ml solution of danazol in 15% HP-β-CD, to provide an administered dose of 2.3 mg/kg) was administered 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 at a constant infusion rate (1 mL/min) 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.

5.4.3 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 as previously described.[220]

5.4.4 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 area under the plasma concentration vs. time profiles ($AUC_{0\text{-}tz}$) was calculated using the linear trapezoidal method. In the absent of ABT treatment, the AUC was in general long with a short elimination phase and the

terminal elimination rate constant from the IV study was used to extrapolate AUC from the last time point to infinity, which was added to $AUC_{0\text{-}tz}$ in order to determine $AUC_{0\text{-}\infty}$. In the presence of ABT, AUC was high generating a good elimination phase and the extrapolated AUC was therefore based on individual plasma profiles. Clearance (Cl), volume of distribution (Vd_{β}) 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.

5.5 IN VITRO EXPERIMENTS

5.5.1 In vitro dispersion of SEDDS formulations

Evaluation of the impact of gastric dispersion was performed by dispersing formulations into either model gastric fluid or authentic gastric fluids obtained from rats. For studies using model gastric fluids, two dilution levels and pH was explored. 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 at 37°C in a glass vessel and the temperature controlled by a thermostatically controlled water jacket. The dispersion was stirred magnetically (disc-shaped Teflon coated stirrer bar, 10 x 14 mm) and samples (200 µL) collected at t = 1, 15 and 30 min.

Subsequently, a low dilution/ intermediate pH model was utilised to better reflect gastric conditions in the rat. Here, 100 mg of lipid-based formulation was weighed into a flat bottom glass vial and 900 µL of buffer (pH 5.5) used as the dispersion medium (i.e. a 1 in 10 dilution). A pH of 5.5 was used based on previously reported values of rat gastric pH[79]. The dispersion was stirred using a magnetic stirrer (Teflon coated stirrer bar, 10 x 6 mm) and samples (200 µL) collected at t = 1, 15 and 30 min.

Finally, experiments were repeated using ex-vivo gastric fluids. To collect gastric fluids from the rat, male Sprague Dawley (SD) rats (300-400 g) were fasted for 12 h prior to surgery but were allowed 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 dispersion experiments using the low volume dispersion protocol described above.

5.5.2 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 5.2 and described below.

5.5.2.1 Dog digestion model – High dilution / High enzyme activity

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

Previous in vitro studies[179] of the formulations utilised in the current studies employed an in vitro model that was originally developed to estimate the impact of digestion on SEDDS formulations in larger species such as dogs or humans.[34] In these studies, 1 g of formulation was dispersed in a total volume of 40 mL of digestion media in a thermostatically controlled (37°C) vessel and digestion initiated by addition of 4 mL of porcine pancreatin extract. These data are reproduced in the current study to provide a point of comparison with the data obtained using the modified models described below.

5.5.2.2 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 firstly carried out in the current studies 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. In these studies, the ratio of formulation: digestion medium was kept constant relative to previous studies using the in vitro dog digestion model and employed a formulation dilution factor of 40, i.e. 250 mg formulation in 9 mL digestion medium with 1 mL of ex vivo rat pancreatic/biliary secretions employed to stimulate digestion. This is consistent with the in vitro dog digestion model where 1 g formulation was examined in a 40 mL digest containing 4 ml porcine lipase extract. Since rat pancreatic/bile secretions contain bile, experiments were conducted in digestion buffer without added bile salt and phospholipid. Otherwise, in vitro digestion experiments were conducted as described previously[109], and 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 (high dilution/low enzyme) but using a quantity of porcine enzyme that provided a similar enzyme activity to that of 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.

5.5.2.3 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 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). This condition was intended to reflect the in vivo studies where administration of the dispersed formulation was expected to mix with endogenous rat bile/pancreatic secretions. An additional 250 µL MilliQ water was added to the vial to simulate the low levels of basal intestinal fluids that might be present in the GI tract during in vivo studies. Due to the low volume it was not possible to employ a pH stat for maintenance of pH during the digestions, and studies were performed in a 10 mL glass vial and media was stirred magnetically (Teflon coated stirrer bar, 10 x 6 mm). The pH of the digest was measured at the end of the experiment to confirm that pH had not dropped to levels that would compromise lipase activity. After 60 min digestion, the pH of the digestion medium was 7.9 and 5.8 for SEDDS-IV and SEDDS-III, respectively. Samples of the digest (300 µL) were collected at t = 10, 20, 30 and 60 min.

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

Finally, the low dilution rat model was re-run but this time substituting a quantity of porcine pancreatic extract that had previously been shown to have similar in vitro enzyme activity to that

in 1 mL of ex vivo rat bile/pancreatic fluid. In vitro digestion experiments were conducted as described above.

5.5.3 Determination of pancreatic lipase activity in rat pancreatic/biliary fluid

The lipolytic activity of enzymes in ex vivo rat bile/pancreatic fluid was determined using an in vitro lipolysis assay and tributyrin as a model substrate. This approach provides enzyme activity in tributyrin units (TBUs) where 1 TBU is defined as the amount of enzyme that releases 1 μmol of titratable butyric acid per minute.[108] The lipase activity was determined as previously described.[97] 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 in a glass vessel, the temperature was controlled by a thermostatically controlled water jacket and the fluids contained in the vessel (i.e. digestion buffer plus tributyrin) were stirred magnetically.

After 2 min of dispersion, digestion was initiated by addition of 1 mL of collected ex vivo pancreatic fluids. Digestion was continuously monitored using a pH-stat titration unit (Radiometer, Copenhagen, Denmark) that maintained the pH at 7.5 via titration of liberated fatty acids with 0.5 M NaOH. Digestion was followed for 30 min (or until 10 mL NaOH had been added). Lipase activity in TBU (μmol titratable butyric acid per minute) was calculated from the initial rate of digestion determined 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 the 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 5 mM NaTDC and 1.25 mM phosphatidycholine (PC) were added to the digestion buffer since the enzyme preparation did not contain bile). The 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 in order to identify 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.

5.5.4 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. There is no gall bladder in a rat and the bile duct also serves as the main duct for the transfer of pancreatic secretions to the GIT. To obtain an accurate secretion ratio between biliary and pancreatic fluids secreted in vivo, fluids were collected together. Pancreatic lipase activity is stable for up to at least 3 h.[208,221,222]

Anaesthesia and surgical procedures in rats were performed as previously described.[127,153,223] Briefly, anaesthesia was induced and maintained during the entire experiment, while a ligature was tied around the bile duct at the point of entry into the duodenum (to allow simultaneous collection of pancreatic fluids) 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 subsequently 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 5.2 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[97]

[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

[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

5.5.5 Sample work up for in vitro dispersion/digestion experiments

The lipase inhibitor 4-bromophenylboronic acid[152] (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) in order to pellet any drug that precipitated on dispersion/digestion. Samples obtained from each phase were diluted (aqueous phase: 50 µL to 950 µL ACN; Precipitate (pellet) and oil phase: dissolved in oil phase: 50 µL chloroform/methanol (2:1 v/v) to 950 µL MeOH) and analysed by HPLC.

5.5.6 Quantification of danazol by HPLC

Danazol concentrations in samples taken from in vitro dispersion and digestion experiments were measured using HPLC as described previously.[179]

5.6 RESULTS

In vivo evaluation

5.6.1 Intravenous pharmacokinetics of danazol

To allow evaluation of the absolute bioavailability of danazol in subsequent oral and ID PK studies, pharmacokinetic data were collected after intravenous administration (IV). 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 5.1 with corresponding mean pharmacokinetic parameters summarised in Table 5.3.

Plasma concentrations of danazol declined bi-exponentially after intravenous administration. 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). Danazol clearance was higher and half-life shorter than that reported previously[129,224] (Cl: 40 - 41 mL min^{-1} kg^{-1}; Vd: 6.1 - 7.5 L kg^{-1}; half-life: 1.8-2.1 h (data re-calculated from the published dose, AUC and elimination rate constants (β)). However, previous studies were performed at higher doses (7 mg/kg) than that utilised here (2 mg/kg), and the lower clearance and higher half-life observed in the previous studies may reflect some non-linearity in metabolism.

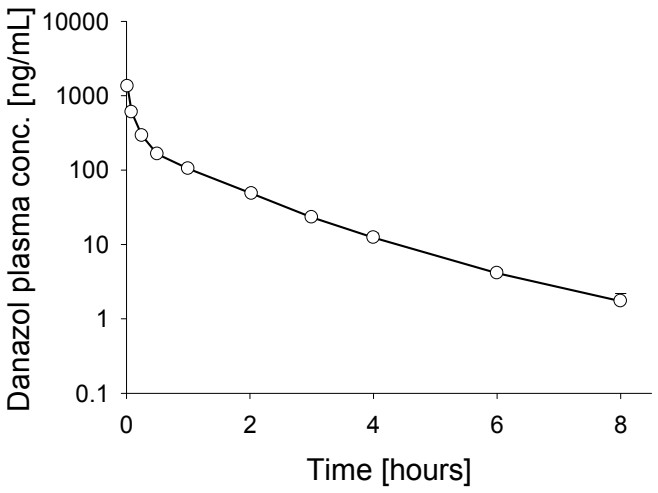

Figure 5.1 Danazol plasma profile after IV administration of 15% HP-β-CD solution containing danazol at 1.2 mg/mL (total dose 2.3 mg kg^{-1}) to rats [mean ± SEM (n = 5)].

Table 5.3 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	$t_{1/2}$
[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

5.6.2 Bioavailability of danazol after oral administration of SEDDS in the presence and absence of PPI

The mean plasma concentration versus time profiles for danazol following oral administration of a BCS type III, lipid-containing SEDDS formulation (SEDDS-III) and a BCS Type IV, lipid-free surfactant/cosolvent formulation (SEDDS-IV) at low drug loading are shown in Figure 5.2**.** 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 5.4.

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 an approximately 2-fold increase in absolute danazol bioavailability was also evident at the higher dose. In all cases, absolute bioavailability was low (< 5%) (Table 5.4).

Previous studies have shown that the addition of polymeric precipitation inhibitors (PPIs) such as HPMC, to lipid based formulations may reduce precipitation and enhance bioavailability.[24-29,179] Bearing in mind the low bioavailability of danazol after administration of SEDDS-III and SEDDS-IV, these formulations were therefore also administered to rats after incorporation of PPI. Mean plasma profiles are shown in Figure 5.2B-D and the summary pharmacokinetic parameters given in Table 5.4.

For $SEDDS_L$-III, a trend towards increased C_{max} was evident on addition of PPI, and for $SEDDS_H$-III, a trend towards decreased bioavailability was apparent. However, across the series addition of 5% w/w HPMC failed to lead to statistically significant differences in danazol bioavailability.

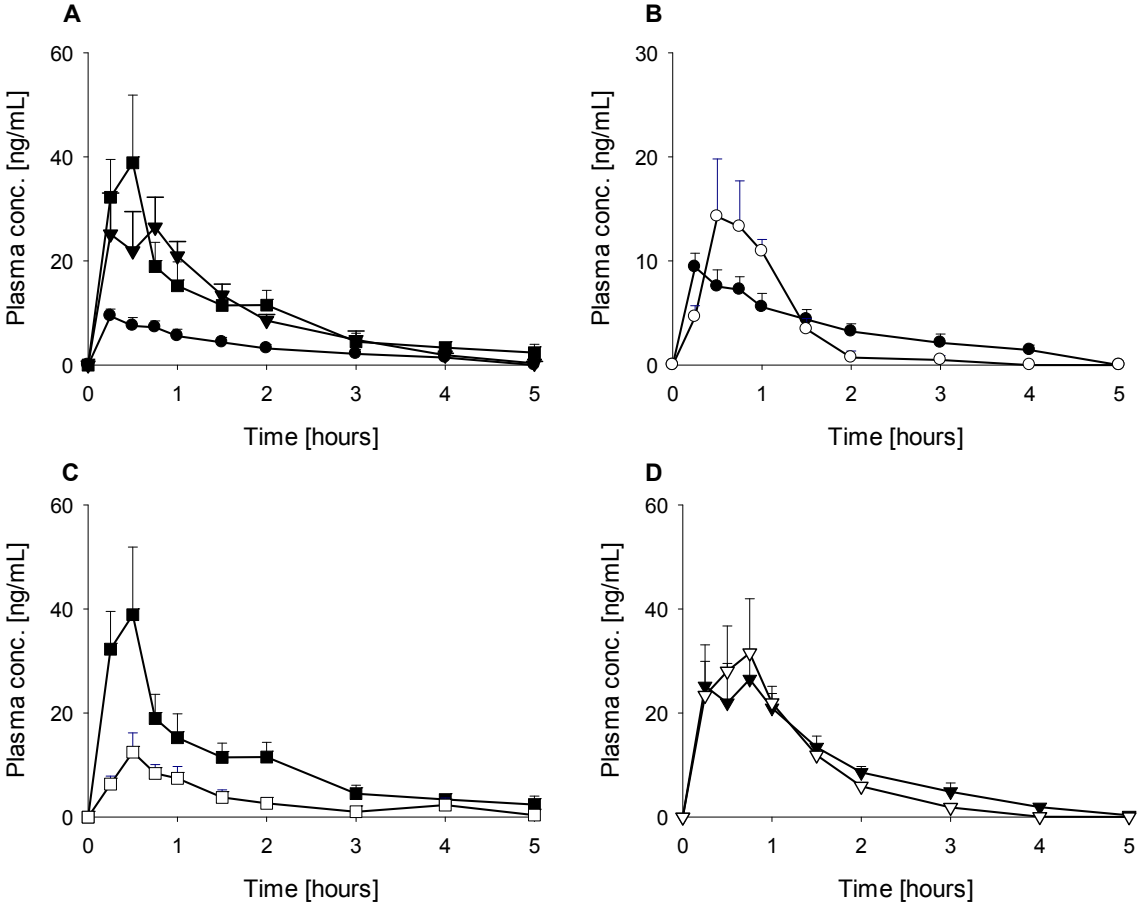

Figure 5.2 Danazol plasma concentration profiles following oral administration to rats of A: $SEDDS_L$-III (circle), $SEDDS_H$-III (square) and SEDDS-IV (triangle) in the absence of PPI, followed by panels of individual formulations in the presence (open) and absence (filled) of 5% (w/w) PPI (HPMC) for B: $SEDDS_L$-III C: $SEDDS_H$-III and D: SEDDS-IV. All plasma concentration profiles are presented as mean ± SEM (n = 4) and the administrated doses are tabulated in Table 5.4.

Table 5.4 Pharmacokinetic parameters for danazol after oral administration to rats [mean ± SEM (n = 4)]

Treatment	Dose [mg kg^{-1}]	$AUC_{0-\infty}$ [ng h mL^{-1}]	C_{max} [ng mL^{-1}]	T_{max} [h]	BA [%]
$SEDDS_L$-III	4.9	19 ± 3	10 ± 1	0.4 ± 0.1	2.0 ± 0.4
$SEDDS_L$-III + 5% HPMC	4.3	18 ± 3	16 ± 4	0.6 ± 0.2	2.2 ± 0.4
$SEDDS_H$-III	8.6	62 ± 13	43 ± 12	0.4 ± 0.1	3.7 ± 0.8[a]
$SEDDS_H$-III + 5% HPMC	8.3	20 ± 4	13 ± 4	0.5 ± 0.0	1.2 ± 0.2
SEDDS-IV	5.8	49 ± 7	32 ± 7	0.7 ± 0.2	4.4 ± 0.6 [ab]
SEDDS-IV + 5% HPMC	5.4	43 ± 8	35 ± 9	0.7 ± 0.2	4.2 ± 0.8

[a] Statistically significant different compared to $SEDDS_H$-III + 5% HPMC ($P < 0.050$)
[b] Statistically significant different compared to $SEDDS_L$-III ($P < 0.050$)

5.6.3 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 5.3 A, B and C, respectively. The corresponding mean pharmacokinetic parameters following ID administration are summarised in Table 5.5. 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 5.5). 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.

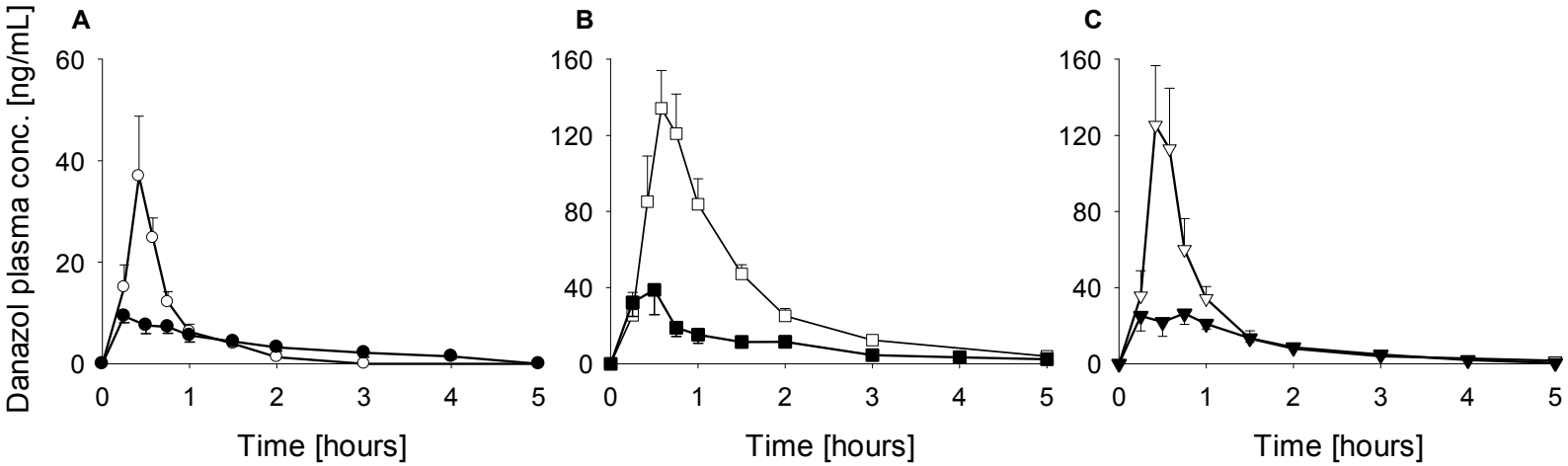

Figure 5.3 Danazol plasma concentration profiles comparing oral (filled) versus intraduodenal (ID)(open) administration of A: $SEDDS_L$-III (circle), B: $SEDDS_H$-III (square), C: SEDDS-IV (triangle). All plasma concentration profiles are illustrated as mean ± SEM (n = 4) and the administrated doses are tabulated in Table 5.5.

Table 5.5 Pharmacokinetic parameters for danazol after ID administration to rats [mean ± SEM (n = 4)]

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

[a] Statistically significant different compared to $SEDDS_L$-III (P <0.050)
[b] Statistically significant different compared to oral administration of equivalent formulation (P < 0.050) (see Figure 5.2 and Table 5.4)

5.6.4 Impact of first pass metabolism on danazol bioavailability from SEDDS

The low bioavailability of danazol (≤ 13%) observed after both oral and ID administration stimulated a subsequent examination of the possible role of first pass metabolism in limiting danazol oral bioavailability in the rat. The impact of first pass metabolism was therefore explored by administration of a non-specific cytochrome P450 inhibitor, 1-aminobenzotriazole (ABT), prior to danazol administration. ABT has been shown previously to almost completely inhibit hepatic and intestinal CYP450s at the dose employed here.[155,158]

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 (Figure 5.9) and the corresponding mean pharmacokinetic parameters (Table 5.8) 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 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 pre-treated ABT rats are shown in Figure 5.4 with mean pharmacokinetic parameters summarised in Table 5.6.

Panel A in Figure 5.4 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 5.2). Higher AUC in the ABT pre-treated groups were expected due to the reduction in systemic clearance under conditions of CYP450 inhibition. However, comparison with IV data also obtained in the presence of ABT, corrects for differences in systemic clearance, and very large increases in oral bioavailability (up to 30 fold) were evident when compared to data obtained in the absence of ABT. The data suggest the presence of a significant first pass effect for danazol in rats.

Data obtained after oral administration in the presence of ABT provides an indication of bioavailability, unaffected by the complications of CYP-mediated first pass metabolism. Under these conditions, oral administration of SEDDS-IV resulted in 45.4% danazol bioavailability compared to only 4.4% in the untreated group (i.e. a 10-fold increase in BA). 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).

In order to evaluate whether the non-linear increase in bioavailability seen after oral and ID administration of SEDDS-III at different danazol doses were a result of saturated first pass metabolism at the higher dose or a result of thermodynamic activity driving increases in drug

absorption, $SEDDS_H$-III was also administered orally in pre-treated ABT rats (Figure 5.4A). In this case, 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 by dose (59.7% vs. 51.6% at low and high dose, respectively).

The increase in exposure evident after ID administration compared to oral administration in the absence of ABT may reflect precipitation in the stomach after oral administration, or the potential for direct introduction of relatively high local drug concentrations in the intestine following ID administration, subsequently leading to saturation of first pass metabolism, thus increased bioavailability. To provide an indication of the role of saturation of first pass metabolism, data were therefore obtained after intraduodenal administration of $SEDS\text{-}III_L$ in the presence of ABT.

Panel B in Figure 5.4 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.

Table 5.6 Pharmacokinetic parameters for danazol after oral and intraduodenal administration to ABT pre-treated rats [mean ± SEM (n = 4)].

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

[a] Oral administration (O),

[b] Intraduodenal administration (I.D.)

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

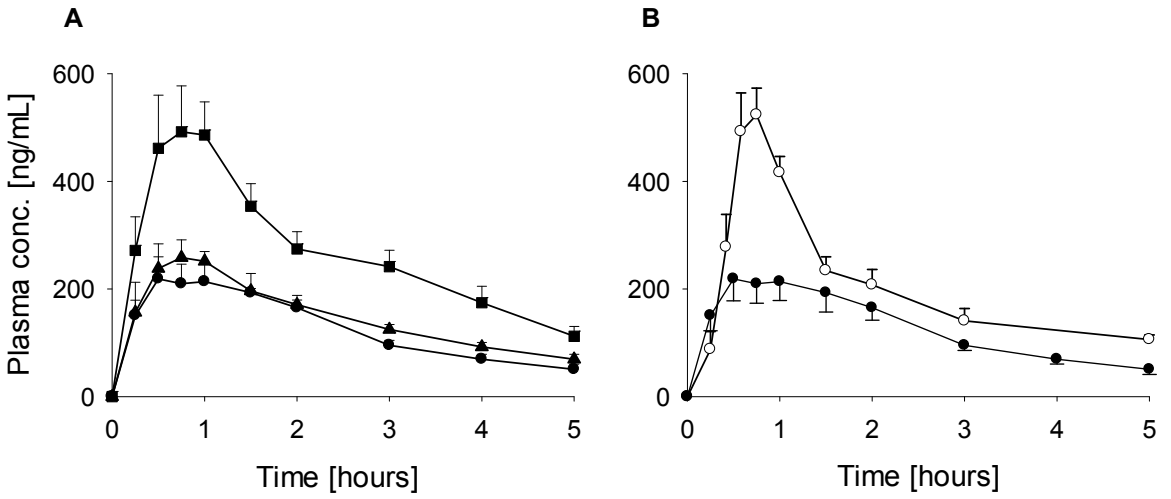

Figure 5.4 Danazol plasma concentration profile from ABT pre-treated rats following A: oral administration of $SEDDS_L$-III (filled circle), $SEDDS_H$-III (filled square), SEDDS-IV (open triangle); B: Intraduodenal (open) versus oral (filled) administration of $SEDDS_L$-III. All plasma concentration profiles are illustrated as mean ± SEM (n = 4).

5.7 IN VITRO EVALUATION

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

The in vivo data suggest that plasma drug exposure following administration of danazol-containing SEDDS is dependent on formulation, drug dose and route of administration, with higher exposure obtained when the formulation is directly administered into the duodenum. Drug dose effects were ameliorated on co-administration with ABT, suggesting an effect largely driven by changes to first pass metabolism. However, formulation effects and the impact of intraduodenal versus oral administration were maintained in the presence of ABT suggesting differences due to absorption rather than first pass metabolism. The comparatively poorer performance of formulations administered orally when compared to ID administration may reflect

drug precipitation in the stomach. The impact of formulation processing under gastric conditions on the in vitro performance of the investigated formulations (SEDDS-IV / $SEDDS_L$-III) was therefore evaluated in a series of dispersion studies (Figure 5.5). Results in the upper panels of Figure 5.5 (A1/B1/C1) correspond to danazol solubilisation/precipitation patterns after dispersion of SEDDS-IV and the lower panels (A2/B2/C2) $SEDDS_L$-III.

Formulations were initially assessed using simulated gastric fluid at pH 1.2 (Figure 5.5 A1/A2) and high dilution conditions. However, the hydrodynamics of formulation dispersion and flow in the rat are likely to differ to that of the dog or human. In particular, the fluid volume is likely to be significant lower in the rat than that in larger species and the pH in the rat stomach has been reported to be higher than pH 1.2.[79] As such, experiments were also conducted under lower dilution conditions (1 in 10) and at pH 5.5 (Figure 5.5 B1/B2).

Dispersion of SEDDS-IV under high dilution conditions at pH 1.2 led to rapid drug precipitation within 10 min and retention of only 40% of the initial drug load in a solubilized state after 30 min dispersion. In comparison, no drug precipitation was observed on dispersion of $SEDDS_L$-III under similar conditions. 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 after 30 min dispersion (panel B2 in Figure 5.5).

To better reflect in vivo conditions, and realizing that in the rat, lingual lipase is secreted in saliva and might be expected to impact on the solubilisation capacity of lipid based formulations (gastric lipase activity is very low in rats[225]), gastric dispersion was also evaluated using ex vivo gastric fluids from rats. The solubilisation/precipitation patterns under these conditions are shown in Figure 5.5C1 and Figure 5.5C2 for $SEDDS_L$-III and SEDDS-IV, respectively.

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[79] and similar to that used in the simulated rat gastric fluid buffer (pH 5.5).

The results of the gastric dispersion studies therefore suggest that the SEDDS-IV formulation was susceptible to drug precipitation under all simulated gastric conditions. In contrast, the SEDDS-III formulation was more resistant to precipitation on dispersion and drug precipitation from this formulation was only evident at higher drug loads (i.e. $SEDDS_H$-III). The use of ex-vivo gastric fluid gave essentially identical results to that obtained using simple dispersion protocols into either pH 1.2 or pH 5.5 buffer.

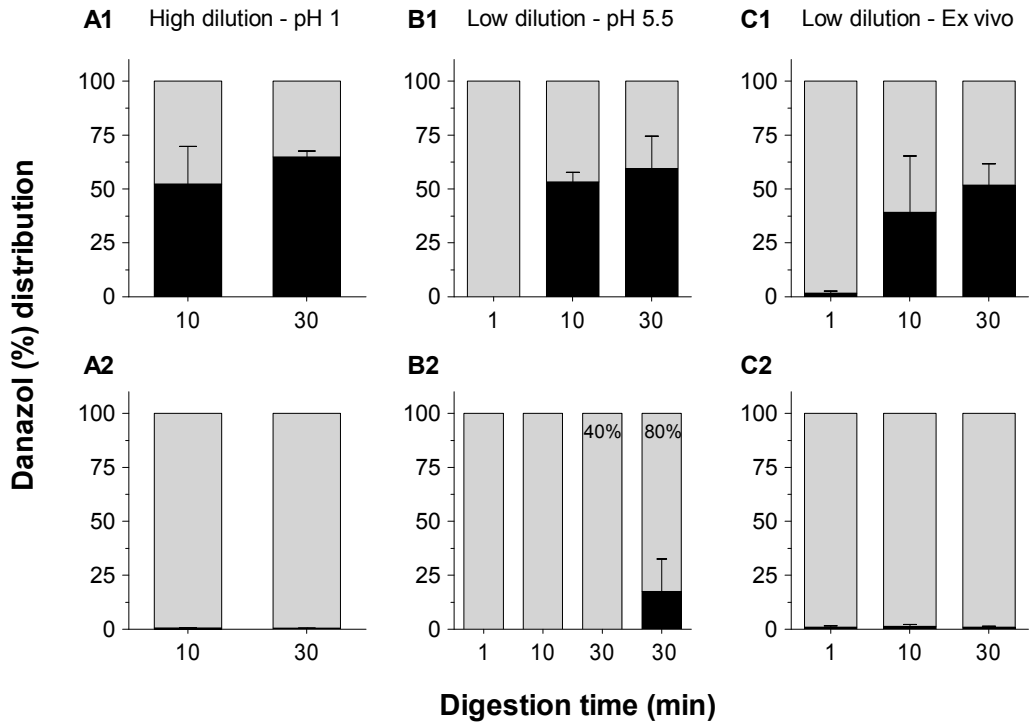

Figure 5.5 The extent of danazol precipitation after 30 min dispersion of SEDDS-IV (upper panels) and $SEDDS_L$-III (lower panels) under gastric condition [mean ± SD (n = 3)] in A: 40 mL 0.1 N HCL pH 1.2; B: 0.9 mL buffer, pH 5.5; C: 2.25 mL ex vivo rat gastric fluid pH 4.8. In addition, panel B2 shows $SEDDS_H$-III and the effect of drug loading on drug precipitation after 30 min of dispersion.

5.7.2 Impact of intestinal digestion on in vitro performance of danazol SEDDS

To further explore the likely behaviour of the SEDDS formulations in vivo, subsequent experiments examined behaviour under digesting conditions, and employed a series of modified in vitro digestion models. In vitro digestion profiles for the formulations examined in the current study have previously been published using an experimental protocol designed to reflect the likely intestinal conditions in the dog.[179] It is apparent, however, that the intestinal conditions in the rat are likely to differ when compared to that in the dog and therefore that in vitro methods designed to mirror those conditions might usefully be changed.

5.7.2.1 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 first conducted using ex vivo rat pancreatic enzymes and biliary fluids rather than the porcine pancreatin extract previously employed. These initial studies were conducted using the pH-stat titration system previously employed, but in the first instance the volume of the test was reduced to 10 mL (Table 5.2 - Rat digestion model (high/Low)). The quantity of formulation tested was reduced proportionally to 250 mg to give a dilution factor of 40, identical to that employed previously in the dog digestion model.

Figure 5.7 A and B show the precipitation profiles for danazol over 60 min during in vitro digestion of $SEDDS_L$-III using the previously published in vitro lipolysis model (dog digestion model (high dilution/high enzyme activity)) and the initial rat digestion model (Table 5.2 - 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.

5.7.2.2 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 and the data is presented in Figure 5.6 and Table 5.7. 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 5.6).

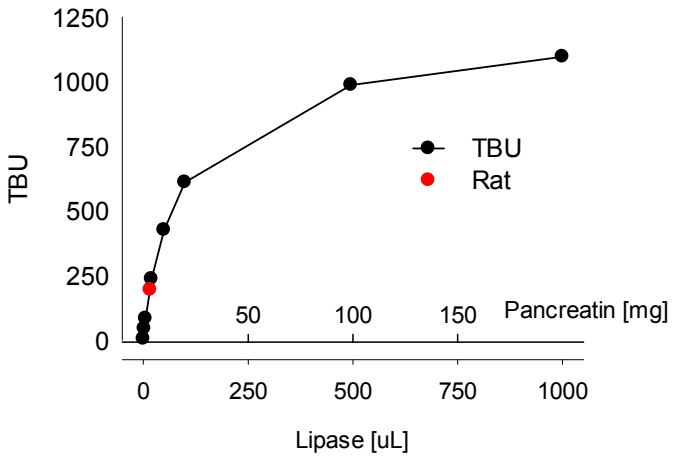

Figure 5.6 Volume of porcine pancreatic extract (and the corresponding quantity of pancreatin powder) added to 10 mL digestions versus the correlating enzyme activity expressed in TBUs. The lipase activity in ex vivo rat pancreatic/biliary fluid is illustrated by the red circle. Similar activity is expected from 17 µL of porcine pancreatic extract (~ 1.7% of the volume utilized previously).

Table 5.7 Activity of ex vivo rat bile and lipase fluids in comparison to the activity of porcine pancreatin extract.

	Porcine							Rat
Volume of pancreatin extract [µL][a]	1000	496	99	50	20	6	3	-
Mass of pancreatin [mg] [b]	202	100	20	10	4	1.1	0.6	-
Activity [TBU] [c]	1097	987	610	426	236	83	44	194 ± 34 [d]

[a] Porcine pancreatic extract was generated by reconstitution of 1 g pancreatin powder in 5 mL buffer followed by centrifugation to separate the aqueous extract.

[b] Quantity of dry porcine pancreatin powder equivalent to the volume of the extract based on the extraction procedure above (i.e. 5 ml extract = 1 g pancreatin).

[c] Activity is measured in tributyrin units where 1 TBU is the amount of enzyme able to liberate 1 µmol fatty acid. Activity for pancreatic extract are mean (n = 2).

[d] Ex vivo rat pancreatic/biliary fluid was collected over 2 h. The data represent activity data obtained across all six animals [mean ± SD (n = 6)]

To examine whether the addition of the equivalent volume of porcine pancreatic extract to the digest led to similar drug distribution patterns to that seen using ex vivo pancreatic/biliary fluids, experiments were repeated under the same conditions but adding 17 µL of porcine pancreatic extract rather than 1 mL of ex vivo rat bile. These data are shown in **Figure 5.7**C. 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.

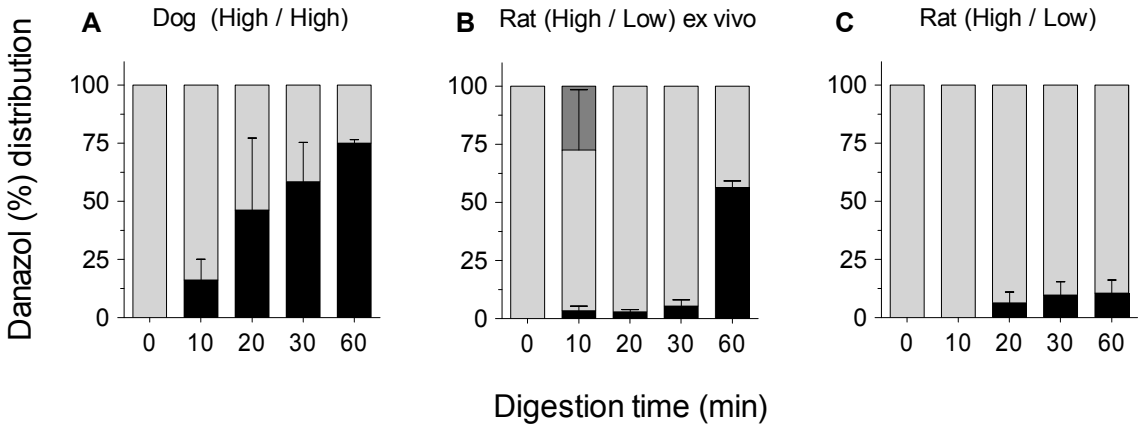

Figure 5.7 Danazol solubilisation during digestion of $SEDDS_L$-III under three conditions; A: In vitro digestion utilizing the previous dog digestion model (high dilution/high enzyme activity) using 4 mL porcine pancreatin extract [data reproduced from Anby et al[179]], B: a rat digestion model (high 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 grey), poorly dispersed oil phase at the surface (dark grey) and precipitate (black) [mean ± SD (n = 3)].

Rat digestion model – low dilution / low enzyme activity

The data obtained using the rat digest model (high dilution/low enzyme activity) allowed an exploration of digestion under the (apparently) much lower enzyme activity conditions in the rat GI tract using the previously used in vitro lipolysis model.[97,108] Whilst this allowed the volumes

examined to be reduced, in reality the volume of the fluids in the rat intestinal tract is likely to be less than 10 mL. In an attempt to better reproduce conditions in the GI tract of the rat, a lower volume (low dilution) rat digestion model was also evaluated.

The low dilution model (Table 5.2 - Rat digestion model (low dilution/low activity)) was set up 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), however in this case, 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 previously published data using the much higher volume digests[179]. These are shown for $SEDDS_L$-III, $SEDDS_H$-III, and SEDDS-IV in (Figure 5.8) where the dog data which has previously has been published.[179] The most notable change was for the SEDDS-III formulation where the decrease in enzyme activity and reduction in volume resulted in the generation of a lipid-rich phase that was more dense than the digestion media and therefore migrated to the bottom of the tube on centrifugation. In contrast, digestion and phase separation under higher enzyme loads and higher dilution was previously shown to result in the production of 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 5.8A2). Similar data were obtained using 1.7% of the standard porcine pancreatic extract (Figure 5.8 panel 3).

Drug loading in the formulation has previously been shown to affect drug precipitation significantly on formulation processing utilizing the dog digestion model (high dilution/high enzyme activity).[179] As such, the impact of drug load was also evaluated using the low volume digestion model. For the $SEDDS_H$-III formulation addition of ex vivo lipase led to some drug precipitation, albeit at a relatively low level (< 20%). However, a similar high density oil phase was generated above the precipitate and this contained the majority of the drug (Figure5.8B2).

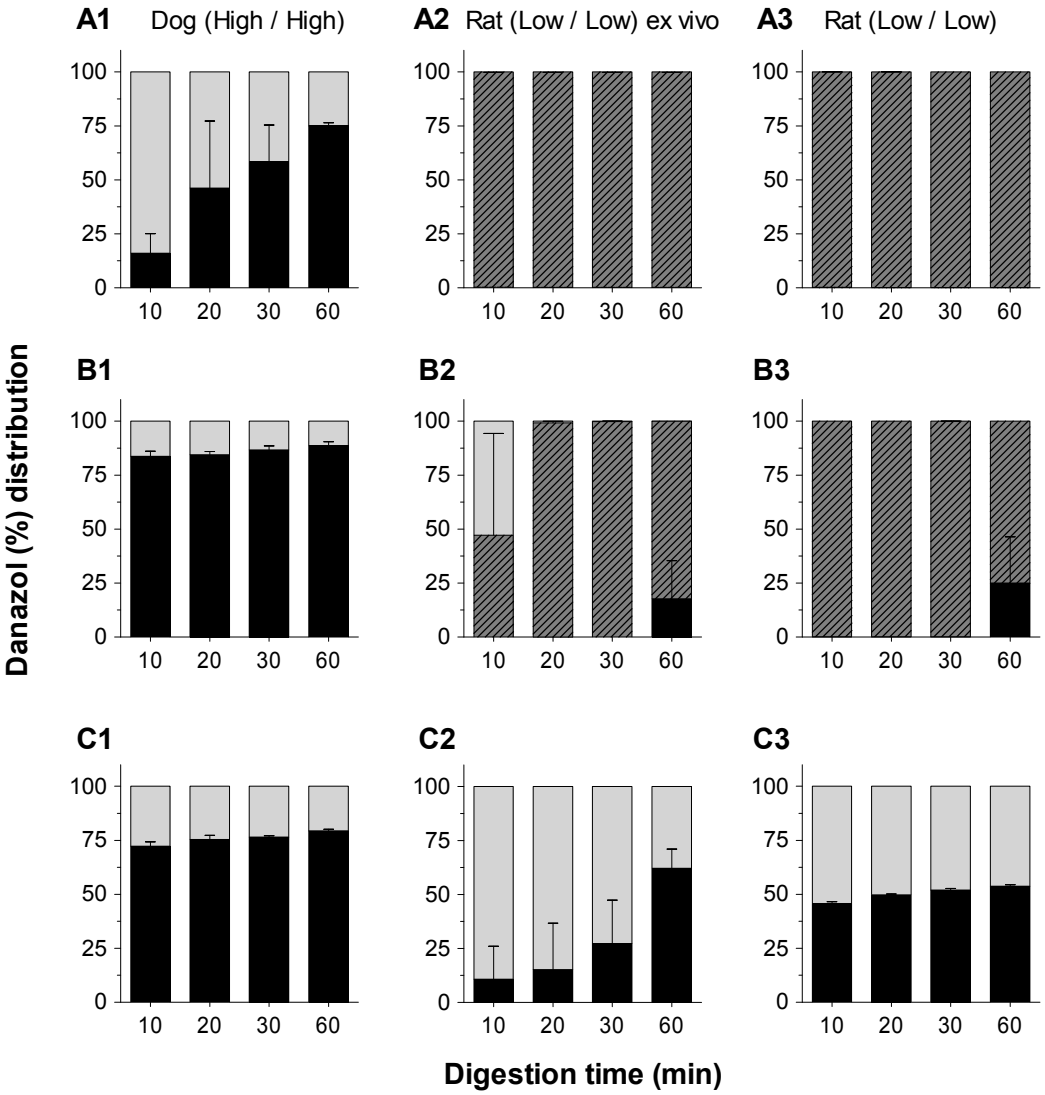

Figure 5.8 Danazol distribution during digestion of A: $SEDDS_L$-III, B: $SEDDS_H$-III and C: SEDDS-IV under three conditions; 1: In vitro dog digestion model (high/high) using 1 mL of porcine pancreatin extract (reproduced from Anby et al[179]), 2: In vitro rat digestion model (low/low) using 1 mL of 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 grey), oil phase below colloidal phase (dark grey pattern) and precipitate (black) [mean ± SD (n = 3)].

The use of porcine pancreatic extract resulted in a similar profile, but in this case precipitation was delayed (Figure 5.8B3). Low dilution rat digestion experiments conducted with formulation SEDDS-IV resulted in high drug precipitation despite the low volume digestion model (Figure 5.8C). The ex vivo rat pancreatic fluid led to lower drug precipitation during the initial 30 min digestion (Figure 5.8C1) when compared to the porcine pancreatic extract (Figure 5.8C2), however, regardless of the source of pancreatic enzymes, only 40-50% of the initial drug loading was recovered in the aqueous phase following 60 min 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 traditional oil in the formulation.

5.8 DISCUSSION

Previous studies of danazol absorption from SEDDS revealed non-linear increases in exposure (bioavailability) with increasing drug dose in dogs.[216] 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 cohort sensitivity of the data suggested the possibility that these events were sensitive to physiological variability such as changes to gastrointestinal conditions (e.g. intestinal bile salt concentrations) or changes to metabolic activity. A series of related studies also examined the potential for polymeric precipitation inhibitors to promote in vitro solubilisation and in vivo bioavailability[179], and unlike several previous studies[24-27], found that the impact of PPI on drug absorption was relatively limited in some cases, and entirely absent in others.

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.

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

The plasma profiles in Figure 5.2A and the pharmacokinetic parameters presented in Table 5.4 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%.[129,224] 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.[216]

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.[179]

The addition of 5% HPMC to the $SEDDS_L$-III formulation was previously shown to result in a moderate increase in danazol oral bioavailability in dogs, whereas PPI addition to the same formulation containing danazol at a higher drug load (i.e. with $SEDDS_H$-III) had little effect. The potential impact of PPI addition was also explored here in the rat (Figure 5.2B, C, D) and in contrast to previous studies, the addition of 5% HPMC to SEDDS-III or SEDDS-IV had either no significant effect on bioavailability, or resulted in a reduction in exposure. Increased viscosity has been reported to decrease absorption following oral administration and it is possible therefore

that the negative effect of the polymer for $SEDDS_H$-III was due to increases in viscosity as a result of the presence of HPMC.[226] This might be expected to be more prevalent in the rat GI tract, where dilution might be expected to be lower and therefore viscosity increases higher than in the dog. It is not clear, however, why this effect was not replicated with the other formulations (where HPMC levels were the same). Since the principle mechanism by which PPI are expected to act is via a reduction in drug precipitation on formulation processing, the species specificity of the PPI effects also suggests that formulation dispersion and digestion events in the rat are somewhat different to that in the dog. This possibility is discussed in detail in later sections, but in short the principle limitation to danazol bioavailability from SEDDS in the rat may not be loss of solubilisation capacity, but rather significant first pass metabolism. This in turn provides some explanation for the lack of effect of the PPI.

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 5.3 and the pharmacokinetic parameters presented in Table 5.5 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), 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 delayed entry into the small intestine).

5.8.2 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.[177] In vitro studies using pooled human liver microsomes and cytochrome P450 isoform specific inhibitors have further shown that danazol is primarily a substrate for CYP3A4 (86%) and to a lesser extent CYP2D6 (11%) and CYP2J2 (3%).[197] Direct, interspecies extrapolations of enzyme activities are difficult due to differences in CYP isoforms between species (a detailed review on species differences in CYP-mediated metabolism can be found in Martignoni et al[63]), however, a significant role for CYP3A4 and CYP2D6-based metabolism in danazol clearance is apparent and consistent with the potential for first pass hepatic and enterocyte-based metabolism.

The role of CYP-mediated danazol metabolism in the rat was explored 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.[155] 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.[155,158] High single oral doses of ABT (100 mg/kg) are also safe and result in greater than 93% reduction in the plasma clearance of the model CYP substrate (antipyrine).[155]

Pre-dosing of ABT to inhibit CYP-mediated metabolism led to a significant increase in danazol plasma concentrations after both intravenous and oral administration. 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 5.4 and Figure 5.2, respectively). First pass metabolism is therefore a very significant limitation to danazol oral bioavailability in rats under these conditions.

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 5.4). 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 5.1) 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 (that is present in high concentration in SEDDS IV) is able to inhibit CYP3A in human and rat liver microsomes.[227,228] 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.

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 further suggests that in this case the differences between ID and oral administration were 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 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 was evident for SEDDS-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 pas 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[179] that showed 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.

5.8.3 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 previously published dispersion data[179] conducted under simulated intestinal conditions (panel 1 in Figure 5.5)**.** To better reflect the conditions expected in the GI tract of the rat, dispersion volume and pH were subsequently lowered 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 (panel 2 in Figure 5.5) 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,[229] and lingual lipase activity is reportedly[225] high in rodents and may extend from the pharynx through the oesophagus and stomach and into the upper small intestine.[230] 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 5.5C)**.** However, previous studies in rats have shown that pre-processing of lipids in the stomach may affect subsequent events in the duodenum, including the stimulation and secretion of digestive enzymes and the rate and extent of lipid digestion.[231-233] As

such, gastric digestion by lingual lipase may indirectly affect drug absorption in the small intestine via a priming effect on intestinal processing, even when gastric digestion is limited.

5.8.4 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.

Several studies have investigated lipolytic activity in ex vivo rat pancreatic fluid, and for example, Nalbone et al[234] report an activity of 1500 U lipase activity/mL pancreatic juice (absent of bile) (where 1 lipase unit was defined as the quantity of enzyme that resulted in the liberation of 1 µmol fatty acid per min). In contrast, Borgström[235] reported somewhat lower activity levels (~600 U/mL of pancreatic juice absent of bile). In both cases, activity was higher than that observed here (~200 TBU/mL pancreatic/biliary fluid). In these previous studies, however, pancreatic fluid was collected directly from the pancreatic duct rather than collection of mixed pancreatic/biliary fluids from the common bile duct. The rate of pancreatic fluid secretion in anaesthetised rats is 540-620

µL/h[236,237] and significantly lower than the rate of secretion of mixed pancreatic/biliary fluids in the current studies (~1.5 mL/h). 1 mL of collected pancreatic/biliary fluid might therefore be expected to contain ~0.4 mL pancreatic fluid and 0.6 mL biliary fluid. The activity measured here (200 TBU/mL pancreatic/biliary fluid) therefore reflects the enzyme activity in ~400 µL of pancreatic fluid and simplistically equates to enzyme activities of ~500 TBU/mL pancreatic fluid. This is more consistent with that reported by Borgström[235]. Recently, Tønsberg et al[238] 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 5.7). 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 5.6). 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, at least initially, 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 drop in pH during digestion of SEDDS-III, and the pH dropped to 5.8 at the end of the 60 min digestion period. Pancreatic enzyme activity, however, was expected to be retained since the drop in pH was limited.[208] Lingual lipase activity is also expected to continue at lower pH in the upper small intestine and may have also contributed to enzyme activity.[230] 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 these observations, Holt et al[239] previously reported the generation of a dense viscous phase following ultracentrifugation of duodenal content in humans, and the sedimentation of a birefringent lamellar liquid crystalline phase was also reported by Patton and Carey[92] in their

seminal microscopic study of lipid digestion. The potential for the generation of liquid crystal phases, including lamellar liquid crystals, during the digestion of formulation lipids has been described previously[123,124] and studied in detail using combinations of medium-chain lipid digestion products and model intestinal fluids.[114] In the latter studies, increasing dilution (i.e. higher proportions of bile salt/phospholipid micelles and lower proportions of lipid digestion products) resulted in an increasing prevalence of highly dispersed micellar phases whereas at higher lipid proportions liquid crystals were more apparent.[114] The current data are consistent with these observations and suggest that in the rat, under conditions of lower dilution in vitro (and potentially in vivo), less readily dispersed lipid phases, including lamellar liquid crystal phases, are formed that are less dense than water. 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[240,241] 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 and suggest that drug precipitation may be lower, at least initially, than that indicated by digestion experiments conducted under high dilution and high enzyme conditions.

5.8.5 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.[216] First impressions therefore indicate that the increase in bioavailability with dose seen previously in dogs may also have been a function of reduced first pass metabolism. However, in vivo studies in other laboratories with beagle dogs suggest that the absolute oral bioavailability of danazol may be quite high, and certainly much higher than that seen here in rats. For example, Liversidge et al[217] reported oral bioavailabilities of 107% for a cyclodextrin-danazol formulation and 82% for a nanoparticle suspension.[217] In these studies, much higher danazol doses were administered than our previous studies (~20 mg/kg rather than doses up to 3.5 mg/kg in Anby et al[216], and therefore more complete saturation of first pass metabolism may have occurred. However, Jain et al[242] also reported relatively high oral bioavailability for danazol in beagle dogs (64%) after administration of a capsule containing a danazol-cyclodextrin complex at doses (5 mg/kg) similar to those employed in Anby et al[216]. Collectively, the data indicate 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 significantly lower. Under those conditions it seems less likely that

the differences in bioavailability seen in previous studies with increases in drug dose were a result of saturation of first pass metabolism, and rather that they reflect enhanced thermodynamic activity at higher drug dose, although a mixed effect remains possible. Interestingly, in the previous dog studies different results were obtained with two different animal cohorts, and an increase in exposure with increasing dose was only evident in older animals. In the younger animals exposure increased linearly with dose. At the time, this was suggested to reflect either differences in metabolic capabilities in the two cohorts (assuming saturation of first pass metabolism as the mechanisms behind the increase in absorption at increased dose) or differences in intestinal conditions resulting in differences in solubilisation capacity (assuming differences in thermodynamic activity as the driver for increased absorption with dose). In support of the latter contention, in vitro studies showed that if intestinal bile salt levels were raised in the older animals, then prolonged supersaturation, and therefore increases in thermodynamic activity, was possible with increasing drug dose. Unfortunately, advancing age in the older cohort precluded their ethical use in further studies. Both possibilities therefore remain possible.

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 where considerable drug precipitation was evident when studies were conducted using conditions modelled on the likely GI environment in a dog.[13,179] High absorption was also not predicted based on previous bioavailability data in the dog[13,106] showing significant formulation effects on bioavailability that were seemingly related to solubilisation changes on in vitro dispersion and digestion. 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. This may also explain the more significant effect of the PPI noted previously on drug solubilisation in the dog[179] rather than the lack of effect noted here in rats.

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.[179] 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 suggest that in spite of the somewhat higher prevalence of precipitation from SEDDS-IV when compared to SEDDS-III, in both dog and rat digest 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[243], and later the basic BSC class II drug mebendazole[244], and simple in vitro models of drug precipitation where the in vitro tests also appeared to over-predict precipitation in vivo.

Whilst the arguments described above suggest that the very large impact of first pass metabolism described here for the rat may not be replicated in the dog, the potential for first pass metabolic

effects to confound previous interpretations of excipient effects on danazol bioavailability in beagle dogs stimulated a re-examination of these earlier data sets. In particular, in studies by Cuine et al[13] danazol bioavailability was previously shown to decrease with the inclusion of increasing proportions of surfactant and cosolvent in SEDDS formulations. These data were also shown to correlate with increases in drug precipitation from the same type of formulations on in vitro digestion. This led to the suggestion that the principle limitation to bioavailability was continued drug solubilisation and that loss of solubilisation capacity on digestion of the surfactant-rich formulation was more evident than that from the formulations containing larger quantities of long-chain lipids. In turn, the increase in precipitation from the surfactant-rich formulations in vitro was suggested to explain the reduction in exposure seen in vivo. Notably, the long-chain lipid-rich formulations were also digested but in this case retained solubilisation capacity on digestion, consistent with increased in vivo exposure.[13]

The current data raise the question as to whether formulation or dose effects on first pass metabolism may have obscured data interpretation in these previous dog studies. This seems unlikely, however, as 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.

5.9 CONCLUSION

Danazol bioavailability in the rat after oral administration of a LFCS class III and Class IV lipid based formulation was extremely low. In contrast, data obtained in the presence of the metabolic inhibitor ABT suggest that the fraction absorbed was high (45-60%) and that the major limitation to oral bioavailability was first pass metabolism. Consistent with these suggestions, PPI had little effect on bioavailability and no significant differences in danazol absorption were evident after administration of two candidate SEDDS, even though differences in solubilisation were evident during the conduct of in vitro digestion tests. 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.

5.10 ACKNOWLEDGEMENT

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5.11 SUPPORTING INFORMATION

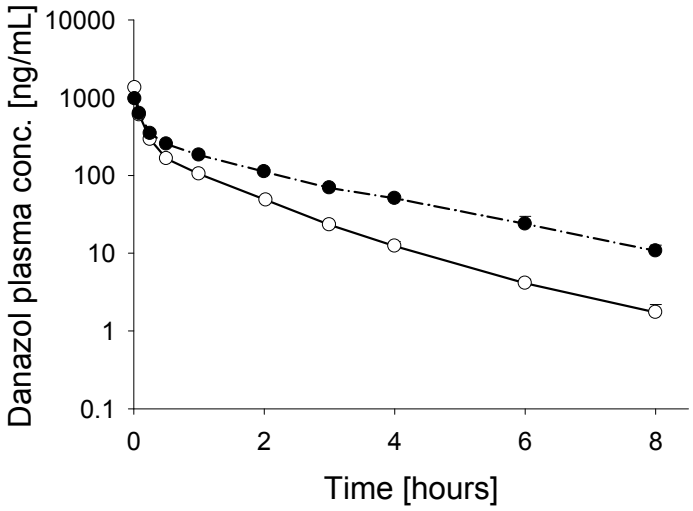

Figure 5.9 Danazol plasma profile after IV administration of 15% HP-β-CD solution containing danazol at 1.2 mg/mL to control (open) [mean ± SD (n = 5)] and pre-treated (filled) ABT rats [mean ± SD (n = 4)].

Table 5.8 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)].

Treatment	Dose [mg kg^{-1}]	$AUC_{0-\infty}$ [ng h mL^{-1}]	Cl [mL min^{-1} kg^{-1}]	Vd [L kg^{-1}]	$t_{1/2}$ [h]
Control	2.3	434 ± 18	87 ± 3.3	8.9 ± 0.5	1.2 ± 0.1
ABT pre-treated	2.3	761 ± 22	50 ± 2.6[a]	7.6 ± 0.4	1.8 ± 0.2[a]

[a] Statistically significant different compared to control ($P < 0.050$)

CHAPTER 6

SUMMARY AND PERSPECTIVES

6 Summary and perspectives

This thesis has explored the mechanisms that underpin drug absorption following the oral administration of lipid-based drug delivery systems (LBDDS). The role of formulation excipients and drug loading in the generation and maintenance of drug supersaturation during LBDDS processing in the gastrointestinal tract has been a particular focus. The work contributes to a better understanding of the mechanisms by which LBDDS promote drug absorption and specifically to the influence of drug dose, the presence or absence of polymeric precipitation inhibitors (PPI) and the role of different in vivo and in vitro models in predicting patterns of drug solubilisation, supersaturation and absorption.

Low systemic exposure after oral administration is a major challenge to effective drug development. For highly hydrophobic drugs, low water-solubility is a particular concern, since in almost all cases drugs must be in solution in the gastrointestinal (GI) fluids to be absorbed. Poor oral absorption due to low GI solubility and slow dissolution may be overcome using a range of approaches including changes to drug solid state properties (e.g. isolation of different salt and crystal forms), changes to particle size (e.g. milling and nano-milling) and the use of specialised formulations, such as solid dispersions, cyclodextrins and lipid-based drug delivery systems (LBDDS). The work described here has focussed on the use of LBDDS to enhance the oral bioavailability of poorly water-soluble drugs (PWSD) and has examined, in detail, LBDDS that contain hydrophilic surfactants and cosolvents, and therefore that self-emulsify on contact with aqueous environments (so called self-emulsifying drug delivery systems (SEDDS)).

LBDDS provide for increased absorption of PWSD by circumventing the traditional dissolution processes that commonly limit the absorption of drugs with low GI solubility. This is achieved by presenting drug to the GI tract in a molecularly dispersed form (i.e. in solution in the formulation) and simultaneously by increasing the solubilisation capacity of the GI fluids. The latter results

from the incorporation of formulation-derived digestion products into endogenous, biliary-derived, bile salt-phospholipid micelles to form a solubilised reservoir of colloidal structures, including mixed micelles, vesicles and microemulsion droplets. These colloidal structures significantly enhance the solubilisation capacity of the GI fluid for PWSD when compared to the fasted GI content.

Historically, attention in the lipid-based formulation field has focused largely on the composition and self-emulsification properties of LBDDS as determinants of formulation performance and drug bioavailability. The research presented here, however, builds on recent work from our laboratories[13-16,167] and elsewhere[130] to suggest that the kinetic events that evolve on oral administration of LBDDS and the patterns of interaction of these systems with pancreatic and biliary secretions may be more important in determining drug absorption patterns in vivo. A more detailed understanding of these events is expected to facilitate a better choice of in vitro measures of formulation performance and therefore a better correlation between in vitro evaluation and in vivo bioavailability.

In the first series of experiments (Chapter 3), an in vitro lipid digestion model was used to explore the impact of changes to the composition of LBDDS, on the solubilisation and/or precipitation patterns of a model PWSD (danazol) under simulated GI conditions. The lipid digestion experiments were designed to model the kinetic changes to formulation properties that occur on interaction with digestive enzymes and biliary-derived solubilizers in vivo (Chapter 3). During the conduct of these studies, and against a backdrop of increased interest in the role of supersaturation in drug absorption[136,138,140,149,170,174,243,245-247], lipid digestion was revealed as a significant 'trigger' for the generation of drug supersaturation during LBDDS processing. Attention subsequently focussed on quantifying the degree of drug supersaturation in the colloidal structures formed during formulation digestion and in relating this to formulation design.

The data obtained suggest that knowledge of the extent to which excipients facilitate the generation of supersaturation, and the stability of this metastable state as the formulation is processed in the GI tract, may be more significant determinants of formulation performance (at least in vitro) than the equilibrium solubilisation capacity of the colloids formed post digestion. The studies further showed that solubilisation/precipitation behaviour was highly correlated with the maximum degree of supersaturation obtained as the formulation was dispersed and digested (as defined by the maximum supersaturation ratio (S^M). Formulations that contained larger quantities of drug, and where lipid digestion led to the most significant drop in solubilisation capacity, resulted in higher degrees of initial supersaturation and above a critical point this initial supersaturation 'pressure' also provided a stimulus for precipitation. A threshold was identified such that formulations resulting in supersaturation ratios of greater than ~3 typically resulted in significant precipitation, whereas formulations where supersaturation was more modest (i.e. < 3), were more stable and protected against precipitation.

Awareness of the likelihood of drug precipitation from highly supersaturated intestinal colloidal species stimulated an examination of the potential for polymers to stabilise supersaturated drug concentrations, and in turn, to enhance absorption (Chapter 3). Several candidate 'polymeric precipitation inhibitors' (PPI) were evaluated, many of which were found to effectively inhibit drug precipitation using the in vitro digestion model. Hydroxypropyl methylcellulose (HPMC) was chosen for more detailed evaluation and was incorporated into a range of SEDDS formulations that had previously been shown to lead to significant drug precipitation on digestion. In almost all cases, the inclusion of HPMC in the formulation, even at relatively low concentrations (< 5% w/w in the formulation) led to a dramatic decrease in the degree of drug precipitation. Subsequent in vivo evaluation in beagle dogs showed that in some cases the addition of HPMC was also able to provide for moderate increases (~65%) in danazol oral bioavailability after the oral administration of lipid formulations when compared to the equivalent formulations without HPMC. However,

this was not always the case and at higher drug loads, where supersaturation was the greatest, HPMC failed to have any impact on bioavailability.

These initial studies suggest that differences in the solubilisation performance of LBDDS stem largely from differences in supersaturation stabilisation. The data further suggest that although PPIs can be very effective in stabilising supersaturation in vitro, these effects are not always apparent in vivo. One additional finding from the studies in Chapter 3 was that at higher drug loads (doses), regardless of the presence or absence of polymer, systemic exposure increased non-linearly when compared to the data obtained at lower drug loads. This apparently anomalous result (since bioavailability, for PWSD, more typically decreases at higher dose) suggested a possible role for factors beyond solubility in determining danazol bioavailability from these formulations and stimulated a series of further studies described in Chapter 4.

Chapter 4 describes a more detailed evaluation of the mechanism(s) by which increases in drug dose result in increased bioavailability from LBDDS in beagle dogs. As part of this investigation, it became evident that the non-linearity in danazol bioavailability observed previously was dependent on the animal cohort in which the study was conducted and was apparent only after administration to an older (but not younger) group of beagle dogs. Thus, in the younger cohort increases in danazol exposure were linear with increases in dose and bioavailability was unchanged across the dosing range. In contrast, in the older dogs, increasing dose resulted in greater than linear increases in bioavailability up to a critical point, above which further increases in dose resulted in a reduction in bioavailability, and a decrease to exposure levels obtained in the young cohort. These patterns were consistent with the in vitro data that suggest that increases in dose lead initially to increases in supersaturation and thermodynamic activity (and therefore potential increases in absorption), but that ultimately, further increases in dose result in drug precipitation and a reduction in bioavailability.

In an attempt to explain the differences in danazol exposure observed across animal cohorts, the potential role of differences in first pass metabolism (as indicated by differences in hepatic function) and differences in intestinal processing of LBDDS (due to differences in bile salt concentration and formulation digestion) were evaluated in vitro. The data revealed no significant differences in hepatic function between cohorts, and instead suggested that age-related changes in GI solubilisation, potentially through increased bile salt secretion in the older cohort, may have led to better supersaturation stabilisation and therefore increases in danazol absorption. Increases in the quantity of drug absorbed at higher doses in the old cohort may also have led to saturation of first pass metabolism, resulting in further increases in bioavailability.

Realising the potential impact of first pass metabolism on danazol bioavailability, further studies were conducted in rats to allow more direct exploration of first pass effects via the use of a chemical inhibitor of CYP-mediated metabolism (Chapter 5). The role of drug dose on the bioavailability of danazol from LBDDS was explored in the presence and absence of the CYP450-mediated enzyme inhibitor, ABT, and in the presence and absence of PPI. Interestingly, danazol bioavailability in the rat following oral administration of two different LBDDS was low (< 12%), and incorporation of PPI had either no effect or limited impact on bioavailability. In contrast, data obtained in the presence of ABT indicated that the fraction of the dose absorbed was high (40-60%) and that first pass metabolism was a major limitation to oral bioavailability.

To evaluate the potential influence of gastric processing on formulation performance, danazol bioavailability was also assessed after intraduodenal and oral administration of the LBDDS formulations to rats. In most cases, circumvention of gastric effects resulted in significant increases in bioavailability, suggesting that formulation dispersion in the stomach or differences in gastric emptying rates after oral versus intraduodenal administration may have had an impact on danazol exposure. As part of this evaluation, the applicability of previous in vitro models of lipid digestion (that were largely based on the conditions in the dog GI tract) to events in the rat was

evaluated and a number of modifications to the model suggested to more effectively mimic events in the rat GI tract. Most notably, evaluation of the activity of rat pancreatic fluids suggested that lipid digestion may be less efficient in rats than in dogs (or human), and therefore that danazol precipitation on formulation digestion may be lower. This was consistent with the in vivo data in rats where danazol absorption from the LBDDS formulations was high (> 50%) and the principle limitation to danazol bioavailability was first pass metabolism. In similar agreement with these suggestions, the addition of PPI to the LBDDS formulations had little effect on bioavailability in the rat, presumably because the fraction absorbed was already high.

In conclusion, the studies undertaken in this thesis have shown that lipid digestion functions as an effective trigger for drug supersaturation in the GIT and that the generation of supersaturation may have a significant impact on formulation performance and bioavailability. As such, the extent to which excipients generate supersaturation and maintain the stability of the metastable state as the formulation is processed in the GI tract may be significant determinants of formulation performance. More specifically, the utilisation of lower drug loads, higher surfactant levels, reduced cosolvent and the addition of PPI all enhanced formulation performance (i.e. drug solubilisation) in vitro. The data further suggest that increases in drug dose are able to increase thermodynamic activity in the aqueous colloidal phase and that to a point this may be sufficiently stable to drive improvements in absorption and therefore in vivo bioavailability. Above a critical drug loading, however, supersaturation 'pressure' appears to promote drug precipitation and therefore counteracts any beneficial effects of increases in thermodynamic activity, therefore resulting in reductions in bioavailability.

The studies further illustrate the impact that variability between animal cohorts and age can play when evaluating bioavailability from LBDDS, and likely reflects differences in the physiological variables that control lipid digestion. Thus, in the rat, formulation dilution and digestive enzyme activity were low, metabolic activity was high and effects on first pass metabolism appeared to

dominate danazol absorption from LBDDS. In contrast, in beagle dogs danazol bioavailability was less dependent on first pass metabolism, more dependent on continued solubilisation, and more sensitive to differential formulation processing. The greater similarity between the GI tract of dogs and humans when compared to rats (with respect to bile salt concentration and pancreatic lipase activity), suggests that for LBDDS, events in humans may more closely reflect formulation processing under in vitro conditions reflective of dog intestinal conditions when compared to experiments conducted using conditions modeled on the rat.

Looking forward, the current studies signal the potential importance of drug supersaturation in determining the performance of LBDDS, but also raise a number of questions that, whilst beyond the scope of the current investigation, might usefully be addressed. Firstly, and perhaps most obviously, the current studies were conducted with a single probe compound, danazol. Whether the patterns of behavior reported here would be replicated with other poorly water-soluble drugs is not possible to say without further experimental data – although the drivers of supersaturation and precipitation are largely physicochemical and therefore likely predictable. As became evident in the final set of studies, danazol is highly first pass metabolized in rats, and in retrospect, is not an ideal probe for differences in drug absorption in the rat under normal conditions. Future studies in rats could be conducted to evaluate formulation effects on danazol absorption, but in this case routine use of ABT as a means of circumventing the complications of first pass metabolism would be beneficial. Broadening the data sets to include a range of PWSD with differing physicochemical properties would also add power to the current data set.

The current studies also explored the use of altered in vitro lipid digestion protocols to better reflect conditions in the rat when compared to the dog, however, the scope and scale of these studies were limited and future studies might usefully generate a larger data base of correlative in vitro-in vivo data to better interrogate the most effective conditions across species.

Finally, an intrinsic limitation of all currently available in vitro lipid digestion models is the lack of sink conditions for both lipid digestion products and drug. For drugs with reasonable aqueous solubility, the conduct of dissolution studies in a significant volume is sufficient to maintain sink conditions. However, this is not possible for lipids and PWSD where only a small proportion of the delivered dose is able to be solubilized in the GI fluids at any one time. Two phase in vitro dispersion and/or digestion tests containing a water immiscible organic phase as a sink might be envisaged (and have been used to good effect in simple dissolution testing for PWSD[248-252]), but are complicated in this case by the potential for solubilisation of the sink in bile salt/digestion product micelles. Alternatively, in vitro dispersion and digestion tests may be coupled to cell culture models or in situ permeability models to better understand drug absorption from LBDDS and to inform the development, and interpretation, of improved in vitro models. However, simple, reproducible, in vitro models of lipid digestion will likely remain as the mainstay of routine in vitro challenge tests for lipid-based formulations as more complex dissolution-permeability models highly influenced by issues like complexity, reliability and difficulties of application in a formulation development environment. Increasingly, however, it seems likely that more detailed interpretation of in vitro digestion data will be required to delineate the role of supersaturation as a driver of both precipitation (and therefore reduced absorption) and thermodynamic activity (and therefore increased absorption).

7 References

(1) Florence, A. T.; Attwood, D., *Physicochemical Principles of Pharmacy*. 3rd Ed. ed.; Pharmaceutical Press: London, 1998.

(2) Gursoy, R. N.; Benita, S. Self-emulsifying drug delivery systems (SEDDS) for improved oral delivery of lipophilic drugs. *Biomedicine & amp; Pharmacotherapy* **2004,** *58*, (3), 173-182.

(3) Lipinski, C. A. Drug-like properties and the causes of poor solubility and poor permeability. *Journal of Pharmacological and Toxicological Methods* **2000,** *44*, (1), 235-249.

(4) Strickley, R. Solubilizing Excipients in Oral and Injectable Formulations. *Pharmaceutical Research* **2004,** *21*, (2), 201-230.

(5) Williams, H. D.; Trevaski, N. L.; Charman, S. A.; Shanker, R. M.; Charman, W. N.; Pouton, C. W.; Porter, C. J. H. Strategies to Address Low Drug Solubility in Discovery and Development. *Pharmacological Review* **2013***, 65,* (1), 315-499.

(6) Pouton, C. W.; Porter, C. J. H. Formulation of lipid-based delivery systems for oral administration: Materials, methods and strategies. *Advanced Drug Delivery Reviews* **2008,** *60*, (6), 625-637.

(7) Charman, S. A.; Charman, W. N.; Rogge, M. C.; Wilson, T. D.; Dutko, F. J.; Pouton, C. W. Self-Emulsifying Drug Delivery Systems: Formulation and Biopharmaceutic Evaluation of an Investigational Lipophilic Compound. *Pharmaceutical Research* **1992,** *9*, (1), 87-93.

(8) Shah, N. H.; Carvajal, M. T.; Patel, C. I.; Infeld, M. H.; Malick, A. W. Self-emulsifying drug delivery systems (SEDDS) with polyglycolyzed glycerides for improving in vitro dissolution and oral absorption of lipophilic drugs. *International Journal of Pharmaceutics* **1994,** *106*, (1), 15-23.

(9) Hauss, D. J.; Fogal, S. E.; Ficorilli, J. V.; Price, C. A.; Roy, T.; Jayaraj, A. A.; Keirns, J. J. Lipid-based delivery systems for improving the bioavailability and lymphatic transport of a poorly water-soluble LTB4 inhibitor. *Journal of Pharmaceutical Sciences* **1998,** *87*, (2), 164-169.

(10) Kim, J. Y.; Ku, Y. S. Enhanced absorption of indomethacin after oral or rectal administration of a self-emulsifying system containing indomethacin to rats. *International Journal of Pharmaceutics* **2000,** *194*, (1), 81-89.

(11) Kang, B. K.; Lee, J. S.; Chon, S. K.; Jeong, S. Y.; Yuk, S. H.; Khang, G.; Lee, H. B.; Cho, S. H. Development of self-microemulsifying drug delivery systems (SMEDDS) for oral bioavailability enhancement of simvastatin in beagle dogs. *International Journal of Pharmaceutics* **2004,** *274*, (1–2), 65-73.

(12) Hong, J.-Y.; Kim, J.-K.; Song, Y.-K.; Park, J.-S.; Kim, C.-K. A new self-emulsifying formulation of itraconazole with improved dissolution and oral absorption. *Journal of Controlled Release* **2006,** *110*, (2), 332-338.

(13) Cuiné, J. F.; Charman, W. N.; Pouton, C. W.; Edwards, G. A.; Porter, C. J. H. Increasing the Proportional Content of Surfactant (Cremophor EL) Relative to Lipid in Self-emulsifying Lipid-

based Formulations of Danazol Reduces Oral Bioavailability in Beagle Dogs. *Pharmaceutical Research* **2007,** *24*, (4), 748-757.

(14) Kaukonen, A.; Boyd, B. J.; Charman, W. N.; Porter, C. J. H. Drug Solubilization Behavior During in Vitro Digestion of Suspension Formulations of Poorly Water-Soluble Drugs in Triglyceride Lipids. *Pharmaceutical Research* **2004b,** *21*, (2), 254-260.

(15) Kossena, G. A.; Charman, W. N.; Boyd, B. J.; Dunstan, D. E.; Porter, C. J. H. Probing drug solubilization patterns in the gastrointestinal tract after administration of lipid-based delivery systems: A phase diagram approach. *Journal of Pharmaceutical Sciences* **2004,** *93*, (2), 332-348.

(16) Porter, C. J. H.; Kaukonen, A. M.; Boyd, B. J.; Edwards, G. A.; Charman, W. N. Susceptibility to Lipase-Mediated Digestion Reduces the Oral Bioavailability of Danazol After Administration as a Medium-Chain Lipid-Based Microemulsion Formulation. *Pharmaceutical Research* **2004a,** *21*, (8), 1405-1412.

(17) Raghavan, S. L.; Kiepfer, B.; Davis, A. F.; Kazarian, S. G.; Hadgraft, J. Membrane transport of hydrocortisone acetate from supersaturated solutions; the role of polymers. *International Journal of Pharmaceutics* **2001a,** *221*, (1-2), 95-105.

(18) Simonelli, A. P.; Mehta, S. C.; Higuchi, W. I. Inhibition of sulfathiazole crystal growth by polyvinylpyrrolidone. *Journal of Pharmaceutical Sciences* **1970,** *59*, (5), 633-638.

(19) Usui, F.; Maeda, K.; Kusai, A.; Nishimura, K.; Keiji, Y. Inhibitory effects of water-soluble polymers on precipitation of RS-8359. *International Journal of Pharmaceutics* **1997,** *154*, (1), 59-66.

(20) Miller, D. A.; DiNunzio, J. C.; Yang, W.; McGinity, J. W.; Williams, R. O. Targeted Intestinal Delivery of Supersaturated Itraconazole for Improved Oral Absorption. *Pharmaceutical Research* **2008a,** *25*, (6), 1450-1459.

(21) Miller, D. A.; DiNunzio, J. C.; Yang, W.; McGinity, J. W.; Williams, R. O. Enhanced In Vivo Absorption of Itraconazole via Stabilization of Supersaturation Following Acidic-to-Neutral pH Transition. *Drug Development and Industrial Pharmacy* **2008b,** *34*, (8), 890 - 902.

(22) Raghavan, S. L.; Schuessel, K.; Davis, A.; Hadgraft, J. Formation and stabilisation of triclosan colloidal suspensions using supersaturated systems. *International Journal of Pharmaceutics* **2003,** *261*, (1-2), 153-158.

(23) Raghavan, S. L.; Trividic, A.; Davis, A. F.; Hadgraft, J. Crystallization of hydrocortisone acetate: influence of polymers. *International Journal of Pharmaceutics* **2001b,** *212*, (2), 213-221.

(24) Gao, P.; Akrami, A.; Alvarez, F.; Hu, J.; Li, L.; Ma, C.; Surapaneni, S. Characterization and optimization of AMG 517 supersaturatable self-emulsifying drug delivery system (S-SEDDS) for improved oral absorption. *Journal of Pharmaceutical Sciences* **2009,** *98*, (2), 516-528.

(25) Gao, P.; Guyton, M. E.; Huang, T.; Bauer, J. M.; Stefanski, K. J.; Lu, Q. Enhanced Oral Bioavailability of a Poorly Water Soluble Drug PNU-91325 by Supersaturatable Formulations. *Drug Development and Industrial Pharmacy* **2004,** *30*, (2), 221 - 229.

(26) Gao, P.; Morozowich, W., Design and Development of Supersaturatable Self-Emulsifying Drug Delivery Systems for Enhancing the Gastrointestinal Absorption of Poorly Soluble Drugs. In

Oral Lipid-Based Formulations: Enhancing the bioavailbility of poorly water-soluble drugs.1st ed.; Hauss, D. J., 'Ed.' Informa Healthcare: New York, 2007; Vol. 170, pp 303-328.

(27) Gao, P.; Rush, B. D.; Pfund, W. P.; Huang, T.; Bauer, J. M.; Morozowich, W.; Kuo, M.-S.; Hageman, M. J. Development of a supersaturable SEDDS (S-SEDDS) formulation of paclitaxel with improved oral bioavailability. *Journal of Pharmaceutical Sciences* **2003,** *92*, (12), 2386-2398.

(28) Wei, Y.; Ye, X.; Shang, X.; Peng, X.; Bao, Q.; Liu, M.; Guo, M.; Li, F. Enhanced oral bioavailability of silybin by a supersaturatable self-emulsifying drug delivery system (S-SEDDS). *Colloids and Surfaces A: Physicochemical and Engineering Aspects* **2012,** *396*, (0), 22-28.

(29) Kataoka, M.; Sugano, K.; da Costa Mathews, C.; Wong, J.; Jones, K.; Masaoka, Y.; Sakuma, S.; Yamashita, S. Application of Dissolution/Permeation System for Evaluation of Formulation Effect on Oral Absorption of Poorly Water-Soluble Drugs in Drug Development. *Pharmaceutical Research* **2012,** *29*, (6), 1485-1494.

(30) Amidon, G. L.; Lennernäs, H.; Shah, V. P.; Crison, J. R. A Theoretical Basis for a Biopharmaceutic Drug Classification: The Correlation of in Vitro Drug Product Dissolution and in Vivo Bioavailability. *Pharmaceutical Research* **1995,** *12*, (3), 413-420.

(31) Zhang, Y.; Benet, L. Z. The Gut as a Barrier to Drug Absorption: Combined Role of Cytochrome P450 3A and P-Glycoprotein. *Clinical Pharmacokinetics* **2001,** *40*, (3), 159-168.

(32) Lipinski, C. A.; Lombardo, F.; Dominy, B. W.; Feeney, P. J. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Advanced Drug Delivery Reviews* **1997,** *23*, (1-3), 3-25.

(33) Rane, S. S.; Anderson, B. D. What determines drug solubility in lipid vehicles: Is it predictable? *Advanced Drug Delivery Reviews* **2008,** *60*, (6), 638-656.

(34) Porter, C. J. H.; Charman, W. N. In vitro assessment of oral lipid based formulations. *Advanced Drug Delivery Reviews* **2001,** *50*, (Supplement 1), S127-S147.

(35) Dressman, J. B.; Amidon, G. L.; Reppas, C.; Shah, V. P. Dissolution Testing as a Prognostic Tool for Oral Drug Absorption: Immediate Release Dosage Forms. *Pharmaceutical Research* **1998,** *15*, (1), 11-22.

(36) Hörter, D.; Dressman, J. B. Influence of physicochemical properties on dissolution of drugs in the gastrointestinal tract. *Advanced Drug Delivery Reviews* **2001,** *46*, (1–3), 75-87.

(37) Washington, N.; Washington, C.; Wilson, C. G., *Physiological Pharmaceutics: barriers to drug absorption*. 2nd ed.; the Taylor & Francis e-Library: 2003.

(38) Grover, A.; Benet, L. Effects of Drug Transporters on Volume of Distribution. *The AAPS Journal* **2009,** *11*, (2), 250-261.

(39) Ganong, W. F., *Review of Medical Physiology*. 22nd ed.; The McGraw-Hill Companies: San Francisco, 2005.

(40) Löbenberg, R.; Amidon, G. L. Modern bioavailability, bioequivalence and biopharmaceutics classification system. New scientific approaches to international regulatory standards. *European Journal of Pharmaceutics and Biopharmaceutics* **2000,** *50*, (1), 3-12.

(41) Aungst, B. J. Novel formulation strategies for improving oral bioavailability of drugs with poor membrane permeation or presystemic metabolism. *Journal of Pharmaceutical Sciences* **1993,** *82*, (10), 979-987.

(42) Ishikawa, T. The ATP-dependent glutathione S-conjugate export pump. *Trends in Biochemical Sciences* **1992,** *17*, (11), 463-468.

(43) Yamazaki, M.; Suzuki, H.; Sugiyama, Y. Recent Advances in Carrier-mediated Hepatic Uptake and Biliary Excretion of Xenobiotics. *Pharmaceutical Research* **1996,** *13*, (4), 497-513.

(44) Riviere, J. E., *Comparative Pharmacokinetics: Principles, Techniques, and Applications*. 2nd ed.; Wiley-Blackwell: Raleigh, North Carolina, 2011.

(45) Evans, W. E.; Relling, M. V. Pharmacogenomics: Translating Functional Genomics into Rational Therapeutics. *Science* **1999,** *286*, (5439), 487-491.

(46) Zuber, R.; Anzenbacherová, E.; Anzenbacher, P. Cytochromes P450 and experimental models of drug metabolism. *Journal of Cellular and Molecular Medicine* **2002,** *6*, (2), 189-198.

(47) Lin, J. H.; Lu, A. Y. H. Role of Pharmacokinetics and Metabolism in Drug Discovery and Development. *Pharmacological Reviews* **1997,** *49*, (4), 403-449.

(48) Shimada, T.; Yamazaki, H.; Mimura, M.; Inui, Y.; Guengerich, F. P. Interindividual variations in human liver cytochrome P-450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: studies with liver microsomes of 30 Japanese and 30 Caucasians. *Journal of Pharmacology and Experimental Therapeutics* **1994,** *270*, (1), 414-423.

(49) Dutton, G. J., *Glucuronidation of drugs and other compounds*. ed.; CRC Press, Inc.: Boca Raton, Florida, 1980.

(50) van de Kerkhof, E. G.; de Graaf, I. A. M.; de Jager, M. H.; Meijer, D. K. F.; Groothuis, G. M. M. Characterizartion of rat small intestinal and colon precision-cut slices as an in vitro system for drug metabolism and induction studies. *Drug Metabolism and Disposition* **2005,** *33*, (11), 1613-1620.

(51) Benet, L. Z.; Cummins, C. L. The drug efflux–metabolism alliance: biochemical aspects. *Advanced Drug Delivery Reviews* **2001,** *50, Suppl 1*, S3-S11.

(52) Lindell, M.; Lang, M.; Lennernäs, H. Expression of genes encoding for drug metabolising cytochrome P450 enzymes and P-glycoprotein in the rat small intestine; comparison to the liver. *European Journal of Drug Metabolism and Pharmacokinetics* **2003,** *28*, (1), 41-48.

(53) Mealey, K. L.; Jabbes, M.; Spencer, E.; Akey, J. M. Differential expression of CYP3A12 and CYP3A26 mRNAs in canine liver and intestine. *Xenobiotica* **2008,** *38*, (10), 1305-1312.

(54) Galetin, A.; Houston, J. B. Intestinal and Hepatic Metabolic Activity of Five Cytochrome P450 Enzymes: Impact on Prediction of First-Pass Metabolism. *Journal of Pharmacology and Experimental Therapeutics* **2006,** *318*, (3), 1220-1229.

(55) Lindell, M.; Karlsson, M. O.; Lennernäs, H.; Påhlman, L.; Lang, M. A. Variable expression of CYP and Pgp genes in the human small intestine. *European Journal of Clinical Investigation* **2003,** *33*, (6), 493-499.

(56) Benet, L. Z. The Drug Transporter–Metabolism Alliance: Uncovering and Defining the Interplay. *Molecular Pharmaceutics* **2009,** *6*, (6), 1631-1643.

(57) Lin, J. H. Species similarities and differences in pharmacokinetics. *Drug Metabolism and Disposition* **1995,** *23*, (10), 1008-1021.

(58) Poggesi, I. Predicting human opharmacokinetics from preclinical data. *Current opinion in drug discovery and development* **2004,** *7*, (1), 100-111.

(59) DeKeyser, J. G.; Shou, M.; Lyubimov, A. V., Species Differences of Drug-Metabolizing Enzymes. In *Encyclopedia of Drug Metabolism and Interactions*.ed.; 'Ed.' John Wiley & Sons, Inc.: 2011; Vol. pp.

(60) Guengerich, F. P. Comparisons of catalytic selectivity of cytochrome P450 subfamily enzymes from different species. *Chemico-Biological Interactions* **1997,** *106*, (3), 161-182.

(61) Iwatsubo, T.; Suzuki, H.; Sugiyama, Y. Prediction of Species Differences (Rats, Dogs, Humans) in theIn Vivo Metabolic Clearance of YM796 by the Liver fromIn Vitro Data. *Journal of Pharmacology and Experimental Therapeutics* **1997,** *283*, (2), 462-469.

(62) Komura, H.; Iwaki, M. Species differences in in vitro and in vivo small intestinal metabolism of CYP3A substrates. *Journal of Pharmaceutical Sciences* **2008,** *97*, (5), 1775-1800.

(63) Martignoni, M.; Groothuis, G. M.; de Kanter, R. Species differences between mouse, rat, dog, monkey and human CYP-mediated drug metabolism, inhibition and induction. *Expert Opinion on Drug Metabolism & Toxicology* **2006,** *2*, (6), 875-894.

(64) Porter, C. J. H.; Trevaskis, N. L.; Charman, W. N. Lipids and lipid-based formulations: optimizing the oral delivery of lipophilic drugs. *Nature Reviews Drug Discovery* **2007,** *6*, (3), 231-248.

(65) Porter, C. J. H.; Kaukonen, A. M.; Taillardat-Bertschinger, A.; Boyd, B. J.; O'Connor, J. M.; Edwards, G. A.; Charman, W. N. Use of *in vitro* lipid digestion data to explain the *in vivo* performance of triglyceride-based oral lipid formulations of poorly water-soluble drugs: Studies with halofantrine. *Journal of Pharmaceutical Sciences* **2004b,** *93*, (5), 1110-1121.

(66) Shiau, Y. F. Mechanisms of intestinal fat absorption. *Am J Physiol Gastrointest Liver Physiol* **1981,** *240*, (1), G1-9.

(67) Carey, M. C.; Small, D. M.; Bliss, C. M. Lipid Digestion and Absorption. *Annual Review of Physiology* **1983,** *45*, (1), 651-677.

(68) Embleton, J. K.; Pouton, C. W. Structure and function of gastro-intestinal lipases. *Advanced Drug Delivery Reviews* **1997,** *25*, (1), 15-32.

(69) Bergström, S.; Borgstrom, B. Metabolism of Lipids. *Annu. Rev. Biochem.* **1956,** *25*, 177-200.

(70) FDA *Guidance for Industry: "Waiver of In Vivo Bioavailability and Bioequivalence Studies for Immediate-Release Solid Oral Dosage Forms Based on a Biopharmaceutics Classification System"*; U.S. Department of Health and Human Services Food and Drug Administration (FDA): http://www.fda.gov/cder/guidance/index.htm, August, 2000; p^pp 1-16.

(71) Pouton, C. W. Formulation of poorly water-soluble drugs for oral administration: Physicochemical and physiological issues and the lipid formulation classification system. *European Journal of Pharmaceutical Sciences* **2006,** *29*, (3-4), 278-287.

(72) Yu, L. X.; Amidon, G. L.; Polli, J. E.; Zhao, H.; Mehta, M. U.; Conner, D. P.; Shah, V. P.; Lesko, L. J.; Chen, M.-L.; Lee, V. H. L.; Hussain, A. S. Biopharmaceutics Classification System: The Scientific Basis for Biowaiver Extensions. *Pharmaceutical Research* **2002,** *19*, (7), 921-925.

(73) Vasconcelos, T.; Sarmento, B.; Costa, P. Solid dispersions as strategy to improve oral bioavailability of poor water soluble drugs. *Drug Discovery Today* **2007,** *12*, (23-24), 1068-1075.

(74) Porter, C. J. H.; Pouton, C. W.; Cuine, J. F.; Charman, W. N. Enhancing intestinal drug solubilisation using lipid-based delivery systems. *Advanced Drug Delivery Reviews* **2008,** *60*, (6), 673-691.

(75) Duchêne, D.; Wouessidjewe, D.; Ponchel, G. Cyclodextrins and carrier systems. *Journal of Controlled Release* **1999,** *62*, (1-2), 263-268.

(76) Merisko-Liversidge, E. M.; Liversidge, G. G. Drug Nanoparticles: Formulating Poorly Water-Soluble Compounds. *Toxicol Pathol* **2008,** *36*, (1), 43-48.

(77) Serajuddin, A. T. M. Solid dispersion of poorly water-soluble drugs: Early promises, subsequent problems, and recent breakthroughs. *Journal of Pharmaceutical Sciences* **1999,** *88*, (10), 1058-1066.

(78) Humberstone, A. J.; Charman, W. N. Lipid-based vehicles for the oral delivery of poorly water soluble drugs. *Advanced Drug Delivery Reviews* **1997,** *25*, (1), 103-128.

(79) Hamosh, M.; Scow, R. O. Lingual Lipase and Its Role in the Digestion of Dietary Lipid. *The Journal of Clinical Investigation* **1973,** *52*, (1), 88-95.

(80) Persson, E.; Nilsson, R.; Hansson, G.; Löfgren, L.; Libäck, F.; Knutson, L.; Abrahamsson, B.; Lennernäs, H. A clinical single-pass perfusion investigation of the dynamic *in vivo* secretory response to a dietary meal in human proximal small intestine. *Pharmaceutical Research* **2006,** *23*, (4), 742-751.

(81) Erlanson-Albertsson, C. Pancreatic colipase. Structural and physiological aspects. *Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism* **1992,** *1125*, (1), 1-7.

(82) Borgström, B. On the Mechanism of the Intestinal Fat Absorption: Metabolism of Lipids 6. *Acta Physiologica Scandinavica* **1952,** *25*, (4), 291-314.

(83) Thomson, A. B. R.; Keelan, M.; Garg, M. L.; Clandinin, M. T. Intestinal aspects of lipid absorption: in review. *Canadian Journal of Physiology and Pharmacology* **1989,** *67*, (3), 179-191.

(84) Mattson, F. H.; Volpenhein, R. A. The Digestion and Absorption of Triglycerides. *J. Biol. Chem.* **1964,** *239*, (9), 2772-2777.

(85) Carriere, F.; Barrowman, J. A.; Verger, R.; Laugier, R. Secretion and contribution to lipolysis of gastric and pancreatic lipases during a test meal in humans. *Gastroenterology* **1993,** *105*, (3), 876-888.

(86) Fernandez, S.; Rodier, J.-D.; Ritter, N.; Mahler, B.; Demarne, F.; Carrière, F.; Jannin, V. Lipolysis of the semi-solid self-emulsifying excipient Gelucire® 44/14 by digestive lipases. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* **2008,** *1781*, (8), 367-375.

(87) Fernandez, S.; Jannin, V.; Rodier, J.-D.; Ritter, N.; Mahler, B.; Carrière, F. Comparative study on digestive lipase activities on the self emulsifying excipient Labrasol®, medium chain glycerides and PEG esters. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* **2007,** *1771*, (5), 633-640.

(88) Hofmann, A. F.; Borgström, B. The Intraluminal Phase of Fat Digestion in Man: The Lipid Content of the Micellar and Oil Phases of Intestinal Content Obtained during Fat Digestion and Absorption. *The Journal of Clinical Investigation* **1964,** *43*, (2), 247-257.

(89) Staggers, J. E.; Hernell, O.; Stafford, R. J.; Carey, M. C. Physical-chemical behavior of dietary and biliary lipids during intestinal digestion and absorption. 1. Phase behavior and aggregation states of model lipid systems patterned after aqueous duodenal contents of healthy adult human beings. *Biochemistry* **1990a,** *29*, (8), 2028-2040.

(90) Kossena, G. A.; Boyd, B. J.; Porter, C. J. H.; Charman, W. N. Separation and characterization of the colloidal phases produced on digestion of common formulation lipids and assessment of their impact on the apparent solubility of selected poorly water-soluble drugs. *Journal of Pharmaceutical Sciences* **2003,** *92*, (3), 634-648.

(91) Hernell, O.; Staggers, J. E.; Carey, M. C. Physical-chemical behavior of dietary and biliary lipids during intestinal digestion and absorption. 2. Phase analysis and aggregation states of luminal lipids during duodenal fat digestion in healthy adult human beings. *Biochemistry* **1990,** *29*, (8), 2041-2056.

(92) Patton, J. S.; Carey, M. C. Watching fat digestion. *Science* **1979,** *204*, (4389), 145-148.

(93) Rigler, M. W.; Honkanen, R. E.; Patton, J. S. Visualization by freeze fracture, in vitro and in vivo, of the products of fat digestion. *Journal of Lipid Research* **1986,** *27*, (8), 836-57.

(94) Pouton, C. W. Lipid formulations for oral administration of drugs: non-emulsifying, self-emulsifying and 'self-microemulsifying' drug delivery systems. *European Journal of Pharmaceutical Sciences* **2000,** *11*, (Supplement 2), S93-S98.

(95) Kossena, G. A.; Charman, W. N.; Wilson, C. G.; O'Mahony, B.; Lindsay, B.; Hempenstall, J.; Davison, C.; Crowley, P.; Porter, C. J. H. Low Dose Lipid Formulations: Effects on Gastric Emptying and Biliary Secretion. *Pharmaceutical Research* **2007,** *24*, (11), 2084-2096.

(96) Kaukonen, A.; Boyd, B. J.; Porter, C. J. H.; Charman, W. N. Drug Solubilization Behavior During in Vitro Digestion of Simple Triglyceride Lipid Solution Formulations. *Pharmaceutical Research* **2004a,** *21*, (2), 245-253.

(97) MacGregor, K. J.; Embleton, J. K.; Lacy, J. E.; Perry, E. A.; Solomon, L. J.; Seager, H.; Pouton, C. W. Influence of lipolysis on drug absorption from the gastro-intestinal tract. *Advanced Drug Delivery Reviews* **1997,** *25*, (1), 33-46.

(98) Sek, L.; Porter, C. J. H.; Kaukonen, A. M.; Charman, W. N. Evaluation of the in-vitro digestion profiles of long and medium chain glycerides and the phase behaviour of their lipolytic products. *Journal of Pharmacy and Pharmacology* **2002,** *54*, 29-41.

(99) Fatouros, D. G.; Bergenstahl, B.; Mullertz, A. Morphological observations on a lipid-based drug delivery system during in vitro digestion. *European Journal of Pharmaceutical Sciences* **2007b,** *31*, (2), 85-94.

(100) Zangenberg, N. H.; Müllertz, A.; Kristensen, H. G.; Hovgaard, L. A dynamic in vitro lipolysis model: I. Controlling the rate of lipolysis by continuous addition of calcium. *European Journal of Pharmaceutical Sciences* **2001a,** *14*, (2), 115-122.

(101) Zangenberg, N. H.; Müllertz, A.; Gjelstrup Kristensen, H.; Hovgaard, L. A dynamic in vitro lipolysis model: II: Evaluation of the model. *European Journal of Pharmaceutical Sciences* **2001b,** *14*, (3), 237-244.

(102) Fatouros, D. G.; Mullertz, A., Using in vitro dynanic lipolysis modeling as a tool for exploring IVIVC relationships for oral lipid-based formulations. In *Oral lipid-based formulations: enhancing the bioavailability of poolrly water-soluble drugs,* .1st ed.; Hauss, D. J., 'Ed.' Informa Healthcare, Inc.: New York, 2007c; Vol. pp 257-271.

(103) Khoo, S.-M.; Humberstone, A. J.; Porter, C. J. H.; Edwards, G. A.; Charman, W. N. Formulation design and bioavailability assessment of lipidic self-emulsifying formulations of halofantrine. *International Journal of Pharmaceutics* **1998,** *167*, (1-2), 155-164.

(104) Williams, H. D.; Sassene, P.; Kleberg, K.; Bakala N'Goma, J. C.; Calderone, M.; Jannin, V.; Igonin, A.; Partheil, A.; Marchaud, D.; Jule, E.; Vertommen, J.; Maio, M.; Blundell, R.; Benameur, H.; Carriere, F.; Mullertz, A.; Porter, C. J. H.; Pouton, C. W. Toward the establishment of standardized in vitro tests for lipid-based formulations, Part 1: Method parameterization and comparison of in vitro digestion profiles across a range of representative formulations. *Journal of Pharmaceutical Sciences* **2012,** *101*, (9), 3360-3380.

(105) Sek, L.; Boyd, B. J.; Charman, W. N.; Porter, C. J. H. Examination of the impact of a range of Pluronic surfactants on the in-vitro solubilisation behaviour and oral bioavailability of lipidic formulations of atovaquone. *Journal of Pharmacy and Pharmacology* **2006,** *58*, (6), 809-820.

(106) Cuiné, J. F.; McEvoy, C. L.; Charman, W. N.; Pouton, C. W.; Edwards, G. A.; Benameur, H.; Porter, C. J. H. Evaluation of the impact of surfactant digestion on the bioavailability of danazol after oral administration of lipidic self-emulsifying formulations to dogs. *Journal of Pharmaceutical Sciences* **2008,** *97*, (2), 995-1012.

(107) Williams, H. D.; Anby, M. U.; Sassene, P.; Kleberg, K.; Bakala N'Goma, J. C.; Calderone, M.; Jannin, V.; Igonin, A.; Partheil, A.; Marchaud, D.; Jule, E.; Vertommen, J.; Maio, M.; Blundell, R.; Benameur, H.; Carriere, F.; Mullertz, A.; Pouton, C. W.; Porter, C. J. H. Toward the establishment of standardized in vitro tests for lipid-based formulations, Part 2: The effect of bile salt concentration and drug saturation level (dose) on the performance of Type I, II, IIIA, IIIB and IV formulations during in vitro digestion. *Molecular Pharmaceutics* **2012,** *9*, (11), 3286-3300.

(108) Alvarez, F. J.; Stella, V. J. The Role of Calcium Ions and Bile Salts on the Pancreatic Lipase-Catalyzed Hydrolysis of Triglyceride Emulsions Stabilized with Lecithin. *Pharmaceutical Research* **1989,** *6*, (6), 449-457.

(109) Sek, L.; Porter, C. J. H.; Charman, W. N. Characterisation and quantification of medium chain and long chain triglycerides and their in vitro digestion products, by HPTLC coupled with in situ densitometric analysis. *Journal of Pharmaceutical and Biomedical Analysis* **2001,** *25*, (3-4), 651-661.

(110) Patton, J. S.; Albertsson, P. A.; Erlanson, C.; Borgstrom, B. Binding of porcine pancreatic lipase and colipase in the absence of substrate studies by two-phase partition and affinity chromatography. *J. Biol. Chem.* **1978,** *253*, (12), 4195-4202.

(111) Aloulou, A.; Rodriguez, J. A.; Fernandez, S.; van Oosterhout, D.; Puccinelli, D.; Carrière, F. Exploring the specific features of interfacial enzymology based on lipase studies. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* **2006,** *1761*, (9), 995-1013.

(112) Christensen, J. Ø.; Schultz, K.; Mollgaard, B.; Kristensen, H. G.; Mullertz, A. Solubilisation of poorly water-soluble drugs during in vitro lipolysis of medium- and long-chain triacylglycerols. *European Journal of Pharmaceutical Sciences* **2004,** *23*, (3), 287-296.

(113) Mohsin, K.; Long, M. A.; Pouton, C. W. Design of lipid-based formulations for oral administration of poorly water-soluble drugs: Precipitation of drug after dispersion of formulations in aqueous solution. *Journal of Pharmaceutical Sciences* **2009,** *98,* (10), 3582-3595.

(114) Kossena, G. A.; Charman, W. N.; Boyd, B. J.; Porter, C. J. H. Influence of the intermediate digestion phases of common formulation lipids on the absorption of a poorly water-soluble drug *J. Pharm. Sci.* **2005,** *94*, (3), 481.

(115) Schick, M. J. E., *Nonionic surfactants*. ed.; Marcel Dekker, Inc.: New York, 1977.

(116) Christiansen, A.; Backensfeld, T.; Weitschies, W. Effects of non-ionic surfactants on in vitro triglyceride digestion and their susceptibility to digestion by pancreatic enzymes. *European Journal of Pharmaceutical Sciences* **2010,** *41*, (2), 376-382.

(117) Anderson, B. D.; Maara, M. T. Chemical and related factors controlling lipid solubility. *Bulletin Technique Gattefosse* **1999,** *92*, 11-19.

(118) Nielsen, P. B.; Müllertz, A.; Norling, T.; Kristensen, H. G. The effect of [alpha]-tocopherol on the in vitro solubilisation of lipophilic drugs. *International Journal of Pharmaceutics* **2001,** *222*, (2), 217-224.

(119) Han, S.-f.; Yao, T.-t.; Zhang, X.-x.; Gan, L.; Zhu, C.; Yu, H.-z.; Gan, Y. Lipid-based formulations to enhance oral bioavailability of the poorly water-soluble drug anethol trithione: Effects of lipid composition and formulation. *International Journal of Pharmaceutics* **2009,** *379*, (1), 18-24.

(120) Dahan, A.; Hoffman, A. The effect of different lipid based formulations on the oral absorption of lipophilic drugs: The ability of in vitro lipolysis and consecutive ex vivo intestinal permeability data to predict in vivo bioavailability in rats. *European Journal of Pharmaceutics and Biopharmaceutics* **2007,** *67*, (1), 96-105.

(121) Fatouros, D. G.; Deen, G.; Arleth, L.; Bergenstahl, B.; Nielsen, F.; Pedersen, J.; Mullertz, A. Structural Development of Self Nano Emulsifying Drug Delivery Systems (SNEDDS) During In Vitro Lipid Digestion Monitored by Small-angle X-ray Scattering. *Pharmaceutical Research* **2007**, *24*, (10), 1844-1853.

(122) Fatouros, D. G.; Walrand, I.; Bergenstahl, B.; Müllertz, A. Colloidal Structures in Media Simulating Intestinal Fed State Conditions with and Without Lipolysis Products. *Pharmaceutical Research* **2009,** *26*, (2), 361-374.

(123) Warren, D. B.; Anby, M. U.; Hawley, A.; Boyd, B. J. Real Time Evolution of Liquid Crystalline Nanostructure during the Digestion of Formulation Lipids Using Synchrotron Small-Angle X-ray Scattering. *Langmuir* **2011,** *27*, (15), 9528-9534.

(124) Müllertz, A.; Fatouros, D. G.; Smith, J. R.; Vertzoni, M.; Reppas, C. Insights into Intermediate Phases of Human Intestinal Fluids Visualized by Atomic Force Microscopy and Cryo-Transmission Electron Microscopy ex Vivo. *Molecular Pharmaceutics* **2012,** *9*, (2), 237-247.

(125) FDA *Extended Release Oral Dosage Forms: Development, Evaluation, and Application of In Vitro/In Vivo Correlations*; U.S. Department of Health and Human Services Food and Drug Administration (FDA), Center for Drug Evaluation and Research (CDER): http://www.fda.gov/cder/guidance/index.htm, September, 1997; p^pp 1-27.

(126) Dahan, A.; Hoffman, A. Use of a Dynamic in Vitro Lipolysis Model to Rationalize Oral Formulation Development for Poor Water Soluble Drugs: Correlation with in Vivo Data and the Relationship to Intra-Enterocyte Processes in Rats. *Pharmaceutical Research* **2006,** *23*, (9), 2165-2174.

(127) Caliph, S. M.; Charman, W. N.; Porter, C. J. H. Effect of short-, medium-, and long-chain fatty acid-based vehicles on the absolute oral bioavailability and intestinal lymphatic transport of halofantrine and assessment of mass balance in lymph-cannulated and non-cannulated rats. *Journal of Pharmaceutical Sciences* **2000,** *89*, (8), 1073-1084.

(128) Fatouros, D. G.; Nielsen, F. S.; Douroumis, D.; Hadjileontiadis, L. J.; Mullertz, A. In vitro-in vivo correlations of self-emulsifying drug delivery systems combining the dynamic lipolysis model and neuro-fuzzy networks. *European Journal of Pharmaceutics and Biopharmaceutics* **2008,** *69*, (3), 887-898.

(129) Larsen, A. T.; Holm, R.; Pedersen, M.; Müllertz, A. Lipid-based Formulations for Danazol Containing a Digestible Surfactant, Labrafil M2125CS: In Vivo Bioavailability and Dynamic In Vitro Lipolysis. *Pharmaceutical Research* **2008,** *25*, (12), 2769-2777.

(130) Thomas, N.; Holm, R.; Müllertz, A.; Rades, T. In vitro and in vivo performance of novel supersaturated self-nanoemulsifying drug delivery systems (super-SNEDDS). *Journal of Controlled Release* **2012**, *160*, (1), 25-32.

(131) Attwood, D.; Florence, A. T., *Surfactant systems: Their chemistry, pharmacy and biology*. ed.; Chapman and Hall: New York, 1983.

(132) Amidon, G. E.; Higuchi, W. I.; Ho, N. F. H. Theoretical and experimental studies of transport of micelle-solubilized solutes. *Journal of Pharmaceutical Sciences* **1982,** *71*, (1), 77-84.

(133) Boyd, B. J.; Porter, C. J. H.; Charman, W. N. Using the polymer partitioning method to probe the thermodynamic activity of poorly water-soluble drugs solubilized in model lipid digestion products. *Journal of Pharmaceutical Sciences* **2003,** *92*, (6), 1262-1271.

(134) Kashchiev, D.; van Rosmalen, G. M. Review: Nucleation in solutions revisited. *Crystal Research and Technology* **2003,** *38*, (7-8), 555-574.

(135) Pedersen, O., Precipitation in limit tests. In *Pharmaceutical Chemical Analysis: methods for identification and limit tests*.ed.; 'Ed.' CRC Press / Taylor & Francis: 2006; Vol. pp 93-96.

(136) Brouwers, J.; Brewster, M. E.; Augustijns, P. Supersaturating drug delivery systems: The answer to solubility-limited oral bioavailability? *Journal of Pharmaceutical Sciences* **2009,** *98*, (8), 2549-2572.

(137) Lindfors, L.; Forssén, S.; Westergren, J.; Olsson, U. Nucleation and crystal growth in supersaturated solutions of a model drug. *Journal of Colloid and Interface Science* **2008,** *325*, (2), 404-413.

(138) Warren, D. B.; Benameur, H.; Porter, C. J. H.; Pouton, C. W. Using polymeric precipitation inhibitors to improve the absorption of poorly water-soluble drugs: A mechanistic basis for utility. *Journal of Drug Targeting* **2010,** *18*, (10), 704-731.

(139) Kashchiev, D. Note: On the critical supersaturation for nucleation. *The Journal of Chemical Physics* **2011,** *134*, (19), 196102-2.

(140) Vandecruys, R.; Peeters, J.; Verreck, G.; Brewster, M. E. Use of a screening method to determine excipients which optimize the extent and stability of supersaturated drug solutions and application of this system to solid formulation design. *International Journal of Pharmaceutics* **2007,** *342*, (1-2), 168-175.

(141) DiNunzio, J. C.; Miller, D. A.; Yang, W.; McGinity, J. W.; Williams, R. O. Amorphous Compositions Using Concentration Enhancing Polymers for Improved Bioavailability of Itraconazole. *Molecular Pharmaceutics* **2008,** *5*, (6), 968-980.

(142) Taylor, L. S.; Zografi, G. Spectroscopic Characterization of Interactions Between PVP and Indomethacin in Amorphous Molecular Dispersions. *Pharmaceutical Research* **1997,** *14*, (12), 1691-1698.

(143) Bakatselou, V.; Oppenheim, R. C.; Dressman, J. B. Solubilization and Wetting Effects of Bile Salts on the Dissolution of Steroids. *Pharmaceutical Research* **1991,** *8*, (12), 1461-1469.

(144) Erlich, L.; Yu, D.; Pallister, D. A.; Levinson, R. S.; Gole, D. G.; Wilkinson, P. A.; Erlich, R. E.; Reeve, L. E.; Viegas, T. X. Relative bioavailability of danazol in dogs from liquid-filled hard gelatin capsules. *International Journal of Pharmaceutics* **1999,** *179*, (1), 49-53.

(145) Armand, M.; Borel, P.; Pasquier, B.; Dubois, C.; Senft, M.; Andre, M.; Peyrot, J.; Salducci, J.; Lairon, D. Physicochemical characteristics of emulsions during fat digestion in human stomach and duodenum. *Am J Physiol Gastrointest Liver Physiol* **1996,** *271*, (1), G172-183.

(146) Lindahl, A.; Ungell, A.-L.; Knutson, L.; Lennernäs, H. Characterization of Fluids from the Stomach and Proximal Jejunum in Men and Women. *Pharmaceutical Research* **1997,** *14*, (4), 497-502.

(147) Pedersen, B. L.; Brøndsted, H.; Lennernäs, H.; Christensen, F. N.; Müllertz, A.; Kristensen, H. G. Dissolution of Hydrocortisone in Human and Simulated Intestinal Fluids. *Pharmaceutical Research* **2000,** *17*, (2), 183-189.

(148) Brouwers, J.; Tack, J.; Lammert, F.; Augustijns, P. Intraluminal drug and formulation behavior and integration in in vitro permeability estimation: A case study with amprenavir. *Journal of Pharmaceutical Sciences* **2006,** *95*, (2), 372-383.

(149) Bevernage, J.; Brouwers, J.; Clarysse, S.; Vertzoni, M.; Tack, J.; Annaert, P.; Augustijns, P. Drug supersaturation in simulated and human intestinal fluids representing different nutritional states. *Journal of Pharmaceutical Sciences* **2010,** *99*, (11), 4525-4534.

(150) Moreno, M. P. d. l. C.; Oth, M.; Deferme, S.; Lammert, F.; Tack, J.; Dressman, J.; Augustijns, P. Characterization of fasted-state human intestinal fluids collected from duodenum and jejunum. *Journal of Pharmacy and Pharmacology* **2006,** *58*, (8), 1079-1089.

(151) Persson, E.; Löfgren, L.; Hansson, G.; Abrahamsson, B.; Lennernäs, H.; Nilsson, R. Simultaneous assessment of lipid classes and bile acids in human intestinal fluid by solid-phase extraction and HPLC methods. *Journal of Lipid Research* **2007,** *48*, (1), 242-251.

(152) Garner, C. W. Boronic acid inhibitors of porcine pancreatic lipase. *J. Biol. Chem.* **1980,** *255*, (11), 5064-5068.

(153) Trevaskis, N.; Porter, C. J. H.; Charman, W. N. Bile Increases Intestinal Lymphatic Drug Transport in the Fasted Rat. *Pharmaceutical Research* **2005,** *22*, (11), 1863-1870.

(154) Nguyen, T.-H. Investigation of novel liquid crystalline materials for the sustained oral delivery of poorly water-soluble drugs. PhD thesis, Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, 2008.

(155) Balani, S. K.; Zhu, T.; Yang, T. J.; Liu, Z.; He, B.; Lee, F. W. Effective Dosing Regimen of 1-Aminobenzotriazole for Inhibition of Antipyrine Clearance in Rats, Dogs, and Monkeys. *Drug Metabolism and Disposition* **2002,** *30*, (10), 1059-1062.

(156) Mico, B. A.; Federowicz, D. A.; Ripple, M. G.; Kerns, W. In vivo inhibition of oxidative drug metabolism by, and acute toxicity of, 1-aminobenzotriazole (ABT): A tool for biochemical toxicology. *Biochemical Pharmacology* **1988,** *37*, (13), 2515-2519.

(157) Mugford, C. A.; Mortillo, M.; Mico, B. A.; Tarloff, J. B. 1-Aminobenzotriazole-induced destruction of hepatic and renal cytochromes P450 in male Sprague-Dawley rats. *Fundamental and Applied Toxicology* **1992,** *19*, (1), 43-49.

(158) Strelevitz, T. J.; Foti, R. S.; Fisher, M. B. In Vivo use of the P450 inactivator 1-aminobenzotriazole in the rat: Varied dosing route to elucidate gut and liver contributions to first-pass and systemic clearance. *Journal of Pharmaceutical Sciences* **2006,** *95*, (6), 1334-1341.

(159) Selinger, K.; Hill, H. M.; Anslow, J. A.; Gash, D. A liquid chromatographic method for the determination of danazol in human serum. *Journal of Pharmaceutical and Biomedical Analysis* **1990,** *8*, (1), 79-84.

(160) de Oca Porto, R. M.; Fernández, A. R.; Brito, D. M.; Vidal, T. C.; Diaz, A. L. Gas chromatography/mass spectrometry characterization of urinary metabolites of danazol after oral administration in human. *Journal of Chromatography B* **2006,** *830*, (1), 178-183.

(161) Leinonen, A.; Kuuranne, T.; Kotiaho, T.; Kostiainen, R. Screening of free 17-alkyl-substituted anabolic steroids in human urine by liquid chromatography-electrospray ionization tandem mass spectrometry. *Steroids* **2004,** *69*, (2), 101-109.

(162) Pozo, O. J.; Van Eenoo, P.; Deventer, K.; Delbeke, F. T. Ionization of anabolic steroids by adduct formation in liquid chromatography electrospray mass spectrometry. *Journal of Mass Spectrometry* **2007,** *42*, (4), 497-516.

(163) Englard, S.; Seifter, S.; Murray, P. D., Precipitation techniques. In *Methods in Enzymology*.ed.; 'Ed.' Academic Press: 1990; Vol. 182, pp 285-300.

(164) Carey, M. C.; Small, D. M. The characteristics of mixed micellar solutions with particular reference to bile. *The American Journal of Medicine* **1970,** *49*, (5), 590-608.

(165) Gibson, L., Lipid-Based Excipients for Oral Drug Delivery. In *Oral Lipid-Based Formulations: Enhancing the bioavailbility of poorly water-soluble drugs*.ed.; Hauss, D. J., 'Ed.' Informa Heathcare: New York, 2007; Vol. 170, pp 33-62.

(166) Larsen, A. T.; Sassene, P.; Müllertz, A. In vitro lipolysis models as a tool for the characterization of oral lipid and surfactant based drug delivery systems. *International Journal of Pharmaceutics* **2011,** *417*, (1-2), 245-255.

(167) Porter, C. J. H.; Anby, M. U.; Warren, D. B.; Williams, H. D.; Benameur, H.; Pouton, C. W. Lipid based formulations: Exploring the link between in vitro Supersaturation and in vivo exposure. *Bulletin Technique Gattefossé.* **2011,** *104*, 61-69.

(168) Guzmán, H. R.; Tawa, M.; Zhang, Z.; Ratanabanangkoon, P.; Shaw, P.; Gardner, C. R.; Chen, H.; Moreau, J.-P.; Almarsson, Ö.; Remenar, J. F. Combined use of crystalline salt forms and precipitation inhibitors to improve oral absorption of celecoxib from solid oral formulations. *Journal of Pharmaceutical Sciences* **2007,** *96*, (10), 2686-2702.

(169) Yamashita, K.; Nakate, T.; Okimoto, K.; Ohike, A.; Tokunaga, Y.; Ibuki, R.; Higaki, K.; Kimura, T. Establishment of new preparation method for solid dispersion formulation of tacrolimus. *International Journal of Pharmaceutics* **2003,** *267*, (1-2), 79-91.

(170) Raghavan, S. L.; Trividic, A.; Davis, A. F.; Hadgraft, J. Effect of cellulose polymers on supersaturation and in vitro membrane transport of hydrocortisone acetate. *International Journal of Pharmaceutics* **2000,** *193*, (2), 231-237.

(171) Reymond, J.-P.; Sucker, H.; Vonderscher, J. In Vivo Model for Ciclosporin Intestinal Absorption in Lipid Vehicles. *Pharmaceutical Research* **1988,** *5*, (10), 677-679.

(172) Martin, A.; Wu, P. L.; Adjei, A.; Lindstrom, R. E.; Elworthy, P. H. Extended hildebrand solubility approach and the log linear solubility equation. *Journal of Pharmaceutical Sciences* **1982,** *71*, (8), 849-856.

(173) Gao, P.; Morozowich, W. Development of supersaturatable self-emulsifying drug delivery system formulations for improving the oral absorption of poorly soluble drugs. *Expert Opinion on Drug Delivery* **2006,** *3*, (1), 97-110.

(174) Bevernage, J.; Forier, T.; Brouwers, J.; Annaert, P.; Tack, J.; Augustijns, P. Excipient-mediated supersaturation stabilization in Human Intestinal Fluids. *Molecular Pharmaceutics* **2011,** *8*, (2), 564-570.

(175) Sassene, P. J.; Knopp, M. M.; Hesselkilde, J. Z.; Koradia, V.; Larsen, A.; Rades, T.; Müllertz, A. Precipitation of a poorly soluble model drug during in vitro lipolysis: Characterization and dissolution of the precipitate. *Journal of Pharmaceutical Sciences* **2010,** *99*, (12), 4982-4991.

(176) Khoo, S.-M.; Porter, C. J. H.; Charman, W. N. The formulation of Halofantrine as either non-solubilising PEG 6000 or solubilising lipid based solid dispersions: Physical stability and absolute bioavailability assessment. *International Journal of Pharmaceutics* **2000,** *205*, (1-2), 65-78.

(177) Davison, C.; Banks, W.; Fritz, A. The absorption, Distribution and Metabolic Fate of Danazol in Rats, Monkeys and human Volunteers. *Arch Int Pharmacodyn* **1976,** *221*, 294-310.

(178) O'Driscoll, C. M.; Griffin, B. T. Biopharmaceutical challenges associated with drugs with low aqueous solubility - The potential impact of lipid-based formulations. *Advanced Drug Delivery Reviews* **2008,** *60*, (6), 617-624.

(179) Anby, M. U.; Williams, H. D.; McIntosh, M.; Benameur, H.; Edwards, G. A.; Pouton, C. W.; Porter, C. J. H. Lipid digestion as a trigger for supersaturation: evaluation of the impact of supersaturation stabilisation on the in vitro and in vivo performance of self-emulsifying drug delivery systems. *Molecular Pharmaceutics* **2012,** *9*, (7), 2063-2079.

(180) Center, S. A.; Baldwin, B. H.; Erb, H. N.; Tennant, B. C. Bile-acid concentrations in the diagnosis of hepatobiliary disease in the dog. *J. Am. Vet. Med. Assoc.* **1985,** *187*, (9), 935-940.

(181) Center, S. A.; Leveille, C. R.; Baldwin, B. H.; Tennant, B. C. Direct spectrometric determination of serum bile acids in the dog and cat *American Journal of Veterinary Research* **1984,** *45*, (10), 2043-50.

(182) Center, S. A.; Manwarren, T.; Slater, M. R.; Wilentz, E. Evaluation of 12-hour preprandial and 2-hour post prandial serum bile-acids concentrations for diagnosis of hepatobiliary diseaase in dogs *J. Am. Vet. Med. Assoc.* **1991,** *199*, (2), 217-226.

(183) Dawson, P. A.; Shneider, B. L.; Hofmann, A. F., Bile Formation and the Enterohepatic Circulation. In *Physiology of the Gastrointestinal Tract (Fourth Edition)*.ed.; 'Ed.' Academic Press: Burlington, 2006; Vol. pp 1437-1462.

(184) Schlesinger, D. P.; Rubin, S. I. Serum bile acids and the assessment of hepatic function in dogs and cats. *Can Vet J.* **1993,** *34*, (4), 215-220.

(185) Strasser, A.; NiedermÜLler, H.; Hofecker, G.; Laber, G. The Effect of Aging on Laboratory Values in Dogs. *Journal of Veterinary Medicine Series A* **1993,** *40*, (1-10), 720-730.

(186) Washizu, T.; Koizumi, I.; Kaneko, J. J. Postprandial Changes in Serum Bile Acids Concentration and Fractionation of Individual Bile Acid by High Performance Liquid Chromatography in Normal Dogs. *The Japanese Journal of Veterinary Science* **1987,** *49*, (4), 593-600.

(187) Charman, W. N.; Rogge, M. C.; Boddy, A. W.; Berger, B. M. Effect of food and a monoglyceride emulsion formulation on danazol bioavailability. *J Clin Pharmacol* **1993,** *33*, (4), 381-386.

(188) Jensen, A. L. Variations in Total Bile Acid Concentration in Serum of Dogs after a Test Meal. *Journal of Veterinary Medicine Series A* **1991,** *38*, (1-10), 241-246.

(189) Buddington, R. K.; Elnif, J.; Malo, C.; Donahoo, J. B. Activities of gastric, pancreatic, and intestinal brush-border membrane enzymes during postnatal development of dogs. *American Journal of Veterinary Research* **2003,** *64*, (5), 627-634.

(190) Akimoto, M.; Nagahata, N.; Furuya, A.; Fukushima, K.; Higuchi, S.; Suwa, T. Gastric pH profiles of beagle dogs and their use as an alternative to human testing. *European Journal of Pharmaceutics and Biopharmaceutics* **2000,** *49*, (2), 99-102.

(191) Evans, M. A.; Triggs, E. J.; Cheung, M.; Broe, G. A.; Creasey, H. Gastric-emptying rate in the elderly - Implcations for drug-therapy. *J. Am. Geriatr. Soc.* **1981,** *29*, (5), 201-205.

(192) Wade, P. R. Aging and neural control of the GI tract - I. Age-related changes in the enteric nervous system. *Am. J. Physiol.-Gastroint. Liver Physiol.* **2002,** *283*, (3), G489-G495.

(193) Wiley, J. W. Aging and neural control of the GI tract - III. Senescent enteric nervous system: lessons from extraintestinal sites and nonmammalian species. *Am. J. Physiol.-Gastroint. Liver Physiol.* **2002,** *283*, (5), G1020-G1026.

(194) Annaert, P.; Brouwers, J.; Bijnens, A.; Lammert, F.; Tack, J.; Augustijns, P. Ex vivo permeability experiments in excised rat intestinal tissue and in vitro solubility measurements in aspirated human intestinal fluids support age-dependent oral drug absorption. *European Journal of Pharmaceutical Sciences* **2010,** *39*, (1-3), 15-22.

(195) Klotz, U. Pharmacokinetics and drug metabolism in the elderly. *Drug Metabolism Reviews* **2009,** *41*, (2), 67-76.

(196) Wilde, P. J.; Chu, B. S. Interfacial & colloidal aspects of lipid digestion. *Advances in Colloid and Interface Science* **2011,** *165*, (1), 14-22.

(197) Lee, C. A.; Neul, D.; Clouser-Roche, A.; Dalvie, D.; Wester, M. R.; Jiang, Y.; Jones, J. P.; Freiwald, S.; Zientek, M.; Totah, R. A. Identification of Novel Substrates for Human Cytochrome P450 2J2. *Drug Metabolism and Disposition* **2010,** *38*, (2), 347-356.

(198) Editorial. Serum Bile Acids in Hepatobiliary Disease. *The Lancet* **1982,** *320*, (8308), 1136-1138.

(199) Parraga, M.; Kaneko, J. Total serum bile acids and the bile acid profile as tests of liver function. *Veterinary Research Communications* **1985,** *9*, (1), 79-88.

(200) Uchida, K.; Chikai, T.; Takase, H.; Nomura, Y.; Seo, S.; Nakao, H.; Takeuchi, N. Age–related changes of bile acid metabolism in rats. *Archives of Gerontology and Geriatrics* **1990,** *10*, (1), 37-48.

(201) Uchida, K.; Nomura, Y.; Kadowaki, M.; Takase, H.; Takano, K.; Takeuchi, N. Age-related changes in cholesterol and bile acid metabolism in rats. *Journal of Lipid Research* **1978,** *19*, (5), 544-52.

(202) Benno, Y.; Nakao, H.; Uchida, K.; Mitsuoka, T. Impact of the Advances in Age on the Gastrointestinal Microflora of Beagle Dogs. *Journal of Veterinary Medical Science* **1992,** *54*, (4), 703-706.

(203) Cusack, B. J. Pharmacokinetics in older persons. *The American Journal of Geriatric Pharmacotherapy* **2004,** *2*, (4), 274-302.

(204) Fahey, G. C.; Barry, K. A.; Swanson, K. S. Age-Related Changes in Nutrient Utilization by Companion Animals. *Annual Review of Nutrition* **2008,** *28*, (1), 425-445.

(205) McLean, A. J.; Le Couteur, D. G. Aging Biology and Geriatric Clinical Pharmacology. *Pharmacological Reviews* **2004,** *56*, (2), 163-184.

(206) Saltzman, J. R.; Kowdley, K. V.; Perrone, G.; Russell, R. M. Changes in small-intestine permeability with aging. *J. Am. Geriatr. Soc.* **1995,** *43*, (2), 160-164.

(207) Yuasa, H.; Soga, N.; Kimura, Y.; Watanabe, J. Effect of Aging on the Intestinal Transport of Hydrophilic Drugs in the Rat Small Intestine. *Biological & Pharmaceutical Bulletin* **1997,** *20*, (11), 1188-1192.

(208) Carriére, F.; Laugier, R.; Barrowman, J. A.; Douchet, I.; Priymenko, N.; Verger, R. Gastric and Pancreatic Lipase Levels during a Test Meal in Dogs. *Scandinavian Journal of Gastroenterology* **1993,** *28*, (5), 443-454.

(209) Swanson, K. S.; Kuzmuk, K. N.; Schook, L. B.; Fahey, G. C. Diet affects nutrient digestibility, hematology, and serum chemistry of senior and weanling dogs. *Journal of Animal Science* **2004,** *82*, (6), 1713-1724.

(210) Le Couteur, D. G.; Cogger, V. C.; Markus, A. M. A.; Harvey, P. J.; Yin, Z.-L.; Ansselin, A. D.; McLean, A. J. Pseudocapillarization and associated energy limitation in the aged rat liver. *Hepatology* **2001,** *33*, (3), 537-543.

(211) Le Couteur, D. G.; McLean, A. J. The Aging Liver: Drug Clearance and an Oxygen Diffusion Barrier Hypothesis. *Clinical Pharmacokinetics* **1998,** *34*, (5), 359-373.

(212) Woodhouse, K. W.; Mutch, E.; Williams, F. M.; Rawlins, M. D.; James, I. F. W. The effect of age on pathways of drug metabolism in human liver *Age and Ageing* **1984,** *13*, (6), 328-334.

(213) Trull, A. K.; Tan, K. K.; Tan, L.; Alexander, G. J.; Jamieson, N. V. Absorption of cyclosporin from conventional and new microemulsion oral formulations in liver transplant recipients with external biliary diversion. *British Journal of Clinical Pharmacology* **1995,** *39*, (6), 627-31.

(214) Vonderscher, J.; Meinzer, A. Rationale for the development of Sandimmune Neoral. *Transplantation proceedings* **1994,** *26*, (5), 2925-7.

(215) Charman, W. N.; Rogge, M. C.; Boddy, A. W.; Barr, W. H.; Berger, B. M. Absorption of danazol after administration to different sites of the gastrointestinal tract and the relationship to single- and double-peak phenomena in the plasma profiles. *The Journal of Clinical Pharmacology* **1993,** *33*, (12), 1207-1213.

(216) Anby, M. U.; Williams, H. D.; Benameur, H.; Edwards, G. A.; Pouton, C. W.; Porter, C. J. H. Non-linear increases in danazol exposure with dose in older vs. younger beagle dogs: the potential role of differences in bile salt concentration, thermodynamic activity and formulation digestion. *In submission* **2013**.

(217) Liversidge, G. G.; Cundy, K. C. Particle size reduction for improvement of oral bioavailability of hydrophobic drugs: I. Absolute oral bioavailability of nanocrystalline danazol in beagle dogs. *International Journal of Pharmaceutics* **1995,** *125*, (1), 91-97.

(218) Nguyen, T.-H.; Hanley, T.; Porter, C. J. H.; Larson, I.; Boyd, B. J. Phytantriol and glyceryl monooleate cubic liquid crystalline phases as sustained-release oral drug delivery systems for poorly water soluble drugs I. Phase behaviour in physiologically-relevant media. *Journal of Pharmacy and Pharmacology* **2010,** *62*, (7), 844-855.

(219) Lyons, K. C.; Charman, W. N.; Miller, R.; Porter, C. J. H. Factors limiting the oral bioavailability of N-acetylglucosaminyl-N-acetylmuramyl dipeptide (GMDP) and enhancement of

absorption in rats by delivery in a water-in-oil microemulsion. *International Journal of Pharmaceutics* **2000,** *199*, (1), 17-28.

(220) Van Speybroeck, M.; Williams, H. D.; Nguyen, T.-H.; Anby, M. U.; Porter, C. J. H.; Augustijns, P. Incomplete Desorption of Liquid Excipients Reduces the in Vitro and in Vivo Performance of Self-Emulsifying Drug Delivery Systems Solidified by Adsorption onto an Inorganic Mesoporous Carrier. *Molecular Pharmaceutics* **2012,** *9*, (9), 2750-2760.

(221) Mattson, F. H.; Volpenhein, R. A. Carboxylic ester hydrolases of rat pancreatic juice. *Journal of Lipid Research* **1966,** *7*, (4), 536-543.

(222) Lairon, D.; Nalbone, G.; Lafont, H.; Domingo, N.; Hauton, J. Protective effect of biliary lipids on rat pancreatic lipase and colipase. *Lipids* **1978,** *13*, (3), 211-216.

(223) Johnson, B. M.; Chen, W.; Borchardt, R. T.; Charman, W. N.; Porter, C. J. H. A Kinetic Evaluation of the Absorption, Efflux, and Metabolism of Verapamil in the Autoperfused Rat Jejunum. *Journal of Pharmacology and Experimental Therapeutics* **2003,** *305*, (1), 151-158.

(224) Badawy, S. I. F.; Ghorab, M. M.; Adeyeye, C. M. I. Characterization and bioavailability of danazol-hydroxypropyl [beta]-cyclodextrin coprecipitates. *International Journal of Pharmaceutics* **1996,** *128*, (1-2), 45-54.

(225) DeNigris, S. J.; Hamosh, M.; Kasbekar, D. K.; Lee, T. C.; Hamosh, P. Lingual and gastric lipases: species differences in the origin of prepancreatic digestive lipases and in the localization of gastric lipase. *Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism* **1988,** *959*, (1), 38-45.

(226) Reppas, C.; Meyer, J. H.; Sirois, P. J.; Dressman, J. B. Effect of Hydroxypropylmethylcellulose on gastrointestinal transit and luminal viscosity in dogs. *Gastroenterology* **1991,** *100*, 1217-1223.

(227) Christiansen, A.; Backensfeld, T.; Denner, K.; Weitschies, W. Effects of non-ionic surfactants on cytochrome P450-mediated metabolism in vitro. *European Journal of Pharmaceutics and Biopharmaceutics* **2011,** *78*, (1), 166-172.

(228) Bravo González, R. C.; Huwyler, J.; Boess, F.; Walter, I.; Bittner, B. In vitro investigation on the impact of the surface-active excipients Cremophor EL, Tween 80 and Solutol HS 15 on the metabolism of midazolam. *Biopharmaceutics & Drug Disposition* **2004,** *25*, (1), 37-49.

(229) Liao, T. H.; Hamosh, P.; Hamosh, M. Fat Digestion by Lingual Lipase: Mechanism of Lipolysis in the Stomach and Upper Small Intestine. *Pediatric Research May* **1984,** *18*, (5), 402-409.

(230) Roberts, I. M.; Montgomery, R. K.; Carey, M. C. Rat lingual lipase: partial purification, hydrolytic properties, and comparison with pancreatic lipase. *American Journal of Physiology - Gastrointestinal and Liver Physiology* **1984,** *247*, (4), G385-G393.

(231) Plucinski, T.; Hamosh, M.; Hamosh, P. Fat digestion in rat: role of lingual lipase. *American Journal of Physiology - Endocrinology And Metabolism* **1979,** *237*, (6), E541-E547.

(232) Ramirez, I. Oral stimulation alters digestion of intragastric oil meals in rats. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology* **1985,** *248*, (4), R459-R463.

(233) Roy, C.; Roulet, M.; Lefebvre, D.; Chartrand, L.; Lepage, G.; Fournier, L. The role of gastric lipolysis on fat absorption and bile acid metabolism in the rat. *Lipids* **1979,** *14*, (9), 811-815.

(234) Nalbone, G.; Lairon, D.; Lafont, H.; Domingo, N.; Hauton, J.; Sarda, L. Behavior of biliary phospholipids in intestinal lumen during fat digestion in rat. *Lipids* **1974,** *9*, (10), 765-770.

(235) Borgström, B. Influence of bile salt, pH, and time on the action of pancreatic lipase; physiological implications. *Journal of Lipid Research* **1964,** *5*, (4), 522-531.

(236) Petersen, H.; Grossman, M. Pancreatic exocrine secretion in anesthetized and conscious rats. *American Journal of Physiology - Gastrointestinal and Liver Physiology* **1977,** *233*, (6), G530-G536.

(237) Grossman, M. L. Pancreatic Secretion in the Rat. *American Journal of Physiology - Legacy Content* **1958,** *194*, (3), 535-539.

(238) Tønsberg, H.; Holm, R.; Mu, H.; Boll, J. B.; Jacobsen, J.; Müllertz, A. Effect of bile on the oral absorption of halofantrine in polyethylene glycol 400 and polysorbate 80 formulations dosed to bile duct cannulated rats. *Journal of Pharmacy and Pharmacology* **2011,** *63*, (6), 817-824.

(239) Holt, P.; Fairchild, B.; Weiss, J. A liquid crystalline phase in human intestinal contents during fat digestion. *Lipids* **1986,** *21*, (7), 444-446.

(240) Grove, M.; Müllertz, A.; Nielsen, J. L.; Pedersen, G. P. Bioavailability of seocalcitol: II: Development and characterisation of self-microemulsifying drug delivery systems (SMEDDS) for oral administration containing medium and long chain triglycerides. *European Journal of Pharmaceutical Sciences* **2006,** *28*, (3), 233-242.

(241) Grove, M.; Müllertz, A.; Pedersen, G. P.; Nielsen, J. L. Bioavailability of seocalcitol: III. Administration of lipid-based formulations to minipigs in the fasted and fed state. *European Journal of Pharmaceutical Sciences* **2007,** *31*, (1), 8-15.

(242) Jain, A. C.; Aungst, B. J.; Adeyeye, M. C. Development and in vivo evaluation of buccal tablets prepared using danazol–sulfobutylether 7 β-cyclodextrin (SBE 7) complexes. *Journal of Pharmaceutical Sciences* **2002,** *91*, (7), 1659-1668.

(243) Carlert, S.; Pålsson, A.; Hanisch, G.; von Corswant, C.; Nilsson, C.; Lindfors, L.; Lennernäs, H.; Abrahamsson, B. Predicting Intestinal Precipitation - A Case Example for a Basic BCS Class II Drug. *Pharmaceutical Research* **2010,** *27*, (10), 2119-2130.

(244) Carlert, S.; Åkesson, P.; Jerndal, G.; Lindfors, L.; Lennernäs, H.; Abrahamsson, B. In Vivo Dog Intestinal Precipitation of Mebendazole: A Basic BCS Class II Drug. *Molecular Pharmaceutics* **2012**, *9,* (10), 2903-2911.

(245) Janssens, S.; Nagels, S.; Armas, H. N. d.; D'Autry, W.; Van Schepdael, A.; Van den Mooter, G. Formulation and characterization of ternary solid dispersions made up of Itraconazole and two excipients, TPGS 1000 and PVPVA 64, that were selected based on a supersaturation screening study. *European Journal of Pharmaceutics and Biopharmaceutics* **2008,** *69*, (1), 158-166.

(246) Miller, M. A.; DiNunzio, J. C.; Matteucci, M. E.; Ludher, B. S.; Williams, R. O.; Johnston, K. P. Flocculated amorphous itraconazole nanoparticles for enhanced in vitro supersaturation and in vivo bioavailability. *Drug Development and Industrial Pharmacy* **2012,** *38*, (5), 557-570.

(247) Psachoulias, D.; Vertzoni, M.; Goumas, K.; Kalioras, V.; Beato, S.; Butler, J.; Reppas, C. Precipitation in and Supersaturation of Contents of the Upper Small Intestine After Administration of Two Weak Bases to Fasted Adults. *Pharmaceutical Research* **2011,** *28*, (12), 3145-3158.

(248) Gibaldi, M.; Feldman, S. Establishment of sink conditions in dissolution rate determinations - Theoretical considerations and applications to non-disintegrating dosage forms. *Journal of Pharmaceutical Sciences* **1967,** *56*, (10), 1238-1242.

(249) Grundy, J. S.; Anderson, K. E.; Rogers, J. A.; Foster, R. T. Studies on dissolution testing of the nifedipine gastrointestinal therapeutic system .1. Description of a two-phase in vitro dissolution test. *Journal of Controlled Release* **1997,** *48*, (1), 1-8.

(250) Heigoldt, U.; Sommer, F.; Daniels, R.; Wagner, K. G. Predicting in vivo absorption behavior of oral modified release dosage forms containing pH-dependent poorly soluble drugs using a novel pH-adjusted biphasic in vitro dissolution test. *European Journal of Pharmaceutics and Biopharmaceutics* **2010,** *76*, (1), 105-111.

(251) Hoa, N. T.; Kinget, R. Design and evaluation of two-phase partition-dissolution method and its use in evaluating artemisinin tablets. *Journal of Pharmaceutical Sciences* **1996,** *85*, (10), 1060-1063.

(252) Shi, Y.; Gao, P.; Gong, Y. C.; Ping, H. L. Application of a Biphasic Test for Characterization of In Vitro Drug Release of Immediate Release Formulations of Celecoxib and Its Relevance to In Vivo Absorption. *Molecular Pharmaceutics* **2010,** *7*, (5), 1458-1465.

APPENDICES

APPENDIX 1

Re-printed with permission from Anby MU, Williams HD, McIntosh M, Benameur H, Edwards GA, Pouton CW & Porter CJH. Lipid digestion as a trigger for supersaturation: evaluation of the impact of supersaturation stabilisation on the in vitro and in vivo performance of self-emulsifying drug delivery systems. Molecular Pharmaceutics. 2012;9(7):2063-79.

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Lipid Digestion as a Trigger for Supersaturation: Evaluation of the Impact of Supersaturation Stabilization on the in Vitro and in Vivo Performance of Self-Emulsifying Drug Delivery Systems

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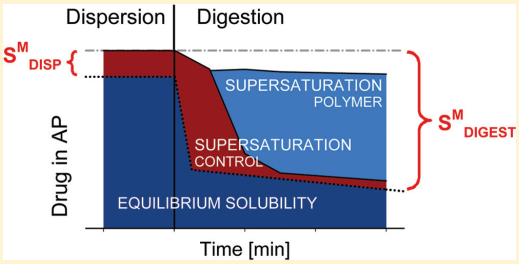
ABSTRACT: The generation of supersaturation in the gastrointestinal (GI) tract is an increasingly popular means of promoting oral absorption for poorly water-soluble drugs. The current study examined the impact of changes to the quantities of medium-chain (MC) lipid (Captex 300:Capmul MCM), surfactant (Cremophor EL) and cosolvent (EtOH), and the addition of polymeric precipitation inhibitors (PPI), on supersaturation during the dispersion and digestion of MC self-emulsifying drug delivery systems (SEDDS) containing danazol. The data suggest that digestion acts as a "trigger" for enhanced supersaturation and that solubilization/precipitation behavior is correlated with the degree of supersaturation on dispersion (S^{M}_{DISP}) or digestion (S^{M}_{DIGEST}). The ability of the formulation to maintain solubilization in vitro decreased as the S^{M} of the formulation increased. PPI significantly increased supersaturation stabilization and precipitation was inhibited where S^{M}_{DISP} < 3.5 and S^{M}_{DIGEST} < 4. In the presence of polymer, some degree of supersaturation was maintained up to S^{M}_{DIGEST} ~ 8. Differentiation in the ability of SEDDS to maintain drug solubilization stems from the ability to stabilize supersaturation and for MC SEDDS, utilization of lower drug loads, higher surfactant levels (balanced against increases in S^{M}_{DISP}), lower cosolvent and the addition of PPI enhanced formulation performance. In vivo studies confirmed the ability of PPI to promote drug exposure at moderate drug loads (40% of saturated solubility in the formulation). At higher drug loads (80% saturation) and in lipid-free SEDDS, this effect was lost, suggesting that the ability of PPIs to stabilize supersaturation in vitro may, under some circumstances, overestimate utility in vivo.



KEYWORDS: *lipid-based drug delivery systems, self-emulsifying drug delivery systems (SEDDS), in vitro digestion, supersaturation, polymeric precipitation inhibitors, hydroxypropyl methylcellulose (HPMC), poorly water-soluble drugs, bioavailability*

INTRODUCTION

The oral absorption of poorly water-soluble drugs (PWSD) is frequently compromised by low gastrointestinal (GI) solubility and slow dissolution. Limited absorption dictates a reduction in oral bioavailability and may eventually limit effective drug development. Self-emulsifying drug delivery systems (SEDDS) are one of several approaches that have been explored to address these issues and improve the oral bioavailability of PWSD.[1]

In contrast to traditional solid dose forms, the drug dose in SEDDS is usually dissolved in the formulation. SEDDS therefore present drug to the GI tract in solution, albeit in nonaqueous solution, and circumvent the conventional dissolution processes that commonly limit the absorption of PWSD. The key to the utility of SEDDS is maintenance of the integrity of drug solubilization as the formulation is processed in the GI tract and delivery of drug, in a solubilized reservoir, to the absorptive surface (enterocyte). There is little evidence to suggest the potential for direct absorption of the solubilized construct (for example, a micelle or an emulsion droplet), and the prevailing view is that drug absorption is facilitated by drug equilibration between the solubilized reservoir and drug in free, intermicellar solution followed by absorption of drug from the intermicellar phase. Drug absorption subsequently shifts the equilibrium between solubilized and free drug, maintaining sink

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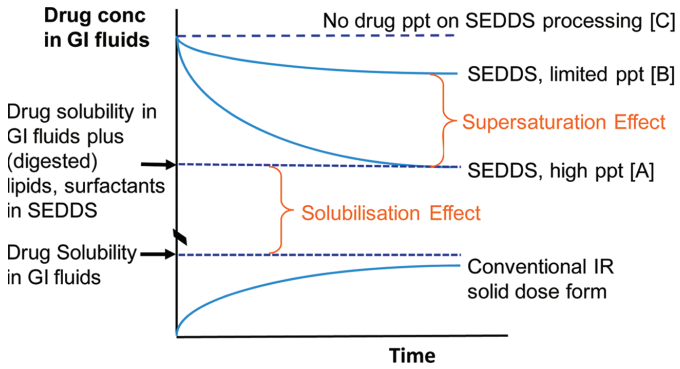

Figure 1. Schematic representation of potential changes to solubilized drug concentration in GI fluids after oral administration of a conventional immediate release (IR) solid oral dose and a SEDDS formulation. Dissolution of a typical solid dose form results in increasing concentrations of drug in the GI fluids that are ultimately limited by drug solubility. In contrast, SEDDS formulations do not undergo traditional dissolution and instead initially disperse to provide high solubilized drug concentrations where drug is present in a molecularly dispersed state in the formulation [C]. However digestion of formulation components typically leads to a reduction in solubilization capacity and drug precipitation (ppt) [A]. In some cases, precipitation to [A] is not immediate, resulting in transient supersaturation [B] (the supersaturation effect). Even in the absence of supersaturation, the presence of lipid and surfactant digestion products results in increases in solubilized drug concentrations [A] when compared to drug solubility in the GI fluids alone (the solubilization effect).

conditions and perpetuating absorption via ongoing release from the solubilized reservoir. The nature of the solubilized reservoir is a critical aspect of the utility of SEDDS and is a composite function of formulation-derived materials such as lipids and surfactants (and their digestion products), and endogenous solubilizers such as bile salts and phospholipids that are secreted in bile in the form of mixed micelles.[2−7]

Historically, the utility of lipid- and surfactant-based formulations such as SEDDS has been thought to lie in their ability to supplement the solubilization capacity of the GI fluids. Solubilization enhancement occurs via the provision of additional surfactants and the generation of lipid digestion products that swell the solubilization capacity of bile salt−phospholipid micelles.[3,4,6−11] It is increasingly apparent, however, that this explanation is incomplete and that a more detailed understanding of the kinetic events that evolve on administration of SEDDS is required to better understand formulation performance.

Figure 1 provides a diagrammatic representation of possible changes to PWSD solubilization in the GI fluids after oral administration of a conventional immediate release (IR) solid oral dose form and a SEDDS formulation. For the IR formulation, drug dissolution is usually slow and ultimately limited by the solubility of the drug in the GI fluids. In contrast, administration of drug in solution in the SEDDS dictates that initial dispersion of the formulation results in the attainment of solubilized drug concentrations that are orders of magnitude higher than the equilibrium solubility of drug in the GI fluids alone (upper dotted line [C] in Figure 1). However, SEDDS usually contain digestible lipids[3,5,12,13] and surfactants,[14] and the nature of these components is expected to change significantly as digestion occurs.[14] Indeed, in many cases, the solubilization capacity of SEDDS formulations reduces on digestion leading to drug precipitation.[15−19] In a classical scenario, digestion reduces the solubilization capacity of the colloidal aqueous phase and drug precipitates in line with the reduction in solubilization capacity. Ultimately, this results in a drop in solubilized drug concentrations to values approximating the equilibrium solubility of drug in the "composite" GI fluids containing bile salt and phospholipid from bile and lipid and surfactant digestion products from the formulation ([A] in Figure 1). However, as we have reported previously[20] and describe in some detail here, certain formulations resist precipitation, even though digestion leads to a decrease in solubilization capacity. As a result, a transiently supersaturated condition is created ([B] in Figure 1).

The overarching goal of the recent work conducted in our laboratories has been to explore the formulation dependence of supersaturation generation during the dispersion and digestion of lipid-based SEDDS formulations, and the studies reported here are the first to explore these trends in detail. In particular, we describe the impact of changes to the relative quantities of lipid, surfactant and cosolvent contained within a SEDDS formulation on supersaturation using a formulation comprising medium-chain triglyceride and a blend of medium-chain mono-, di- and triglycerides as a lipid source, Cremophor EL as a surfactant and ethanol as a cosolvent. In addition to the inherent potential of conventional SEDDS components such as lipids, surfactants and cosolvents to promote supersaturation on formulation dispersion or digestion, several authors have also shown that supersaturation, in both lipidic[21−24] and non-lipidic[25−31] formulations, can be stabilized by polymers. The additional effect of polymers on supersaturation in lipid-based formulations has therefore also been explored. The data suggest that much of the differentiation between solubilization properties for SEDDS stems from differences in the ability to stabilize supersaturation and that although polymers can be very effective in stabilizing supersaturation in vitro, these effects are not always apparent in vivo.

MATERIALS AND METHODS

Materials. Danazol (pregna-2,4-dien-20-yno[2,3-*d*]isoxazol-17-ol) was kindly supplied by Sterling Pharmaceuticals (Sydney, Australia), and progesterone was from Sigma-Aldrich (St. Louis, MO, USA). Captex 300, a medium-chain triglyceride (MCT), and Capmul MCM, a blend of medium-chain mono-,

di- and triglycerides, were donated by Abitec Corporation (Janesville, WI, USA). Soybean oil (predominantly C18 triglycerides), Cremophor EL (polyoxyl 35 castor oil), sodium taurodeoxycholate 97% (NaTDC) and porcine pancreatin (8xUSP specification activity) 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), 4-bromophenylboronic acid (4-BPB) was obtained from Sigma Aldrich (St. Louis, MO, USA) and 1 M sodium hydroxide, which was diluted to obtain 0.6 M NaOH titration solution, was purchased from Merck (Darmstadt, Germany). Water was obtained from a Milli-Q (Millipore, Bedford, MA, USA) purification system. All other chemicals and solvents were of analytical purity or high performance liquid chromatography (HPLC) grade.

The following polymers were obtained from Sigma Aldrich Pty Ltd., Australia: Hydroxypropyl methylcellulose (HPMC) E4M, polyvinylpyrrolidone (PVP) 10 and PVP 360. Hercules Chemical Company Inc., c/o APS Healthcare, Nuplex Industries Pty Ltd., Australia, supplied hydroxyethylcellulose (HEC) 250GF, hydroxypropyl cellulose (HPC) HXF, methylcellulose (MC) A4C and HPMC E5. Eudragit E100 and L100 were supplied by Evonik Degussa Australia Pty Ltd., Aerosil 200 was supplied by Degussa AG, Frankfurt am Main, and HPMC phthalate (HPMC-P) 55S and HPMC acetate succinate (HPMC-AS) LF and HF were supplied by ShinEtsu Chemical Co. Ltd.

Preparation of SEDDS Formulations Containing Danazol. All formulations were prepared as previously described,[32] and danazol, a synthetic steroid originally developed to treat endometriosis, was utilized as a model poorly water-soluble drug (aqueous solubility; 0.59 μg/mL,[33] log *P*; 4.53[34]). Danazol was incorporated into formulations at 40% or 80% of saturated solubility in the formulation (based on measured values at equilibrium at 37 °C). Drug solubility in each of the formulations was assessed using standard methodologies, and all experiments were performed in triplicate.[35] Equilibrium solubility was defined as the value attained when at least three consecutive solubility samples varied by ≤5%. This was typically reached after equilibration times of between 48 and 72 h.

Formulations were constructed to contain (i) varying ratios of medium-chain (MC) lipid to surfactant at a constant cosolvent level (10% v/v) and (ii) varying ratios of cosolvent to MC lipid at approximately constant surfactant levels (64−65% v/v). A ternary plot of the composition of the individual formulations is shown in Figure 2, and numeric values are given in Table 1.

Formulations containing polymeric precipitation inhibitors (PPI) were assembled using the formulations presented in Table 1 and subsequent addition of the PPIs at the stated concentrations (% w/w). The formulations were vortexed for 30 s and equilibrated overnight at 37 °C. The following polymers were utilized: Aerosil 200, Eudragit E100, Eudragit L100, hydroxypropyl methylcellulose (HPMC) (E4M), HPMC (E5), HPMC-AS (LF), HPMC-AS (HF), methylcellulose (MC) (A4C), hydroxypropyl cellulose (HPC), hydroxyethylcellulose (HEC), HPMC-P (55S) and polyvinylpyrrolidone (PVP) 10 or PVP360.

In Vitro Evaluation. *Drug Solubilization during Formulation Dispersion and Digestion.* In vitro experiments were conducted using a modification of a previously described lipid digestion model, although in this case a dispersion step was

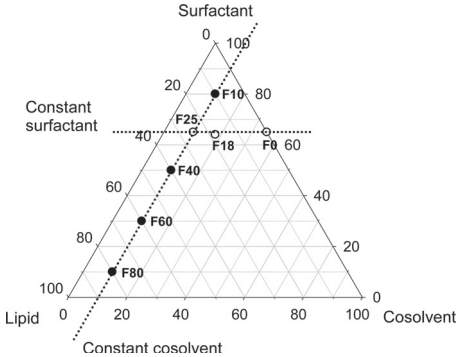

Figure 2. Ternary plot of formulation composition at approximately constant surfactant (64−65%) or constant cosolvent (10%) for formulations comprising medium-chain lipid (Captex 300: Capmul MCM 50:50), surfactant (Cremophor EL) and cosolvent (ethanol). Exact compositions are shown in Table 1.

added prior to initiation of digestion to allow for evaluation of the potential for drug precipitation on formulation dispersion.[32,38] To simplify evaluation, formulation dispersion properties were assessed under intestinal conditions. This allowed distinction between the contribution of dispersion versus digestion events as a driver of supersaturation or precipitation, but does not provide an indication of the potential impact of dispersion under gastric (acidic) conditions. Previous studies, however, suggest relatively moderate differences between formulation dispersion properties under intestinal or gastric conditions, especially for un-ionized drugs.[32] The impact of pH on the dispersion properties of danazol will be examined in more detail in subsequent publications.

Briefly, 1 g of lipid-based formulation was dispersed in 36 g of digestion buffer (50 mM TRIS maleate, 150 mM NaCl, 5 mM $CaCl_2.2H_2O$, pH 7.5) containing 5 mM NaTDC and 1.25 mM phosphatidycholine (PC) to reflect typical fasted state intestinal conditions.[39−45] Experiments were performed at 37 °C in a glass vessel, the temperature was controlled by a thermostatically controlled water jacket and the fluids contained in the vessel (i.e., digestion buffer plus formulation) were stirred magnetically. The first experimental period (0−30 min) comprised the dispersion stage of the study, where the formulation was simply dispersed in the digestion medium by stirring. After 30 min, digestion was initiated by addition of 4 mL of pancreatin extract containing 40,000 TBU (to provide approximately 10,000 TBU per mL of extract and approximately 1000 TBU per mL of digest) of pancreatic enzyme. Digestion was followed for 60 min using a pH-stat titration unit (Radiometer, Copenhagen, Denmark) that maintained the pH at 7.5 via titration of liberated fatty acids with 0.6 M NaOH. Aliquots (4.2 mL) were taken from the dispersion/digestion media throughout the 90 min experimental period at t = 10, 30, 40, 50, 60, and 90 min. Lipid digestion inhibitor (4-BPB, 9 μL of a 0.5 M solution in methanol per mL of dispersion/digestion medium) was added to each sample immediately after sampling to prevent further lipolysis.[4,46] Dispersion samples were centrifuged for 10 min at 21000*g* (Heraeus Fresco 21 microcentrifuge, Thermo Scientific, Langenselbold, Germany) in order to pellet any drug that precipitated on dispersion.

Table 1. Composition (% w/w) of SEDDS and Danazol Solubility Data in the Formulation and in the Aqueous Phase (AP) Formed during in Vitro Formulation Digestion[a]

Formulation components	F0	F10	F18	F25	F40	F60	F80
Captex 300: Capmul MCM (1:1) (% w/w)	0	10	18	25	40	60	80
Cremophor EL (% w/w)	65	80	64	65	50	30	10
Ethanol (absolute) (% w/w)	35	10	18	10	10	10	10
LCFS classification type[b]	IV	IV/IIIB	IV/IIIB	IIIB	IIIA	IIIA	IIIA
Danazol solubility in formulation [mg/g]	45 ± 1.3	36 ± 0.6	39 ± 0.8	35 ± 2.3	32 ± 0.7	30 ± 0.7	25 ± 1.0
AP_{max} (@ 80% sat.) [μg/mL][c]	994	800	860	784	718	674	544
AP_{max} (@ 40% sat.) [μg/mL][c]	497	400	430	392	359	337	272
Equilibrium solubility [μg/mL]							
AP_{DISP}[d]	178 ± 6.8	259 ± 5.1	243 ± 2.5	282 ± 5.5	306 ± 3.7	301 ± 3.9	269 ± 15.9
AP_{DIGEST} (5 min)[e]	134 ± 5.0	155 ± 2.5	126 ± 1.0	132 ± 0.3	118 ± 2.9	106 ± 3.6	88 ± 0.6
AP_{DIGEST} (60 min)[e]	89 ± 2.8	102 ± 0.9	81 ± 0.8	91 ± 2.7	71 ± 3.2	56 ± 0.9	46 ± 1.0
AP_{DIGEST} + HPMC (60 min)[e]	82 ± 0.3	98 ± 3.3	80 ± 1.7	81 ± 2.5	67 ± 2.6	59 ± 0.3	38 ± 1.1

[a]Captex 300 (medium-chain triglycerides); Capmul MCM (medium-chain mono-, di- and triglycerides); Cremophor EL (surfactant); ethanol (cosolvent); HPMC (hydroxypropyl methylcellulose); AP, aqueous colloidal phase present in in vitro digest; w/w %. [b]Classification scheme taken from Pouton et al.[36,37] [c]Represents the AP concentration that would result if no precipitation occurred on either formulation dispersion or digestion, thus the maximum AP concentration possible. Calculations shown for formulations containing danazol drug loads representing 40% and 80% of danazol saturated solubility in the formulation. [d]Data represent equilibrium solubility of danazol in the AP produced by dispersion of blank (drug-free) formulation for 10 min. [e]Data represent equilibrium solubility of danazol in the AP produced by dispersion and digestion of blank (drug-free) formulation for either 5 or 60 min. Data at 60 min also generated for formulations containing HPMC.

Digestion samples were centrifuged more thoroughly via ultracentrifugation for 30 min at 37 °C and 400000*g* (Optima xL-100 K centrifuge, SW-60 rotor, Beckman, Palo Alto, CA, USA) to separate the digestion sample into an aqueous phase and a pellet phase. Since all formulations dispersed to form microemulsified dispersions, an undispersed "oil" phase was not evident on digestion. Samples obtained from each phase were assayed for danazol content by HPLC.

X-ray Powder Diffraction (XRPD). The pellet phase obtained after digestion and ultracentrifugation was examined by X-ray powder diffraction to investigate the influence of HPMC on the physical form of precipitated danazol. Samples of HPMC and crystalline danazol were measured as controls using a quartz sample plate. The pellet phase obtained post digestion of the F60 formulation (± 5% (w/w) HPMC E4M) was placed on a zero background silicone holder. XRPD was performed using a Philips 1140 vertical diffractometer (Philips, Holland) for scanning from 2° to 60°, with an angular increment of 0.02° and a speed of 2°/min. A copper (Cu) tube anode and Kα radiation (λ 1.54184 Å) were used. The voltage and current applied were 45 kV and 40 mA. The data were acquired and analyzed using the Visual XRD and traces software package, version 6.6.1 (GBD Scientific Equipment).

Solubility in Aqueous Phase Predigestion (Dispersion Phase) (AP_{DISP}). The solubility of danazol in dispersed emulsified blank (i.e., drug-free) formulation was evaluated. Triplicate samples of the AP were taken after dispersion of drug-free (blank) formulations for 10 min, excess danazol was added and the samples were incubated at 37 °C. Solubility samples were subsequently taken over 6 h and centrifuged for 30 min at 21000*g*, 37 °C. The equilibrium solubility of danazol in the dispersed aqueous colloidal phase was defined as the mean solubility value obtained after 2, 4, and 6 h incubation.

Solubility in Aqueous Phase Post Digestion (AP_{DIGEST}). The solubility of danazol in the aqueous colloidal phase generated by digestion of blank (i.e., drug-free) formulation was evaluated after 5 and 60 min digestion. Conditions for digestion of the blank formulation were as described above, and digestion samples were ultracentrifuged and the aqueous phase was separated prior to measurement of drug solubility. Triplicate samples comprising danazol in excess were incubated at 37 °C and centrifuged for 30 min at 21000*g*, 37 °C. As the solubility was found to decrease up to 20% over 24 h for some of the formulations, presumably due to instability of the colloids present, the apparent equilibrium solubility in the aqueous colloidal phase post digestion was defined as the mean solubility value obtained after 2, 4, and 6 h incubation following attainment of the maximum solubility.

Quantification of Danazol in in Vitro Experiments by HPLC. Aqueous phase samples obtained during dispersion experiments and after initiation of digestion were diluted 1:10 (v/v) with acetonitrile before HPLC analysis. Samples of danazol in pellets were first dissolved in 5 mL of chloroform/methanol (2:1 v/v) and subsequently diluted 1:10 (v/v) in acetonitrile prior to analysis by HPLC.

Danazol chromatography was conducted using a Waters 2690 separation module and a Waters 486 tunable absorbance detector (Waters, MA, USA). A Waters symmetry C18 column (3.9 × 150 mm) (Waters, MA, USA) was maintained at 25 °C. The mobile phase consisted of 75% (v/v) methanol and 25% (v/v) milli-Q water. The flow rate was 1 mL/min. All samples were maintained at 10 °C in the autosampler prior to injection of 50 μL. Detection of danazol was conducted by single wavelength monitoring at 286 nm.

The assay was validated by analysis of n = 5 replicate standards made up at three different concentrations (0.5, 5, 50 μg/mL). Intra-assay variability was accurate to 96.6, 101.1 and 99.7% and precise to ±0.5, 0.3 and 3.8% of 0.5, 5 and 50 μg/mL. Interassay variability was assessed over three separate days and was accurate to 100.5, 99.3 and 100.3% and precise to ±2.9, 0.2 and 0.1% at 0.5, 5 and 50 μg/mL. The recovery of danazol spiked into aqueous and pellet phases obtained from blank digests of soybean oil was 96.4 ± 0.3% and 100.0 ± 0.8% (n = 6), respectively.

Data Analysis for in Vitro Experiments. *Solubilization Profiles.* The ability of excipients to maintain danazol in a metastable, supersaturated state during kinetic dispersion and digestion experiments was assessed using solubilization/

precipitation profiles. Figure 3 shows a typical profile of a SEDDS during dispersion and digestion including the potential

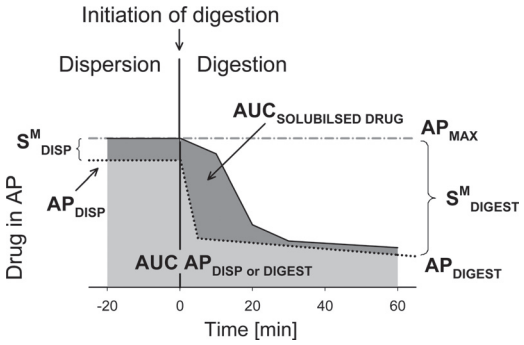

Figure 3. Supersaturation during dispersion and digestion of SEDDS formulations. The upper (dashed) line indicates the potential maximum concentration in the aqueous colloidal phase in the absence of drug precipitation (AP_{MAX}). The lower (dotted) line indicates drug solubility in the colloidal phase produced by dispersion (AP_{DISP}) or digestion (AP_{DIGEST}) of drug-free SEDDS. S^{M}_{DISP} and S^{M}_{DIGEST} are the maximum supersaturation ratios for each formulation on dispersion or digestion, respectively (eq 2).

maximum concentration in the aqueous colloidal phase assuming no precipitation (AP_{MAX}) and the apparent equilibrium solubility in the aqueous colloidal phase post dispersion (AP_{DISP}) and digestion (AP_{DIGEST}). The maximum concentration that could be obtained in the aqueous colloidal phase (AP_{MAX}) was determined from the mass of drug contained in the 1 g of formulation added to the dispersion/digestion experiment, divided by the volume of the digest. Since the solubilized concentrations varied as a function of time, an indication of changes to solubilization/precipitation across the entire experiment was gained via the use of areas under the solubilization curves (AUC).

The ability of the formulation to maintain supersaturation during dispersion and digestion was expressed as a supersaturation ratio, S (eq 1). S was determined as the area under the solubilized drug concentration versus time profile divided by the AUC of the apparent equilibrium solubility of danazol in the AP during dispersion (AP_{DISP}) and digestion (AP_{DIGEST}). To allow representation of AP_{DIGEST} in the current plots, the change in solubility (during the course of digestion) was assumed to be linear between 5 and 60 min post digestion (see Figure 3).

$$S = \frac{\text{AUC}_{\text{solubilized drug}}}{\text{AUC AP}_{\text{DISP or DIGEST}}} \quad (1)$$

The ratio of the potential maximum drug levels in the aqueous colloidal phase in the absence of drug precipitation (AUC AP_{MAX}) and the equilibrium solubility in the AP obtained after dispersion/digestion of a drug-free formulation (AUC $AP_{DISP\ or\ DIGEST}$) provides a measure of the theoretical maximum thermodynamic instability of the drug in the dispersed or digested formulation, i.e., the potential maximum supersaturation ratio (S^{M}) (eq 2) that the formulation may generate.

$$S^{M} = \frac{\text{AUC AP}_{\text{max}}}{\text{AUC AP}_{\text{DISP or DIGEST}}} \quad (2)$$

A measure of how effectively solubilization was maintained was gained by comparing the AUC for the solubilized drug concentration as a function of time, divided by the area under the maximum drug concentration that may be attained in the absence of precipitation (eq 3).

$$\%\ \text{solubilized} = \frac{\text{AUC}_{\text{solubilized drug}}}{\text{AUC AP}_{\text{max}}} \times 100\% \quad (3)$$

Bioavailability Studies in Beagle Dogs. *Administration and Sampling.* Based on the in vitro experiments, six formulations with varying drug load and polymer (HPMC E4M) concentration were evaluated in vivo to examine the impact of the addition of polymeric material and the potential role of drug saturation in the formulation on in vivo exposure.

All experiments were approved by the local animal ethics committee and were conducted in accordance with the Australian and New Zealand Council for the Care of Animals in Research and Teaching guidelines. Treatments were based on two formulation types, F0 and F60 (Table 1). F0, a lipid-free SEDDS, contained danazol at nominally 40% of saturated solubility in the formulation. F60, a MC lipid-based SEDDS, contained danazol at nominally 40% or 80% of saturated solubility in the formulation. All treatments were evaluated in the presence and absence of 5% (w/w) HPMC E4M.

The studies were conducted as a four-way crossover (7 days washout) in male beagle dogs (13−23 kg). Treatments were hand filled into gelatin capsules 16 h prior to dosing as previously described.[47] Each treatment was administered in two capsules (2 × 800 mg formulation) with approximately 50 mL of water. The actual dose administered for F0 (nominally at 40% saturation in the formulation) was 26.3 mg (representing 37% saturation) in the absence of polymer and 23.6 mg (33% saturation) in the presence of polymer. For F60 (nominal 40%), the actual dose was 17.3 mg (36% saturation) and 17.2 mg (35% saturation) in the absence and presence of polymer. For F60 (nominal 80%), the actual dose was 34.2 mg (70%) and 34.1 mg (70%) in the absence and presence of polymer respectively. The dogs were fasted for at least 20 h prior to dosing. After dosing, animals remained fasted until 10 h postdosing, after which they were fed on a daily basis. Water was available ad libitum during the study.

Blood samples of approximately 3 mL were collected before dosing and at 15, 30, 45, 60 and 90 min, then at 2, 3, 4, 6, 8 and 10 h postdosing. Samples were collected via an indwelling catheter inserted in the cephalic vein prior to dosing, and additional samples were obtained by individual venipuncture at 24, 32 and 48 h postdose. Blood samples were collected into 4 mL tubes containing dipotassium EDTA. Plasma was separated within 2 h by centrifugation for 10 min at 1328*g* (Eppendorf 5702 R/A-4-38 centrifuge, Eppendorf AG, Hamburg, Germany) and stored at −80 °C until sample analysis.

Plasma Sample Preparation. Calibration standards for danazol were prepared by spiking 250 μL aliquots of blank beagle plasma with 10 μL of an acetonitrile (ACN) solution containing 25, 62.5, 125, 250, 1250, 2500 and 6250 ng/mL danazol. This provided spiked plasma concentrations in the range of 1−250 ng/mL danazol. Ten microliters of an internal standard (IS) solution (2000 ng/mL of progesterone in ACN) was also added to each plasma sample or standard, the tubes were vortexed for 30 s (s) and 125 μL of aqueous ammonium sulfate solutions (saturated at room temperature) were added. After an additional 30 s of vortexing, 250 μL of ACN was

added. The tubes were vortexed again for 30 s and left to equilibrate for 20 min at room temperature prior to centrifugation for 5 min at 21000*g* (Eppendorf 5804 R centrifuge, Eppendorf AG, Hamburg, Germany). 100 μL of the organic phase was transferred into autosampler vials and 10 μL injected onto the HPLC−MS. Unknown concentrations were determined by comparison of the unknown danazol:IS peak area ratio with a calibration curve of danazol:IS peak area ratio vs danazol concentration constructed using the calibration standards.

Quantification of Danazol in Plasma Samples by LC−MS. Plasma analysis was conducted using an LCMS 2020 system (Shimadzu, Japan) which included an LC-20AD binary pump, a SiL-20AC refrigerated autosampler, a mobile phase vacuum degassing unit (DGU-20A_3) and a temperature-controlled column compartment (CTO-20A), coupled with a single-quadrupole mass spectrometer (Shimadzu LCMS 2020) equipped with an electrospray ionization source. The autosampler was maintained at 4 °C and the column at 40 °C. A Phenomenex Gemini C6-phenyl column (50 × 2.0 mm, 3 μm) was used to allow separation. Samples were eluted via gradient elution at a flow rate of 300 μL/min. The mobile phases consisted of a mixture of solvent A (95% v/v Milli-Q water:5% v/v MeOH) and solvent B (5% v/v Milli-Q water:95% v/v MeOH) both containing 1 mM ammonium formate and 0.1% formic acid. The initial percentage of solvent B was 60%. The proportion of solvent B was linearly increased to 100% over 6.5 min and was held at 100% for 7 min. After 14.25 min, the gradient was returned to 60% solvent B until the end of the 17 min run time to achieve re-equilibration. Under the above-mentioned conditions, the retention times of danazol and progesterone were 4.59 and 4.35 min, respectively. The MS conditions were as follows: drying gas flow, 20 L/min; nebulizing gas flow, 1.5 L/min; drying gas temperature, 200 °C; interface voltage, 3.5 kV; and detector voltage, 1.0 kV. Selected-ion monitoring was accomplished at m/z 338.2 for danazol, and m/z 314.9 for the internal standard, progesterone. The chromatographic data were acquired and analyzed using the LabSolution software package, 5.31.277 (Shimadzu).

The plasma assay was validated by analysis of $n = 6$ quality control samples containing 1, 10 and 100 ng/mL danazol in blank plasma. Intra-assay variability was accurate to 113.5, 103.8 and 111.8% and precise to 11.2, 9.2 and 4.4% of 1, 10 and 100 ng/mL, respectively. The lower limit of quantification for the plasma assay (1 ng/mL) was determined by replicate analysis ($n = 6$) of spiked plasma samples and defined as the lowest concentration at which appropriate accuracy and precision was obtained. Calibration curves and regression parameters were calculated by least-squares linear regression analysis using a weighting factor of $1/x$.

Pharmacokinetic Data Analysis. The maximum plasma concentrations (C_{max}) and the time for their occurrence (T_{max}) were noted directly from the individual plasma concentration vs time profiles. The area under the plasma concentration vs time profiles (AUC_{0-10h}) were calculated using the linear trapezoidal method. Danazol plasma concentrations were typically below the limit of quantification of the assay at 24, 32 and 48 h postdose, and accurate determination of the terminal elimination rate constant and ($AUC_{0-\infty}$) was not possible. Since the danazol plasma concentrations at 10 h were low, the extrapolated AUC ($AUC_{10-\infty}$) was expected to contribute only a minor proportion of the total AUC ($AUC_{0-\infty}$). Relative bioavailability comparisons were therefore performed using (AUC_{0-10h}). 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.

RESULTS

In Vitro Evaluation. *Characterization of SEDDS Formulations Containing Danazol.* Table 1 provides the exact composition of the model formulations shown in the ternary plot in Figure 2 and also danazol solubility data in the formulations and in the aqueous phases formed by in vitro dispersion or digestion of the blank formulations. The in vitro performance of the formulations was evaluated using formulations containing drug (danazol) loads equivalent to 40% and 80% of danazol saturated solubility in the formulation. The maximum solubilized dose that the formulations could provide at low (40% of saturated solubility) and high (80% of saturated solubility) drug loading in the formulation is also shown. As the lipid/surfactant ratio in the formulation increased at constant cosolvent levels (i.e., F10 − F25 − F40 − F60 − F80), danazol solubility in the formulation decreased. Therefore, the potential maximum drug concentration that could be attained in the aqueous phase of the digest in the absence of drug precipitation (AP_{MAX}) also decreased at drug loads representing either 40% or 80% of danazol solubility in the formulations. The same trend was observed for formulations containing decreasing cosolvent/lipid ratios at constant surfactant levels (i.e., F0 − F18 − F25).

Polymeric Inhibition of Drug Precipitation from SEDDS Formulations. To evaluate the potential effect of polymeric precipitation inhibitors (PPI) on the performance of danazol-containing SEDDS, F60 was used as a typical formulation composition (60% lipid, 30% surfactant, 10% cosolvent), and a screening study was conducted to explore the effect of 12 different polymers (and Aerosil) on drug precipitation during formulation dispersion and digestion. In this screening exercise, danazol was included at 40% of saturated solubility in the formulation. Figure 4 shows the percentage of the original quantity of drug dissolved in the formulation that precipitated after dispersion and a period of 60 min digestion in the absence (control) and presence of the different polymers. Consistent

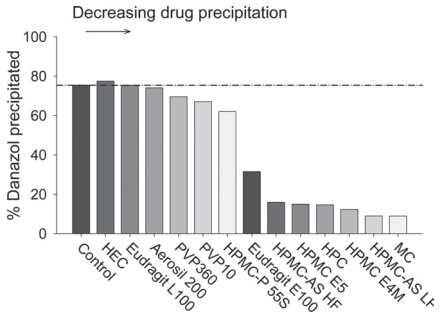

Figure 4. Screening of various polymers as precipitation inhibitors after 60 min in vitro digestion of a model SEDDS (F60) containing danazol. The dotted line represents the extent of drug precipitation from the formulation in the absence of polymer. Data below the line represent formulations where polymer addition leads to lower drug precipitation on formulation digestion.

with previous studies describing similar medium-chain SEDDS formulations of danazol,[18] digestion led to significant drug precipitation. After a 60 min digestion period, 75% of the initial drug load in the formulation had precipitated. The addition of polymer to the formulation, however, had in many cases a profound effect on drug precipitation. Data below the dotted line in Figure 4 show polymers that resulted in lower drug precipitation compared to the control formulation. Seven of the polymers examined (Eudragit E100, HPMC (E4M), HPMC (E5), HPMC-AS (LF), HPMC-AS (HF), MC (A4C) and HPC) reduced drug precipitation by more than 50%, and the cellulose derivatives led to the greatest reduction in drug precipitation. In contrast, Eudragit L100, Aerosil 200, HEC, HPMC-P (55S) and PVP10 or PVP360 had little effect on drug precipitation (Aerosol was included in the current comparison, even though it is not a polymer, since this high surface area material was seen as a prospective precipitation inhibitor). HPMC (E4M grade) was chosen as a candidate PPI for further evaluation as it more easily dispersed in the SEDDS formulation when compared to, e.g., HPMC-AS, which resulted in formulations with much higher viscosity that were more difficult to manipulate.

Effect of Polymer Concentration on Drug Precipitation. To probe the impact of HPMC (E4M) concentration on drug precipitation, two SEDDS formulations were examined at different HPMC loads. F60 was again employed as a typical SEDDS formulation, and formulation F0 was explored as an example of a formulation containing much higher surfactant and cosolvent levels. Figure 5 shows the effect of polymer on drug precipitation from the formulations as the HPMC concentration in the formulations was altered. For F60 (Figure 5, black bars), a greater than 3-fold decrease in drug precipitation was evident when HPMC was added to the formulation at levels of 0.5% w/w or higher. In contrast, the polymer effect on drug precipitation from the formulation containing high surfactant and cosolvent [F0] was more (polymer) concentration dependent, and larger quantities of polymer (5% w/w) were required to inhibit precipitation to a similar extent (Figure 5, gray bars). Subsequent studies included HPMC at 5% w/w in all formulations.

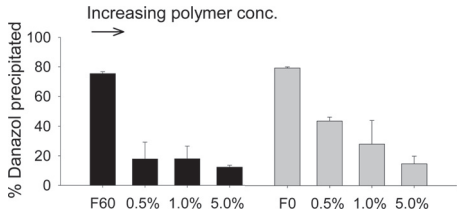

Figure 5. Effect of polymer concentration on danazol precipitation from F60 (black bars) and F0 (gray bars) after 60 min digestion. HPMC was incorporated at 0, 0.5, 1 and 5% (w/w) in F0 and F60 [mean ± SD (n = 3)].

Drug Solubilization during in Vitro Dispersion and Digestion. After identification of the most appropriate PPI (HPMC E4M) and an appropriate polymer concentration (5% w/w) for the model drug and formulations investigated here, subsequent studies sought to explore the impact of systematic changes to the nature of the SEDDS formulation (including the presence and absence of polymer) on drug precipitation during dispersion and digestion and in particular the potential for stabilization of supersaturation. Experiments were conducted using formulations containing drug at two different loads: 40% and 80% of drug solubility in the formulation, since increasing drug load was expected to increase the likelihood of drug precipitation and decrease the potential for either excipients or polymer to maintain supersaturation for extended periods. Data exploring the impact of changes to the lipid/surfactant ratio (at fixed cosolvent) are presented in Figure 6. Similar data exploring the effect of changes to the ratio of cosolvent/lipid at fixed surfactant are shown in Figure 7.

Impact of Lipid/Surfactant Ratio on Performance of SEDDS ± HPMC. Low Drug Loading (40% Saturation). Drug precipitation profiles from formulations with varying lipid/surfactant ratios containing danazol at 40% of saturated solubility are shown in the left-hand panels of Figure 6 in the presence and absence of 5% w/w HPMC. The potential maximum drug concentration that could be attained in the aqueous phase (in the absence of drug precipitation) (AP_{MAX}) is shown as the top horizontal dashed line in each plot. Consistent with the decrease in drug solubility in the formulations, increasing the ratio of lipid/surfactant led to lower AP_{MAX} values from 400 μg/mL for F10 to 272 μg/mL for F80.

To gain a better understanding of the drivers of drug precipitation during dispersion and digestion and to evaluate the possibility of supersaturation during formulation processing, the apparent equilibrium solubility of danazol in the AP generated by dispersion (AP_{DISP}) of blank (drug free) formulations and digestion (AP_{DIGEST}) of blank formulations (at 5 and 60 min post digestion) was also measured. Only moderate decreases in drug solubility were evident over the 5–60 min digestion period, and the equilibrium drug solubility in the dispersed and digested AP is shown as the lower dotted line in Figures 6 and 7.

The ratio between AP_{MAX} and drug solubility in the aqueous phase (see Figure 3) provides a measure of the maximum degree of supersaturation that each formulation is able to generate and is described by the maximum supersaturation ratio (S^M) (see eq 2). S^M quantifies the degree of thermodynamic instability generated by formulation dispersion or digestion (when S^M is >1) and is expected to influence the degree of drug precipitation. The S^M values are annotated in Figures 6 and 7 for both dispersion (S^M_{DISP}) and digestion (S^M_{DIGEST}) phases.

All the formulations dispersed readily, and no evidence of drug precipitation was apparent during the initial 30 min dispersion period at low drug loading despite the level of supersaturation generated. As such, AP concentrations over the 30 min dispersion period were consistent with AP_{MAX} (dashed line).

Examination of the drug concentrations measured in the AP of the digest after initiation of digestion (filled symbols in Figure 6) reveals a very rapid drop in drug solubilization on digestion initiation for all formulations and approach toward the equilibrium solubility of drug in the phases formed post digestion. A brief period of supersaturation was evident in all cases, but by 30 min digestion drug precipitation had lowered the AP concentration to a level consistent with drug solubility in the phase formed, effectively removing the driving force for further precipitation.

As digestion was initiated, the equilibrium solubilization capacity of the system also rapidly decreased due to changes in the physical and chemical properties of the aqueous colloidal phase upon digestion. This is reflected in the data describing

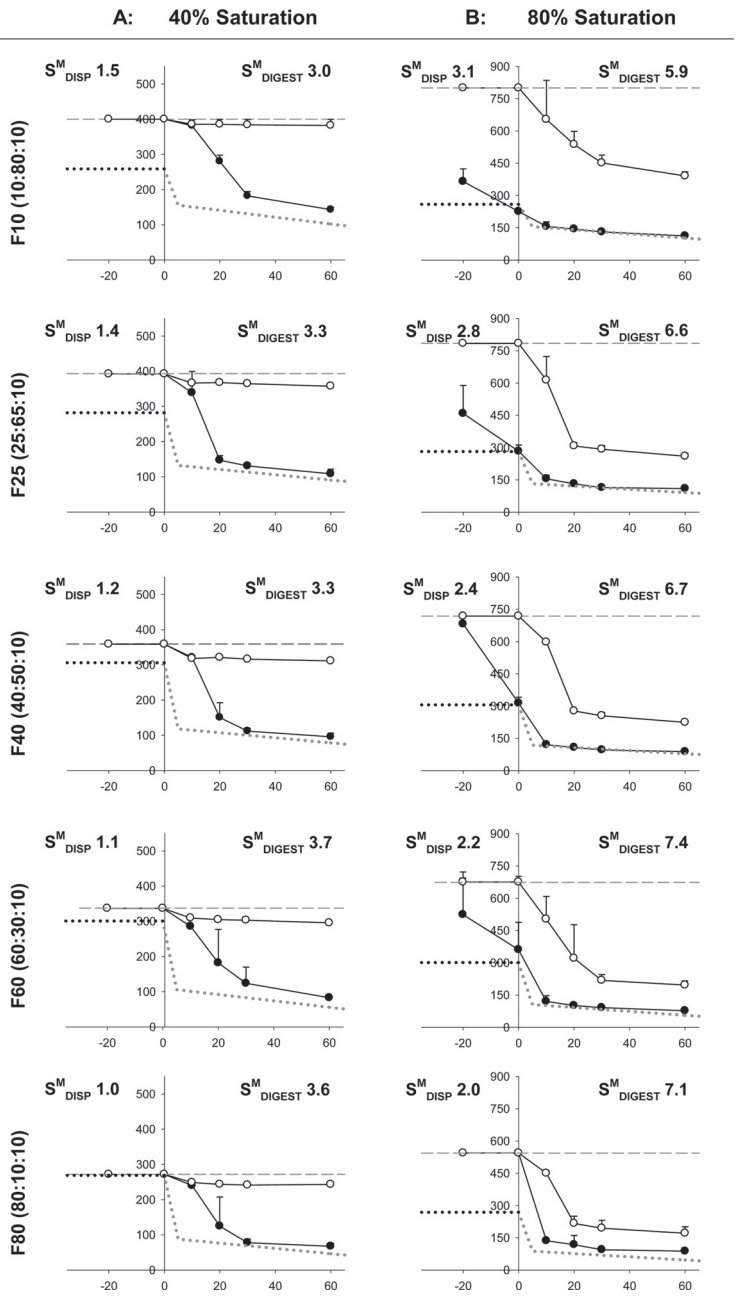

Figure 6. Drug precipitation profiles during dispersion and digestion of SEDDS formulations (FX (*X*:*Y*:*Z*) where *X* is the % composition of lipid, *Y* is surfactant and *Z* is cosolvent) when decreasing lipid/surfactant ratio at constant cosolvent (10%) [mean ± SD (n = 3)]. Danazol was incorporated at 40% (panel A) and 80% (panel B) of the equilibrium solubility in the formulation and in the absence (filled symbols) and presence (open symbols) of 5% (w/w) HPMC. The upper dashed line indicates AP_{MAX}, and the lower dotted line indicates the drug solubility in the aqueous colloidal phase produced by dispersion (AP_{DISP}) and digestion (AP_{DIGEST}) of drug-free SEDDS. S^{M}_{DISP} and S^{M}_{DIGEST} are the maximum supersaturation ratios for each formulation on dispersion or digestion respectively. Axis labels are not shown on the plots to improve clarity; *x*-axis represents digestion time [min]; *y*-axis represents danazol concentration in AP [μg/mL].

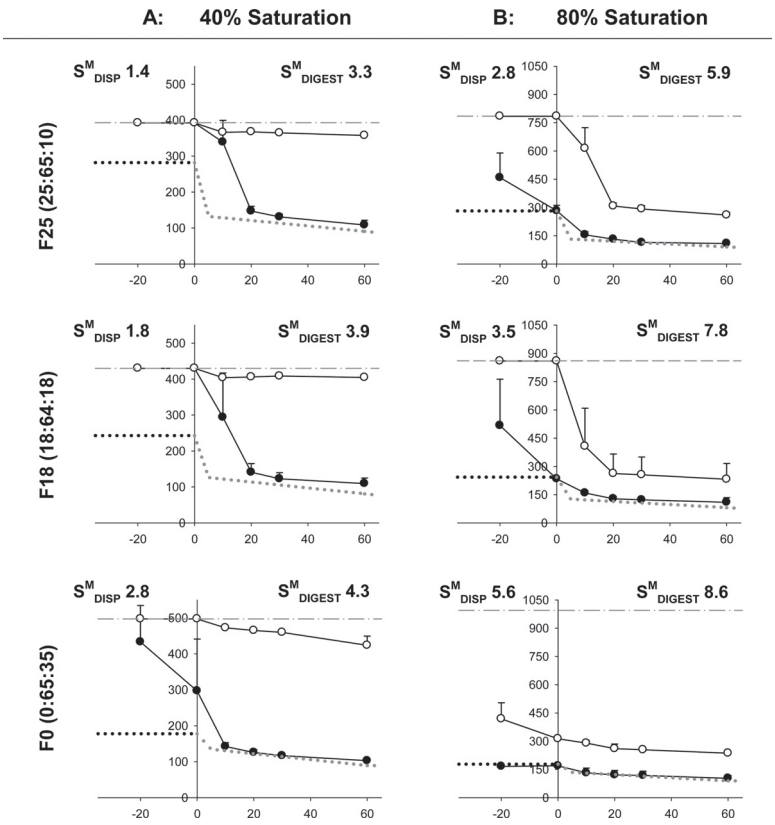

Figure 7. Drug precipitation profiles during dispersion and digestion of SEDDS formulations (FX (*X*:*Y*:*Z*) where *X* is the % composition of lipid, *Y* is surfactant and *Z* is cosolvent) when increasing cosolvent/lipid ratio at constant surfactant [mean ± SD (n = 3)]. Danazol was incorporated at 40% (panel A) and 80% (panel B) of the equilibrium solubility in the formulation and in the absence (filled symbols) and presence (open symbols) of 5% (w/w) HPMC. Figure labels as described in the caption for Figure 6.

equilibrium solubility of drug in the AP after 5 min of digestion (AP_{DIGEST} 5 min in Table 1).

Addition of 5% (w/w) HPMC to all formulations (open circles in Figure 6) resulted in greatly reduced drug precipitation and maintenance of supersaturation for extended periods. This occurred regardless of lipid/surfactant ratio. To evaluate whether the enhanced drug solubilization that was obtained in the presence of HPMC reflected stabilization of a supersaturated state or changes to the inherent solubilization capacity of the post digestion AP, the equilibrium solubility of danazol in blank (drug-free) AP was measured in the presence of polymer. Table 1 summarizes the equilibrium solubility values obtained after 60 min digestion in the presence of 5% w/w HPMC, and no significant difference was evident between drug solubility in the presence and absence of HPMC. The impact of the polymer on drug solubilization during digestion was therefore to stabilize a supersaturated state rather than to enhance intrinsic solubility.

High Drug Loading (80% Saturation). Panel B in Figure 6 shows the drug precipitation profiles of SEDDS with varying lipid/surfactant ratio at high drug loading (80%) in the presence and absence of 5% w/w HPMC during in vitro dispersion and digestion. Doubling the drug load in the formulations resulted in a corresponding 2-fold increase in S^M. In the absence of polymer, all formulations with S^M_{DISP} above 2 resulted in rapid drug precipitation during the initial 30 min dispersion period. Precipitation continued with the initiation of digestion, and within 10 min, the drug concentration in the aqueous colloidal phase reduced to the level of the equilibrium solubility in the AP regardless of the lipid/surfactant ratio or S^M_{DIGEST}.

At the high drug load, addition of 5% (w/w) HPMC prevented drug precipitation on dispersion of all formulations, maintaining supersaturated conditions. However, initiation of digestion led to drug precipitation despite the presence of HPMC. The presence of the polymer slowed drug precipitation, resulting in profiles similar to that obtained at the lower drug loading in the absence of polymer. Interestingly, after 20−30 min digestion, drug concentrations appeared to plateau at a level above the equilibrium solubility in the AP.

Impact of Increasing Cosolvent/Lipid Ratio in SEDDS ± HPMC. Low Drug Loading (40% Saturation). Panel A in Figure 7 shows the drug precipitation profiles of SEDDS with varying cosolvent/lipid ratio at low drug loading (40%) in the presence and absence of 5% w/w HPMC during in vitro dispersion and digestion. At constant surfactant levels,

increasing the cosolvent/lipid ratio in the formulation increased drug solubility in the formulation and therefore AP_{MAX}. In contrast, increasing cosolvent resulted in little difference to the equilibrium solubility of drug in the post digestion AP (consistent with the decrease in solubilization capacity for diluted cosolvents[48]). As a result, S^{M}_{DIGEST} increased as the cosolvent/lipid ratio in the formulation was increased.

In the absence of polymer, formulations containing low levels of cosolvent were able to prevent drug precipitation during dispersion and maintain supersaturation, however as cosolvent concentrations increased (>18% in the formulation), precipitation was evident, even during the dispersion phase. After initiation of digestion, rapid drug precipitation occurred for all formulations, resulting in limited supersaturation and drug concentrations in the AP approaching equilibrium solubility after 10−20 min digestion. Addition of 5% (w/w) HPMC to all formulations containing danazol at low drug loads significantly reduced drug precipitation on digestion and stabilized supersaturation regardless of cosolvent levels and S^{M}_{DIGEST} at low drug loading (Figure 7A).

High Drug Loading (80% Saturation). As the drug loading in the formulations was increased, S^{M} increased, as did the extent of drug precipitation from the formulations. In the absence of polymer, significant precipitation was evident even on dispersion and drug concentrations in the post digestion aqueous phase approached equilibrium solubility more quickly than was evident in the lower dose profiles, regardless of the cosolvent/lipid ratio (Figure 7B).

Addition of 5% w/w HPMC to the formulations containing lower cosolvent (F25, F18) prevented precipitation on dispersion, however, the increased level of cosolvent in F0 resulted in rapid precipitation within 15 min of dispersion, despite the presence of polymer. In all formulations, initiation of digestion led to rapid drug precipitation, although the presence of polymer appeared to result in a plateau in the AP concentrations at a level slightly above the equilibrium solubility of drug in the post digestion AP, similar to the results presented in Figure 6.

Figure 8 shows the XRPD diffractograms of the pellet phase obtained after digestion and ultracentrifugation of F60.

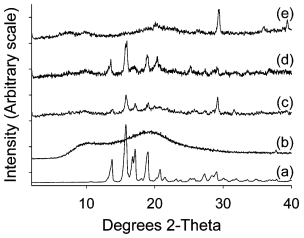

Figure 8. XRPD profiles of (a) crystalline danazol, (b) HPMC, (c) pellet after digestion of F60 comprising danazol and 5% HPMC, (d) pellet after digestion of F60 comprising danazol and (e) blank pellet after digestion of drug-free F60.

Physicochemical examination of precipitated danazol in both the presence and absence of HPMC revealed similar XRPD signals to the original crystalline material, suggesting that polymer addition did not affect the nature of the crystallized material that precipitated out of solution. The polymer effect was therefore more likely to be an effect of retardation of crystal nucleation and growth rather than the promotion of phase separation of amorphous drug in the precipitate.

Bioavailability Data. *Effect of Formulation Composition and the Presence of PPI in Vivo.* The mean plasma concentration versus time profiles for danazol following oral administration of a high cosolvent formulation (F0) and high lipid formulation (F60) in the presence and absence of 5% w/w HPMC (E4M grade) to fasted beagle dogs are shown in Figure 9, and the corresponding mean pharmacokinetic parameters are summarized in Table 2. Danazol plasma concentrations at 24, 32 and 48 h were typically below the limit of quantification of the analytical assay, and data were therefore truncated to 10 h for pharmacokinetic evaluation.

Panel A in Figure 9 shows the administration of F0 containing danazol at 40% saturated solubility. In the absence of polymer, the dose normalized plasma profile and AUC of danazol after oral administration were consistent with a previously reported study using the same formulation, providing confidence in reproducibility.[32] In contrast to the in vitro precipitation profiles, however, the addition of 5% w/w HPMC to the formulation failed to increase danazol bioavailability and resulted in a decrease in C_{max} and similar exposure as expressed by AUC.

The mean plasma concentration time profiles for F60 are shown in Figure 9 at low (panel B) and high (panel C) drug loading, and in the presence and absence of HPMC. At low drug loads, addition of HPMC to F60 led to a 65% increase in danazol exposure. Increasing the drug loading from 40% to 80%, however, resulted in loss of the polymer effect, and AUCs in the presence and absence of polymer were similar.

To compare excipient effects on bioavailability across formulations and at different drug loads, the AUC_{0-10h} was also normalized to a standardized 15 mg dose, and these data are compared in Figure 10. The administration of F0 [high cosolvent/lipid-free] and F60 [high lipid] resulted in similar drug exposure at the low drug load in the absence of polymer. In the presence of HPMC, however, bioavailability from F60 was higher than that of F0 (although statistical significance was not reached), reflecting the effect of the polymer in increasing exposure for F60 but not F0.

At the higher drug load, the polymer had no effect on bioavailability for F60. Interestingly, however, bioavailability was increased when compared to the low drug dose formulation regardless of the presence of polymer.

DISCUSSION

Self-emulsifying drug delivery systems (SEDDS) show great potential for improvements in oral bioavailability for poorly water-soluble drugs. The ability of SEDDS to maintain drug in a solubilized state and to prevent drug precipitation is highly dependent on the nature of included formulation components, and this is further complicated by the realization that the properties of lipid and surfactant based excipients can change markedly during dispersion and digestion in the GI tract.[18,32,49−51] In particular, digestion may lead to a loss in solubilization capacity of the formulation and a decrease in bioavailability.[16,18,32,35,52]

The loss of solubilization capacity that results from either dispersion or digestion of a SEDDS formulation also results in the generation of transient supersaturation. Where precipitation can be delayed for a period sufficient to allow absorption, supersaturation increases thermodynamic activity and may promote absorption. In recent years, considerable efforts have

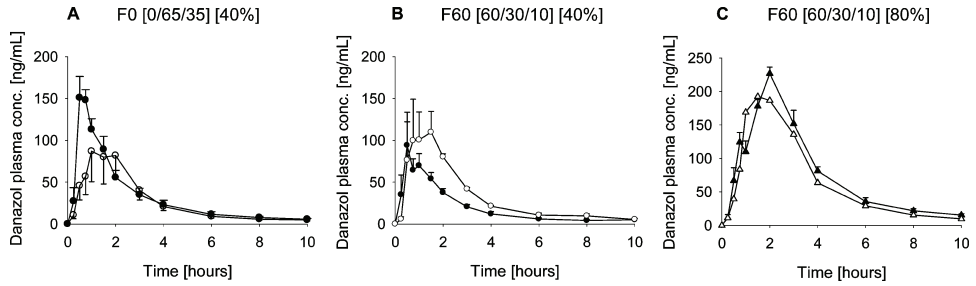

Figure 9. Mean plasma concentration vs time profiles for danazol after oral administration to fasted beagle dogs in the presence (open) and absence (filled) of 5% (w/w) HPMC. (A) Formulations F0 [0/65/35; lipid/CrEL/ethanol], danazol incorporated at 40% saturated solubility [mean ± SEM (n = 4)], (B) F60 [60/30/10], danazol at 40% saturated solubility [mean ± SEM (n = 3)], (C) F60 [60/30/10] in the absence of polymer (filled), [mean ± SEM (n = 3)] and in the presence of polymer (open), [mean (n = 2)] with danazol incorporated at 80% saturated solubility.

Table 2. Pharmacokinetic Parameters for Danazol after Oral Administration of F0 [Mean ± SEM (n = 4)] and F60 [Mean ± SEM (n = 3)] to Fasted Beagle Dogs

	F0		F60			
	40%[a]	40% + HPMC	40%	40% + HPMC	80%	80% + HPMC[c]
AUC_{0-10h} (ng·h/mL)	325 ± 42	267 ± 45	194 ± 40	316 ± 48	762 ± 48	738.6: 628.9
AUC_{0-10h} (normalized)[b]	185 ± 24	170 ± 29	168 ± 35	277 ± 42	334 ± 21	320.1: 272.6
C_{max} (ng/mL)	160 ± 22	127 ± 26	104 ± 30	124 ± 37	227 ± 10	196.7: 249.2
T_{max} (h)	0.7 ± 0.1	1.3 ± 0.2	0.8 ± 0.3	1.4 ± 0.4	2.0 ± 0.0	3.0: 1.5
$t_{1/2}$ (h)	2.5 ± 0.1	2.1 ± 0.2	4.4 ± 0.7	2.9 ± 0.4	3.3 ± 0.2	2.4: 2.5

[a]Nominal % saturation shown here. Accurate doses and saturation level for each dose based on variation in fill concentrations and fill weights detailed in Materials and Methods. [b]AUC normalized to 15 mg dose to compare relative exposure across different drug doses. [c]Dog 3 was excluded due to vomiting after dosing. Data presented for the individual dogs (n = 2).

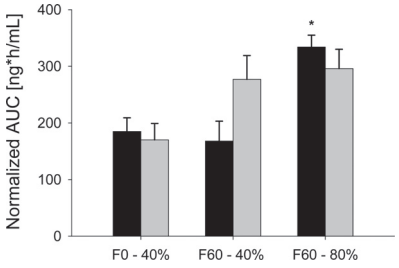

Figure 10. Normalized AUC_{0-10h} [ng·h/mL] to 15 mg dose. Formulation F0 with danazol incorporated at 40% saturated solubility and F60 with danazol incorporated at 40% and 80% saturated solubility in the absence (black) and presence (gray) of 5% (w/w) HPMC. *Statistically significantly different from F0 (40%) and F60 (40%) (p < 0.050).

therefore been directed toward the design and development of drug formulations that promote the formation and stabilization of supersaturation during GI formulation processing. Hydrophilic polymers (e.g., hydroxypropyl methylcellulose (HPMC)) have been shown to be effective in stabilizing supersaturation in several applications, perhaps most notably the marketed itraconazole formulation (Sporanox), and as such have also started to be explored for their potential to stabilize supersaturation during the dispersion/digestion of SEDDS formulations.[21−23,53]

The balance between, on the one hand, increasing supersaturation and thermodynamic activity and, on the other, avoiding precipitation is further complicated by differences in dose, where increasing dose will increase the potential for supersaturation but also the risk of precipitation.

The current studies were conducted to evaluate the potential for supersaturation generation during the processing of SEDDS formulations, to probe the ability of polymers to enhance the performance of SEDDS by stabilizing supersaturation, and to evaluate the effect on solubilization and supersaturation of increasing drug dose.

Polymers as Drug Precipitation Inhibitors. The cellulose-based polymers (e.g., HPMC, HPC and MC) were the most effective in preventing drug precipitation during the dispersion and digestion of medium-chain SEDDS containing danazol (Figure 4). This is consistent with recent data from our laboratories that detailed the effect of polymers on drug precipitation for danazol using a solvent shift method[54] and also the work of others.[55,56] Data obtained using simple in vitro methods to screen for polymeric precipitation inhibitors (PPIs) therefore appears to translate into utility in more complex in vitro dispersion and digestion evaluation protocols for SEDDS formulations (at least for danazol).

Evaluation of the impact of increasing HPMC concentration on the ability of the polymer to maintain danazol in a solubilized state post digestion revealed differences in polymer performance depending on the nature of the formulation. For the F60 formulation, addition of 0.5%, 1% or 5% (w/w) HPMC resulted in a significant decrease in drug precipitation, however, no differences were seen as a function of polymer concentration, suggesting that only low quantities of polymer were sufficient to inhibit precipitation. In contrast, for formulation F0, a concentration dependency in polymer effect was evident, and maximum precipitation inhibition occurred only at the highest polymer concentration (5% w/w). These

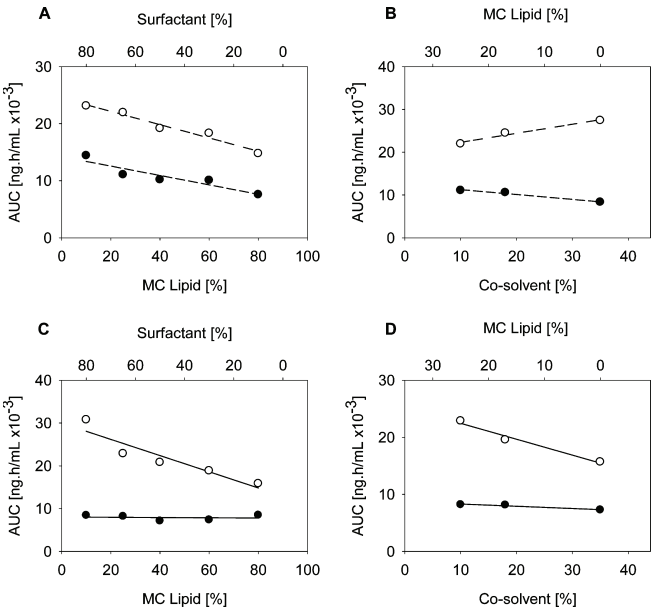

Figure 11. Area under the solubilization profile after initiation of digestion (AUC) in the presence (open) and absence (filled) of 5% w/w HPMC versus (A) increasing lipid/surfactant ratio, (B) increasing cosolvent/lipid ratio in the formulation with danazol incorporated at 40% saturated solubility, (C) increasing lipid/surfactant ratio and (D) increasing cosolvent/lipid ratio in the formulation with danazol incorporated at 80% saturated solubility.

data are consistent with the findings of Raghavan et al.,[26] where the flux of hydrocortisone acetate across a silicone membrane was used to evaluate the potential for different polymers to stabilize a supersaturated cosolvent solution. In these studies, the utility of the added polymer reached a maximum effect at polymer concentrations that varied depending on polymer type (1% for HPMC, 1% for HPC and 10% for PVP), although in this case further increases in polymer concentrations were shown to reduce drug flux.[26]

Formulation Sensitivity of Polymer Precipitation Effect. Data describing the formulation dependency of the ability of 5% HPMC to prevent drug precipitation on dispersion and digestion of SEDDS formulations is shown in Figures 6 and 7. To facilitate easier cross-formulation comparison, the area under the AP drug concentration versus time profiles after initiation of digestion was utilized as a composite measure of drug solubilization or total drug "exposure" to the AP over time. Figure 11 shows the in vitro AP AUC as a function of formulation composition in the presence and absence of HPMC. In all cases, the addition of polymer significantly enhanced total drug solubilization. The addition of HPMC to the formulations did not, however, affect drug solubility in the AP produced by digestion of blank formulations (Table 1). The effect of the polymer on drug solubilization during digestion was therefore due to stabilization of a transiently supersaturated state rather than an increase in solubilization capacity of the aqueous colloidal phase. Polymer addition has been suggested to prevent drug precipitation via a reduction in crystal nucleation and/or crystal growth, and intermolecular interactions (e.g., hydrogen bonding) between drug and polymer implicated in mediation or accentuation of the precipitation inhibition effect.[25−27,57−60] The utility of polymeric precipitation inhibitors is therefore likely to be highly dependent on the physicochemical properties of both drug molecule and polymer.[55]

Drug solubilization patterns after digestion of SEDDS formulations have also been suggested to reflect changes in crystal form, and the prospect of drug precipitation in the amorphous form after digestion of SEDDS formulation has been raised by Sassene et al.[61] The presence of polymer has also been suggested to change the crystal form, and Gao et al.[21] previously showed that polymer addition to SEDDS formulations of the poorly water-soluble drug AMG 517 did not prevent precipitation on formulation dispersion, but instead led to drug precipitation in the amorphous rather than the crystalline form, thereby enhancing the resolubilization potential by forming a more rapidly dissolving amorphous drug precipitate. In the current studies, danazol precipitated out of solution in a crystalline form on in vitro digestion both in the absence of HPMC (consistent with previous studies[17]) and in the presence of HPMC. In the case of danazol, therefore, the data reported here suggest that polymer addition was more likely to be an effect of retardation of crystal nucleation and growth rather than the promotion of phase separation of amorphous danazol (Figure 8). More detailed discussion of the potential mechanism of action of PPIs is available in the following reviews.[54,62]

Increasing surfactant and decreasing lipid resulted in greater in vitro drug exposure at low drug loading (40% of saturated solubility in the formulation) in the absence and presence of polymer (Figure 11A), and at high drug loading in the presence of polymer (Figure 11C). At higher drug loads and in the absence of polymer, precipitation was rapid and drug solubilization was low and invariant for all formulations (Figure

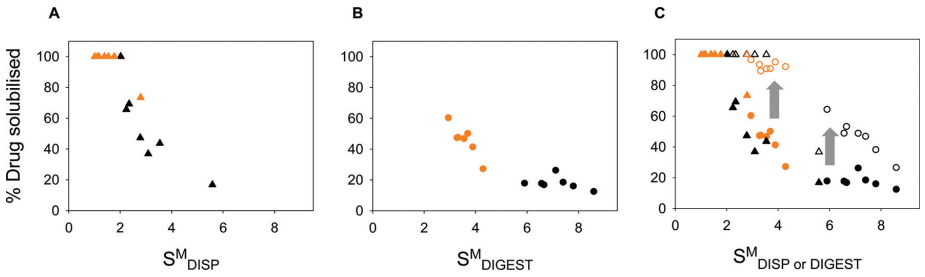

Figure 12. Percentage drug solubilized versus S^M at 40% (orange) and 80% (black) on (A) dispersion and (B) digestion. Panel C shows percent drug solubilized versus $S^M_{DISP\&DIGEST}$ at 40% (orange) and 80% (black) in the presence (open) and absence (closed) of PPI. Arrow illustrates the effect of PPI on percentage of solubilized drug at constant $S^M_{DISP\&DIGEST}$.

11C). A similar trend was observed when the cosolvent/lipid ratio in the formulation was increased (Figure 11B,D), consistent with a drop in the solubilization capacity of cosolvents on dilution. One exception was the data obtained at low drug loads and in the presence of polymer where a small increase in drug solubilization was evident at higher cosolvent (Figure 11B).

The trends in in vitro exposure are a function of both the quantity of drug initially dissolved in the formulation and the subsequent ability of the formulation to maintain solubilization on GI processing. Thus, at the 40% drug load and in the presence of polymer, precipitation was almost entirely inhibited, and therefore the solubilized drug concentrations in the AP (in vitro "exposure") reflected the initial drug load in the formulation. Increasing AP exposure was therefore evident on increasing surfactant and cosolvent levels in the formulation since these components result in greater drug solubility in the formulation. In contrast, at the 80% drug load and in the absence of polymer, precipitation was rapid, exposure was low and differences in formulation had little impact on solubilization profiles.

Cuine et al.[32] previously showed that the inclusion of Cremophor EL (CrEL) and ethanol in danazol SEDDS containing long-chain (rather than medium-chain) lipids increased drug solubility in the formulation and contributed to enhanced formulation dispersion. However, Cremophor EL and ethanol did not enhance solubilization post digestion, and instead a reduction in lipid content and an increase in surfactant and cosolvent composition resulted in a significant reduction in drug solubilization post digestion and a reduction in in vivo exposure.[32] Comparison of the data across the two studies suggests that increasing surfactant may be beneficial in formulations comprising medium-chain lipids (where the lipids provide limited ongoing solubilization capacity post digestion), however, for long-chain lipid-containing formulations, the inclusion of high quantities of surfactants (or at least digestible surfactants such as Cremophor EL) may be counterproductive.

In all cases evaluated here, differences in danazol equilibrium solubility in the AP formed on dispersion or digestion of the model formulations (lower dotted lines in Figures 6 and 7) were relatively small when compared across the different formulations, and differences in formulation performance were largely evident in the level of supersaturation seen after dispersion or initiation of formulation digestion. Consistent with the data reported here, analysis of danazol equilibrium solubility in the AP formed on digestion of similar long-chain SEDDS formulations has also shown that differences in formulation performance are driven largely by differences in supersaturation post digestion, rather than differences in the intrinsic solubilization capacity of the colloids formed.[20] Collectively, these data suggest the possibility that design specifications for lipid-based formulations might be reorientated such that emphasis is placed on the ability of excipients to maintain drug in a solubilized and supersaturated state, rather than focused on changing the equilibrium solubilization capacity of the colloids that are formed post digestion.

Impact of S^M on Drug Solubilization and Supersaturation. More detailed examination of the patterns of in vitro solubilization of danazol during dispersion and digestion of medium-chain lipid-containing SEDDS formulations reveals the importance of the initial (maximum) supersaturation ratio generated by formulation dispersion or digestion, prior to drug precipitation (a property we have defined as S^M).

Figures 6 and 7 show that danazol precipitation on dispersion of the evaluated formulations was only evident when S^M_{DISP} values were higher than 2, and the presence of polymer was able to prevent drug precipitation on dispersion for formulations up to a S^M_{DISP} of 3.5. Indeed, drug precipitation on formulation dispersion in the presence of polymer was only evident for the F0 formulation that contained surfactant and cosolvent alone and had the highest S^M_{DISP} (5.6).

A notable observation from examination of Figures 6 and 7 is the significant increase in S^M that occurred on initiation of digestion. In these formulations, therefore, digestion acts as a very effective supersaturation "trigger", and S^M increased by a factor of at least 2 and often 3−4-fold within 5 min of initiation of digestion. Generation of supersaturation subsequently predisposed the formulations to crystallization and precipitation. Whether precipitation occurred and how rapidly precipitation occurred was highly formulation dependent, however, trends similar to those seen on formulation dispersion were evident, and increased precipitation was associated with formulations that led to higher S^M_{DIGEST} values (Figures 6 and 7). For the formulations examined, increasing surfactant resulted in higher S^M_{DISP} and lower S^M_{DIGEST}, trends reflecting greater loss in solubilization capacity on dispersion of formulations containing greater quantities of surfactant rather than lipid, whereas on digestion, the loss in solubilization capacity was greater for MC lipid-rich formulations rather than surfactant-rich systems.

In an attempt to illustrate the impact of the degree of supersaturation on the potential for drug precipitation, Figure 12 provides a correlation between the S^M for the different formulations and a measure of the ability of the formulation to

resist precipitation on dispersion and digestion (% solubilized, eq 3). The data show that the ability to maintain in vitro exposure decreases as the S^M of the formulation increases (Figure 12), consistent with the realization that, at higher S^M, the increase in supersaturation provides a greater driving force for nucleation and drug precipitation.[63] This relationship appears to hold equally true in either the dispersion (Figure 12A) or digestion (Figure 12B) period. It is also apparent from Figure 12C that the presence of polymer significantly increases the ability of the formulation to maintain supersaturation close to maximum levels. Indeed, up to S^M of approximately 4 (consistent with conditions encountered at drug loadings of 40% of saturated solubility in the formulation), precipitation was largely suppressed. Above S^M of 6, however (conditions in line with the higher drug loading), increases in S^M led to significant decreases in solubilization.

Comparison with previous data for danazol obtained for similar long-chain formulations suggests that the trends revealed here may be applicable beyond medium-chain formulations.[32] Thus, previous studies have shown increases in oral bioavailability for poorly water-soluble drugs when administered in lipid-based formulations comprising long-chain rather than medium-chain lipids.[18,64] In the past, this has been ascribed to the presence of long-chain lipid digestion products more effectively swelling the solubilization capacity of intestinal mixed micelles. We recently reported, however, that retrospective analysis of at least one of these studies,[32] including generation of equilibrium solubility data for danazol in the colloids formed by digestion of the long-chain SEDDS employed, suggests that much of the difference in formulation performance was also driven by differences in supersaturation, rather than equilibrium solubility.[20] Further evaluation of these data and applying a similar analysis to that described here suggests that the AP_{MAX} values for the long-chain formulations are typically lower than that for medium-chain formulations (reflecting lower drug solubility in long-chain rather than medium-chain lipids) and that AP_{DIGEST} values are somewhat higher than that observed here for analogous medium-chain SEDDS (presumably due to the improved micelle swelling properties of long-chain digestion products). As such, the combination of lower AP_{MAX} and higher AP_{DIGEST} values on digestion of the long-chain SEDDS formulations results in lower S^M, i.e., a lower degree of supersaturation on digestion. This is entirely consistent with the trends observed here. The common observation of improved performance of long-chain lipid-based SEDDS formulations when compared to analogous medium-chain formulations[18,64] may therefore reflect an inherent tendency toward lower S^M on dispersion and digestion, reducing the drive toward drug precipitation and increasing in vivo exposure.

Effect of Formulation Composition on in Vivo Performance. The plasma profiles in Figure 9 and the pharmacokinetic parameters presented in Table 2 reveal limited differences in in vivo exposure of danazol after oral administration of formulations F0 and F60. In spite of the considerable difference in formulation composition between the two formulations, the in vitro digestion profiles share similarities with S^M_{DIGEST} values of 4.3 and 3.7 for F0 (Figure 7) and F60 (Figure 6), and in vitro solubilization AUCs of 8.3 and 11.2 (Figure 11). The similarity in in vivo exposure is therefore largely consistent with the in vitro data. Addition of polymer to F60 also resulted in a moderate increase in in vivo exposure, again consistent with the in vitro data, however, for F0, no increase in in vivo exposure was seen, in spite of significant increases in in vitro solubilization in the presence of polymer. Indeed, the addition of polymer appeared to reduce C_{max} in vivo. The lack of in vivo benefit provided by the addition of polymer to formulation F0 was consistent with the much greater dependency on polymer concentration for precipitation inhibition when compared to F60 (Figure 5) and a higher S^M_{DISP}, perhaps pointing to a greater inclination toward precipitation. For F0 in the presence of polymer, the in vitro digestion experiments therefore appear to overestimate in vivo solubilization. It is also possible that drug precipitation from a supersaturated state is more likely in the less well controlled, highly kinetic environment of the GI tract when compared with a relatively "clean" in vitro digestion experiment, such that higher concentrations of polymer may be required to prevent precipitation. A complete explanation for the lack of correlation between in vitro solubilization and in vivo exposure for F0 is not apparent at this time. However, this observation is consistent with previous studies by Gao and co-workers,[21,22,24] where the incorporation of PPIs into SEDDS led to increased drug exposure only in selected studies and where the polymer effect was dependent on the formulation, drug and animal species used.

In an attempt to further evaluate the effect of drug loading (and therefore S^M) on SEDDS performance, the in vivo performance of F60 was also evaluated in the presence and absence of polymer at higher drug loads. In contrast to the F60 data obtained at lower drug load, the addition of polymer at the higher drug load had little effect on bioavailability. The in vitro data suggest an increase in supersaturation stabilization when assessed by in vitro AUC (Figure 11), however, even in the presence of polymer the extent of danazol precipitation was high and the ability to stabilize the supersaturated state was far less efficient than was the case at the lower dose. Consistent with the data obtained with F0, it seems likely that polymeric stabilization of danazol supersaturation on digestion of F60 is more readily achieved in vitro than in vivo, and therefore that care should be taken when looking to extrapolate from in vitro to in vivo data, especially where the extent of stabilization is relatively low (e.g., F60 high dose and F0).

Evaluation of the dose normalized AUC data for the high dose and low dose data obtained for F60 also revealed a significant nonlinear increase in bioavailability at the higher dose (Figure 10). This nonlinear increase in bioavailability may be due to increased initial supersaturation driving increases in thermodynamic activity in the aqueous colloidal phase at the higher drug load. The in vitro data, however, suggest rapid precipitation, and whether the higher thermodynamic activity is maintained in vivo for long enough to enhance bioavailability is not known. Alternatively, danazol has previously been shown to be subject to extensive hepatic metabolism,[65] and the increase in bioavailability with increasing dose may also reflect saturation of first pass metabolism. Studies are ongoing to explore these possibilities.

CONCLUSION

The current studies have explored in detail the impact of supersaturation on the performance of self-emulsifying drug delivery systems. The data suggest that knowledge of the extent to which supersaturation is generated and the stability of the metastable state as the formulation is processed in the GI tract may be more significant determinants of formulation performance than the equilibrium solubilization capacity of the colloids

that are formed post digestion. The current data also illustrate that digestion can act as an effective supersaturation "trigger" for lipid based formulations and that solubilization/precipitation behavior is highly correlated with the maximum supersaturation ratio obtained on dispersion and digestion (as defined by S^{M}_{DISP} and S^{M}_{DISP}). Thus, reducing the degree of generated supersaturation protects against precipitation, at least in vitro. To this end, utilization of lower drug loads, higher surfactant levels, reduced cosolvent and the addition of polymer all enhanced performance in vitro. Correlation between in vitro solubilization and in vivo exposure was evident at moderate drug loads, and the inclusion of HPMC in the formulation enhanced drug exposure. In contrast, at higher drug loads, in vivo exposure increased in both the presence and absence of polymer, and the stabilizing effects of polymer on supersaturation in vitro were not replicated in vivo. An explanation for the increase in exposure at higher dose is not clear at this time but may reflect changes to first pass metabolism or thermodynamic activity.

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Notes

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REFERENCES

(1) Strickley, R. G. Solubilizing Excipients in Oral and Injectable Formulations. *Pharm. Res.* **2004**, *21* (2), 201−230.

(2) Porter, C. J. H.; Trevaskis, N. L.; Charman, W. N. Lipids and lipid-based formulations: optimizing the oral delivery of lipophilic drugs. *Nat. Rev. Drug Discovery* **2007**, *6* (3), 231−248.

(3) Patton, J. S.; Carey, M. C. Watching fat digestion. *Science* **1979**, *204* (4389), 145−148.

(4) Staggers, J. E.; Hernell, O.; Stafford, R. J.; Carey, M. C. Physical-chemical behavior of dietary and biliary lipids during intestinal digestion and absorption. 1. Phase behavior and aggregation states of model lipid systems patterned after aqueous duodenal contents of healthy adult human beings. *Biochemistry* **1990**, *29* (8), 2028−2040.

(5) Carey, M. C.; Small, D. M. The characteristics of mixed micellar solutions with particular reference to bile. *Am. J. Med.* **1970**, *49* (5), 590−608.

(6) Fatouros, D. G.; Bergenstahl, B.; Mullertz, A. Morphological observations on a lipid-based drug delivery system during in vitro digestion. *Eur. J. Pharm. Sci.* **2007**, *31* (2), 85−94.

(7) Kossena, G. A.; Charman, W. N.; Boyd, B. J.; Porter, C. J. H. Influence of the intermediate digestion phases of common formulation lipids on the absorption of a poorly water-soluble drug. *J. Pharm. Sci.* **2005**, *94* (3), 481.

(8) Hofmann, A. F.; Borgström, B. The Intraluminal Phase of Fat Digestion in Man: The Lipid Content of the Micellar and Oil Phases of Intestinal Content Obtained during Fat Digestion and Absorption. *J. Clin. Invest.* **1964**, *43* (2), 247−257.

(9) MacGregor, K. J.; Embleton, J. K.; Lacy, J. E.; Perry, E. A.; Solomon, L. J.; Seager, H.; Pouton, C. W. Influence of lipolysis on drug absorption from the gastro-intestinal tract. *Adv. Drug Delivery Rev.* **1997**, *25* (1), 33−46.

(10) Müllertz, A.; Fatouros, D. G.; Smith, J. R.; Vertzoni, M.; Reppas, C. Insights into Intermediate Phases of Human Intestinal Fluids Visualized by Atomic Force Microscopy and Cryo-Transmission Electron Microscopy ex Vivo. *Mol. Pharmaceutics* **2012**, *9* (2), 237−247.

(11) Fatouros, D.; Deen, G.; Arleth, L.; Bergenstahl, B.; Nielsen, F.; Pedersen, J.; Mullertz, A. Structural Development of Self Nano Emulsifying Drug Delivery Systems (SNEDDS) During In Vitro Lipid Digestion Monitored by Small-angle X-ray Scattering. *Pharm. Res.* **2007**, *24* (10), 1844−1853.

(12) Carey, M. C.; Small, D. M.; Bliss, C. M. Lipid Digestion and Absorption. *Annu. Rev. Physiol.* **1983**, *45* (1), 651−677.

(13) Gibson, L. Lipid-Based Excipients for Oral Drug Delivery. In *Oral Lipid-Based Formulations: Enhancing the bioavailbility of poorly water-soluble drugs* Hauss, D. J., Ed.; Informa Heathcare: New York, 2007; Vol. *170*, pp 33−62.

(14) Schick, M. J. E. *Nonionic surfactants*; Marcel Dekker, Inc.: New York, 1977; Vol. *1*.

(15) Kaukonen, A.; Boyd, B. J.; Porter, C. J. H.; Charman, W. N. Drug Solubilization Behavior During in Vitro Digestion of Simple Triglyceride Lipid Solution Formulations. *Pharm. Res.* **2004**, *21* (2), 245−253.

(16) Kaukonen, A.; Boyd, B. J.; Charman, W. N.; Porter, C. J. H. Drug Solubilization Behavior During in Vitro Digestion of Suspension Formulations of Poorly Water-Soluble Drugs in Triglyceride Lipids. *Pharm. Res.* **2004**, *21* (2), 254−260.

(17) Larsen, A. T.; Sassene, P.; Müllertz, A. In vitro lipolysis models as a tool for the characterization of oral lipid and surfactant based drug delivery systems. *Int. J. Pharm.* **2011**, *417* (1−2), 245−255.

(18) Porter, C. J. H.; Kaukonen, A. M.; Boyd, B. J.; Edwards, G. A.; Charman, W. N. Susceptibility to Lipase-Mediated Digestion Reduces the Oral Bioavailability of Danazol After Administration as a Medium-Chain Lipid-Based Microemulsion Formulation. *Pharm. Res.* **2004**, *21* (8), 1405−1412.

(19) Porter, C. J. H.; Kaukonen, A. M.; Taillardat-Bertschinger, A.; Boyd, B. J.; O'Connor, J. M.; Edwards, G. A.; Charman, W. N. Use of *in vitro* lipid digestion data to explain the *in vivo* performance of triglyceride-based oral lipid formulations of poorly water-soluble drugs: Studies with halofantrine. *J. Pharm. Sci.* **2004**, *93* (5), 1110−1121.

(20) Porter, C. J. H.; Anby, M. U.; Warren, D.; Williams, H. D.; Benameur, H.; Pouton, C. W. Lipid based formulations: Exploring the link between in vitro Supersaturation and in vivo exposure. *Bull. Tech. Gattefosse* **2011**, *104*, 61−69.

(21) Gao, P.; Akrami, A.; Alvarez, F.; Hu, J.; Li, L.; Ma, C.; Surapaneni, S. Characterization and optimization of AMG 517 supersaturatable self-emulsifying drug delivery system (S-SEDDS) for improved oral absorption. *J. Pharm. Sci.* **2009**, *98* (2), 516−528.

(22) Gao, P.; Guyton, M. E.; Huang, T.; Bauer, J. M.; Stefanski, K. J.; Lu, Q. Enhanced Oral Bioavailability of a Poorly Water Soluble Drug PNU-91325 by Supersaturatable Formulations. *Drug Dev. Ind. Pharm.* **2004**, *30* (2), 221−229.

(23) Gao, P.; Rush, B. D.; Pfund, W. P.; Huang, T.; Bauer, J. M.; Morozowich, W.; Kuo, M.-S.; Hageman, M. J. Development of a supersaturable SEDDS (S-SEDDS) formulation of paclitaxel with improved oral bioavailability. *J. Pharm. Sci.* **2003**, *92* (12), 2386−2398.

(24) Gao, P.; Morozowich, W. Design and Development of Supersaturatable Self-Emulsifying Drug Delivery Systems for Enhancing the Gastrointestinal Absorption of Poorly Soluble Drugs. In *Oral Lipid-Based Formulations: Enhancing the bioavailbility of poorly water-soluble drugs*, 1st ed.; Hauss, D. J., Ed.; Informa Healthcare: New York, 2007; Vol. *170*, pp 303−328.

(25) Miller, D.; DiNunzio, J.; Yang, W.; McGinity, J.; Williams, R. Targeted Intestinal Delivery of Supersaturated Itraconazole for Improved Oral Absorption. *Pharm. Res.* **2008**, *25* (6), 1450−1459.

(26) Raghavan, S. L.; Kiepfer, B.; Davis, A. F.; Kazarian, S. G.; Hadgraft, J. Membrane transport of hydrocortisone acetate from supersaturated solutions; the role of polymers. *Int. J. Pharm.* **2001**, *221* (1−2), 95−105.

(27) Simonelli, A. P.; Mehta, S. C.; Higuchi, W. I. Inhibition of sulfathiazole crystal growth by polyvinylpyrrolidone. *J. Pharm. Sci.* **1970**, *59* (5), 633−638.
(28) Guzmán, H. R.; Tawa, M.; Zhang, Z.; Ratanabanangkoon, P.; Shaw, P.; Gardner, C. R.; Chen, H.; Moreau, J.-P.; Almarsson, Ö.; Remenar, J. F. Combined use of crystalline salt forms and precipitation inhibitors to improve oral absorption of celecoxib from solid oral formulations. *J. Pharm. Sci.* **2007**, *96* (10), 2686−2702.
(29) Vasconcelos, T.; Sarmento, B.; Costa, P. Solid dispersions as strategy to improve oral bioavailability of poor water soluble drugs. *Drug Discovery Today* **2007**, *12* (23−24), 1068−1075.
(30) Yamashita, K.; Nakate, T.; Okimoto, K.; Ohike, A.; Tokunaga, Y.; Ibuki, R.; Higaki, K.; Kimura, T. Establishment of new preparation method for solid dispersion formulation of tacrolimus. *Int. J. Pharm.* **2003**, *267* (1−2), 79−91.
(31) Raghavan, S. L.; Trividic, A.; Davis, A. F.; Hadgraft, J. Effect of cellulose polymers on supersaturation and in vitro membrane transport of hydrocortisone acetate. *Int. J. Pharm.* **2000**, *193* (2), 231−237.
(32) Cuiné, J. F.; Charman, W. N.; Pouton, C. W.; Edwards, G. A.; Porter, C. J. H. Increasing the Proportional Content of Surfactant (Cremophor EL) Relative to Lipid in Self-emulsifying Lipid-based Formulations of Danazol Reduces Oral Bioavailability in Beagle Dogs. *Pharm. Res.* **2007**, *24* (4), 748−757.
(33) Erlich, L.; Yu, D.; Pallister, D. A.; Levinson, R. S.; Gole, D. G.; Wilkinson, P. A.; Erlich, R. E.; Reeve, L. E.; Viegas, T. X. Relative bioavailability of danazol in dogs from liquid-filled hard gelatin capsules. *Int. J. Pharm.* **1999**, *179* (1), 49−53.
(34) Bakatselou, V.; Oppenheim, R. C.; Dressman, J. B. Solubilization and Wetting Effects of Bile Salts on the Dissolution of Steroids. *Pharm. Res.* **1991**, *8* (12), 1461−1469.
(35) Kossena, G. A.; Charman, W. N.; Boyd, B. J.; Dunstan, D. E.; Porter, C. J. H. Probing drug solubilization patterns in the gastrointestinal tract after administration of lipid-based delivery systems: A phase diagram approach. *J. Pharm. Sci.* **2004**, *93* (2), 332−348.
(36) Pouton, C. W. Lipid formulations for oral administration of drugs: non-emulsifying, self-emulsifying and 'self-microemulsifying' drug delivery systems. *Eur. J. Pharm. Sci.* **2000**, *11* (Suppl. 2), S93−S98.
(37) Pouton, C. W. Formulation of poorly water-soluble drugs for oral administration: Physicochemical and physiological issues and the lipid formulation classification system. *Eur. J. Pharm. Sci.* **2006**, *29* (3−4), 278−287.
(38) Sek, L.; Porter, C. J. H.; Charman, W. N. Characterisation and quantification of medium chain and long chain triglycerides and their in vitro digestion products, by HPTLC coupled with in situ densitometric analysis. *J. Pharm. Biomed. Anal.* **2001**, *25* (3−4), 651−661.
(39) Armand, M.; Borel, P.; Pasquier, B.; Dubois, C.; Senft, M.; Andre, M.; Peyrot, J.; Salducci, J.; Lairon, D. Physicochemical characteristics of emulsions during fat digestion in human stomach and duodenum. *Am. J. Physiol.* **1996**, *271* (1), G172−G183.
(40) Lindahl, A.; Ungell, A.-L.; Knutson, L.; Lennernäs, H. Characterization of Fluids from the Stomach and Proximal Jejunum in Men and Women. *Pharm. Res.* **1997**, *14* (4), 497−502.
(41) Pedersen, B. L.; Brøndsted, H.; Lennernäs, H.; Christensen, F. N.; Müllertz, A.; Kristensen, H. G. Dissolution of Hydrocortisone in Human and Simulated Intestinal Fluids. *Pharm. Res.* **2000**, *17* (2), 183−189.
(42) Brouwers, J.; Tack, J.; Lammert, F.; Augustijns, P. Intraluminal drug and formulation behavior and integration in in vitro permeability estimation: A case study with amprenavir. *J. Pharm. Sci.* **2006**, *95* (2), 372−383.
(43) Bevernage, J.; Brouwers, J.; Clarysse, S.; Vertzoni, M.; Tack, J.; Annaert, P.; Augustijns, P. Drug supersaturation in simulated and human intestinal fluids representing different nutritional states. *J. Pharm. Sci.* **2010**, *99* (11), 4525−4534.
(44) Moreno, M. P. d. l. C.; Oth, M.; Deferme, S.; Lammert, F.; Tack, J.; Dressman, J.; Augustijns, P. Characterization of fasted-state human intestinal fluids collected from duodenum and jejunum. *J. Pharm. Pharmacol.* **2006**, *58* (8), 1079−1089.
(45) Persson, E.; Löfgren, L.; Hansson, G.; Abrahamsson, B.; Lennernäs, H.; Nilsson, R. Simultaneous assessment of lipid classes and bile acids in human intestinal fluid by solid-phase extraction and HPLC methods. *J. Lipid Res.* **2007**, *48* (1), 242−251.
(46) Reymond, J.-P.; Sucker, H.; Vonderscher, J. In Vivo Model for Ciclosporin Intestinal Absorption in Lipid Vehicles. *Pharm. Res.* **1988**, *5* (10), 677−679.
(47) Khoo, S.-M.; Humberstone, A. J.; Porter, C. J. H.; Edwards, G. A.; Charman, W. N. Formulation design and bioavailability assessment of lipidic self-emulsifying formulations of halofantrine. *Int. J. Pharm.* **1998**, *167* (1−2), 155−164.
(48) Martin, A.; Wu, P. L.; Adjei, A.; Lindstrom, R. E.; Elworthy, P. H. Extended hildebrand solubility approach and the log linear solubility equation. *J. Pharm. Sci.* **1982**, *71* (8), 849−856.
(49) Mattson, F. H.; Volpenhein, R. A. The Digestion and Absorption of Triglycerides. *J. Biol. Chem.* **1964**, *239* (9), 2772−2777.
(50) Porter, C. J. H.; Pouton, C. W.; Cuine, J. F.; Charman, W. N. Enhancing intestinal drug solubilisation using lipid-based delivery systems. *Adv. Drug Delivery Rev.* **2008**, *60* (6), 673−691.
(51) Dahan, A.; Hoffman, A. The effect of different lipid based formulations on the oral absorption of lipophilic drugs: The ability of in vitro lipolysis and consecutive ex vivo intestinal permeability data to predict in vivo bioavailability in rats. *Eur. J. Pharm. Biopharm.* **2007**, *67* (1), 96−105.
(52) Cuiné, J. F.; McEvoy, C. L.; Charman, W. N.; Pouton, C. W.; Edwards, G. A.; Benameur, H.; Porter, C. J. H. Evaluation of the impact of surfactant digestion on the bioavailability of danazol after oral administration of lipidic self-emulsifying formulations to dogs. *J. Pharm. Sci.* **2008**, *97* (2), 995−1012.
(53) Gao, P.; Morozowich, W. Development of supersaturatable self-emulsifying drug delivery system formulations for improving the oral absorption of poorly soluble drugs. *Expert Opin. Drug Delivery* **2006**, *3* (1), 97−110.
(54) Warren, D. B.; Benameur, H.; Porter, C. J. H.; Pouton, C. W. Using polymeric precipitation inhibitors to improve the absorption of poorly water-soluble drugs: A mechanistic basis for utility. *J. Drug Targeting* **2010**, *18* (10), 704−731.
(55) Vandecruys, R.; Peeters, J.; Verreck, G.; Brewster, M. E. Use of a screening method to determine excipients which optimize the extent and stability of supersaturated drug solutions and application of this system to solid formulation design. *Int. J. Pharm.* **2007**, *342* (1−2), 168−175.
(56) Bevernage, J.; Forier, T.; Brouwers, J.; Annaert, P.; Tack, J.; Augustijns, P. Excipient-mediated supersaturation stabilization in Human Intestinal Fluids. *Mol. Pharmaceutics* **2011**, *8* (2), 564−570.
(57) Miller, D. A.; DiNunzio, J. C.; Yang, W.; McGinity, J. W.; Williams, R. O. Enhanced In Vivo Absorption of Itraconazole via Stabilization of Supersaturation Following Acidic-to-Neutral pH Transition. *Drug Dev. Ind. Pharm.* **2008**, *34* (8), 890−902.
(58) Raghavan, S. L.; Schuessel, K.; Davis, A.; Hadgraft, J. Formation and stabilisation of triclosan colloidal suspensions using supersaturated systems. *Int. J. Pharm.* **2003**, *261* (1−2), 153−158.
(59) Raghavan, S. L.; Trividic, A.; Davis, A. F.; Hadgraft, J. Crystallization of hydrocortisone acetate: influence of polymers. *Int. J. Pharm.* **2001**, *212* (2), 213−221.
(60) Usui, F.; Maeda, K.; Kusai, A.; Nishimura, K.; Keiji, Y. Inhibitory effects of water-soluble polymers on precipitation of RS-8359. *Int. J. Pharm.* **1997**, *154* (1), 59−66.
(61) Sassene, P. J.; Knopp, M. M.; Hesselkilde, J. Z.; Koradia, V.; Larsen, A.; Rades, T.; Müllertz, A. Precipitation of a poorly soluble model drug during in vitro lipolysis: Characterization and dissolution of the precipitate. *J. Pharm. Sci.* **2010**, *99* (12), 4982−4991.
(62) Brouwers, J.; Brewster, M. E.; Augustijns, P. Supersaturating drug delivery systems: The answer to solubility-limited oral bioavailability? *J. Pharm. Sci.* **2009**, *98* (8), 2549−2572.

(63) Kashchiev, D.; van Rosmalen, G. M. Review: Nucleation in solutions revisited. *Cryst. Res. Technol.* **2003**, *38* (7−8), 555−574.
(64) Khoo, S.-M.; Porter, C. J. H.; Charman, W. N. The formulation of Halofantrine as either non-solubilising PEG 6000 or solubilising lipid based solid dispersions: Physical stability and absolute bioavailability assessment. *Int. J. Pharm.* **2000**, *205* (1−2), 65−78.
(65) Davison, C.; Banks, W.; Fritz, A. The absorption, Distribution and Metabolic Fate of Danazol in Rats, Monkeys and human Volunteers. *Arch. Int. Pharmacodyn.* **1976**, *221*, 294−310.

APPENDIX 2

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Real Time Evolution of Liquid Crystalline Nanostructure during the Digestion of Formulation Lipids Using Synchrotron Small-Angle X-ray Scattering

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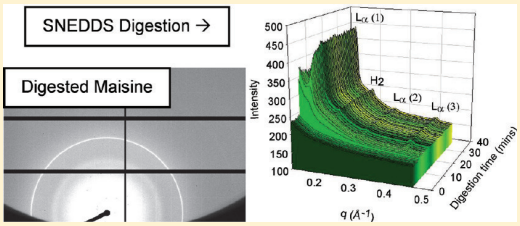
ABSTRACT: The role of the digestion of lipids in facilitating absorption of poorly water-soluble compounds, such as vitamins, is not only an important nutritional issue but is increasingly being recognized as an important determinant in the effectiveness of lipid-based drug formulations. It has been known for some time that lipids often form complex liquid crystalline structures during digestion and that this may impact drug solubilization and absorption. However, until recently we have been unable to detect and characterize those structures in real time and have been limited in establishing the interplay between composition, digestion, and nanostructure. Here, we establish the use of an in vitro lipid digestion model used in conjunction with synchrotron small-angle X-ray scattering by first confirming its validity using known, nondigestible liquid crystalline systems, and then extend the model to study the real time evolution of nanostructure during the digestion of common formulation lipids. The formation of liquid crystalline structures from unstructured liquid formulations is discovered, and the kinetics of formation and dependence on composition is investigated.



INTRODUCTION

The study of lipid-based drug formulations has intensified in recent years, in a large part driven by the discovery of increasingly lipophilic candidate compounds from drug discovery programs. Lipid-based formulations often comprise triglycerides, partially digested glyceride mixtures, and/or nonionic surfactants.[1] They improve drug bioavailability in the gastrointestinal tract primarily by maintaining the drug in a soluble form, both in the dispersed formulation and also during lipid digestion, via the formation of dispersed colloidal structures, including vesicles and mixed micelles composed of lipid digestion products and endogenous amphiphilic compounds. The formation of intermediate liquid crystalline structures, using model lipid compositions representative of partially digested systems, has also been correlated to changes in drug solubilization and absorption.[2] Hence, there is a need to link digestion kinetics and composition to liquid crystalline nanostructures formed during digestion to understand how to assemble systems to provide the optimal structural attributes to facilitate quantitative drug absorption and, therefore, the best therapeutic outcome.

Approaches to study transient liquid crystalline nanostructure formation during the digestion process have been limited primarily to optical microscopic studies[3] or studies on model lipid compositions believed to be present during the process.[4−8] Real time in vitro lipolysis models have been reported that allow chemical composition to be followed during the digestion process. However, real time structural data has thus far been inaccessible. A recent report by Fatouros et al. used "quenched" samples removed at certain times during in vitro digestion of a self-nanoemulsifying drug delivery system (SNEDDS) for later analysis by small-angle X-ray scattering (SAXS).[9] The SNEDDS formulation comprised two commonly employed formulation lipids, sesame oil and Maisine 35-1, and contained long chain triglycerides, di- and monoglycerides, as well as fatty acids in a complex mixture. Fatouros et al. found liquid crystalline structures to be present during digestion and, interestingly, an inverse hexagonal phase structure which developed over time in response to compositional changes. While this study has progressed the field in terms of showing the likely presence of nanostructure during the lipolysis process, the studies were not performed in situ, making it difficult to draw definitive conclusions about the validity of the findings to the evolving structure in dynamic digesting systems. However, the presence of both lamellar and inverse hexagonal phase structures during digestion highlights the likelihood of observation of interesting dynamic phase structures in such systems and was the primary motivation for these studies.

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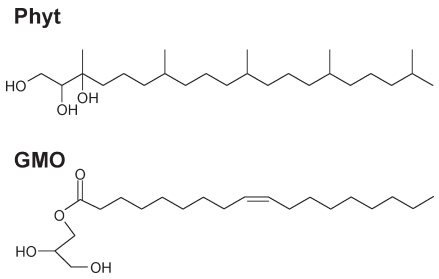

Figure 1. Chemical structures of phytantriol and GMO.

In these studies, we have approached real time structure evolution during digestion in pharmaceutically relevant systems using synchrotron SAXS with similar methodology to that recently reported by Salentinig et al.[10] A previously reported in vitro lipolysis model[11] was modified to enable continuous flow through a glass capillary placed in the path of the X-ray beam and structural evolution followed without the need to quench and store samples. Short acquisition times facilitate true real time kinetic events to be observed and correlated to changes in composition during digestion. In initial experiments, we validate the relevance of the model using nondigestible and digestible liquid crystalline systems of known structure (prepared using phytantriol and glyceryl monooleate respectively, Figure 1) in simulated fasted intestinal fluid (SEIF) subjected to lipolytic enzymes. We then demonstrate the kinetics of structural evolution during digestion of sesame oil and Maisine individually, as mixtures of the two lipids, and, finally, in the form of the SNEDDS formulation reported previously. We report a correlation between previous structural analysis with real time in situ measurements.

■ EXPERIMENTAL SECTION

Materials. Phytantriol (3,7,11,15-tetramethyl-1,2,3-hexadecanetriol) was a gift from DSM Nutritional (Singapore). Rylo MG19 Pharma grade was a gift from Danisco (Grindsted, Denmark). It contains primarily glyceryl monooleate (92.5%).[12] Maisine 35-1 was a gift from Gattefossé, France. Sesame oil was from Sigma (St. Louis, MO). Cremophor RH40 was from BASF (Ludwigshafen, Germany). Egg yolk lecithin (consisting of approximately 60% phosphatidylcholine (PC) by dry weight) was from Sigma (St. Louis, MO). Sodium taurodeoxycholate (NaTDC), Trizma maleate, porcine pancreatin (8 × USP specification), and 4-bromophenylboronic acid (4-BPB) were also obtained from Sigma (St. Louis, MO). Ammonium dihydrogen orthophosphate was from BDH (Poole, England). Ethanol (99% purity) was obtained from CSR distilleries (Yarraville, VIC, Australia). Water was obtained from a Milli-Q filtration/purification system (Millipore, Billerica, MA).

Formulation Preparation. Liquid crystalline cubosome formulations were prepared by prior dispersion of phytantriol or Rylo MG19 (GMO) to facilitate efficient mixing with simulated intestinal fluid in the digestion experiments. To prepare cubosome dispersions, Pluronic F127 solution (9 mL of 1% w/w in digestion buffer described below) was added to 1 g of lipid in a 20 mL glass vial and immediately ultrasonicated for 15 min using a Misonix XL 2000 ultrasonicator at 40% power with a 2 s on, 2 s off sequence. Other lipid formulations were prepared by weight and introduced to the digestion vessel without prior dispersion prior to addition of SEIF. The SNEDDS formulation contained 30% w/w each of Maisine 35-1, sesame oil, and Cremophor RH40, with the remaining 10% comprising ethanol.

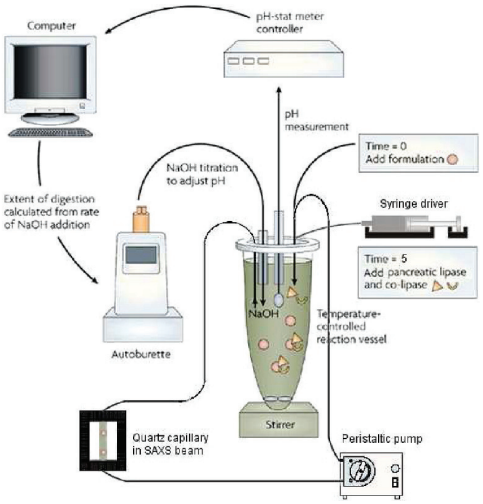

Figure 2. Schematic of the in vitro digestion apparatus coupled to a synchrotron SAXS flow through cell (adapted from ref 33). Reprinted by permission from Macmillan Publishers Ltd: *Nat. Rev. Drug Discovery 6*, 231−248, copyright 2007.

In Vitro Lipolysis Model. In vitro digestion studies were undertaken using a digestion model based on a pH-stat auto titrator (Radiometer, Copenhagen, Denmark) as described previously.[11,13] The digestion apparatus is illustrated schematically in Figure 2. Briefly, simulated fasted intestinal fluid, comprising 5 mM sodium taurodexycholate and 1.25 mM phosphatidylcholine micellar solution in Trizma maleate "digestion buffer" (8 mL for dispersed liquid crystalline systems for which 1 mL of liquid crystalline dispersion was added, or 9 mL for formulations added to the vessel by weight), was added to the formulation in the thermostatted digestion vessel at 37 °C. The sample was magnetically stirred for 5 min for complete mixing and thermal equilibration, and the pH was adjusted to 7.500 ± 0.002. A remotely operated syringe driver was used to deliver 1 mL of pancreatin extract over approximately 20 s into the vessel to initiate digestion. On addition of pancreatin (20 000 TBU), the pH-stat titrated the digestion mixture with 0.2 M NaOH in order to maintain the system at pH = 7.500 ± 0.002. Digestion was allowed to proceed for up to 60 min, with which the degree of enzymatic digestion of the lipid reflected in the volume of NaOH used to neutralize the fatty acids (FA) liberated during the digestion process. For the digestion containing the inhibitor, 100 μL of 4-BPB at 0.5 M in methanol was added to the 9 mL fasted micelles prior to addition of the pancreatin.

Synchrotron Small-Angle X-ray Scattering (SSAXS). To enable real-time monitoring of nanostructure in the digesting medium, the in vitro digestion vessel was fitted with silicon tubing (total volume approximately 1 mL) and the digestion medium drawn through a 2 mm diameter quartz capillary using a peristaltic pump and returned to the digestion medium, at a rate of 5 mL/min. The capillary was fixed in the X-ray beam at the SAXS/WAXS beamline at the Australian Synchrotron. An X-ray beam with a wavelength of 0.83 Å (15 keV) was selected. The 2D SAXS patterns were collected using a Pilatus 1 M (170 mm × 170 mm) detector which was located 650 mm from the sample position. Scattering patterns were acquired for 5 s at each 30 s interval, with enzyme addition occurring after acquisition of the 10th frame (i.e., 5 min after commencing the titration). Typically, no titrant was required during the initial 5 min interval as the pH stat did not detect a change in pH until lipolysis commenced. The software programs SAXS15ID[5]

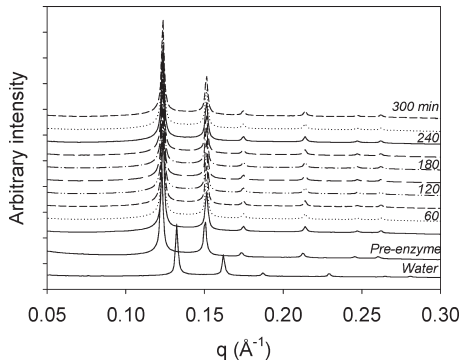

Figure 3. Synchrotron SAXS intensity versus scattering vector profiles for phytantriol cubosomes in fasted micellar solution over time after addition of lipase to the digestion mixture. Italicized annotations indicate time in minutes after lipase addition.

and Fit2D[6] were used to acquire and reduce 2D patterns to 1D curves. The mean lattice parameter, a, was calculated from the interplanar distance, d, using the appropriate scattering law for the phase structure.[14] For cubic phases $a = d(h^2 + k^2 + l^2)^{1/2}$, while for H_2 phase $a = 4d/3(h^2 + k^2)^{1/2}$, where h, k, and l are the Miller indices for the particular structure present.

RESULTS AND DISCUSSION

Validation Using Nondigestible (Phytantriol) and Digestible (GMO) Cubosome Formulations. Dispersed phytantriol particles were used as a negative control for their well established structure and because the phytantriol is nondigestible. Dispersion of the cubosomes in bile salt medium did not induce a change in phase identity from the *Pn*3*m* cubic phase structure (indicated by spacing ratios of $\sqrt{2}$, $\sqrt{3}$, and $\sqrt{4}$) observed for phytantriol in excess water in Figure 3, but did induce a shift in peak positions to lower q values, indicating an interaction with bile salts. This has been observed previously by us for phytantriol cubosomes[15] and by others in glyceride-based lipid systems.[16] Addition of the pancreatin induced no change in the phase structure, evident from the static peak positions in q, indicating that no change in lipid structure or composition had occurred.

In contrast, subjecting the GMO cubosomes to an identical digestion procedure induced significant changes in phase structure (Figure 4). The v_2 bicontinuous cubic phase (*Im*3*m* space group, identified by peak spacing ratios $\sqrt{2}$, $\sqrt{4}$, and $\sqrt{6}$), commonly observed for GMO-based cubosomes dispersed using Pluronic F127, was observed prior to digestion. The lattice parameter was 124 Å prior to digestion, in agreement with previous studies.[17,18] Digestion induced a steady shift in the lattice parameter for the *Im*3*m* structure to larger lattice dimensions over approximately 20 min, with decreasing overall intensity for peaks identifying the cubic phase, with a concurrent gradual increase in the intensity of a diffuse peak at approximately 0.05 Å^{-1} (Figure 4A). The relative intensity of the diffuse peak after 50 min rivaled that of the initial cubic phase (Figure 4B). The increase in lattice parameter correlated strongly with the digestion profile (Figure 4C), with both profiles tending toward a plateau. However, a loss of sensitivity to detect the cubic phase meant that lattice parameter could only be tracked for 20 min. This is strongly indicative of the link between the digestion

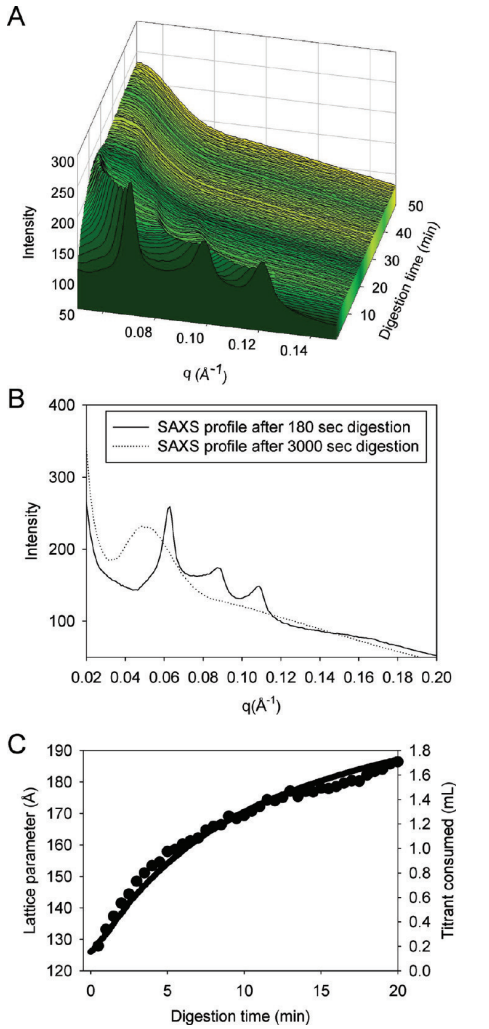

Figure 4. Synchrotron SAXS intensity versus scattering vector profiles for GMO cubosomes prepared from Rylo MG after addition of lipase to the digestion mixture. (A) Evolution of SAXS profiles over time. (B) SAXS scattering profile for cubosomes (*Im*3*m* space group) at start and end of digestion. (C) Evolution of lattice parameter for cubosomes over time during digestion (filled circles) and digestion kinetic profile (titrant added by pH stat in response to fatty acid production) solid line.

process and evolution of nanostructure in the self-assembled lipid system, and we believe that this is the first time that such an in situ correlation and quantitation has been provided.

Structural Evolution during Digestion of Initially Unstructured Sesame Oil and Maisine, and Their Mixtures. Having established that the model provides structural detail consistent with lipid nanostructure evolution, the structure and kinetics of formulation lipids (sesame oil, Maisine, and mixtures thereof) was assessed. Sesame oil displayed a single ring at $q = 0.125$ Å^{-1} that formed over the first few minutes of digestion and was

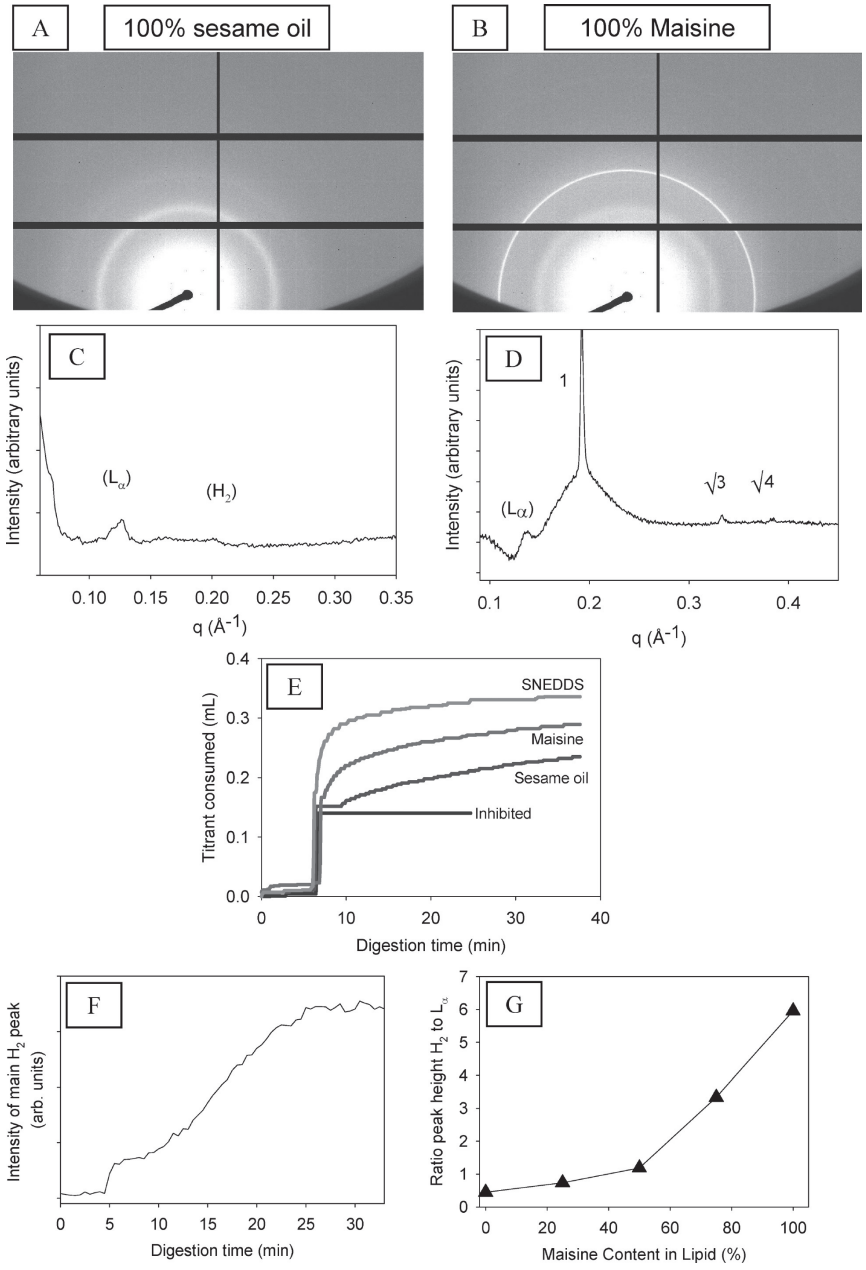

Figure 5. SAXS data for digestion of sesame oil, Maisine 35-1, and their mixtures. (A) SAXS detector view for sesame oil at end of digestion process (40 min). (B) SAXS detector view for Maisine 35-1 at end of digestion process (40 min). (C) Synchrotron SAXS intensity versus scattering vector profiles at the end of digestion of sesame oil (40 min), with assignments for weak peaks representing the L_α and H_2 phase. (D) Synchrotron SAXS intensity versus scattering vector profiles at the end of digestion of Maisine 35-1 (40 min), with peak assignments for the H_2 phase indicated by 1, $\sqrt{3}$, and $\sqrt{4}$ indicating the relative spacing ratios. (E) Digestion profiles for sesame oil, Maisine 35-1, the SNEDDS formulation, and sesame oil in the presence of the digestion inhibitor. (F) Change in intensity for H_2 peak at $q = 0.192$ Å^{-1} during digestion of Maisine 35-1. (G) Relative height of the first H_2 peak compared to L_α peak at end of digestion of sesame oil + Maisine mixtures with increasing Maisine content.

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persistent over the duration of the experiment. Figure 5A shows the detector view at the end of the digestion process, after 45 min, with the main ring being evident. This ring is attributed to the formation of the lamellar phase (evidence for this assignment is provided later) with a lattice parameter of 49.6 Å. There is also a very weak reflection at $q = 0.192$ Å^{-1} in panel (A) that appears late in the digestion process and is attributed to the inverted hexagonal phase (H_2). The intensity versus scattering vector (q) plot in Figure 5C indicates the (albeit weak) peaks for these two reflections, the identity of which are further supported below. When the experiment was repeated in the presence of an enzyme inhibitor, the titration profile in Figure 5E indicated that no digestion occurred and no Bragg reflections were present in the scattering patterns.

The reflection at $q = 0.192$ Å^{-1} was much stronger during the digestion of Maisine (Figure 5B) than sesame oil. After background subtraction to obtain the profile in Figure 5D, the second and third reflections, correlating to peak positions at $\sqrt{3}$ and $\sqrt{4}$, indicate unequivocally that the peak at $q = 0.192$ Å^{-1} arises from the presence of the hexagonal phase. The evolution of the intensity of the hexagonal phase peak during the digestion of Maisine is presented in Figure 5F, which again follows a similar kinetic profile to that observed for the digestion. Unlike the cubosomes, there was no shift in the peak position during digestion, only an increase in intensity, suggestive of production of increasing quantity of the same phase structure.

The difference between phase composition for the sesame oil and Maisine system was reflected in their mixtures; the relative intensity of the hexagonal phase compared to the lamellar phase increased gradually as the proportion of Maisine was increased in the initial reaction mixture (Figure 5G).

Structural Evolution during Digestion of the SNEDDS Formulation. The digestion of the SNEDDS formulation was the most rapid (Figure 5E), although the SNEDDS contains a 50:50 w/w mixture of sesame oil and Maisine and might otherwise have been expected to digest at a rate similar to or between the two lipid components. The presence of Cremophor RH40 and ethanol facilitates dispersion as a submicrometer emulsion with very large surface area for substrate binding, leading to an enhanced digestion rate. The more rapid digestion also leads to very fast appearance of the H_2 phase; within 5−10 min of enzyme addition (Figure 6), its evolution is complete. The intensity of the H_2 peak is then stagnant, indicating no further change in lipid composition, which is in general agreement with the digestion kinetics. The lower intensity of the H_2 peak relative to the first lamellar peak is in broad agreement with that expected at the end of digestion for a 50:50 ratio of the lipids from Figure 5G. This is important as it indicates that Cremophor RH40 in the formulation does not significantly influence the lipid self-assembly structure in digested formulations, but solely enhances kinetic evolution through facilitating digestion. The appearance of the second and third reflection for the lamellar phase, at spacing ratios 2 and 3, is apparent in the SAXS profile for the SNEDDS, confirming the initial assignment of lamellar phase to the peak at $q = 0.125$ Å^{-1} in the sesame oil/Maisine mixtures.

Discussion. The relatively recent availability of synchrotron X-ray sources has stimulated a significant body of research into the kinetic aspects of evolution of nanostructure in lipid systems.[10,19−21] Most of this research has been aimed at understanding the mechanism of transition between different self-assembled structures, with a view to understanding the biological relevance in processes such as cell fusion and fission. However, the kinetic aspects of self-assembly of lipid systems also has more applied relevance in the physiological aspects of digestion, development of functional foods, and, as highlighted in this study, lipid-based drug delivery systems.

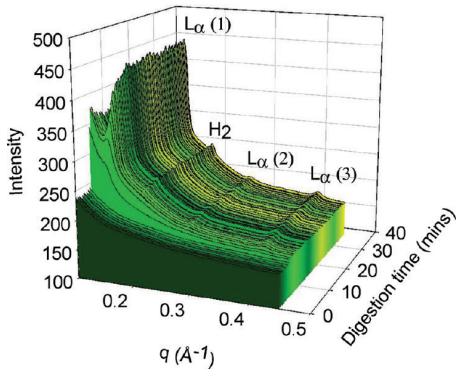

Figure 6. Synchrotron SAXS intensity versus scattering vector profiles during digestion for the SNEDDS formulation, indicating the three reflections for lamellar phase and single reflection for H_2 phase, again at $q = 0.192$ Å^{-1}.

The use of in vitro digestion models to follow the kinetics in lipid systems has been state of the art for several decades; however, the variables and means of data interpretation are still under debate.[22−24] It is clear however that understanding structure in digesting systems is ultimately the key to moving from empirical formulation development to engineering of highly effective formulation approaches from a position of understanding and producing optimal preabsorptive structural conditions. The progression of lipid nanostructure during digestion has been studied by sampling and subsequent SAXS analysis for the same composition SNEDDS as studied in the current work.[25] Pleasingly, the structural evolution observed here for the SNEDDS system agrees broadly with that obtained using a sampling regime. The coexisting lamellar and hexagonal phases and their change over time were in agreement with the kinetics shown in Figure 6; however, the digestion occurred over a slower time scale likely due to subtle differences in the digestion models themselves such as calcium concentration. The application of the real time model may be more useful in rapidly digesting systems, such as those employing medium chain triglycerides, where sampling and inhibition protocols may lead to artifacts. Very recently, the real-time digestion of triolein was studied using in-line SAXS using a benchtop source of X-rays.[10] The structural evolution observed was quite different from that seen for the sesame oil in the current study. The model employed was very different; the pH was not maintained using a pH stat, different bile salt and phospholipid compositions were employed, and different temperatures were used, all of which would impact enzyme activity, fatty acid ionization, and consequent structure formation. Patterns were acquired over 2 min, making it difficult to compare peak positions with lower resolution. Sesame oil typically also only contains approximately 40% triolein.[26] Hence, it is important which model is chosen in such studies, perhaps more so than the SAXS instrumentation used, to enable comparison across digesting lipid systems.

The structural behavior during digestion of the GMO cubosomes deserves some discussion. The inverted bicontinuous cubic phase with the *Im*3*m* space group was evident initially in Figure 4A. As digestion proceeded, the progression to a larger lattice parameter was indicative of swelling of the cubic phase matrix. This is to be understood as the production of oleic acid, evident from the titration curve in Figure 4C, which at pH 7.5 is expected to be in a primarily ionized state, inducing electrostatic repulsion and swelling of the matrix (similar to that observed for bile salts in Figure 3 for phytantriol cubosomes). This effect has been reported by Borne et al., through addition of oleic acid to GMO in increasing proportion.[27] The swelling increased with oleic acid production, and at a critical point there is a transformation to a diffuse scattering peak. The diffuse peak is reminiscent of the behavior of the GMO system on addition of octyl glucoside.[28] In this case, modeling revealed the presence of a swollen sponge phase. While the system here is further complicated by the presence of bile salts and phospholipids, with potential to also form swollen micelles via solubilization of the fatty acids to form mixed micelles, there is sufficient evidence that the system is likely exhibiting a similar swollen sponge topology. This interesting transition requires further investigation. It is also worth noting that the hexagonal phase observed in the GMO + OA + water system is absent, due to the absence of protonated oleic acid which would otherwise induce negative curvature at lower pH.

Pluronic F127, used to impart colloidal stability to dispersions of cubosomes, does not appear to play any specific role in the evolution of nanostructure during digestion. In the case of phytantriol, there is clearly no role of F127 in the nanostructure evolution; the space group or lattice parameter does not change and has been previously reported to not influence phase structure in phytantriol systems.[18] F127 is well-known to induce a change in space group from the double diamond (*Pn*3*m*) bicontinuous structure observed for the nondispersed cubic phase formed by GMO in excess water to the primitive bicontinuous structure (*Im*3*m*);[17] however, there is no reversion to the *Pn*3*m* structure during digestion and only a steady change in dimensions of the *Im*3*m* lattice up to the phase transition, indicating F127 is not degraded during the digestion process (as anticipated from its block copolymer structure). Hence, while F127 appears not to influence nanostructure *during* the digestion of GMO cubosomes, decoupling the effect of F127 from the digestion of GMO cubosomes would require dispersion using a different stabilizer, which may be investigated in future studies.

The behavior of drug in digesting lipid systems is also of great interest, as drug precipitation can lead to poor bioavailability. Precipitation is a consequence of the capacity of lipid species to solubilize and transport drug for absorption in vivo, but it has been used in vitro as an indicator (under some circumstances) of likely in vivo performance. The distribution of drug within digesting oil droplets has recently been tracked using CARS, and it offers a potential complementary technique to scattering methods in understanding drug behavior in digesting lipid systems.[29] However, the kinetics of drug precipitation and morphology of precipitated drug during digestion has received no attention in the literature until very recently.[30] It is a natural progression to utilize the model described herein to enable the lipid nanostructure and drug precipitation to be correlated in real time, as a leap forward in correlating drug solubilization with target structures to ultimately enable reverse engineering of drug formulations for optimal drug solubilization and bioavailability.

The use of self-dispersing systems in lipid-based drug delivery has been increasing for a number of reasons, including perceptions of improved bioavailability with small particle size, often increased drug solubility in surfactant-containing lipid mixtures, and because the dispersible formulations are amenable to a "dispersibility" quality control test not able to be applied to regular oil formulations. There has been some debate around the role of surfactants in such formulations, particularly Cremophor-based surfactants, which may also impart some other physiological and possibly toxicological effects on such drug products. The structural aspects of digestion of the SNEDDS formulation in this study showed that Cremophor plays little if any role in the structures formed during digestion and that the ratio of the lipid components was more closely correlated to the structural evolution. Further, the formation of the hexagonal phase structure was not a consequence of the SNEDDS formulation as previously hypothesized,[9] but it is inherent in the use of increasing amounts of Maisine. Although it would be premature to speculate as to the absolute dependence of the relative proportion of the lamellar and hexagonal phases with increasing Maisine content in Figure 5F, there is a definitive trend to increased proportion of hexagonal phase. Whether the dependence is approximately linear or there is a rational basis for a nonlinear dependence is unclear at this stage. Maisine contains more free fatty acids and is more readily digested than sesame oil to produce further fatty acids. The evolution of the hexagonal phase is apparently related to fatty acid production, and the formation of the hexagonal phase would tend to indicate that the fatty acids are acting to increase the curvature toward oil, imparting an overall inverted wedge shape to the lipid component of the bilayer. This is however somewhat at odds with the swelling and putative formation of the sponge phase on digestion of the GMO cubosomes; further studies with varying pH conditions are planned to clarify this behavior.

The limited influence of Cremophor on structure is also borne out in the in vivo performance of Cremophor containing formulations; Cremophor is often found to have little impact or even to decrease oral bioavailability where the permeability of drug is not the limiting factor.[31] On the other hand, Cremophor is known to enhance the permeability of drug compounds by inhibiting intestinal metabolism,[32] which is a controversial physiological effect. Hence, isolating the true role of surfactants in formulations from a performance perspective is important, and understanding their influence on structural aspects of lipid processing in vivo will help in optimizing their contribution.

CONCLUSIONS

The findings presented in this paper highlight that synchrotron SAXS coupled with in vitro digestion models can provide information on real time structural evolution in digesting lipid systems. Such information is of importance for pharmaceutical formulation, functional foods, and nutritional diseases. A broader understanding of the dependence of lipid type on nanostructure will now be sought to provide the link between structure, lipid, and drug absorption.

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■ REFERENCES

(1) Charman, W. N. *J. Pharm. Sci.* **2000**, *89*, 967–978.
(2) Kossena, G. A.; Charman, W. N.; Boyd, B. J.; Porter, C. J. H. *J. Pharm. Sci.* **2005**, *94*, 481–492.
(3) Patton, J.; Carey, M. *Science* **1979**, *204*, 145–148.
(4) Kossena, G. A.; Boyd, B. J.; Porter, C. J. H.; Charman, W. N. *J. Pharm. Sci.* **2003**, *92*, 634–648.
(5) http://www.synchrotron.org.au/index.php/aussyncbeamlines/saxswaxs/saxs-data-a-processing.
(6) Hammersley, A. P. ESRF Internal Report, ESRF98HA01T 1998, FIT2D V9.129, Reference Manual V3.1.
(7) Borne, J.; Nylander, T.; Khan, A. *Langmuir* **2000**, *16*, 10044–10054.
(8) Borne, J.; Nylander, T.; Khan, A. *Langmuir* **2002**, *18*, 8972–8981.
(9) Fatouros, D. G.; Deen, G. R.; Arleth, L.; Bergenstahl, B.; Nielsen, F. S.; Pedersen, J. S.; Mullertz, A. *Pharm. Res.* **2007**, *24*, 1844–1853.
(10) Salentinig, S.; Sagalowicz, L.; Leser, M. E.; Tedeschi, C.; Glatter, O. *Soft Matter* **2011**, *7*, 650–661.
(11) Sek, L.; Porter, C. J. H.; Kaukonen, A.; Charman, W. N. *J. Pharm. Pharmacol.* **2002**, *54*, 29–41.
(12) Esposito, E.; Eblovi, N.; Rasi, S.; Drechsler, M.; Di Gregorio, M.; Menegatti, E.; Cortesi, R. *AAPS PharmSci* **2003**, *5*, 30.
(13) Kaukonen, A. M.; Boyd, B. J.; Porter, C. J. H.; Charman, W. N. *Pharm. Res.* **2004**, *21*, 245–253.
(14) Hyde, S. *Identification of lyotropic liquid crystalline mesophases*; John Wiley & Sons: Chichester, U.K., 2002.
(15) Nguyen, T.; Hanley, T.; Porter, C. J. H.; Larson, I.; Boyd, B. J. *J. Pharm. Pharmacol.* **2010**, *62*, 844–855.
(16) Gustafsson, J.; Nylander, T.; Almgren, M.; Ljusberg-Wahren, H. *J. Colloid Interface Sci.* **1999**, *211*, 326–335.
(17) Gustafsson, J.; Ljusberg-Wahren, H.; Almgren, M.; Larsson, K. *Langmuir* **1997**, *13*, 6964–6971.
(18) Dong, Y. D.; Larson, I.; Hanley, T.; Boyd, B. J. *Langmuir* **2006**, *22*, 9512–9518.
(19) López, O.; Cócera, M.; Pons, R.; Amenitsch, H.; Caelles, J.; Parra, J. L.; Coderch, L.; Maza, A. d. l. *Spectroscopy* **2002**, *16*, 343–350.
(20) Squires, A.; Templer, R. H.; Ces, O.; Gabke, A.; Woenckhaus, J.; Seddon, J. M.; Winter, R. *Langmuir* **2000**, *16*, 3578–3582.
(21) Tilley, A.; Dong, Y.-D.; Amenitsch, H.; Rappolt, M.; Boyd, B. J. *Phys. Chem. Chem. Phys.* **2011**, *13*, 3026–3032.
(22) McClements, D. J.; Li, Y. *Food Funct.* **2010**, *1*, 32–59.
(23) Fatouros, D.; Mullertz, A. *Expert Opin. Drug Metab. Toxicol.* **2008**, *4*, 65–76.
(24) Dahan, A.; Hoffman, A. *J. Controlled Release* **2008**, *129*, 1–10.
(25) Fatouros, D.; Deen, G.; Arleth, L.; Bergenstahl, B.; Nielsen, F.; Pedersen, J.; Mullertz, A. *Pharm. Res.* **2007**, *24*, 1844–1853.
(26) Uzun, B.; Arslan, C.; Furat, S. *J. Am. Oil Chem. Soc.* **2008**, *85*, 1135–1142.
(27) Borne, J.; Nylander, T.; Khan, A. *Langmuir* **2001**, *17*, 7742–7751.
(28) Angelov, B.; Angelova, A.; Garamus, V.; Lebas, G.; Lesieur, S.; Ollivon, M.; Funari, S.; Willimeit, R.; Couvreur, P. *J. Am. Chem. Soc.* **2007**, *129*, 13474–13479.
(29) Day, J. P. R.; Rago, G.; Domke, K. F.; Velikov, K. P.; Bonn, M. *J. Am. Chem. Soc.* **2010**, *132*, 8433–8439.
(30) Sassene, P. J.; Knopp, M. M.; Hesselkilde, J. Z.; Koradia, V.; Larsen, A.; Rades, T.; Mullertz, A. *J. Pharm. Sci.* **2010**, *99*, 4982–4991.
(31) Porter, C. J. H.; Kaukonen, A.; Boyd, B. J.; Edwards, G. A.; Charman, W. N. *Pharm. Res.* **2004**, *21*, 1405–1412.
(32) Nerurkar, M. M.; Burton, P. S.; Borchardt, R. T. *Pharm. Res.* **1996**, *13*, 528–34.
(33) Porter, C. J. H.; Trevaskis, N. L.; Charman, W. N. *Nat. Rev. Drug Discovery* **2007**, *6*, 231–248.

APPENDIX 3

Re-printed with permission from Porter CJH, Anby MU, Warren DB, Williams HD, Benameur H, Pouton CW. Lipid based formulations: Exploring the link between in vitro Supersaturation and in vivo exposure. Bulletin Technique Gattefossé. 2011;104:61-9.

LIPID BASED FORMULATIONS: EXPLORING THE LINK BETWEEN *IN VITRO* SUPERSATURATION AND *IN VIVO* EXPOSURE

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Abstract

Lipid based drug delivery systems (LBDDS) hold great promise as vehicles to enhance the bioavailability of drugs where low aqueous solubility provides a barrier to effective exposure after oral administration. Unlike traditional solid formulations, LBDDS typically present drugs to the gastrointestinal tract (GIT) in solution, albeit in non-aqueous solution, and the challenge to the formulator is to identify approaches that maintain drug in a solubilized form throughout GI transit. Within the GIT, however, LDBBS are diluted and digested – conditions which typically reduce solublization capacity and promote drug precipitation. Inherent in this process is a period of supersaturation prior to drug precipitation. Approaches to kinetically stabilize this supersaturated state provide a viable approach to the generation of more robust formulations. Here we address the conditions under which the processing of lipid based formulations is most likely to lead to supersaturation, the factors that impact on the attainment of a supersaturated state, the conditions under which polymeric excipients are most likely to aid in supersaturation stabilization and finally the link between *in vitro* supersaturation and *in vivo* exposure.

Keywords

Lipid based drug delivery systems, self emulsifying drug delivery systems (SEDDS), in vitro lipolysis, supersaturation, polymeric precipitation inhibitors.

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1. Drug absorption from lipid based delivery systems

The absorption of poorly water soluble drugs and drug candidates is often low and variable after oral administration, and limited by both finite solubility and slow dissolution. Several formulation approaches have been suggested to overcome these issues, including; particle size manipulation, solid dispersions, inclusion complexes and a plethora of nanoparticle-based options [1-3]. Co-administration with lipids may also be effective, and in particular the combinations of lipids, surfactants and co-solvents that spontaneously emulsify on contact with gastrointestinal (GI) fluids (so called self-emulsifying drug delivery systems or SEDDS) [4-6]. SEDDS have been shown to enhance the oral bioavailability of a range of poorly water soluble drugs including cyclosporine, saquinavir, fenofibrate, danazol, halofantrine and many others [7-11]. These formulations are most commonly filled as liquids into soft or hard gelatin capsules (the latter requiring the use of capsule sealing technology) and as such administer the drug to the GI tract in solution in a molecularly dispersed state, albeit in a non-aqueous vehicle. Drug absorption from the SEDDS formulation is facilitated via formulation dispersion and the generation of a high surface area drug reservoir within the GI tract. Drug in this lipid reservoir is in rapid equilibrium with drug in true free solution. Our current understanding of the process of drug absorption is that drug is absorbed via the free fraction, and that the equilibrium between drug in the solubilized reservoir and in free solution is rapidly re-established. The driving force for drug absorption is therefore the free concentration of drug that exists in equilibrium with the dispersed reservoir.

This seemingly simple situation is complicated by the realization that lipids, and many surfactants that comprise of polyethoxylated lipid esters, are substrates for digestive enzymes, including pancreatic lipase (and others), in the GI tract [12-14]. The digestion process can dramatically alter the physicochemical nature of the formulation, and therefore its solubilization capacity. This is further complicated by the ability of lipids to stimulate bile secretion and the potential to change patterns of lipid and lipid digestion product solubilization via interaction with endogenous bile salt micelles.

The concentration of drug that exists in free solution in the GI tract (and that provides the concentration gradient driving flux across the absorptive membrane) is therefore dependent on that nature of the colloidal species that are present in the intestine at that time and the equilibrium that exists between free and solubilized drug. Since the nature and solubilization capacity of these colloidal species is changing rapidly as a function of time (due to dispersion, digestion and bile salt interaction), it seems likely that the free concentration, and thus the driving force for absorption, also varies significantly as a function of time.

In recent years we and others have utilised *in vitro* models of lipid digestion to try to model the events that occur on digestion of candidate drug formulations to gain a better appreciation of the likely limiting steps to formulation performance. It is apparent from these studies that the components that make up SEDDS have

the capacity to integrate into endogenous bile salt / phospholipid / cholesterol mixed micelles that are secreted in bile, and in doing so significantly increase the equilibrium solubilization capacity of the GI fluids. This is highly dependent on the nature of the lipids and surfactants that are employed and can change dramatically as these materials are digested. Nonetheless, the components added to lipid formulations typically increase the drug solubilization capacity of the GI fluids, even after formulation digestion. This provides an advantage over most solid dose forms, which, even where dissolution is effective, are unable to change the solubilization capacity of the GI fluids. This advantage is shown as the 'solubilization effect' in Figure 1. In addition, as SEDDS formulations are dispersed, digested and diluted with biliary lipids, the rapidly changing nature of the intestinal colloidal environment also provides the opportunity for a period of transient supersaturation that may further promote drug absorption (shown as the "supersaturation effect" in Figure 1).

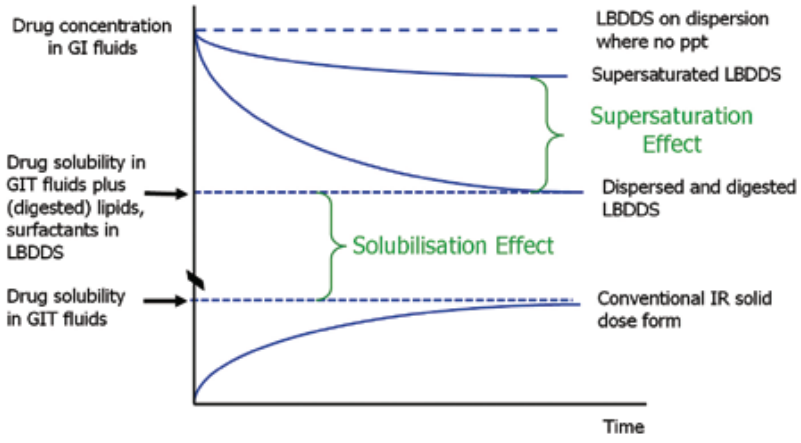

Figure 1. Summary of the impact of formulation excipients (and their digestion products) included in lipid based drug delivery systems (LBDDS) on equilibrium drug solubility in the GI tract, and the potential for LBDDS to promote additional benefit via the generation of transiently supersaturated intestinal species.

2. Supersaturation during dispersion and digestion of lipid based drug delivery systems

Considerable attention has been directed to better understanding the solubilization capacity of SEDDS formulations as they are dispersed and digested in the GI tract. To this point, however, little effort has been expended in order to define whether supersaturation eventuates, or whether the concentration changes that occur simply reflect changes to the equilibrium solubilization capacity of the colloidal species formed. In recent years, therefore, we have increasingly focussed on gaining a better understanding of supersaturation under these conditions using *in vitro* models of lipid digestion.

Interestingly, our data seem to show that the differences we see in solubilized drug concentrations in *in vitro* digests after dispersion and digestion of different SEDDS formulations are in many cases driven by changes in supersaturation, rather than changes in equilibrium solubility. This raises the possibility that the ability of SEDDS formulations to simply avoid precipitation (and therefore to provide a solubilized reservoir) may be an incomplete indicator of the potential for absorption, since the free concentration in equilibrium with the solubilized reservoir will vary as a function of the extent of supersaturation – and this may be markedly different for different formulations.

For example, in Figure 2A the total solubilized quantity of a model drug danazol is shown as a function of time during *in vitro* digestion of a series of four SEDDS formulations (see [12] for full details). Blank digests have subsequently also been generated for each formulation by digesting drug free formulations under identical conditions and equilibrium drug solubility then measured in these blank digests. These data are reproduced as the dotted lines in figure 2A. It is apparent that across the different formulations, equilibrium solubility in the digested formulations is similar, whereas the concentration attained during the digestion experiment differs widely. As such, the ability of the formulations to resist precipitation on dispersion and digestion appears to be better correlated with the ability of the formulation to support supersaturation, rather than the equilibrium solubility of the colloids formed – which varied surprisingly little.

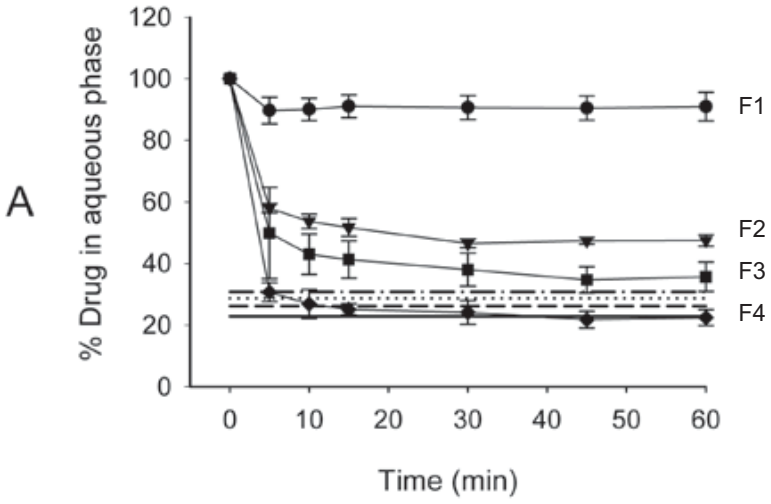

Figure 2A. % drug solubilised in the aqueous phase of an in vitro lipid digestion experiment for four candidate lipid formulations (F1 ●; F2 ▼; F3 ■; F4 ♦). Dotted lines provide the levels equivalent to the equilibrium drug solubility in blank digests for each formulation and rank in the same order with F1 showing the highest equilibrium solubility followed by F2, F3 and F4.

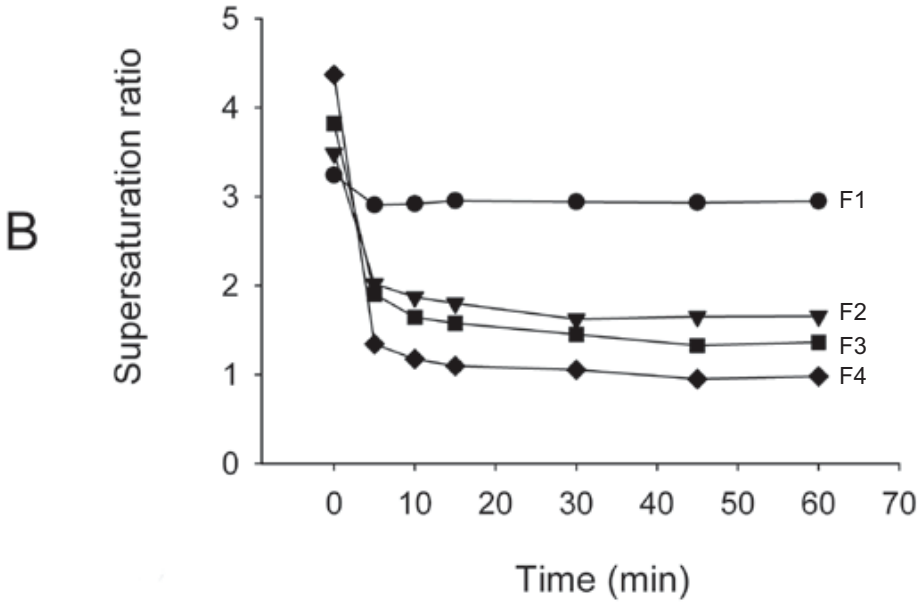

Figure 2B. Supersaturation ratio for each formulation as a function of time, calculated as the drug concentration in the digests at each time point divided by the equilibrium solubility of drug in blank digest obtained at 60 minutes.

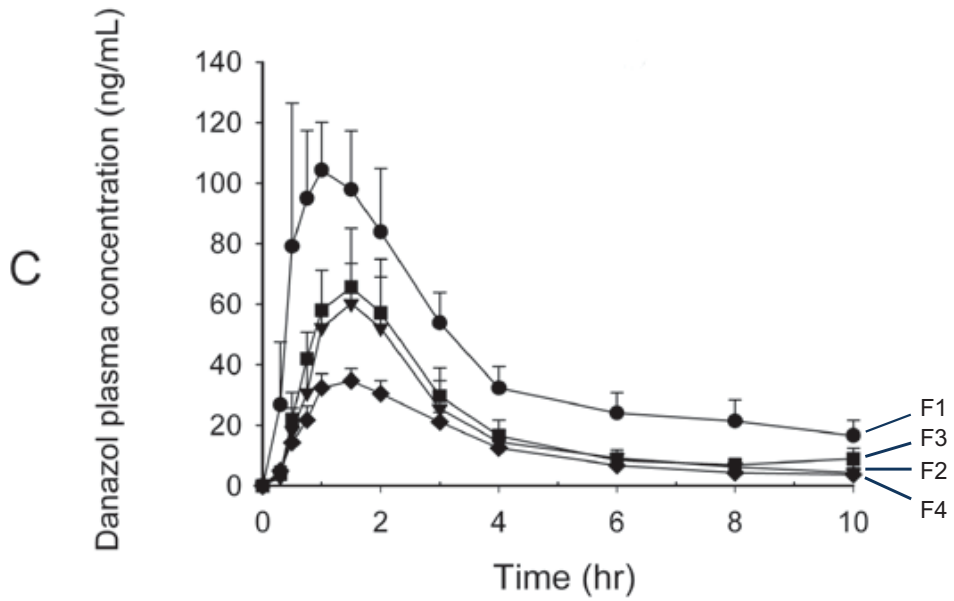

Figure 2C. In vivo plasma level time profiles after oral administrations of formulations 1-4 to male beagle dogs. Figure modified from [12].

Calculation of the degree of supersaturation at each time point (relative to the equilibrium solubility in a blank 60 min digest) results in Figure 2B. Comparison with the *in vivo* exposure of danazol after administration of the same formulation (Figure 2C) suggests broad correlation between either the supersaturation ratio (panel B) or % solubilized drug (panel A) and exposure (panel C), but that equilibrium solubility of drug in the colloids formed post-digestion distinguishes less readily between formulations.

3. Stabilizing supersaturation using polymeric excipients

Increasing realization of the importance of stabilizing the supersaturated state as a driver of drug solubilization in SEDDS formulation has led ourselves and others to explore the potential utility of polymers to inhibit drug precipitation and maintain supersaturation. For example, we recently explored the potential utility of ~70 polymers as potential polymeric precipitation inhibitors (PPIs), using a simple solvent shift method with danazol as a model poorly water soluble drug [16]. In summary, the most effective polymers in this simple challenge test were cellulose derivatives, including hydroxypropyl methylcellulose (HPMC) and HPMC acetate succinate (HPMCAS); consistent with the finding of others [17-19]. Promising data were also obtained with Eudragit E100 (poly(butyl methacrylate-co-(2-dimethylaminoethyl) methacrylate-co-methyl methacrylate) 1:2:1). Exemplar data illustrating the ability of HPMC to inhibit danazol precipitation in the solvent shift assay are shown in Figure 3 [16], with polymer concentrations above ~ 0.001% w/v resulting in almost complete precipitation inhibition.

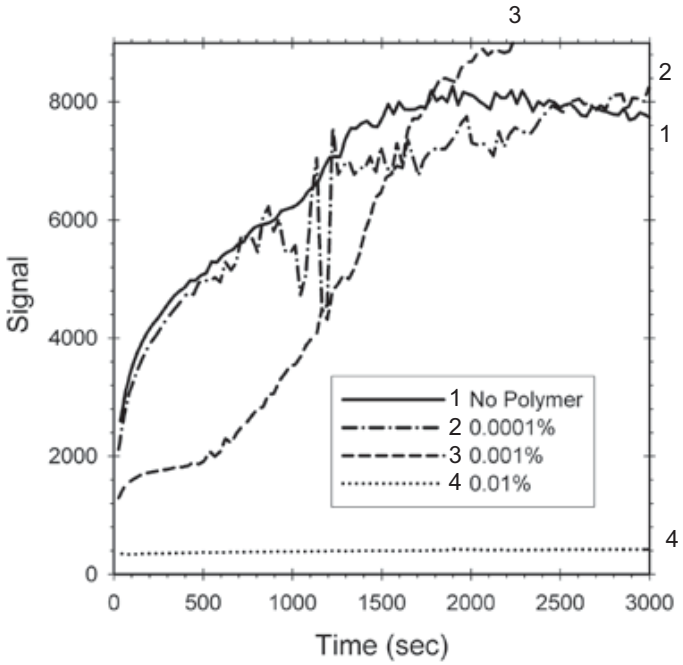

Figure 3. Turbidity (expressed as arbitrary signal) as a function of time after generating a supersaturated danazol solution (degree of supersaturation = 145) in an aqueous buffer containing 0 to 0.01% w/v HPMC E4M. Data from [16].

Subsequently, these studies have been extended to explore the ability of polymers to inhibit drug precipitation during digestion of medium chain lipid based SEDDS formulations. Inclusion of ~5% w/w HPMC in the formulation was sufficient to prevent drug precipitation from formulations where digestion typically resulted in precipitation of > 50% of the drug initially dissolved in the formulation [20]. Perhaps unsurprisingly, the capability of the polymer to prevent precipitation was related to the drug loading in the formulation where increases in drug load towards the maximum quantity soluble in the formulation (i.e. 100% saturation in the formulation) led to a reduction in the efficiency of the PPI.

4. Correlation between *in vitro* supersaturation and *in vivo* exposure

Successful *in vitro* inhibition of drug precipitation from digesting SEDDS formulations prompted an evaluation of the utility of HPMC to prevent precipitation and to promote the absorption of a model poorly water soluble drug (danazol) after administration. These studies were conducted at two levels of drug loading – 40% and 80% of the saturated solubility of drug in the formulation - and in the presence and absence of 5% w/w HPMC in the formulation. At the lower drug loading levels the inclusion of the polymer in the formulation increased danazol exposure by approximately 35% (manuscript in preparation). In contrast, and consistent with the *in vitro* trends, at the higher drug load, polymer inclusion failed to increase danazol bioavailability.

However, drug exposure was significantly (and non-linearly) increased at the higher drug load when compared with the lower drug load resulting in an approximate doubling of relative bioavailability [21]. A complete explanation for these data are not evident at this time, but may reflect changes to absorption, first pass metabolism, or both. One potential explanation is that changes to the level of saturation of drug in the formulation may have led to transient increases in drug thermodynamic activity in the colloids formed in the GI tract during formulation digestion at the higher drug load – in turn resulting in increases to absorption. Studies are on going to explore this hypothesis in more detail.

5. Conclusions

Lipid based formulations have the potential to significantly enhance the oral bioavailability of poorly water soluble drugs. Absorption is thought to be enhanced via the provision of a series of colloidal lipophilic microenvironments in the gastrointestinal tract that consist of a combination of formulation derived lipids and surfactants (and their digestion products) and biliary lipids. These colloids assist in drug solubilization, prevent drug precipitation and provide a readily accessible, molecularly dispersed reservoir that is in rapid equilibrium with drug in free solution. Drug absorption is thought to be mediated via the concentration of drug in free solution and subsequent replenishment of the available free concentration via re-establishment of the equilibrium between solubilized and free drug. In recent years, our focus has been on the engineering of lipid based formulations that provide

for efficient drug solubilization and limited drug precipitation as the formulation is processed in the gastrointestinal tract and using these fundamental principles reasonable rank order correlation has been possible between the proportion of dissolved drug that remains solubilized in a lipid based formulation after *in vitro* dispersion and digestion and *in vivo* exposure [12, 13, 22]. Our recent data, and that of others, however, suggests that this model may be refined further via a realization of the potential importance of the generation of intestinal colloidal species where drug is supersaturated and therefore where supersaturation drives increases to thermodynamic activity and ultimately free concentration. The conditions that promote transient supersaturation, however, also lead to an increased likelihood of drug precipitation. The eventual drug flux across the absorptive membrane is likely a net function of the timescale of stabilized supersaturation, the rate of drug precipitation and the absorptive sink provided by the intrinsic intestinal permeability of the drug candidate. Further examination of the interplay of these three factors will provide a more complete understanding of the process of drug absorption from lipid based formulations.

6. Acknowledgement

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7. References

[1] M.E. Davis, M.E. Brewster. Cyclodextrin-based pharmaceutics: Past, present and future, Nature Rev. Drug Discov. 3 (2004) 1023-35.

[2] C. Leuner, J. B. Dressman. Improving drug solubility for oral delivery using solid dispersions, Eur. J. Pharm. Biopharm. 50 (2000) 47-60.

[3] E. Merisko-Liversidge, G.G. Liversidge, E.R. Cooper ER. Nanosizing: a formulation approach for poorly-water-soluble compounds, Eur. J. Pharm. Sci. 18 (2003) 113-20.

[4] C.M. O'Driscoll, B.T. Griffin. Biopharmaceutical challenges associated with drugs with low aqueous solubility - The potential impact of lipid-based formulations, Adv. Drug Deliv. Rev. 60 (2008) 617-24.

[5] C.J.H. Porter, N.L. Trevaskis, W.N. Charman. Lipids and lipid-based formulations: optimizing the oral delivery of lipophilic drugs, Nature Rev. Drug Discov. 6 (2007) 231-48.

[6] C.W. Pouton, C.J.H. Porter. Formulation of lipid-based delivery systems for oral administration: Materials, methods and strategies, Adv. Drug Deliv. Rev. 60 (2008) 625-37.

[7] W.N. Charman, M.C. Rogge, A.W. Boddy, B.M. Berger. Effect of food and a monoglyceride emulsion formulation on danazol bioavailability, J. Clin. Pharmacol. 33 (1993) 381-6.

[8] S.M. Khoo, A.J. Humberstone, C.J.H. Porter, G.A. Edwards, W.N. Charman. Formulation design and bioavailability assessment of lipidic self-emulsifying formulations of halofantrine, Int. J. Pharm. 167 (1998) 155-64.

[9] C.J.H. Porter, C.W. Pouton, J.F. Cuine, W.N. Charman. Enhancing intestinal drug solubilization using lipid-based delivery systems. Adv. Drug Deliv. Rev. 60 (2008) 673-91.

[10] R.G. Strickley. Solubilizing excipients in oral and injectable formulations, Pharm. Res. 21 (2004) 201-30.

[11] J. Vonderscher, A. Meinzer. Rationale for the development of Sanimmune-Neoral, Transplant. Proc. 26 (1994) 2925-7.

[12] J.F. Cuine, W.N. Charman, C.W. Pouton, G.A. Edwards, C.J.H. Porter. Increasing the proportional content of surfactant (Cremophor EL) relative to lipid in self-emulsifying lipid-based formulations of danazol reduces oral bioavailability in beagle dogs, Pharm. Res. 24 (2007) 748-57.

[13] J.F. Cuine, C.L. McEvoy, W.N. Charman, C.W. Pouton, G.A. Edwards, C.J.H. Porter. Evaluation of the impact of surfactant digestion on the bioavailability of danazol after oral administration of lipidic self-emulsifying formulations to dogs, J. Pharm. Sci. 97 (2008) 995-1012.

[14] S. Fernandez, S. Chevrier, N. Ritter, B. Mahler, F. Demarne, F. Carriere, V. Jannin. *In vitro* gastrointestinal lipolysis of four formulations of piroxicam and cinnarizine with the self emulsifying excipients labrasol (R) and gelucire (R) 44/14. Pharm. Res. 26 (2009) 1901-10.

[15] S. Fernandez, J.D. Rodier, N. Ritter, B. Mahler, F. Demarne, F. Carriere, V. Jannin. Lipolysis of the semi-solid self-emulsifying excipient Gelucire (R) 44/14 by digestive lipases. Biochim. Biophys. Acta 1781 (2008) 367-75.

[16] D.B. Warren, H. Benameur, C.J.H. Porter, C.W. Pouton. Using polymeric precipitation inhibitors to improve the absorption of poorly water-soluble drugs: A mechanistic basis for utility. J. Drug Target. 18 (2010) 704-31.

[17] J. Bevernage, T. Forier, J. Brouwers, J. Tack, P. Annaert, P. Augustijns. Excipient-mediated supersaturation stabilization in human intestinal fluids, Mol. Pharmaceutics 8 (2011) 564-70.

[18] J. Brouwers, M.E. Brewster, P. Augustijns. Supersaturating drug delivery systems: The answer to solubility-limited oral bioavailability? J. Pharm. Sci. 98 (2009) 2549-72.

[19] W. Curatolo, J.A. Nightingale, S.M. Herbig. Utility of hydroxypropylmethylcellulose acetate succinate (HPMCAS) for initiation and maintenance of drug supersaturation in the GI milieu, Pharm. Res. 26 (2009) 1419-31.

[20] M.U. Anby, D.B. Warren, H. Benameur, C.W. Pouton, C.J.H. Porter. Using polymers to enhance the utility of lipid-based delivery systems. The 37th Annual Meeting & Exposition of the Controlled Release Society (CRS), July 10-14 (2010) Abstract 559. Portland, OR, USA.

[21] M.U. Anby, D.B. Warren, H. Benameur, C.W. Pouton, C.J.H. Porter. Thermodynamic activity versus solubility as a driver of drug absorption from lipid-based drug delivery systems. The AAPS Journal (2010) 12 (S2): R6005.

[22] A.M. Kaukonen, B.J. Boyd, C.J.H. Porter, W.N. Charman. Drug solubilization behavior during *in vitro* digestion of simple triglyceride lipid solution formulations Pharm. Res. 21 (2004) 245-53.

APPENDIX 4

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Incomplete Desorption of Liquid Excipients Reduces the *in Vitro* and *in Vivo* Performance of Self-Emulsifying Drug Delivery Systems Solidified by Adsorption onto an Inorganic Mesoporous Carrier

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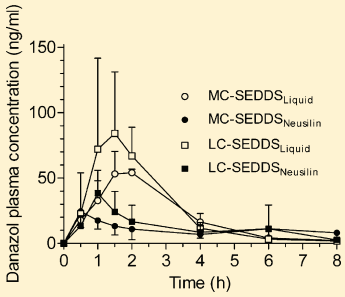
ABSTRACT: The purpose of the current study was to provide a mechanistic basis for *in vitro* and *in vivo* performance differences between lipid-based formulations solidified by adsorption onto a high surface area material and their respective liquid (i.e., nonadsorbed) counterparts. Two self-emulsifying formulations (based on either medium-chain or long-chain lipids) of the poorly water-soluble drug danazol were solidified by adsorption onto Neusilin US2. Liquid and adsorbed lipid-based formulations were subjected to *in vitro* dispersion−digestion tests, and additional *in vitro* experiments were performed to elucidate the cause of performance differences. The bioavailability of danazol after oral administration to rats was also assessed. The percentage of the dose solubilized in the aqueous phase during *in vitro* dispersion−digesting was ∼35% lower for the adsorbed formulations when compared to their liquid counterparts. This trend was also reflected *in vivo*, where the bioavailability of danazol after administration of the adsorbed formulations was ∼50% lower than that obtained after administration of the equivalent liquid formulation. Incomplete desorption of the microemulsion preconcentrate from the carrier on dispersion−digestion was identified as the main contributor to the reduced pharmaceutical performance of the adsorbed formulations. The results of the current study indicate that solidification of lipid-based formulations through adsorption onto a high surface area carrier may limit formulation (and drug) release *in vivo* and thereby reduce oral bioavailability.



KEYWORDS: *lipid-based formulations, self-emulsifying drug delivery systems, danazol, Neusilin, adsorption*

1. INTRODUCTION

Lipid-based formulations (LBFs) present a valuable means by which the oral bioavailability of poorly water-soluble drugs may be improved.[1−3] LBFs promote drug bioavailability through a series of well-described mechanisms that include (i) the ability to circumvent the often slow and potentially absorption-limiting dissolution of solid drug particles by presenting the drug at the likely site of absorption (typically the small intestine) in a predissolved form,[4−8] (ii) the capacity to stimulate lipid digestion and the secretion of endogenous detergents (bile salts and phospholipids) for enhanced solubilization in the gastrointestinal tract[9−12] and (iii) the potential recruitment of lymphatic transport processes and the subsequent bypass of first pass metabolic pathways.[13−15]

Many of the excipients used to construct LBFs are liquid or semisolid at ambient temperature, and the production of unit dose forms for LBFs is therefore typically achieved by filling the liquid (or molten) excipient blend into soft (gelatin) or hard (gelatin or hydroxypropyl methylcellulose) capsules. While there are currently a variety of such liquid or semisolid formulations on the market (e.g., Neoral, Avodart, Accutane),[16] liquid filling of capsules on an industrial scale may present a number of challenges. These have been reviewed in detail elsewhere,[17] and are largely related to issues of compatibility between fill components and the shell.

An alternative approach to circumvent the challenges associated with liquid capsule filling involves the adsorption of liquid excipients onto solid materials with high specific surface area, e.g., colloidal silicon dioxide (Aerosil), calcium silicate (Hubersorb) or magnesium aluminometasilicate (Neusilin).[18−21] These materials are capable of adsorbing large quantities of liquids while retaining the intrinsic bulk properties of the powder. Liquid-loaded powders allow facile filling of capsules or alternatively may be compressed into tablets. For example, the material Neusilin US2 has been reported to retain excellent flowability when loaded with twice its own weight of a

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microemulsion preconcentrate.[20] Similar findings for a variety of other high surface area materials have been reported.[19,22−25]

Although promising studies on the utility of high surface area materials as carriers for LBFs continue to emerge, there is a current bias in the state of the art of this technology toward demonstration of improved technological outcomes such as flowability and compressibility. In contrast, literature data on the effect of such an adsorption process on biopharmaceutical performance (e.g., the ability of the formulation to present the drug in a solubilized state on contact with aqueous media) is less prevalent and often contradictory. For example, several papers have reported comparable or equal performance of liquid and adsorbed formulations,[24,26,27] whereas others show pronounced performance differences, most notably inferior performance of adsorbed formulations when compared to the original liquid formulation.[20,28]

The mechanisms that underpin the potential differences in biopharmaceutical performance of adsorbed versus non-adsorbed LBFs, however, remain unclear.[20,28] In particular, no prior studies have attempted to utilize biorelevant *in vitro* and *in vivo* methods to probe performance differences between liquid and adsorbed LBFs, and a generally accepted picture of how LBF adsorption onto a high surface area carrier affects performance has not been established.

The current study therefore sought to assess the impact of adsorption onto a high surface area carrier on LBF performance during *in vitro* dispersion−digestion testing and to relate these differences to patterns of *in vivo* exposure. Self-emulsifying formulations of the poorly water-soluble steroid danazol (nonionizable, log *P* 4.5[29]), which have been thoroughly characterized in prior work,[4] were solidified by adsorption onto Neusilin US2, a spherical granular material built up of mesoporous magnesium aluminometasilicate. Neusilin US2 was selected as the adsorbent as it is one of the most widely studied materials for the solidification of LBFs. Liquid and adsorbed LBFs were assessed using *in vitro* dispersion−digestion tests, and additional *in vitro* experiments performed to elucidate the causes of performance differences. The bioavailability of danazol after oral administration of liquid and adsorbed LBFs to rats was also assessed.

The current *in vitro* and *in vivo* data suggest that adsorption of self-emulsifying formulations onto Neusilin US2 impacts performance significantly, due to incomplete desorption of the liquid formulation components from the Neusilin US2 carrier. The data illustrate the need for caution when converting liquid LBFs to solid forms using adsorbents.

2. MATERIALS AND METHODS

2.1. Materials. Neusilin US2 was donated by Fuji Chemical Industry Co. (Toyama, Japan). Danazol was kindly supplied by Sterling Pharmaceuticals (Sydney, Australia). Progesterone, sodium taurodeoxycholate (>97%), porcine pancreatin extract (P7545, 8 × USP specifications activity) and 4-bromophenylboronic acid were from Sigma Chemical Co. (St. Louis, MO). Captex 355, a medium-chain triglyceride, and Capmul MCM EP, a blend of mostly medium-chain mono- and diglycerides (with some triglycerides), were donated by Abitec Corporation (Columbus, Ohio) and were used as received. Soybean oil, a long-chain triglyceride, was obtained from Sigma Chemical Co. Maisine 35-1, a blend of long-chain mono-, di- and triglycerides, was a generous gift from Gattefossé (Saint-Priest, France). Phosphatidylcholine (lipoid E PC S, approximately 99.2% pure, from egg yolk) was obtained from Lipoid GmbH (Ludwigshafen, Germany). Sodium hydroxide 1 M (Titrisol), was obtained from Merck KGaA (Darmstadt, Germany). Heparin (1000 IU/mL) was obtained from DBL (Mulgrave, Australia) and normal saline (0.9%) from Baxter Healthcare (Old Toongabbie, Australia). Water was obtained from a Milli-Q purification system (Millipore, Bedford, MA). All other chemicals and solvents were of analytical purity or high performance liquid chromatography (HPLC) grade, respectively.

2.2. Preparation of Self-Emulsifying Drug Delivery Systems (SEDDS). *2.2.1. Liquid SEDDS ($SEDDS_{liquid}$).* The composition of SEDDS utilized in this work was consistent with those described previously by Porter et al.[4] The medium-chain (MC) $SEDDS_{liquid}$ consisted of Captex 355 (36% w/w), Capmul MCM (18% w/w), Cremophor EL (36% w/w) and absolute ethanol (10% w/w), while the corresponding long-chain (LC) formulation contained soybean oil (30% w/w), Maisine 35-1 (30% w/w), Cremophor EL (30% w/w) and absolute ethanol (10% w/w). Isotropic formulations that were liquid at room temperature ($SEDDS_{liquid}$) were obtained by briefly vortex-mixing the individual excipients at the above compositions.

The equilibrium solubility of danazol in the $SEDDS_{liquid}$ was determined using a previously described method,[10] and was defined as the value attained when consecutive solubility values differed by ≤5%. This was typically reached after equilibration times of between 48 and 72 h. The MC- and LC-$SEDDS_{liquid}$ were loaded with 12.5 mg/g of danazol, corresponding to 40% and 49% of the danazol saturated solubility in the formulation, respectively. Danazol was incorporated into $SEDDS_{liquid}$ by adding the required mass of drug into clean screw-top glass vials and subsequently adding drug-free formulation up to the target mass. Vials were sealed, vortex-mixed and incubated at 37 °C for at least 24 h (to ensure complete dissolution of the solid drug) prior to further use. Danazol content in the $SEDDS_{liquid}$ formulations was verified (n = 3) by removing accurately weighed samples from the formulation and transferring to 5 mL volumetric flasks. Samples were made up to volume with chloroform:methanol (2:1 v/v). Aliquots (50−100 μL) were diluted >10-fold with methanol and analyzed for danazol content by HPLC (see section 2.8).

2.2.2. SEDDS Adsorbed onto Neusilin US2 ($SEDDS_{Neusilin}$). $SEDDS_{liquid}$ prepared as described above (either drug-free or danazol-containing) was used to prepare corresponding adsorbed formulations ($SEDDS_{Neusilin}$). Prior to adsorption, Neusilin US2 was dried at 60 °C for 2 h to remove physically adsorbed water. The required mass of $SEDDS_{liquid}$ was pipetted onto Neusilin US2, and this mixture was stirred with a spatula until a homogeneous, dry powder was obtained. The ratio of SEDDS:Neusilin was 2:1 (w/w) for all $SEDDS_{Neusilin}$ investigated in the current study. The $SEDDS_{Neusilin}$ powders were slightly more "tacky" than Neusilin US2 alone, but retained the flow characteristics of the absorbent. These observations are consistent with previous work by other authors.[20] Danazol content in the $SEDDS_{Neusilin}$ formulations was verified (n = 3) using a method similar to that used for equivalent $SEDDS_{liquid}$ formulations (see section 2.2.1), with the exception that $SEDDS_{Neusilin}$ samples were suspended in chloroform:methanol (2:1 v/v) for at least 1 h to ensure a sufficient time for dissolution of the liquid formulation components and danazol from the Neusilin US2 pores. The time required for complete liberation of the formulation

components was confirmed by the conduct of experiments over different time scales (data not shown).

2.3. *In Vitro* Evaluation of $SEDDS_{liquid}$ and $SEDDS_{Neusilin}$ Formulations. *2.3.1. Dispersion and Digestion Testing.* The experimental conditions used to assess the performance of $SEDDS_{Neusilin}$ and $SEDDS_{liquid}$ during *in vitro* digestion were based on those proposed by the LFCS Consortium reported by Williams et al.[30] In brief, the experimental setup consisted of a pH-stat apparatus (Metrohm AG, Herisau, Switzerland), comprising a Titrando 802 propeller stirrer/804 Ti Stand combination, a glass pH electrode (iUnitrode) and two 800 Dosino dosing units coupled to 10 mL autoburets (Metrohm AG). The apparatus was connected to a computer and operated using Tiamo 2.0 software.

$SEDDS_{liquid}$ or $SEDDS_{Neusilin}$ formulations were weighed directly into thermostat-jacketed glass reaction vessel (Metrohm AG) and initially dispersed in 39 mL of digestion medium (pH 6.5, 2 mM Tris-maleate, 150 mM NaCl, 1.4 mM $CaCl_2$ 3 mM sodium taurodeoxycholate and 0.75 mM phosphatidylcholine, 37 °C) with continuous mixing using an overhead propeller stirrer 25 mm in diameter rotating at a speed of ~450 min^{-1}. The pH was manually adjusted to pH 6.5 ± 0.05 using small quantities of NaOH or HCl. The duration of the dispersion phase was 30 min (unless stated otherwise). During this period formulations dispersed to form a homogeneous mixture and three 1 mL samples were removed (at 10 min, 30 min and one additional sample for quality control purposes: see section 2.3.2). Digestion was initiated after 30 min by addition of 4 mL of pancreatin extract (prepared as described previously[30]) to provide concentrations of pancreatic lipase in the digestion vessel equivalent to ~1200 tributyrin units (TBU) per mL of digest. Sodium hydroxide titration solutions (titrants) of 0.2 and 0.6 M were utilized for digests containing LC and MC formulations respectively. Titrants were automatically added (controlled via the pH-stat controller) to the reaction vessel to maintain constant pH during digestion. The period of digestion was fixed at 30 min, during which time two samples were taken (15 min and 30 after initiation of digestion: see section 2.3.3).

The target mass of formulation in these experiments was 0.54 and 0.81 g for $SEDDS_{liquid}$ and $SEDDS_{Neusilin}$, respectively, and ensured that the total mass of liquid formulation components at the commencement of digestion for both $SEDDS_{liquid}$ and $SEDDS_{Neusilin}$ (following removal of samples during the dispersion phase) was 0.50 g.

2.3.2. Collection and Separation of Dispersion Samples. During initial dispersion (nondigestion conditions), samples (1 mL) were removed by pipet at 10 and 30 min and immediately centrifuged (21000*g*) at 37 °C for 10 min using a benchtop centrifuge (Fresco 21, Heraeus, Thermo Scientific, Bremen, Germany). Samples postcentrifugation consisted of a homogeneous aqueous phase (containing the finely dispersed oil droplets) and in the case of $SEDDS_{Neusilin}$ a solid pellet (containing the Neusilin US2 carrier). Aliquots (50−100 μL) of the aqueous phase were subsequently removed and diluted 20-fold with mobile phase before analysis for danazol content by HPLC (see section 2.8). An additional 1 mL sample was withdrawn during the initial dispersion for the determination of the maximum attainable concentration during the test. This sample was collected in between 10 and 30 min, transferred without centrifugation to a 5 mL volumetric flask and made up to volume with methanol. An aliquot of this sample was diluted 10-fold in mobile phase and assayed for danazol by HPLC (see section 2.8). The total amount of danazol recovered was used to calculate the percentage of the dose recovered in the dispersion and digestion samples. The lack of a centrifugation step ensured that all of the drug in the sample (including solubilized, precipitated and carrier-associated drug) was sampled and analyzed, allowing accurate mass balance assessment.

2.3.3. Collection and Separation of Digestion Samples. After 15 and 30 min of digestion, samples were withdrawn by pipet and immediately treated with a lipolysis inhibitor (1.0 M 4-bromophenylboronic acid in methanol, of which 5 μL was used per ml of digest). Samples were then separated by centrifugation. In line with previous guidance,[30] a benchtop centrifuge was used to separate the products of digestion from the MC lipid formulations (both $SEDDS_{liquid}$ and $SEDDS_{Neusilin}$) as samples postcentrifugation consisted of a colloidal aqueous phase and a solid pellet phase. In contrast, samples from the LC lipid formulations also contained a poorly dispersed "oily" phase. The latter required separation by ultracentrifugation. Samples for benchtop centrifugation (MC formulations) were 1 mL and centrifuged at 21000*g* (Fresco 21, Heraeus, Thermo Scientific). Samples for ultracentrifugation (LC formulations) were 4 mL and were centrifuged at 400000*g* (Optima XL-100K centrifuge, SW-60 rotor, Beckman, Palo Alto, CA). In both methods, samples were centrifuged at 37 °C for 30 min. The digestion phases postcentrifugation were recovered according to the protocol described by Williams et al.,[30] and were diluted at least 10-fold with mobile phase before centrifugation (10000*g*, 20 °C, 10 min) and assay for danazol by HPLC (see section 2.8).

2.4. Determination of Danazol Solubility in Dispersed and Digested $SEDDS_{liquid}$ and $SEDDS_{Neusilin}$. The solubilization capacity of dispersed and digested $SEDDS_{liquid}$ and $SEDDS_{Neusilin}$ for danazol were compared via the conduct of equilibrium solubility measurements. Drug-free (blank) formulations were dispersed and digested using the method described in section 2.3. After 30 min of dispersion or 30 min of digestion, samples were removed (and digestion was inhibited in the case of samples removed during the digestion phase). The aqueous phases predigestion (AP_{disp}) and postdigestion (AP_{digest}) were subsequently isolated as described in sections 2.3.2 and 2.3.3, respectively.

Crystalline danazol was added in excess to AP_{disp} and AP_{digest} before vortex-mixing and incubation at 37 °C. After 24 h of equilibration, mixtures were centrifuged (21000*g*) at 37 °C for 10 min, and aliquots (50−100 μL) of the supernatant were subsequently diluted >10-fold with mobile phase prior to analysis for danazol content by HPLC (see section 2.8).

2.5. Determination of the Particle Size of Dispersed $SEDDS_{liquid}$ and $SEDDS_{Neusilin}$. Photon correlation spectroscopy (Malvern Nano-ZS Zetasizer, Malvern Instruments, Worcestershire, U.K.) was used to determine the average particle size (reported throughout this manuscript as the average particle diameter based on the light scattering intensity) and the polydispersity index (PDI) of the self-emulsified oil droplets on dispersion of drug-free $SEDDS_{liquid}$ and $SEDDS_{Neusilin}$. Formulations were dispersed in water (25 °C) to achieve formulation concentrations equivalent to those obtained in the dispersion experiments. After 30 min equilibration, during which time the samples were periodically subjected to mild manual agitation, dispersions from the $SEDDS_{Neusilin}$ formulations were centrifuged at low speed (450*g*, 25 °C) for 5 min (Eppendorf 5804 R) to sediment the

suspended Neusilin US2 particles. This short and mild centrifugation step did not result in pelleting or phase-separation of solubilized lipids. Subsequently, the supernatant was assessed for particle size. The particle size of corresponding $SEDDS_{liquid}$ dispersions was determined without this centrifugation step.

2.6. Determination of the Extent of Residual Adsorption of Formulation Surfactant Cremophor EL (CREL) onto Neusilin. The propensity of the formulation surfactant CrEL to remain adsorbed onto Neusilin US2 during dispersion was estimated as follows. A concentrated stock solution of CrEL in ethanol (5 mg/mL) was added to Neusilin US2 to obtain samples of increasing CrEL:Neusilin weight ratio (ranging from 10 to 50% w/w). In some cases (particularly at high CrEL concentrations), excess ethanol was added during this impregnation step to ensure homogeneous wetting of the Neusilin US2 powder (and thus, homogeneous distribution of CrEL over the surface). These CrEL:Neusilin systems were stored overnight at 60 °C to remove ethanol from the Neusilin US2 pores (when impregnating an accurately weighed and predried quantity of Neusilin US2 with ethanol, 12 h drying at 60 °C was sufficient to bring the Neusilin US2 powder back to its original mass, suggesting complete ethanol evaporation). Subsequently, an accurately weighed quantity of each CrEL:Neusilin system was dispersed in digestion buffer (37 °C) for 30 min, after which Neusilin US2 was pelleted under mild centrifugation conditions as described earlier (see section 2.5) and the solubilization capacity of the supernatant for danazol was measured (as described in section 2.4). The solubilization capacity of the supernatants was referenced against that of solutions with known concentrations of CrEL to provide an indication of the loss of CrEL from the supernatant due to "irreversible" adsorption to the carrier.

2.7. Determination of the Extent of Danazol Adsorption onto the Surface of Neusilin. To determine whether danazol in solution shows the potential to adsorb onto Neusilin US2, thus reducing the concentration of drug in the colloidal aqueous phase, the concentrations of danazol in AP_{disp} samples exposed to Neusilin US2 and those not exposed to Neusilin US2 were directly compared. Neusilin US2 was added at a concentration of 7 mg/mL (equivalent to the concentrations of Neusilin US2 obtained during the dispersion−digestion testing of $SEDDS_{Neusilin}$, see section 2.3.1) to 1 mL samples (n = 4) of the dispersed drug-free $SEDDS_{liquid}$ (AP_{disp}). Samples were subsequently spiked with a concentrated danazol stock solution in DMSO to obtain concentrations in AP_{disp} that were ∼80% of the saturated drug solubility in this phase (determined as described in section 2.4). The final DMSO concentration in all samples was 1% v/v and therefore too low to influence danazol solubility. In the control experiments, danazol was added at equivalent concentrations to Neusilin US2-free AP_{disp} samples. All samples were subsequently equilibrated for 30 min at 37 °C with intermittent gentle agitation after which samples containing suspended Neusilin US2 were centrifuged at low speed (see section 2.5) to sediment the carrier particles. The danazol concentration of the supernatant was determined by HPLC (section 2.8). The mean danazol concentration of this "test" group was directly compared to the danazol concentration in the "control" group.

2.8. HPLC Analysis of Danazol in *in Vitro* Samples. All HPLC analyses of *in vitro* samples were conducted using a Waters Alliance 2695 separation module (Waters Alliance Instruments, Milford, MA) with a reverse-phase C_{18} column (150 × 15 mm, 5 μm, Waters Symmetry) protected by a C_{18} security guard cartridge (4 × 2.0 mm, Phenomenex, Torrence, CA). The injection volume was 50 μL, and UV detection was conducted at 286 nm. The mobile phase consisted of methanol and water in a 75:25 v/v ratio and was pumped through the column at a 1 mL/min flow rate. Validation details for the danazol assays have been previously reported.[31]

2.9. *In Vivo* Evaluation of $SEDDS_{liquid}$ and $SEDDS_{Neusilin}$. All *in vivo* experiments were approved and conducted in accordance with the guidelines of the Institutional Animal Ethics Committee.

2.9.1. Animals. Male Sprague−Dawley rats weighing between 250 and 300 g were used for the oral bioavailability studies. A cannula [0.96 × 0.58 mm (od × id) polyethylene tubing] was inserted into the carotid artery under general inhalation anesthesia (isoflurane 2.5%) to allow for serial blood collection.[32] Rats were connected to a harness and swivel system and allowed to recover overnight prior to dosing. Rats were fasted for at least 12 h prior to dosing and did not have access to feed during the sample collection period. Water was provided *ad libitum*.

2.9.2. Treatments. All animals received a nominal dose of 3 mg of danazol, administered as 240 mg of microemulsion preconcentrate in liquid form ($SEDDS_{liquid}$) or adsorbed onto Neusilin at a 2:1 w/w ratio ($SEDDS_{Neusilin}$). Both MC and LC variants of $SEDDS_{liquid}$ and $SEDDS_{Neusilin}$ were administered, making a total of four treatments. Four animals were dosed for each treatment.

2.9.3. Dosing. Five minutes prior to dosing, formulations were dispersed in a suspension medium (0.5% w/v sodium carboxymethyl cellulose, 0.5% w/v benzyl alcohol, 0.4% w/v Tween 80 and 0.9% w/v NaCl in water) to facilitate dosing. All formulations were administered via oral gavage. All animals were dosed with 1.8 mL of the predispersed formulation. The actual dose was accurately assessed by replicate analyses (n = 4) of the danazol content in the predispersed formulation.

2.9.4. Sample Collection. Blood samples (350 μL) were withdrawn at predose and 0.5, 1, 1.5, 2, 4, 6, and 8 h postdose. Prior to sample collection, cannulas were flushed with 2 IU/mL sodium heparin saline solution after each sample to ensure patency. Collected blood samples were transferred to 1.5 mL microcentrifuge tubes containing 10 IU of sodium heparin and centrifuged at room temperature for 5 min at 6700*g* (Eppendorf MiniSpin). Plasma (125 μL) was collected and stored at −20 °C pending analysis.

2.9.5. Sample Preparation. Calibration standards were prepared by spiking 125 μL aliquots of blank rat plasma with 5 μL of an acetonitrile solution containing 25, 62.5, 125, 250, 1250, 2500, and 6250 ng/mL danazol, yielding standards containing danazol in the range of 1−250 ng/mL. After addition of the internal standard (5 μL of a 2000 ng/mL progesterone solution in acetonitrile), samples were vortex mixed for 30 s and supplemented with 62.5 μL of a saturated aqueous ammonium sulfate solution followed by 30 s of vortex mixing. Subsequently, 125 μL of acetonitrile was added, and samples were vortex mixed again for 30 s after which they were equilibrated for 20 min at room temperature followed by centrifugation (Eppendorf 5804 R, 21000*g*, 5 min, 20 °C). 100 μL of the upper phase obtained postcentrifugation was transferred into autosampler vials prior to analysis of danazol content as described in section 2.9.6.

2.9.6. Quantification of Danazol Content in Plasma Samples Using High Performance Liquid Chromatography

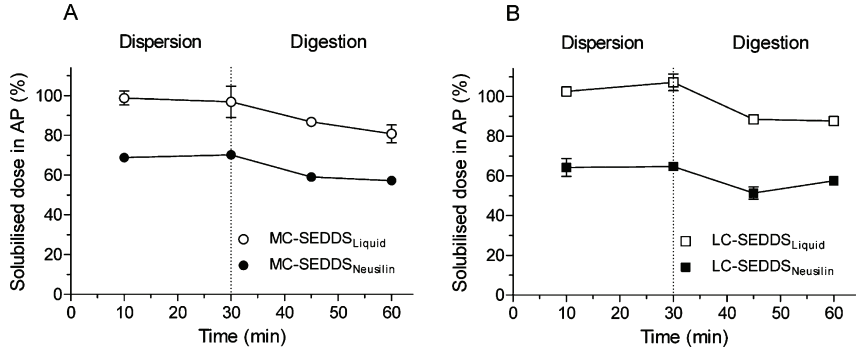

Figure 1. Percentage of the danazol dose recovered in the aqueous phase during *in vitro* evaluation, consisting of a 30 min dispersion followed by a 30 min digestion phase. (A) Medium-chain (MC) lipid SEDDS formulations. (B) Long-chain (LC) lipid SEDDS formulations. Values are expressed as means (n = 3) ± SD. The vertical dotted line indicates the point at which pancreatin was added to initiate digestion.

Coupled to Mass Spectrometric Detection (LC–MS). All analyses were performed on an LC–MS 2020 system (Shimadzu, Kyoto, Japan), comprising an LC-20AD binary pump, a SiL-20AC HT refrigerated autosampler, a DGU-20A$_3$ mobile phase vacuum degassing unit and a CTO-20A temperature-controlled column compartment. The system was coupled to an LC–MS single-quadrupole mass spectrometric detector (Shimadzu) equipped with an electrospray ionization source. The autosampler was maintained at 4 °C and the column at 40 °C. A Gemini C$_6$-phenyl column (50 × 2 mm, 3 μm) (Phenomenex), protected by a Gemini C$_6$-phenyl security guard cartridge (4 × 2.0 mm), was used for chromatographic separation. The injection volume was 10 μL. The mobile phase consisted of 95/5 water/methanol (v/v) (A) and 5/95 water/methanol (v/v) (B), both including 1 mM ammonium formate and 0.1% formic acid. Gradient elution at a constant flow rate of 0.3 mL/min was performed as follows: 60% B for 1 min; linear increase to 100% B in 6.5 min; 100% B for 7.0 min; decrease to 60% B in 30 s and ultimately re-equilibration with 60% B for 2 min. The total run time was 17 min. Under these conditions the retention times of danazol and progesterone were 4.6 and 4.3 min, respectively. The MS conditions were as follows: drying gas flow, 20 L/min; nebulizing gas flow, 1.5 L/min; drying gas temperature, 200 °C; interface voltage, 3.5 kV; detector voltage, 1.0 kV. The MS was operated in selection ion monitoring mode at m/z 338.2 for danazol and m/z 314.9 for the internal standard, progesterone. Data acquisition and peak integration were performed using the LabSolution software package, version 5.31.277 (Shimadzu). Unknown concentrations were determined by interpolation from a calibration curve, constructed using the calibration standards, where danazol:IS peak area ratio was plotted as a function of danazol concentration.

The assay was validated by replicate analyses of quality control samples (n = 6) containing 1, 10, and 100 ng/mL danazol in blank plasma. Intra-assay variability was accurate to 97.6, 117.5 and 118.1% and precise to 5.6, 9.8 and 2.6%. Interassay variability was accurate to 92.9, 104.8 and 113.9% and precise to 10.2, 7.1 and 6.0% for the quality control samples containing 1, 10 and 100 ng/mL, respectively. The lower limit of quantification for the plasma assay (1 ng/mL) was determined by replicate analyses (n = 6) of spiked plasma samples and defined as the lowest concentration at which appropriate accuracy and precision were obtained.

2.9.7. Data Analysis. The observed maximum plasma concentration (C_{max}) and the time of its occurrence (T_{max}) were noted directly from the individual plasma concentration versus time profiles. The area under the plasma concentration versus time curve was calculated from time zero to the last sampling point (AUC_{0-8h}) via linear-trapezoidal integration. The pharmacokinetic parameters for SEDDS$_{liquid}$ were compared against those of their corresponding SEDDS$_{Neusilin}$ using a Mann–Whitney test. P-values <0.05 were considered significant. All statistical analyses were performed using GraphPad Prism for Windows, version 5.00 (GraphPad Software, San Diego, CA).

3. RESULTS

3.1. *In Vitro* Dispersion–Digestion of SEDDS$_{liquid}$ and SEDDS$_{Neusilin}$. The percentage of the initial danazol dose that remained solubilized in the aqueous phase during *in vitro* dispersion and digestion of medium-chain (MC) SEDDS$_{liquid}$ formulations and the equivalent SEDDS$_{Neusilin}$ formulations are shown in Figure 1A. Similar data describing the performance of long-chain (LC) SEDDS formulations are shown in Figure 1B. The vertical dotted line indicates the point at which the pancreatin extract was added to initiate digestion.

During the dispersion of SEDDS$_{liquid}$ formulations (containing either MC or LC lipids), solubilized drug levels in the aqueous phase were maintained at ∼100%, indicating that both formulations could resist precipitation during dispersion. Initiation of digestion of the SEDDS$_{liquid}$ formulations, however, led to a reduction in solubilized drug in the aqueous phase (∼10% decrease after 30 min). This reduction was due to an increase in drug precipitation, as evidenced by an increase in drug recovery in the solid pellet phase postcentrifugation. In the case of LC formulations, some phase separation of undigested/partially digested lipids was also apparent post-centrifugation. A small fraction of the drug (∼2% of the original dose) was recovered in this poorly dispersed oil phase.

The aqueous phase concentrations following dispersion and digestion of the SEDDS$_{Neusilin}$ formulations are also shown in Figure 1. The levels of drug solubilized after 30 min dispersion were ∼70% and 65% of the original dose for MC (Figure 1A) and LC (Figure 1B) SEDDS$_{Neusilin}$ respectively, and therefore in contrast to the complete danazol solubilization of drug observed for the corresponding SEDDS$_{liquid}$. On digestion, a

parallel decrease in aqueous phase concentrations was observed. The magnitude of the drop in aqueous phase drug concentration between 30 and 60 min (i.e., that stimulated by digestion) was similar for $SEDDS_{liquid}$ and $SEDDS_{Neusilin}$.

In an attempt to explain the mechanism behind the lower concentrations of solubilized danazol observed on dispersion of the $SEDDS_{Neusilin}$ formulations (i.e., precipitation versus nonrelease from the carrier), the potential for changes to drug loading in the formulation to influence performance during dispersion was subsequently explored for the LC system. The percentage dose recovered in the aqueous phase following dispersion of LC-$SEDDS_{Neusilin}$ containing danazol at 7.5, 12.5 and 20.5 mg/g is shown in Figure 2 and reveals that the extent

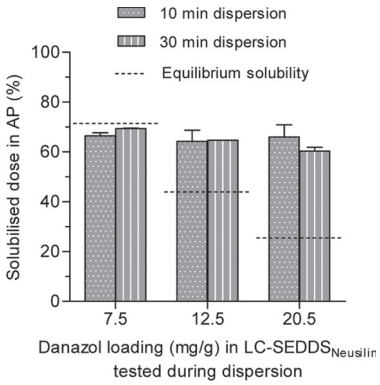

Figure 2. Danazol dose recovered in the aqueous phase (AP) after dispersion of LC-$SEDDS_{Neusilin}$ containing different danazol loadings. Bars represent means (n = 3) ± SD. Dashed lines indicate the percentage solubilized corresponding to the equilibrium solubility of crystalline danazol in the AP following dispersion of drug-free LC-$SEDDS_{Neusilin}$.

of drug solubilization (or conversely recovery in the pellet) was unaffected by the drug load and the dispersion time. This suggests that differences in recovery in the pellet between LC-$SEDDS_{liquid}$ LC-$SEDDS_{Neusilin}$ are unlikely to reflect differences in danazol precipitation as these events would be expected to be highly dose and time dependent.

To exclude the possibility that adsorption of danazol from solution (free or solubilized in an oil-droplet phase or micellar phase) to the surface of Neusilin US2 may be responsible for the decrease in danazol concentrations in the aqueous phase, dispersed drug-free $SEDDS_{liquid}$ formulations were spiked with danazol or danazol plus Neusilin US2 (see section 2.7 for more detailed description of the methods). The results of these experiments are shown in Figure 3 and reveal that danazol concentrations in AP_{disp} were slightly lower in the samples containing Neusilin US2, but that the decrease was small (<4%). The data suggest that the propensity for danazol to irreversibly adsorb to Neusilin US2 is low and unlikely to contribute significantly to the much larger decrease in danazol AP concentration observed for the $SEDDS_{Neusilin}$ formulations during *in vitro* dispersion−digestion testing.

3.2. Effect of Formulation Adsorption onto Neusilin US2 on the Properties of the Aqueous Phase Generated during *in Vitro* Dispersion and Digestion Testing. To further explore the effect of SEDDS adsorption onto Neusilin

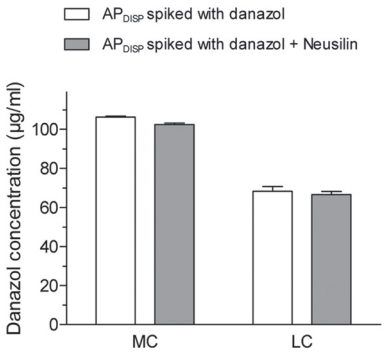

Figure 3. Changes in danazol concentration in the AP_{disp} of $SEDDS_{liquid}$ resulting from the addition of Neusilin US2 (mean ± SD, n = 4).

US2, the solubilization capacity (section 3.2.1) and oil droplet particle size (section 3.2.2) of the $SEDDS_{liquid}$ and $SEDDS_{Neusilin}$ formulations were compared.

3.2.1. Solubilization Capacity of AP_{disp} and AP_{digest}. The equilibrium solubility of crystalline danazol in the dispersed and digested SEDDS is shown in Figure 4, with white and gray bars

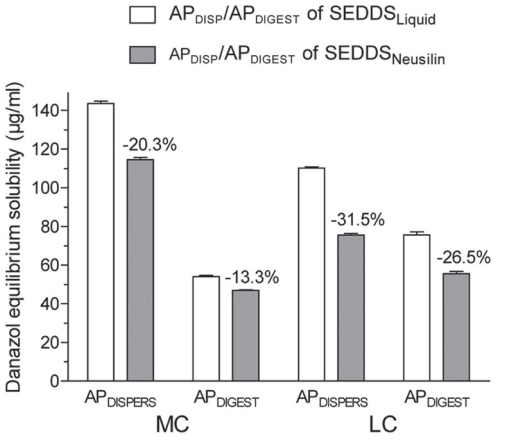

Figure 4. The equilibrium solubility of crystalline danazol in AP_{disp}/AP_{digest} of $SEDDS_{liquid}$ and $SEDDS_{Neusilin}$. Bars represent means (n = 3) ± SD. Numerical values refer to the percentage difference in solubilization capacity between $SEDDS_{liquid}$ and $SEDDS_{Neusilin}$.

describing observations for $SEDDS_{liquid}$ and $SEDDS_{Neusilin}$ respectively. Consistent with previous studies,[4,33,34] digestion of all formulations led to a decrease in solubilization capacity. This reflects changes to the physicochemical properties of the self-emulsified colloidal droplet phase during enzymatic processing by pancreatic lipases.[2,30,35] It was also apparent that the solubilization capacities of AP_{disp} and AP_{digest} generated by $SEDDS_{Neusilin}$ were lower than those generated by the corresponding $SEDDS_{liquid}$. This observation was consistent for formulations containing either MC or LC lipids. As Neusilin US2 was physically removed from the AP_{disp} and AP_{digest} samples prior to solubility determination,the lower solubilization capacity of the samples obtained after dispersing or

digesting $SEDDS_{Neusilin}$ suggests that the presence of Neusilin US2 indirectly altered (reduced) the solubilization capacity of the aqueous phase, most likely via incomplete desorption of the formulation during dispersion/digestion of $SEDDS_{Neusilin}$.

3.2.2. Particle Size of AP_{disp}. $SEDDS_{liquid}$ formulations self-emulsified rapidly in the aqueous media used during *in vitro* testing and formed ultrafine and near-transparent dispersions. In contrast, the emulsions formed by dispersion of the equivalent $SEDDS_{Neusilin}$ formulations (and subsequent removal of Neusilin US2 by low-speed centrifugation) appeared more turbid, particularly those containing LC lipid, suggesting differences in the oil droplet size.

To confirm this observation, the average particle size and polydispersity index (PDI) of dispersions generated by $SEDDS_{liquid}$ and $SEDDS_{Neusilin}$ were determined. These data are shown in Figure 5. The average particle size for all formulations (MC and LC lipid, liquid and adsorbed) were in the nanometer range, however, the particle size and PDI values for $SEDDS_{Neusilin}$ formulations were greater than those of their corresponding $SEDDS_{liquid}$, confirming initial observations. The extent of this increase in particle size was ~140% for MC formulations (35.2 nm cf. 83.8 nm) and ~180% for LC formulations (45.6 nm cf. 125.0 nm).

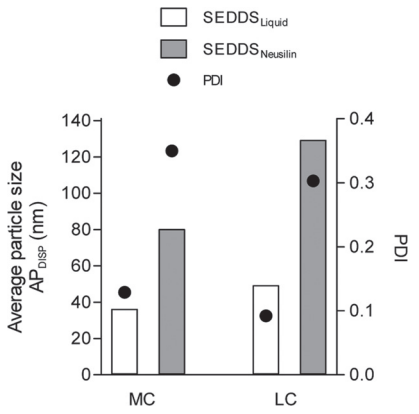

Figure 5. Average particle size (bars, left axis) and polydispersity index (PDI) (dots, right axis) of the microemulsions generated by dispersion of drug-free $SEDDS_{liquid}$ and $SEDDS_{Neusilin}$ in water at 25 °C.

Adsorption of components of the formulation preconcentrate onto Neusilin US2 was thought to be responsible for the change to dispersion properties. Since the degree of emulsification was reduced (and manifest as an increase in particle size), our initial working hypothesis to explain these changes was that preferential affinity of the formulation surfactant (Cremophor EL) for Neusilin US2 led to a decrease in the surfactant:lipid ratio in the aqueous phase, and consequently to an increase in particle size and a reduction in solubilization capacity.

The potential for adsorption to Neusilin US2 to decrease Cremophor EL (CrEL) content in the aqueous phase on dispersion and digestion of the $SEDDS_{Neusilin}$ formulations and to subsequently reduce the efficiency of emulsification was examined by generation of a series of $SEDDS_{liquid}$ formulations with varying surfactant concentrations and comparison of the particle size of these dispersions with that obtained after dispersion of $SEDDS_{Neusilin}$ (Figure 6). A reduction in the surfactant:lipid ratio in $SEDDS_{liquid}$ led to an increase in average particle size of the resultant dispersions. Further scrutiny of the results for the MC formulations (Figure 6A) revealed that the particle sizes of $SEDDS_{liquid}$ containing 20% (w/w) CrEL and $SEDDS_{Neusilin}$ containing 36% CrEL were comparable (77.8 and 79.6 nm, respectively), although the PDI value for $SEDDS_{Neusilin}$ was significantly higher (0.350 cf. 0.177).

For LC formulations, the effect of lowering CrEL concentrations on particle size was consistent with the results obtained for MC formulations. The extent of this effect, however, was more pronounced, as evidenced by a dramatic increase in particle size (and PDI) on decreasing surfactant concentration below 25% (Figure 6B). Again, the particle size and PDI of the dispersed $SEDDS_{Neusilin}$ formulation were higher than those of the equivalent $SEDDS_{liquid}$ formulation and more consistent with $SEDDS_{liquid}$ containing 20−25% CrEL.

3.2.3. Estimation of the Residual Adsorption of CrEL after Dispersion of $SEDDS_{Neusilin}$. To further probe the propensity for CrEL to remain adsorbed to Neusilin US2, experiments were carried out where Neusilin US2 powder containing increasing quantities of adsorbed CrEL was dispersed in digestion buffer, and the solubilization capacity of the dispersions obtained

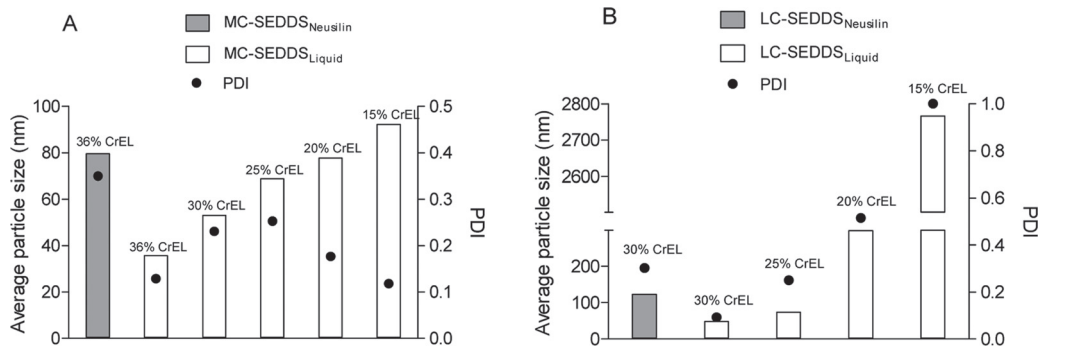

Figure 6. Average particle size (left axis) and polydispersity index (PDI) (right axis) of the microemulsions generated on dispersion of (A) drug-free MC-$SEDDS_{Neusilin}$ (containing 36% w/w CrEL) and MC-$SEDDS_{liquid}$ with decreasing CrEL content and (B) drug-free LC-$SEDDS_{Neusilin}$ (containing 30% w/w CrEL) and LC-$SEDDS_{liquid}$ with decreasing CrEL content.

(following removal of Neusilin US2 by centrifugation) was determined and compared with the solubilization capacity of solutions containing a known quantity of CrEL (see section 2.6 for more detailed description of methods). Danazol solubility in the latter solutions increased in a linear fashion with surfactant concentration (filled triangles, Figure 7), indicative of tradi-

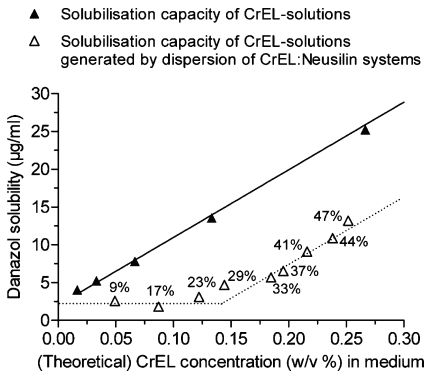

Figure 7. Solubilization capacity of CrEL solutions for danazol. Black triangles represent the solubilization capacity of CrEL solutions that were prepared by dissolving a known amount of CrEL in digestion buffer. The solid line is the linear regression for data points obtained above the critical micelle concentration and reflects increasing micellar solubilization (CMC CrEL ~0.01%). Open triangles represent the solubilization capacity of CrEL solutions that were generated by exposing CrEL:Neusilin samples (i.e., samples that were prepared by adsorbing known quantities of CrEL onto Neusilin US2) to digestion buffer. The percentages next to each symbol indicate the CrEL load (w/w %) on Neusilin US2 and the *X*-axis values represent the theoretical solution concentration in the event that all CrEL desorbed from the Neusilin US2 surface. Dashed lines are a guide for the eye and highlight the shift in solubilization capacity of CrEL in response to adsorption onto Neusilin US2. Increases in solubilization capacity are evident only above CrEL loads of ~30% w/w.

tional micellar solubilization. In contrast, the solubilizing capacity of the CrEL solutions generated by exposure of CrEL:Neusilin samples to digestion buffer were lower than the values that would be expected if all the CrEL desorbed from Neusilin US2 (open triangles, Figure 7). Indeed, a linear increase in solubilization capacity was only apparent for the samples with a CrEL loads above ~30% w/w. In contrast, for samples with lower CrEL, the solubilization capacity of the aqueous phases produced was low, and largely invariant. The data suggest that, below approximately 30% CrEL, little desorption of CrEL occurs and the solubilization capacity of the aqueous phase produced is not increased significantly beyond that of buffer alone.

3.3. *In Vivo* Data. The mean plasma concentration versus time profiles for danazol following oral administration of $SEDDS_{liquid}$ and equivalent $SEDDS_{Neusilin}$ formulations (for both MC and LC systems) are depicted in Figure 8. A summary of the pharmacokinetic parameters is provided in Table 1. To permit cross-comparison, a constant drug load of 12.5 mg/g in all formulations was maintained. The results of the *in vivo* study revealed that AUC_{0-8h} and C_{max} for danazol after administration of $SEDDS_{Neusilin}$ were significantly lower ($p < 0.05$) than those obtained after administration of the corresponding $SEDDS_{liquid}$ formulations and that this trend was replicated for both

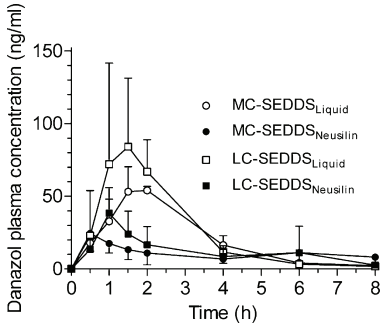

Figure 8. Plasma concentration vs time profiles for danazol after oral administration to overnight fasted rats. Treatments consisted of MC-$SEDDS_{liquid}$, MC-$SEDDS_{Neusilin}$, LC-$SEDDS_{liquid}$ or LC-$SEDDS_{Neusilin}$. All animals received a nominal dose of 3 mg. Average values ($n = 4$) ± SD are depicted.

medium-chain and long-chain lipid-based formulations. Indeed, the relative bioavailability (defined as the ratio of AUC_{0-8h} for $SEDDS_{Neusilin}$ and $SEDDS_{liquid}$) was only 50.7% and 41.8% for the MC and LC $SEDDS_{Neusilin}$ formulations respectively, illustrating that adsorption on Neusilin US2 was detrimental to SEDDS performance and consequently danazol bioavailability. No significant differences in T_{max} between $SEDDS_{liquid}$ and corresponding $SEDDS_{Neusilin}$ formulations were observed.

4. DISCUSSION

Self-emulsifying drug delivery systems (SEDDS) are widely used to improve the oral bioavailability of drugs with low aqueous solubility.[4−8,36,37] SEDDS are composed of a mixture of oils and surfactants, often supplemented with cosolvent, and are typically liquid or semisolid at room temperature. Unit dose forms of SEDDS are usually produced by filling liquid (or molten) excipient blends into soft or hard gelatin capsules. As summarized in the Introduction and reviewed in detail elsewhere,[18] there are a number of challenges associated with this manufacturing process. Increasing interest in overcoming the complexities of working with liquid-filled capsules has led to recent efforts to convert liquid LBFs into solid products through adsorption onto materials with a high specific surface area. Much of the published work on this emerging technology, however, has been biased toward technological outcomes such as improved flow and compression characteristics. In contrast, the potential impact of such an adsorption process on biopharmaceutical performance has remained largely under-investigated.

The main objective of the current study was to explore how adsorption of lipid formulations (SEDDS in this case) onto an inert carrier affects *in vitro* and *in vivo* performance. Effects on processability measures such as flowability and compressibility are well described elsewhere[19−21,23,24] and are not discussed further here. The performance of two model SEDDS, constructed as both liquid formulations ($SEDDS_{liquid}$) and liquid-loaded powder formulations ($SEDDS_{Neusilin}$) has been compared. The formulations investigated were based on those previously described by Porter et al.[4] and consisted of 54−60% w/w lipid [soybean and Maisine 35-1 (1:1) in long-chain (LC) lipid formulations, Captex and Capmul (1:1) in medium-chain (MC) formulations], 30−36% Cremophor EL and 10% ethanol. Neusilin US2, a spherical granular material consisting

Table 1. Summary of the Pharmacokinetic Parameters for Danazol after Oral Administration to Overnight Fasted Rats as MC-SEDDS$_{liquid}$, MC-SEDDS$_{Neusilin}$, LC-SEDDS$_{liquid}$ or LC-SEDDS$_{Neusilin}$[a,b]

	AUC_{0-8h} (ng·h/mL)	C_{max} (ng/mL)	T_{max} (h)	rel bioavailability[c] (%)
MC-SEDDS$_{liquid}$	139.4 ± 14.7	50.1 ± 6.1	1.8 ± 0.3	NA
MC-SEDDS$_{Neusilin}$	70.7 ± 18.0[d]	22.9 ± 8.4[d]	2.0 ± 2.7	50.7
LC-SEDDS$_{liquid}$	183.1 ± 82.0	89.2 ± 39.0	1.4 ± 0.5	NA
LC-SEDDS$_{Neusilin}$	76.6 ± 33.2[d]	37.2 ± 14.8[d]	1.1 ± 0.3	41.8

[a]Normalized to a 10 mg/kg dosage. The actual dosage varied across groups due to minor differences in the dosed mass of formulation and in weights of the animals. [b]Mean values (n = 4) and standard deviations are tabulated. [c]Relative bioavailability (%) of the SEDDS$_{Neusilin}$ in comparison to the corresponding SEDDS$_{liquid}$, expressed as the ratio of the dosage-normalized AUC_{0-8h}. [d]Statistically significant difference ($p < 0.05$) in comparison to the corresponding SEDDS$_{liquid}$.

of mesoporous magnesium aluminometasilicate, was used as adsorbent, as it is the most widely studied carrier for the solidification of LBFs. Danazol was used as a model drug. All formulations were subjected to *in vitro* evaluation in dynamic dispersion−digestion tests, and *in vivo* evaluation after oral administration to rats. Both the *in vitro* and *in vivo* data suggest pronounced performance differences between SEDDS$_{liquid}$ and SEDDS$_{Neusilin}$.

4.1. *In Vitro* and *in Vivo* Performance Differences between SEDDS$_{liquid}$ and SEDDS$_{Neusilin}$. The *in vitro* testing protocol used in the current study involved a 30 min dispersion phase followed by a 30 min digestion phase. Conditions employed during the *in vitro* digestion phase were consistent with those recently suggested by the LFCS Consortium.[30] On dispersion, the SEDDS$_{Neusilin}$ resulted in ∼35% lower solubilized danazol concentrations than the corresponding SEDDS$_{liquid}$ (Figure 1), an effect that was consistent for both MC and LC lipid formulations. The difference in aqueous phase concentration between SEDDS$_{liquid}$ and SEDDS$_{Neusilin}$ was also retained after initiation of digestion under simulated intestinal conditions.

The *in vitro* data suggested a reduced ability of SEDDS$_{Neusilin}$ to present danazol in a solubilized state on dispersion and digestion. These trends were also reflected in the *in vivo* studies (Figure 8; Table 1) where the relative bioavailability of danazol after administration of MC-SEDDS$_{Neusilin}$ and LC-SEDDS$_{Neusilin}$ was only 50.7% and 41.8%, respectively, when compared to the equivalent liquid formulation. While lipid chain length is a critical determinant of the ability of a formulation to retain solubilization capacity on digestion,[4,34,36−38] it proved less critical in determining patterns of desorption from the Neusilin US2 surface. Indeed the extent to which the lipid formulation desorbed from the Neusilin US2 pores on dispersion was approximately 65% for both MC- and LC-SEDDS$_{Neusilin}$.

4.2. Proposed Mechanism for the Decreased Performance of SEDDS$_{Neusilin}$. In the *in vitro* dispersion−digestion experiments (Figure 1), the SEDDS$_{Neusilin}$ formulation gave rise to lower aqueous phase danazol concentrations and higher amounts of drug recovered in the solid pellet phase when compared to the corresponding SEDDS$_{liquid}$. Importantly, the quantity of danazol in the pellet phase did not correlate with that expected based on typical precipitation behavior since the proportion of the drug dose recovered in the pellet was largely consistent (∼30−40% of the total dose) across a series of LC-SEDDS$_{Neusilin}$ with different drug loads (7.5, 12.5 and 20.5 mg/g) (Figure 2). Rather, the incomplete but equal percentage of the dose recovered in the pellet across formulations with different drug loads suggests that a fixed percentage of the dose remained entrapped inside the Neusilin US2 pores during dispersion.

In view of the very low propensity for danazol to adsorb directly onto the Neusilin US2 surface (Figure 3), the most plausible explanation for the incomplete recovery of drug in the aqueous phase was incomplete desorption of the adsorbed liquid formulation. This suggestion is supported by the much lower solubilization capacity of the drug-free SEDDS$_{Neusilin}$ formulation after dispersion and digestion when compared to the corresponding SEDDS$_{liquid}$ (Figure 4). The data in Figure 4 also show that the proportional loss in solubilization capacity on digestion is less pronounced for SEDDS$_{Neusilin}$ than for SEDDS$_{liquid}$, which may reflect a decrease in the concentration of digestible formulation components (CrEL and lipid) in the aqueous phase of dispersed SEDDS$_{Neusilin}$ (due to incomplete desorption of these excipients).

Photon correlation spectroscopy also revealed that the average particle size and polydispersity index of SEDDS$_{Neusilin}$ postdispersion was markedly higher than that of corresponding SEDDS$_{liquid}$ (Figure 5). While the relationship between the particle size of a lipid emulsion and *in vivo* performance remains poorly established,[39] the particle size data collected in the current study does provide insight into the effect of adsorption onto Neusilin US2 on SEDDS performance. The working hypothesis to explain the less effective self-emulsification of SEDDS$_{Neusilin}$ (higher average particle size, higher PDI) was a decrease in the effective concentration of surfactant in the aqueous phase due to irreversible adsorption to the carrier. This hypothesis is supported by the data in Figure 6 that show a similar increase in particle size for dispersed SEDDS$_{liquid}$ when the ratio of surfactant:lipid in the formulation was deliberately reduced.

Estimates of the extent of surfactant adsorption to carriers such as Neusilin US2 may be obtained via the construction of adsorption isotherms.[40−42] In the current application, however, the chemical complexity of CrEL (which consists of many different components) precluded facile quantitative analysis.[43] As such, an indirect estimate of changes to CrEL concentrations in solution as a function of adsorption to Neusilin US2 was gained via the use of functional (solubilization) end points. The data in Figure 7 compare the solubilization capacities for danazol, of CrEL solutions of known composition, with solutions obtained by preadsorption of equivalent masses of CrEL to Neusilin US2 and desorption via dispersion testing. The data show that the solubilization capacities of the dispersed CrEL:Neusilin systems were much lower than the equivalent quantities of CrEL in solution. The data also suggest that, below approximately 30% CrEL, very little CrEL is released, which may be indicative of a surface-associated layer that exhibits poor desorption properties. This data further supports the hypothesis that incomplete desorption of the liquid formulation components, and in particular the formulation

surfactant CrEL, from Neusilin US2 is responsible for the incomplete desorption of danazol on dispersion of $SEDDS_{Neusilin}$.

Further elucidation of the molecular determinants of the adsorption/desorption behavior of CrEL on Neusilin US2 was beyond the scope of the current study, but is the subject of ongoing investigations. The hydrophilicity of the Neusilin US2 surface[44] suggests that interactions with the more polar components of CrEL (polyethylene oxide) may play a role. Surfactant adsorption to inorganic surfaces has not been explored in detail in the pharmaceutical sciences, but has been well studied in many other industrial applications.[40−42,45,46] These studies suggest that nonionic surfactants may adsorb onto silica surfaces either as a monolayer or as aggregates at concentrations below the critical micelle concentration (CMC). In contrast, at concentrations close to the CMC, intercalated structures form that may evolve into bilayer structures at concentrations above the CMC.[42] A better understanding of the relationship between surfactant structure and adsorption behavior on pharmaceutical-grade high surface area materials and drug solubilization is clearly required in order to optimize the performance of adsorbed LBFs.

4.3. Conclusion. The results of the current study illustrate the need for caution when using adsorbents to facilitate the "solidification" of lipid formulations. The performance of two model self-emulsifying formulations of danazol adsorbed onto Neusilin US2 was significantly lower than that of the corresponding liquid formulations, a behavior consistent with data obtained using an *in vitro* dispersion−digestion model. The mechanism proposed to explain this behavior is the incomplete desorption of formulation excipients, and therefore incomplete desorption of drug (since drug is dissolved in the liquid excipient blend) as the formulation is processed in the gastrointestinal tract. While the extent to which this effect occurs is expected to be formulation and adsorbent dependent, the current data suggest that the potential advantages of solidification of lipid based formulations by adsorption onto high surface area excipients must be balanced carefully against the potential impact on *in vivo* performance.

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Notes

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■ REFERENCES

(1) Porter, C. J. H.; Trevaskis, N. L.; Charman, W. N. Lipids and lipid-based formulations: optimizing the oral delivery of lipophilic drugs. *Nat. Rev. Drug Discovery* **2007**, *6*, 231−248.

(2) Porter, C. J. H.; Wasan, K. M.; Constantinides, P. Lipid-based systems for the enhanced delivery of poorly water soluble drugs. *Adv. Drug Delivery Rev.* **2008**, *60*, 615−6.

(3) Porter, C. J. H.; Pouton, C. W.; Cuine, J. F.; Charman, W. N. Enhancing intestinal drug solubilisation using lipid-based delivery systems. *Adv. Drug Delivery Rev.* **2008**, *60*, 673−691.

(4) Porter, C. J. H.; Kaukonen, A. M.; Boyd, B. J.; Edwards, G. A.; Charman, W. N. Susceptibility to lipase-mediated digestion reduces the oral bioavailability of danazol after administration as a medium-chain lipid-based microemulsion formulation. *Pharm. Res.* **2004**, *21*, 1405−1412.

(5) Charman, S. A.; Charman, W. N.; Rogge, M. C.; Wilson, T. D.; Dutko, F. J.; Pouton, C. W. Self-Emulsifying Drug Delivery Systems: Formulation and Biopharmaceutic Evaluation of an Investigational Lipophilic Compound. *Pharm. Res.* **1992**, *9*, 87−93.

(6) Larsen, A.; Holm, R.; Pedersen, M. L.; Müllertz, A. Lipid-based formulations for danazol containing a digestible surfactant, Labrafil M2125CS: in vivo bioavailability and dynamic in vitro lipolysis. *Pharm. Res.* **2008**, *25*, 2769−2777.

(7) Sek, L.; Boyd, B. J.; Charman, W. N.; Porter, C. J. H. Examination of the impact of a range of Pluronic surfactants on the in-vitro solubilisation behaviour and oral bioavailability of lipidic formulations of atovaquone. *J Pharm. Pharmacol.* **2006**, *58*, 809−820.

(8) Chiang, P.-C.; Thompson, D. C.; Ghosh, S.; Heitmeier, M. R. A formulation-enabled preclinical efficacy assessment of a farnesoid X receptor agonist, GW4064, in hamsters and cynomolgus monkeys. *J. Pharm. Sci.* **2011**, *100*, 4722−4733.

(9) Aungst, B. J. Intestinal permeation enhancers. *J. Pharm. Sci.* **2000**, *89*, 429−442.

(10) Kossena, G. A.; Boyd, B. J.; Porter, C. J. .; Charman, W. N. Separation and characterization of the colloidal phases produced on digestion of common formulation lipids and assessment of their impact on the apparent solubility of selected poorly water-soluble drugs. *J. Pharm. Sci.* **2003**, *92*, 634−648.

(11) Constantinides, P. P.; Scalart, J.-P.; Lancaster, C.; Marcello, J.; Marks, G.; Ellens, H.; Smith, P. L. Formulation and Intestinal Absorption Enhancement Evaluation of Water-in-Oil Microemulsions Incorporating Medium-Chain Glycerides. *Pharm. Res.* **1994**, *11*, 1385−1390.

(12) Burcham, D. L.; Aungst, B. A.; Hussain, M.; Gorko, M. A.; Quon, C. Y.; Huang, S. M. The effect of absorption enhancers on the oral absorption of the GP IIB/IIIA receptor antagonist, DMP 728, in rats and dogs. *Pharm. Res.* **1995**, *12*, 2065−2070.

(13) Trevaskis, N. L.; Charman, W. N.; Porter, C. J. H. Lipid-based delivery systems and intestinal lymphatic drug transport: a mechanistic update. *Adv. Drug Delivery Rev.* **2008**, *60*, 702−716.

(14) Trevaskis, N.; McEvoy, C.; McIntosh, M.; Edwards, G.; Shanker, R.; Charman, W.; Porter, C. The Role of the Intestinal Lymphatics in the Absorption of Two Highly Lipophilic Cholesterol Ester Transfer Protein Inhibitors (CP524,515 and CP532,623). *Pharm. Res.* **2010**, *27*, 878−893.

(15) Seeballuck, F.; Lawless, E.; Ashford, M. B.; O'Driscoll, C. M. Stimulation of triglyceride-rich lipoprotein secretion by polysorbate 80: in vitro and in vivo correlation using Caco-2 cells and a cannulated rat intestinal lymphatic model. *Pharm. Res.* **2004**, *21*, 2320−2326.

(16) Strickley, R. G. Solubilizing excipients in oral and injectable formulations. *Pharm. Res.* **2004**, *21*, 201−230.

(17) Cole, E. T.; Cadé, D.; Benameur, H. Challenges and opportunities in the encapsulation of liquid and semi-solid formulations into capsules for oral administration. *Adv. Drug Delivery Rev.* **2008**, *60*, 747−756.

(18) Jannin, V.; Musakhanian, J.; Marchaud, D. Approaches for the development of solid and semi-solid lipid-based formulations. *Adv. Drug Delivery Rev.* **2008**, *60*, 734−746.

(19) Nokhodchi, A.; Hentzschel, C. M.; Leopold, C. S. Drug release from liquisolid systems: speed it up, slow it down. *Expert Opin. Drug Delivery* **2011**, 1−15.

(20) Agarwal, V.; Siddiqui, A.; Ali, H.; Nazzal, S. Dissolution and powder flow characterization of solid self-emulsified drug delivery system (SEDDS). *Int. J. Pharm.* **2009**, *366*, 44−52.
(21) Sander, C.; Holm, P. Porous Magnesium Aluminometasilicate Tablets as Carrier of a Cyclosporine Self-Emulsifying Formulation. *AAPS PharmSciTech* **2009**, *10*, 1388−1395.
(22) Dixit, R. P.; Nagarsenker, M. S. Optimized microemulsions and solid microemulsion systems of simvastatin: Characterization and in vivo evaluation. *J. Pharm. Sci.* **2010**, *9999*, 1−11.
(23) Hentzschel, C. M.; Sakmann, A.; Leopold, C. S. Suitability of various excipients as carrier and coating materials for liquisolid compacts. *Drug Dev. Ind. Pharm.* **2010**, 1−8.
(24) Mura, P.; Valleri, M.; Cirri, M.; Mennini, N. New solid self-microemulsifying systems to enhance dissolution rate of poorly water soluble drugs. *Pharm. Dev. Technol.* **2010**, 1−8.
(25) Hansen, T.; Holm, P.; Schultz, K. Process characteristics and compaction of spray-dried emulsions containing a drug dissolved in lipid. *Int. J. Pharm.* **2004**, *287*, 55−66.
(26) Balakrishnan, P.; Lee, B.-J.; Oh, D. H.; Kim, J. O.; Hong, M. J.; Jee, J.-P.; Kim, J. A.; Yoo, B. K.; Woo, J. S.; Yong, C. S.; Choi, H.-G. Enhanced oral bioavailability of dexibuprofen by a novel solid Self-emulsifying drug delivery system (SEDDS). *Eur. J. Pharm. Biopharm.* **2009**, *72*, 539−545.
(27) Shanmugam, S.; Baskaran, R.; Balakrishnan, P.; Thapa, P.; Yong, C. S.; Yoo, B. K. Solid Self-Nanoemulsifying Drug Delivery System (S-SNEDDS) Containing Phosphatidylcholine for Enhanced Bioavailability of Highly Lipophilic Bioactive Carotenoid Lutein. *Eur. J. Pharm. Biopharm.* **2011**, *79* (2), 250−257.
(28) Kang, M. J.; Jung, S. Y.; Song, W. H.; Park, J. S.; Choi, S. U.; Oh, K. T.; Choi, H. K.; Choi, Y. W.; Lee, J.; Lee, B. J. others Immediate release of ibuprofen from Fujicalin®-based fast-dissolving self-emulsifying tablets. *Drug Dev. Ind. Pharm.* **2011**, 1−8.
(29) Bakatselou, V.; Oppenheim, R. C.; Dressman, J. B. Solubilization and Wetting Effects of Bile Salts on the Dissolution of Steroids. *Pharm. Res.* **1991**, *8*, 1461−1469.
(30) Williams, H. D.; Sassene, P.; Kleberg, K.; Bakala N'Goma, J. C.; Calderone, M.; Jannin, V.; Igonin, A.; Partheil, A.; Marchaud, D.; Jule, E.; Vertommen, J.; Maio, M.; Blundell, R.; Benameur, H.; Carriere, F.; Mullertz, A.; Porter, C. J. H.; Pouton, C. W. Toward the establishment of standardized in vitro tests for lipid-based formulations, part 1: Method parameterization and comparison of in vitro digestion profiles across a range of representative formulations. *J. Pharm. Sci.* **2012**, *101* (9), 3360−3380.
(31) Cuiné, J. F.; Charman, W. N.; Pouton, C. W.; Edwards, G. A.; Porter, C. J. H. Increasing the Proportional Content of Surfactant (Cremophor EL) Relative to Lipid in Self-emulsifying Lipid-based Formulations of Danazol Reduces Oral Bioavailability in Beagle Dogs. *Pharm. Res.* **2007**, *24*, 748−757.
(32) Nguyen, T. H.; Hanley, T.; Porter, C. J. H.; Boyd, B. J. Nanostructured liquid crystalline particles provide long duration sustained-release effect for a poorly water soluble drug after oral administration. *J. Controlled Release* **2011**, *153* (2), 180−186.
(33) Anby, M. U.; Williams, H. D.; McIntosh, M.; Benameur, H.; Edwards, G. A.; Pouton, C. W.; Porter, C. J. H. Lipid digestion as a trigger for supersaturation: In vitro and in vivo evaluation of the utility of polymeric precipitation inhibitors in self emulsifying drug delivery systems. *Mol. Pharmaceutics* **2012**, *9* (7), 2063−2079.
(34) Kaukonen, A. M.; Boyd, B. J.; Porter, C. J. .; Charman, W. N. Drug solubilization behavior during in vitro digestion of simple triglyceride lipid solution formulations. *Pharm. Res.* **2004**, *21*, 245−253.
(35) Pouton, C. W. Formulation of poorly water-soluble drugs for oral administration: Physicochemical and physiological issues and the lipid formulation classification system. *Eur. J. Pharm. Sci.* **2006**, *29*, 278−287.
(36) Dahan, A.; Hoffman, A. Use of a Dynamic In Vitro Lipolysis Model to Rationalize Oral Formulation Development for Poor Water Soluble Drugs: Correlation with In Data and the Relationship to Intra-Enterocyte Processes in Rats. *Pharm. Res.* **2006**, *23*, 2165−2174.
(37) Han, S.; Yao, T.; Zhang, X.; Gan, L.; Zhu, C.; Yu, H.; Gan, Y. Lipid-based formulations to enhance oral bioavailability of the poorly water-soluble drug anethol trithione: Effects of lipid composition and formulation. *Int. J. Pharm.* **2009**, *379*, 18−24.
(38) Williams, H. D.; Sassene, P.; Kleberg, K.; Bakala N'Goma, J. C.; Calderone, M.; Jannin, V.; Igonin, A.; Partheil, A.; Marchaud, D.; Jule, E.; Vertommen, J.; Maio, M.; Blundell, R.; Benameur, H.; Carriere, F.; Mullertz,, C. J. H.; Pouton, C. W.; Porter, C. J. H. Towards the establishment of standardized in vitro tests for lipid-based formulations: 2) The effect of bile salt concentration and drug saturation level on the in vitro digestion of a range of representative lipid-based formulations. Unpublished results.
(39) Pouton, C. W.; Porter, C. J. H. Formulation of lipid-based delivery systems for oral administration: materials, methods and strategies. *Adv. Drug Delivery Rev.* **2008**, *60*, 625−637.
(40) Somasundaran, P.; Snell, E.; Fu, E.; Xu, Q. Effect of adsorption of non-ionic surfactant and non-ionic−anionic surfactant mixtures on silica−liquid interfacial properties. *Colloids Surf.* **1992**, *63*, 49−54.
(41) Somasundaran, P.; Krishnakumar, S. Adsorption of surfactants and polymers at the solid-liquid interface. *Colloids Surf., A* **1997**, *123*, 491−513.
(42) Rutland, M. W.; Senden, T. J. Adsorption of the poly (oxyethylene) nonionic surfactant C12E5 to silica: a study using atomic force microscopy. *Langmuir* **1993**, *9*, 412−418.
(43) Rowe, R. C.; Sheskey, P. J.; Owen, S. C. *Handbook of pharmaceutical excipients*; Pharmaceutical press: London, 2003; Vol. *4*.
(44) Gupta, M. K.; Tseng, Y.-C.; Goldman, D.; Bogner, R. H. Hydrogen bonding with adsorbent during storage governs drug dissolution from solid-dispersion granules. *Pharm. Res.* **2002**, *19*, 1663−1672.
(45) Paria, S.; Khilar, K. C. A review on experimental studies of surfactant adsorption at the hydrophilic solid−water interface. *Adv. Colloid Interface Sci.* **2004**, *110*, 75−95.
(46) Chang, C. H.; Franses, E. I. Adsorption dynamics of surfactants at the air/water interface: a critical review of mathematical models, data, and mechanisms. *Colloids Surf., A* **1995**, *100*, 1−45.

APPENDIX 5

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Toward the Establishment of Standardized *in Vitro* Tests for Lipid-Based Formulations. 2. The Effect of Bile Salt Concentration and Drug Loading on the Performance of Type I, II, IIIA, IIIB, and IV Formulations during *in Vitro* Digestion

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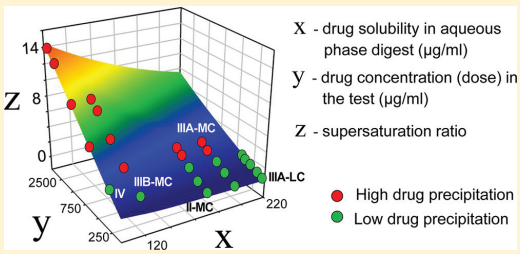
Supporting Information

ABSTRACT: The LFCS Consortium was established to develop standardized *in vitro* tests for lipid-based formulations (LBFs) and to examine the utility of these tests to probe the fundamental mechanisms that underlie LBF performance. In this publication, the impact of bile salt (sodium taurodeoxycholate, NaTDC) concentration and drug loading on the ability of a range of representative LBFs to generate and sustain drug solubilization and supersaturation during *in vitro* digestion testing has been explored and a common driver of the potential for drug precipitation identified. Danazol was used as a model poorly water-soluble drug throughout. In general, increasing NaTDC concentrations increased the digestion of the most lipophilic LBFs and promoted lipid (and drug) trafficking from poorly dispersed oil phases to the aqueous colloidal phase (AP_{DIGEST}). High NaTDC concentrations showed some capacity to reduce drug precipitation, although, at NaTDC concentrations ≥3 mM, NaTDC effects on either digestion or drug solubilization were modest. In contrast, increasing drug load had a marked impact on drug solubilization. For LBFs containing long-chain lipids, drug precipitation was limited even at drug loads approaching saturation in the formulation and concentrations of solubilized drug in AP_{DIGEST} increased with increased drug load. For LBFs containing medium-chain lipids, however, significant precipitation was evident, especially at higher drug loads. Across all formulations a remarkably consistent trend emerged such that the likelihood of precipitation was almost entirely dependent on the maximum supersaturation ratio (SR^M) attained on initiation of digestion. SR^M defines the supersaturation "pressure" in the system and is calculated from the maximum attainable concentration in the AP_{DIGEST} (assuming zero precipitation), divided by the solubility of the drug in the colloidal phases formed post digestion. For LBFs where phase



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separation of oil phases did not occur, a threshold value for SR^M was evident, regardless of formulation composition and drug solubilization reduced markedly above $SR^M > 2.5$. The threshold SR^M may prove to be an effective tool in discriminating between LBFs based on performance.

KEYWORDS: *poorly water-soluble drug, LFCS Consortium, lipid-based drug delivery systems, SEDDS, drug solubilization, in vitro digestion testing, solubility, bioavailability, in vitro models, precipitation, supersaturation*

INTRODUCTION

The delivery of poorly water-soluble drugs within a lipid-based formulation (LBF) can lead to an enhancement in oral bioavailability via several well-described processes.[1] These include improvements in drug solubilization in the intestinal milieu,[2−4] increased intestinal drug permeability,[5−7] and decreased first-pass metabolism in the enterocyte[8] or liver[9]—the latter via an increase in drug transport to the systemic circulation via the intestinal lymph.[10,11]

There are a number of different types of LBFs, including lipid suspensions, solid and semisolid systems, liquid crystalline materials such as liposomes, hexosomes, and cubosomes, and lipid complexes such as cochleates. To this point, however, LBFs comprising simple isotropic liquids, that may be subsequently filled into gelatin capsules, have been the most widely used in oral drug delivery. These LBFs typically contain a mixture of excipients including nonpolar and polar oils, nonionic surfactants, and cosolvents.[12] There are many different examples of excipients in each of these classes, and the prospective design of optimized LBFs is complex. Excipient choice is usually based on the need to dissolve the required drug dose in a unit dosage form, the ability to promote rapid initial dispersion and emulsification of the formulation in the gastrointestinal (GI) fluids, and ultimately, the capacity to maintain effective drug solubilization during dispersion and digestion of the formulation.[12,13] To assist description and comparison of the potentially large number of different LBFs, Pouton[14] recently proposed the Lipid Formulation Classification System (LFCS). This classification tool allows formulations to be described according to both basic composition and general behavior during *in vitro* dispersion and digestion. Although the LFCS has allowed a somewhat more harmonized discussion of LBFs, more widespread comparison of formulation performance has, until recently, been limited by the lack of standardized conditions for testing. To address this limitation, groups in academia and industry established the LFCS Consortium in 2009, with the key objectives of developing standardized *in vitro* methods for LBF assessment. In the first communication from the LFCS Consortium,[15] the preliminary conditions employed within the consortium for *in vitro* digestion testing were described, and a series of eight exemplar LBFs that include representatives of each LFCS class were introduced. Guidance for *in vitro* testing of LBFs was also provided, namely, the most appropriate model bile salt, the utility of back-titrations to better estimate the extent of LBF digestion, and the potential use of a benchtop centrifugation to more efficiently process samples removed during *in vitro* digestion experiments.[15]

In the present study, the *in vitro* performance of the eight LBFs, containing the model poorly water-soluble drug danazol (log *P* 4.5, solubility in water 1 μg/mL,[16] melting temperature 229 °C[17]), has been evaluated under conditions of increasing bile salt concentration and following increases to the drug saturation level in the formulation (drug load). Increasing drug load was explored as it afforded a more detailed evaluation of the propensity for LBFs to generate and maintain supersaturation during digestion. The consideration of supersaturation is timely given the increasing realization that the efficiency of many formulation strategies for poorly water-soluble drugs (including LBFs) is enhanced by the generation (and maintenance) of transiently supersaturated conditions in the GI tract or conversely limited by rapid drug recrystallization in order to adopt a lower-energy state.[18−21] The data presented here suggest that the concentration of bile salt employed in *in vitro* digestion tests has a moderate effect on the rate and extent of LBF digestion, but a significant impact on drug solubilization, and that the most critical driver of drug precipitation from digestible LBFs is the degree of supersaturation generated *in situ*.

MATERIALS AND METHODS

Materials. Maisine 35-1 and Transcutol HP were kind gifts of Gattefossé (Saint-Priest, France). Captex 300 and Capmul MCM EP were supplied by Abitec Corp (Columbus, Ohio). Cremophor EL was a gift from BASF Corporation (Washington, NJ). Corn oil and Tween 85 were purchased from Sigma Chemical Co. (St. Louis, Missouri). Further details of the lipidic excipients used within LFCS Consortium can be found in earlier work.[15] Danazol was from Sterling Pharmaceuticals (Sydney, Australia) and Indis (Aartselaar, Belgium). Sodium taurodeoxycholate >95% (NaTDC), 4-bromophenylboronic acid, and the porcine pancreatin extract (P7545, 8 × USP specifications activity) were all obtained from Sigma Chemical Co. (St. Louis, Missouri). Phosphatidylcholine (Lipoid E PC S, approximately 99.2% pure, from egg yolk) was obtained from Lipoid (Lipoid GmbH, Ludwigshafen, Germany). 1 M sodium hydroxide, which was diluted to obtain 0.2 and 0.6 M NaOH titration solutions, was purchased from Merck (Darmstadt, Germany). Water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA).

Selection of LBFs for Investigation in the LFCS Consortium. The compositions of the eight lipid-based formulations (LBFs) investigated by the consortium are shown in Table 1. Formulations were chosen to span the four classes of the Lipid Formulation Classification System (LFCS)[14] and contained either long-chain (LC) or medium-chain (MC) lipid excipients. Type I or type II formulations consist of water-insoluble components and are highly lipophilic. Type I systems show limited/no intrinsic self-emulsification capacity while type II systems self-emulsify to form turbid emulsions with oil droplets 0.25−2 μm in size.[14] Type III A/B formulations consist of oils mixed with a hydrophilic (i.e., high HLB) surfactant(s) and without/with cosolvent. The distinction between IIIA and IIIB formulations is based on the quantity of hydrophilic components where type IIIB contain larger quantities of cosolvent and surfactant, and typically self-emulsify to form dispersions with smaller particle sizes (<100 nm). Type IV formulations are essentially lipid-free and are miscible with aqueous fluids. Further details of the composition and rationale for choice of these formulations have been published previously.[15]

Drug Incorporation into the LBFs. Formulations were loaded with danazol at 80% of the saturated solubility of the drug in the formulations (which was measured at 37 °C) in the bile salt

Table 1. Composition of Eight Exemplar LBFs Investigated in This Study and Corresponding Danazol Equilibrium Solubility Values for Each LBF[a]

LBF type	composition (w/w)	danazol solubility in the LBF (mg/g)[b]
I-LC	50% corn oil	8.4 ± 1.1 at 37 °C
	50% Maisine 35-1	8.9 ± 0.5[c] at 25 °C
II-LC	65% Corn oil: Maisine 35-1 (1:1)	16.9 ± 1.7 at 37 °C
	35% Tween 85	10.8 ± 0.2 at 25 °C
IIIA-LC	65% Corn oil: Maisine 35-1 (1:1)	19.0 ± 1.6 at 37 °C
	35% Cremophor EL	16.9 ± 07 at 25 °C
I-MC	50% Captex 300	24.8 ± 0.9 at 37 °C
	50% Capmul MCM EP	22.6 ± 0.9 at 25 °C
II-MC	65% Captex 300:Capmul MCM EP (1:1)	27.2 ± 2.1 at 37 °C
	35% Tween 85	22.6 ± 1.1 at 25 °C
IIIA-MC	65% Captex 300:Capmul MCM EP (1:1)	28.3 ± 1.1 at 37 °C
	35% Cremophor EL	26.0 ± 0.8 at 25 °C
IIIB-MC	75% Capmul MCM EP:Cremophor EL (1:2)	48.7 ± 0.6 at 37 °C
	25% Transcutol HP	44.5 ± 0.9 at 25 °C
IV	50% Cremophor EL	65.5 ± 3.4 at 37 °C
	50% Transcutol HP	60.0 ± 2.7 at 25 °C

[a]Corn oil and Captex 300 are triglycerides of LC and MC lipids, respectively. Maisine 35-1 and Capmul MCM EP are blends of predominantly mono- and diglyceride and some triglyceride, of LC and MC lipids respectively. Tween 85 is a polyoxyethylene sorbitan trioleate surfactant (HLB 11). Cremophor EL is a polyethoxylated castor oil surfactant (HLB 14−16). Transcutol HP is diethylene glycol monoethyl ether cosolvent. See ref 15 for further details. LC, long-chain; MC, medium-chain. [b]Solubility values at 37 °C are taken from Williams et al.[15] [c]During the equilibration period, there was some evidence of phase separation of the lipids in the formulation, presumably reflecting a melting temperature of the formulation that was close to 25 °C.

study, and at 20%, 40%, 60%, 80%, and 90% of saturation level in the drug saturation study. Danazol solubility values are shown in Table 1. The required mass of danazol was weighed directly into clean screw-top glass vials, and drug-free LBF was added up to the target mass loading. Vials were sealed, vortex-mixed, and incubated at 37 °C for at least 24 h prior to testing. The danazol content in each manufactured formulation was verified (in triplicate) on the day of testing where accurately weighed samples were removed from the formulation, transferred to 5 mL volumetric flasks, and made up to volume with chloroform:methanol (2:1, v/v). Aliquots (50−100 μL) were diluted >10-fold with methanol and analyzed for danazol content by high performance liquid chromatography (HPLC)

***In Vitro* Evaluation of the LBFs.** *Digestion Experiments.* *In vitro* digestion experiments were performed as described previously by Williams et al.[15] In brief, the experimental setup employed by the LFCS Consortium consisted of a pH-stat apparatus (Metrohm AG, Herisau, Switzerland), comprising a Titrando 802 propeller stirrer/804 Ti Stand combination, a glass pH electrode (iUnitrode), and two 800 Dosino dosing units coupled to 10 mL autoburets (Metrohm AG). The apparatus was connected to a PC and operated using Tiamo 2.0 software (Metrohm AG).

The digestion buffer (pH 6.5) contained 2 mM Tris-maleate, 1.4 mM $CaCl_2 \cdot 2H_2O$, and 150 mM NaCl. Digestion buffer was supplemented with sodium taurodeoxycholate (NaTDC) and the phospholipid, phosphatidylcholine (PC). In the bile salt study, concentrations of NaTDC were 0 mM, 3 mM, 5 mM, and 10 mM and PC concentrations chosen such that a 4:1 bile salt:phospholipid (NaTDC:PC) concentration ratio was maintained. In the danazol saturation study, the NaTDC and PC concentrations were fixed at 3 mM and 0.75 mM respectively. Previous work has shown little difference between digestion rates for studies conducted in the presence of NaTDC or a mixture of bile salts more representative of those present in the small intestine,[15] and therefore, in the interests of cost and complexity, NaTDC was used throughout these studies.

LBFs (1 g) were weighed directly into a thermostat-jacketed glass reaction vessel (Metrohm AG) and dispersed for 10 min in 36 mL of digestion medium (37 °C). Continuous mixing was achieved using an overhead propeller stirrer 25 mm in diameter and rotating at a speed of ∼450 min^{-1}. The pH of the medium during the initial dispersion phase was manually adjusted to pH 6.5 ± 0.05 using small quantities of NaOH or HCl. Digestion was subsequently initiated via addition of 4 mL of pancreatin from porcine pancreas prepared as described previously[15] and providing ∼1000 TBU per mL of digest. Sodium hydroxide titration solutions (titrants) of 0.2 and 0.6 M were utilized for digestions containing LC and MC LBFs, respectively. Titrants were automatically added (controlled via the pH-stat) to the reaction vessel to maintain constant pH during digestion, with the rate of titrant addition reflecting the digestibility of the LBFs. Digestion tests were terminated after 30 min. To determine the total amount of fatty acid released (both ionized and un-ionized at pH 6.5), back-titrations were performed at the end of the digestion experiments.[15,22,23] In these experiments, the pH-stat was programmed to add 1 M sodium hydroxide to the reaction vessel at the end of the experimental period to increase the pH to 9 (and therefore to completely ionize all fatty acids present). The volume of 1 M sodium hydroxide required to raise the pH (corrected for the amount required to increase pH to 9 in the absence of lipid substrate in separate control experiments) reflects the quantity of un-ionized fatty acid present at the end of the experimental period. The quantity of ionized fatty acid originally detected at pH 6.5 plus the concentration of un-ionized fatty acid (determined by back-titration) provides the total amount of fatty acid released in response to digestion. This value was compared to the theoretical quantity of fatty acid that could be liberated if the lipid excipients were completely hydrolyzed to provide an estimation of the extent of LBF digestion using eq 1, and assuming that, on digestion, one triglyceride molecule released two fatty acid molecules and that one molecule of diglyceride or monoglyceride (initially present in the formulation) liberated a single fatty acid molecule.[24,25]

$$\text{extent (\%) of digestion} = \frac{\text{ionized fatty acid} + \text{un-ionized fatty acid}}{\text{theoretical maximum fatty acid from the LBF}} \times 100 \quad (1)$$

Collection and Separation of Samples Removed during in Vitro Digestion. Digestion samples were immediately treated with a digestion inhibitor (5 μL per 1 mL of digestion medium of 1.0 M 4-bromophenylboronic acid in methanol) to arrest digestion. Samples were then separated by centrifugation into three phases, namely, a poorly dispersed oily phase, a dispersed aqueous colloidal phase (AP_{DIGEST}), and a precipitated pellet phase. In line with previous recommendations,[15] the centrifugation step (ultracentrifugation or benchtop centrifugation) was tailored to the LBF type. The centrifugation method was also varied as a function of bile salt concentration. These details are summarized in Table 2. The drug content in the digestion

Table 2. Summary of Centrifugation Method Used To Separate Digestion Samples with Respect to LBF Type and Digestion Conditions[a]

LBF type	method of centrifugation used to separate digestion samples in this study[b]
I-LC	ultracentrifugation
II-LC	ultracentrifugation
IIIA-LC	ultracentrifugation; 0, 3, and 5 mM NaTDC
	bench-top centrifugation; 10 mM NaTDC
I-MC	ultracentrifugation; 0, 3, and 5 mM NaTDC
	bench-top centrifugation; 10 mM NaTDC
II-MC	ultracentrifugation; 0 mM NaTDC
	bench-top centrifugation; 3, 5, and 10 mM NaTDC
IIIA-MC	bench-top centrifugation
IIIB-MC	bench-top centrifugation
IV	bench-top centrifugation

[a]LC, long-chain; MC, medium-chain. NaTDC was the bile salt used in this work. [b]The method of centrifugation employed was dependent on the appearance of the digestion sample postcentrifugation. Samples showing evidence of an oily phase were separated by ultracentrifugation. Ultracentrifuge conditions were 400000*g* at 37 °C (Optima XL-100K centrifuge, SW-60 rotor, Beckman, Palo Alto, California) for 30 min. Bench-top centrifuge conditions were 21000*g* at 37 °C (Heraeus, Fresco 21, Thermo Scientific, Langenselbold, Germany) for 30 min.

phases obtained postcentrifugation was analyzed as described previously.[15]

Determination of Drug Solubility in AP_{DIGEST}. The solubility of danazol in the AP_{DIGEST} generated by digestion of blank (i.e., drug free) LBFs was evaluated after 30 min of digestion as described above. At this time point, samples of the digestion medium were removed and digestion was halted with digestion inhibitor (1.0 M 4-bromophenylboronic acid in methanol) before centrifugation as described in the section Collection and Separation of Samples Removed during in Vitro Digestion. The APDIGEST of each sample was collected and transferred to an Eppendorf tube that contained excess crystalline danazol. Vials were briefly vortex mixed and incubated at 37 °C. At time intervals, mixtures were centrifuged (21000*g*, Heraeus Fresco 21, Thermo Scientific) at 37 °C for 30 min to sediment undissolved drug and 50−100 μL of the supernatant was removed and diluted >10-fold with methanol prior to analysis by HPLC

Solubility in the AP_{DIGEST} was assessed over a 24 h period of equilibration. For some LBFs, solubility values decreased over time. In the most extreme example, danazol solubility at 24 h in the AP_{DIGEST} from the type IIIA-LC formulation digested in the presence of 3 mM NaTDC was approximately 50% of the value obtained at 4 h. Consistent with previous studies,[18] the decrease was attributed to a kinetic instability of the colloids formed following digestion. This was most pronounced at 0 and 3 mM NaTDC concentrations. To preclude complications arising from physical instability of the isolated digests, solubility was frequently assessed during the initial 4 h equilibration period followed by later sampling typically after 8 or 24 h. In incidences where the solubility values decreased at later time periods during the solubility study, values obtained during the first 4 h were utilized. Where the solubility did not decrease over time (presumably reflecting a higher stability of the colloids), solubility values at 8 and 24 h were typically used. Solubility was defined as the mean value obtained over at least two time points where the change in solubility was less than 5%.

Solubility values in $AP_{DIGESTS}$ were used to calculate the supersaturation ratio (SR) obtained at 30 min digestion via eq 2:

$$SR = \frac{\text{solubilized drug concentration in } AP_{DIGEST}}{\text{drug solubility in } AP_{DIGEST}} \quad (2)$$

Equation 3 was used to calculate the maximum supersaturation ratio (SR^M), which is the ratio between the maximum theoretical concentration of solubilized drug (in the absence of any drug precipitation; AP_{MAX}) and drug solubility in the AP_{DIGEST}:

$$SR^M = \frac{\text{maximum drug concentration in } AP_{DIGEST}(AP_{MAX})}{\text{drug solubility in } AP_{DIGEST}} \quad (3)$$

HPLC Detection of Danazol. All HPLC analyses were conducted using a Waters Alliance 2695 Separation Module (Waters Alliance Instruments, Milford, MA) with a reverse-phase C_{18} column (150 × 15 mm^2, 5 μm, Waters Symmetry) and C_{18} security guard cartridge (4 × 2.0 mm, Phenomenex, Torrence, CA). The injection volume was 50 μL, and UV detection was at 286 nm. The mobile phase consisted of methanol and water in a 75:25 v/v ratio and was pumped through the column at a 1 $mL{\cdot}min^{-1}$ flow rate. Validation details for the danazol assays have been previously reported.[26]

Statistics. One-way ANOVA tests (followed by Tukey's test for significance) were performed using GraphPad Prism 5.04 (GraphPad Software, La Jolla, CA). Statistical significance was identified at a level of $p < 0.05$.

RESULTS

Effect of Increasing Bile Salt on the *in Vitro* Performance of LBFs. *Effects on Rate and Extent of Digestion.* The effect of increasing bile salt (NaTDC) concentration on the liberation of titratable fatty acid during *in vitro* digestion of the eight LBFs is shown in Figure 1. The titration profiles in Figure 1 are corrected for background quantities of fatty acid arising from digestion of the phospholipid in the medium (see Supporting Information for the titration profiles of NaTDC/phospholipid micelles alone). The data obtained at 3 mM NaTDC are reproduced from ref 15. Since the ionization of liberated fatty acids was incomplete at pH 6.5 (the pH of the digestion experiments),[15] complete ionization was forced via rapid elevation of the pH of the digest to pH 9 at the end of the experimental period via back-titration experiments.[23,27] Values for total fatty acid (ionized plus un-ionized) were incorporated into eq 1 to estimate the total extent of digestion for the evaluated formulations over 30 min. Data describing titrated fatty acid, total fatty acid (in bold), and the extent of LBF digestion are presented in Table 3.

For several LBFs, and in particular the LC formulations (type I, II, and IIIA) and the type I-MC formulation (upper panels, Figure 1), increasing NaTDC concentration increased the initial rate of fatty acid titration and/or increased in the duration over which additional liberated fatty acid was detected. In the absence of NaTDC, the digestion of the LC formulations (type I, II, and IIIA) and type I-MC and II-MC formulations was much slower compared to equivalent tests conducted in the presence of NaTDC. In the case of the MC formulations, however, no differences were evident across the different NaTDC concentrations (lower panel, Figure 1). Small differences in titrated fatty acid were observed for the type IV formulation, however accurate attribution of this to increased LBF digestion in the presence of

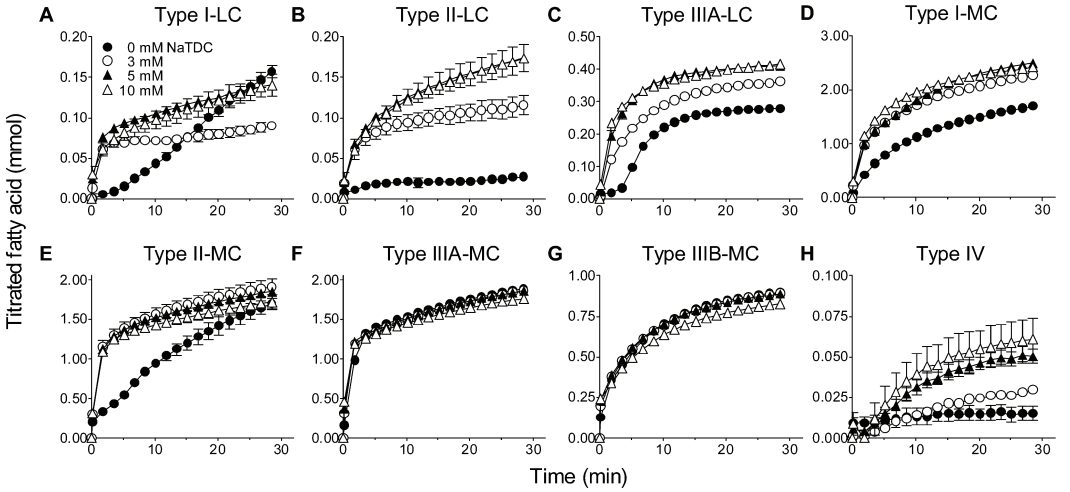

Figure 1. Apparent titration of fatty acids released during *in vitro* digestion of eight lipid-based formulations (LBFs) in conditions of increasing bile salt (NaTDC) concentration. Digestion was initiated at $t = 0$ min by the addition of pancreatin, and pH was maintained constant at pH 6.5 during LBF digestion. Titrated fatty acid was detected by pH titration. Values are expressed as means ($n = 3$) ± SD with background correction for the level of fatty acid released in background digestion tests (i.e., in the absence of LBFs). The formulation compositions can be found in Table 1. Formulations contained danazol at 80% of its saturated solubility in the formulation.

Table 3. The Effect of Bile Salt (NaTDC) Concentration (0, 3, 5, and 10 mM) on the Quantity of Titrated Fatty Acid (Ionized), Total Fatty Acid (Un-Ionized plus Ionized) Liberated Following 30 min Digestion of a Range of LBFs, the Un-Ionized:Ionized Fatty Acid Ratio, and Estimations for the Extent of Total LBF Digestion[a]

	titrated (ionized)/total fatty acid released[b] (mmol)				un-ionized:ionized fatty acid ratio[c]				extent of digestion[d] (%)			
LBF type	0[e]	3	5	10	0	3	5	10	0	3	5	10
I-LC	0.16/**0.65**	0.09/**0.54**	0.14/**0.73**	0.15/**0.86**	3.06	5.00	4.21	4.73	30	25	33	39
II-LC	0.03/**0.43**	0.12/**0.54**	0.18/**1.01**	0.18/**1.05**	13.33	3.50	4.61	4.83	22–30	27–38	51–71	53–73
IIIA-LC	0.28/**1.37**	0.36/**1.32**	0.42/**1.40**	0.42/**1.35**	3.89	2.67	2.33	2.21	74–96	71–92	76–98	73–95
I-MC	1.73/**2.73**	2.30/**3.44**	2.52/**3.70**	2.45/**3.51**	0.58	0.50	0.47	0.43	70	88	95	90
II-MC	1.70/**2.53**	1.93/**2.53**	1.87/**2.50**	1.73/**2.66**	0.49	0.31	0.34	0.54	81–100	82–100	80–98	86–105
IIIA-MC	1.91/**2.70**	1.88/**2.67**	1.88/**2.61**	1.78/**2.44**	0.41	0.42	0.39	0.37	91–106	90–105	88–103	82–96
IIIB-MC	0.90/**1.30**	0.90/**1.16**	0.90/**1.15**	0.84/**1.12**	0.44	0.29	0.28	0.33	83–136	74–121	72–120	72–117
IV	0.02/**0.04**	0.03/**0.10**	0.05/**0.07**	0.06/**0.14**	1.00	2.33	0.40	1.33	7	16	12	23

[a]The formulation compositions can be found in Table 1. [b]Titrated (ionized) fatty acid at pH 6.5. Total fatty acid is ionized plus fatty acid that is un-ionized (and not quantified) at pH 6.5, but ionized and quantified by back-titration at pH 9. [c]Un-ionized fatty acid divided by ionized fatty acid. [d]Values for the extent of digestion of type II and IIIA/B lipid-based formulations (LBFs) are provided as a range spanning values calculated using lower and upper theoretical maximum FA levels based on complete/incomplete digestion of the formulation surfactant.[15] [e]NaTDC concentration (mM).

increasing NaTDC was made difficult by the very low fatty acid concentrations (<0.1 mmol).

The data in Table 3 compares the final concentration of titrated fatty acid (i.e., the concentration of ionized fatty acid at 30 min) with total fatty acid released (i.e., ionized plus un-ionized), and reveals first that quantification by titration underestimated digestion in all cases by virtue of incomplete fatty acid ionization at pH 6.5. Second, differences were also evident in the degree to which the titration profiles captured patterns of digestion as a function of NaTDC concentration, when compared to calculations of total fatty acid release.

For the type I-LC and II-LC and type I-MC formulations, the addition of NaTDC gave rise to an increase in the concentration of titrated fatty acid and also an increase in the extent of LBF digestion calculated via back-titration (Table 3). This was most marked for the type II-LC formulation where increasing NaTDC from 0 mM to 10 mM led to a 30–40% increase in digestion of the LBFs (Table 3). However, despite showing increased levels of titrated fatty acid with increasing NaTDC, total digestion of the type IIIA-LC formulation did not increase (Table 3). Rather, the differences observed in the titration profiles appear to reflect a change (decrease) in the un-ionized:ionized fatty acid ratio with increasing NaTDC (from 3.9 to 2.2), indicative of a change to the apparent pK_a of the liberated fatty acid with change in NaTDC concentration. This observation was evident for all MC or LC lipid-containing LBFs (Table 3), although the effects of NaTDC on the un-ionized:ionized fatty acid ratio were more pronounced for those formulations containing LC lipid and those formulations that showed a low extent of digestion (i.e., type I-LC and II-LC formulations).

At NaTDC concentrations of up to 10 mM, type I-LC and II-LC formulations and the lipid-free type IV formulation

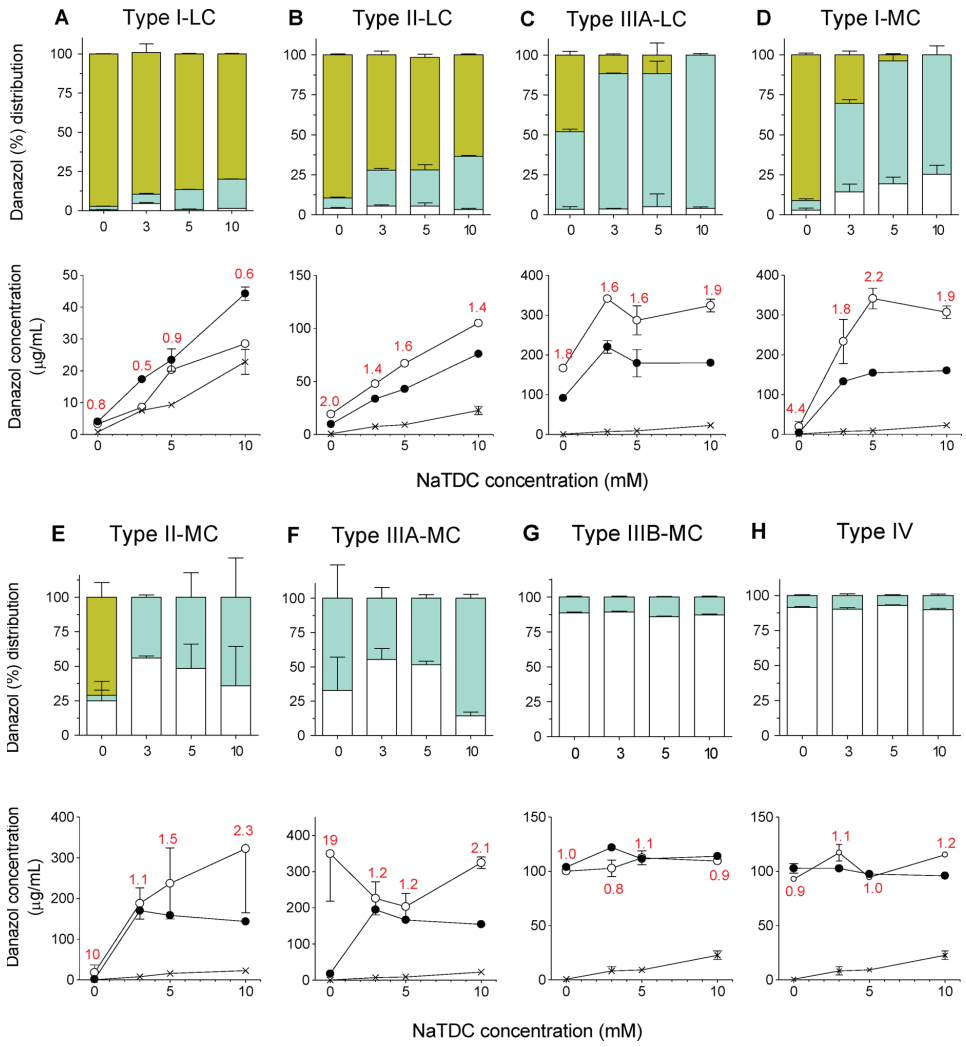

Figure 2. Effect of increasing bile salt (NaTDC) concentration on the performance of eight lipid-based formulations (LBFs) following 30 min *in vitro* digestion. Upper panels for each LBF type show the distribution of drug across partially digested oil phase (dark shaded bars), colloidal aqueous phase, AP_{DIGEST} (lightly shaded bars), and pellet phase (white bars). Lower panels plot danazol concentrations in the colloidal AP_{DIGEST} during the kinetic digestion experiments (○), danazol solubilities in the colloidal AP_{DIGEST} (●), and danazol solubilities in NaTDC/phospholipid micelles (no LBFs) (×).Values are expressed as means ($n = 3$) ± SD. Numerical values in red indicate the supersaturation ratio (SR) at 30 min (eq 2). Formulations contained a danazol load that was equivalent to 80% of its saturated solubility in the formulation, which, along with the formulation compositions, can be found in Table 1.

remained partially digested. For the LC formulations, the extent of digestion at each NaTDC concentration decreased from type IIIA-LC > II-LC > I-LC becoming less efficient as the lipid concentration in the formulation increased. For MC formulations, digestion was largely complete under all NaTDC conditions.

Aside from changes to the total extent of digestion and the unionized:ionized fatty acid ratio, the presence of bile salt in some cases also affected the titration profile shape. Notable examples include type I-LC and IIIA-LC formulations and the type II-MC formulation, with all showing a decrease in the relative rate of digestion and/or a lag in the period between initiation of digestion and detection of fatty acid in the absence of NaTDC (Figure 1). This was not observed after the addition of NaTDC, even at the lowest 3 mM concentration where the same LBF types exhibited a rapid initial rate of fatty acid release.

Drug Distribution and Solubilization Patterns Postdigestion. The effect of NaTDC concentration on the solubilization patterns of incorporated drug (danazol) following 30 min digestion of the eight LBFs is shown in Figure 2. To support the interpretation of the results of drug distribution in the upper panels in Figure 2, danazol solubility was also determined in the colloidal aqueous phase (AP_{DIGEST}) obtained following digestion of drug-free (blank) LBFs. Solubility values are plotted as a function of NaTDC concentration in the lower panels in Figure 2

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(filled circles) and are compared to the danazol concentrations measured in the AP_{DIGEST} during kinetic digestion experiments (open circles). The supersaturation ratio (SR) i.e., the concentration attained in the AP_{DIGEST} at 30 min relative to the drug solubility in the equivalent AP_{DIGEST}, is shown in red in Figure 2. Data describing drug solubility in simple NaTDC/phospholipid micelles (i.e., in the absence of formulation) is also presented (crosses, Figure 2).

For type I formulations constructed with either MC or LC lipid, and type II/IIIA formulations with LC lipids, the quantity of drug in the poorly dispersed oil phase decreased with increasing NaTDC concentration, and this was reflected in corresponding increases in drug concentrations in the AP_{DIGEST} (Figure 2A−D). These observations were consistent with visual assessment of digestion samples postcentrifugation where the quantity of the poorly dispersed oil phase decreased with increasing NaTDC. As increasing NaTDC concentration increased the digestion of type I-LC, I-MC, and II-LC LBFs (Table 3), it seems likely that NaTDC decreased the proportion of drug in the oil phase for these LBFs by improving digestion and by enhancing the solubilization of the lipid digestion products, which in turn promoted drug transfer from the less completely digested (and less readily dispersed) oil phase to the AP_{DIGEST}. While the type IIIA-LC formulation also showed evidence of progressively lower drug concentrations in the oil phase with increasing NaTDC (Figure 2C), the extent of digestion of this formulation was largely consistent across the NaTDC concentration range (Table 3). The data suggest that in this case the oil phase consisted primarily of phase-separated lipid digestion products (rather than undigested lipid), and that drug transfer to the AP_{DIGEST} with increasing NaTDC reflected increasing solubilizing power in the AP_{DIGEST} (due to enhanced solubilization of digestion products and drug in the presence of higher concentrations of NaTDC), rather than more efficient digestion.

The pellet phase in centrifuged digestion samples consists of insoluble calcium soaps of fatty acid and any precipitated drug. The quantity of drug in the pellet phase obtained from digestion of the LC-lipid formulations was low (<10%) across the NaTDC concentration range (Figure 2A−C). Determination of danazol solubility in the colloidal AP_{DIGEST} obtained from the type I-LC and II-LC formulations revealed that the solubilization capacity of this phase increased with increasing NaTDC concentration. The extent of solubility increase (i.e., the gradient in the plots in lower panels; Figure 2) was greater than that observed for simple NaTDC/phospholipid media (closed circles vs cross symbols; Figure 2), suggesting that solubilization of progressively higher concentrations of digestion products in the AP_{DIGEST} (resulting from increased digestion) was possible and contributed significantly to drug solubilization. For the type I-LC formulation, danazol solubility in the AP_{DIGEST} (i.e., the solubilization capacity of this phase) across the NaTDC concentration range was above the drug concentrations measured in this phase during drug distribution experiments, which ensured that the AP_{DIGEST} was not supersaturated (SR < 1) and explains the lack of drug precipitation. For most other formulations, however, the measured danazol concentrations in the AP_{DIGEST} were higher than the equivalent solubility value in this phase resulting in AP_{DIGEST} that were supersaturated (SR >1). The degree of supersaturation for type II-LC and IIIA-LC formulations was largely independent of NaTDC concentration since the trends in danazol solubility in the AP_{DIGEST} and the danazol concentrations measured in this phase in the drug distribution experiments were similar (as seen in Figure 2B,C; lower panels).

The AP_{DIGEST} obtained on digestion of the type I-MC formulation also contained progressively more drug with increasing NaTDC concentration up to 5 mM (Figure 2D). In this case, however, and indeed, with all the MC-lipid containing formulations, there was also evidence of drug precipitation. For the type I-MC formulation, precipitation occurred in parallel with a decrease in the quantity of drug in the poorly dispersed oil phase (Figure 2D), and a change in the appearance of the AP_{DIGEST} from turbid at 0 and 3 mM NaTDC to a clearer solution at higher (5 and 10 mM) NaTDC concentrations suggested greater solubilization of the digestion products in this phase.

For the type II-MC formulation (Figure 2E), digestion in the absence of NaTDC resulted in the formation of a dense oil phase that phase-separated forming a layer immediately above the pellet on centrifugation. This oil phase contained ∼70% of the drug in the digest whereas the AP_{DIGEST} component contained <5% of incorporated drug. Danazol solubility in the AP_{DIGEST} was also very low ($1.2 \pm 0.3\ \mu g/mL$) and comparable to the solubility in simple digestion buffer ($0.8 \pm 0.2\ \mu g/mL$), yet the extent of digestion of this formulation was high (>80%; Table 3). The high drug concentrations in the dense lipid phase suggest that this phase contained the majority of the hydrophobic digestion products from the type II-MC formulation (i.e., protonated fatty acid and monoglyceride) and that this oil phase retained much of the solubilization capacity of the digested formulation, whereas the AP_{DIGEST} contained the more hydrophilic digestion products (i.e., ionized fatty acids), and these failed to significantly increase the solubilization capacity of what remained of the AP_{DIGEST}. This oil phase was not observed in the presence of NaTDC (Figure 2E), and therefore, consistent with the trends observed with the type IIIA-LC formulation, increasing NaTDC appeared to enhance AP_{DIGEST} solubilization of lipid digestion products,[28] rather than increase total digestion (Table 3). The increase in solubilization of the AP_{DIGEST} in the presence of 3 mM NaTDC also occurred coincidently with higher levels of drug precipitation, indicating that better dispersion of the digestion products in the presence of NaTDC forced a distinction between drug solubilization in the AP_{DIGEST} or precipitation.

A trend toward decreasing drug precipitation for the type II-MC LBFs at 5 mM and 10 mM NaTDC was apparent, although the large error bars in Figure 2E reflect a significant variability in this decrease. For the type IIIA-MC formulation, there was a more marked decrease in danazol precipitation on increasing NaTDC (Figure 2F), with levels of precipitated drug decreasing from 56% at 5 mM bile salt to <15% at 10 mM. Increasing NaTDC concentration did not lead to a corresponding increase in danazol solubility in the AP_{DIGEST} for either type II-MC or IIIA-MC formulations (lower panels; Figure 2E,F), and the higher drug concentrations in the AP_{DIGEST} at 10 mM NaTDC resulted in increased supersaturation.

In contrast, increasing NaTDC did not affect the performance of type IIIB-MC and IV formulations during digestion (upper panels; Figure 2G,H). In these systems, the majority (>85%) of danazol precipitated within 30 min digestion, regardless of NaTDC concentration, and the drug concentration in the AP_{DIGEST} was essentially identical to the solubility of drug in this phase.

Effect of Drug Saturation Level (Load) on the *in Vitro* Performance of LBFs. *Effect on Formulation Digestion.* The effect of incorporation of danazol at various drug loadings (expressed as a % of the drug solubility in the LBF) on the digestion of type II-LC and II-MC LBFs is shown in Figure 3. It is evident that the presence of the drug and increasing drug loading in the type II formulations had negligible effect on the level of

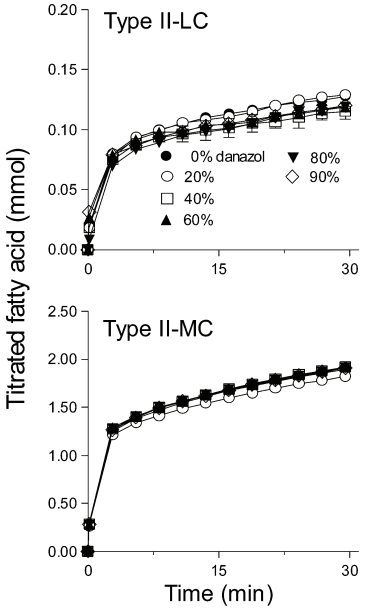

Figure 3. Apparent titration of fatty acid release during *in vitro* digestion of type II-LC and II-MC lipid-based formulations (LBFs) containing an increasing danazol saturation level (drug loading). Values are expressed as means ($n = 3$) ± SD with background correction for the level of fatty acid released in background digestion tests (i.e., in the absence of LBFs). The formulation compositions can be found in Table 1. The effect of danazol on the digestion of all other LBFs was also limited and can be found in the Supporting Information.

titrated fatty acid released from the formulation. The total level of formulation digested (estimated by back-titrations) also varied little across the drug loadings. Similar results were obtained for all other LBF types investigated in this study (see Supporting Information). Taken together, these results confirmed that digestion (rate/extent) of these LBF types was largely unaffected by the drug.

Effect on Drug Distribution and Solubilization Patterns. The effect of danazol loading in LBFs at 20%, 40%, 60%, 80%, and 90% saturation level on the *in vitro* performance of the LBFs following digestion is shown in Figure 4. For each formulation, the effect of increasing drug load on the pattern of drug distribution across the digestion phases (upper panels) and the effect on solubilized danazol concentrations in the colloidal AP_{DIGEST} at 30 min (lower panels) is shown.

For the type I-LC formulation, initial observation of the data in Figure 4A (upper panel) suggests limited effects of drug load on the patterns of drug distribution across the oil phase, AP_{DIGEST}, and pellet phase. Closer observation, however, reveals a slight increase in the proportion of drug in the oil phase and a corresponding decrease in the proportion of drug in the AP_{DIGEST} with increasing drug load. As such, the decrease in proportional distribution into the AP_{DIGEST} is offset by the increase in absolute drug quantity in the formulation, and the concentration of drug in the AP_{DIGEST} was quite consistent across the differing levels of saturation (lower panel; Figure 4A).

The patterns of drug distribution across the digestion phases for the type II-LC and IIIA-LC formulations were remarkably consistent with increasing drug load (Figure 4B,C), and as a result, drug concentrations in the AP_{DIGEST} progressively increased in a dose-dependent manner, eventually leading to the generation of colloidal phases in the AP_{DIGEST} that were supersaturated. This was first evident at 60% drug saturation in the formulation for both the type II-LC and IIIA-LC formulations. The absolute drug concentrations in the colloidal AP_{DIGEST}, however, were significantly higher for the type IIIA-LC formulation, reflecting the higher drug solubility, and therefore drug load, in this formulation, and the fact that there was less phase-separated oil. Since there was little or no drug precipitation, the supersaturation ratio (SR) 30 min postdigestion increased with increasing drug load in type II-LC and IIIA-LC formulations. The highest SR value was obtained at the highest drug load in both formulations and was ∼1.7.

The drug distribution patterns for the type I-MC LBFs were unchanged at danazol drug loads equivalent to 20−60% saturation in the formulation, and the majority (∼70%) of the drug was solubilized in the AP_{DIGEST} (upper panel; Figure 4D). The consistency in % distribution into the AP_{DIGEST} in parallel with an increase in drug load resulted in a progressive increase in drug concentrations in the AP_{DIGEST} from 81.6 ± 2.6 μg/mL at 20% saturation in the formulation to 214.4 ± 4.9 μg/mL at 60% saturation. Drug concentrations exceeded the solubility limit in the AP_{DIGEST} (132.8 ± 1.7 μg/mL) at drug loads in excess of 40% saturation. An increase in drug precipitation was evident at higher drug loads (>60% saturation), and therefore, the use of high drug loadings did not lead to better performance (i.e., increased drug concentration in the AP_{DIGEST}).

A similar trend toward an initial resistance to precipitation on increasing drug dose (and therefore increase in drug concentration in the AP_{DIGEST}) followed by the attainment of a "tipping point", where drug precipitation is initiated, was evident for the type II-MC (Figure 4E) and type IIIA-MC formulations (Figure 4F). However, in contrast to the type I-MC formulation, the extent of drug precipitation from type II-MC and IIIA-MC formulations was greater, and sufficient to rapidly decrease danazol concentrations in the AP_{DIGEST} toward the solubility limit, effectively removing any supersaturation pressure to precipitation. For the LBFs where significant precipitation occurred, the critical drug load (expressed as % saturation) required to stimulate precipitation followed the rank order type II-MC (>60%) > type IIIA-MC (>40%) > type IIIB-MC (>20%) > type IV (20%). The highest drug concentrations in the AP_{DIGEST} following 30 min digestion were therefore evident at these critical drug loads as increasing drug loading further led to precipitation. The respective SR values at these critical drug loads were 2.1 (II-MC), 1.5 (IIIA-MC), 1.9 (IIIB-MC), and 1.7 (IV). At higher drug loads, drug precipitation occurred to an extent that drug concentrations in the AP_{DIGEST} were similar to the solubilities in the respective AP_{DIGEST}, and therefore, SR values were close to unity.

Comparison of LBF performance with increasing drug load using the % of drug solubility in the formulation (i.e., saturation level) as a measure of drug load allows for a mechanistic evaluation of formulation performance at constant thermodynamic activity, but fails to acknowledge that the absolute drug load and, therefore, the absolute drug concentration that may be attained in the AP_{DIGEST} are likely to be different across LBF types. In an attempt to provide this additional comparison, Figure 5 summarizes the performance of LBFs using absolute drug load in milligrams and the concentration attained in the AP_{DIGEST}. The highest danazol AP_{DIGEST} concentration achieved

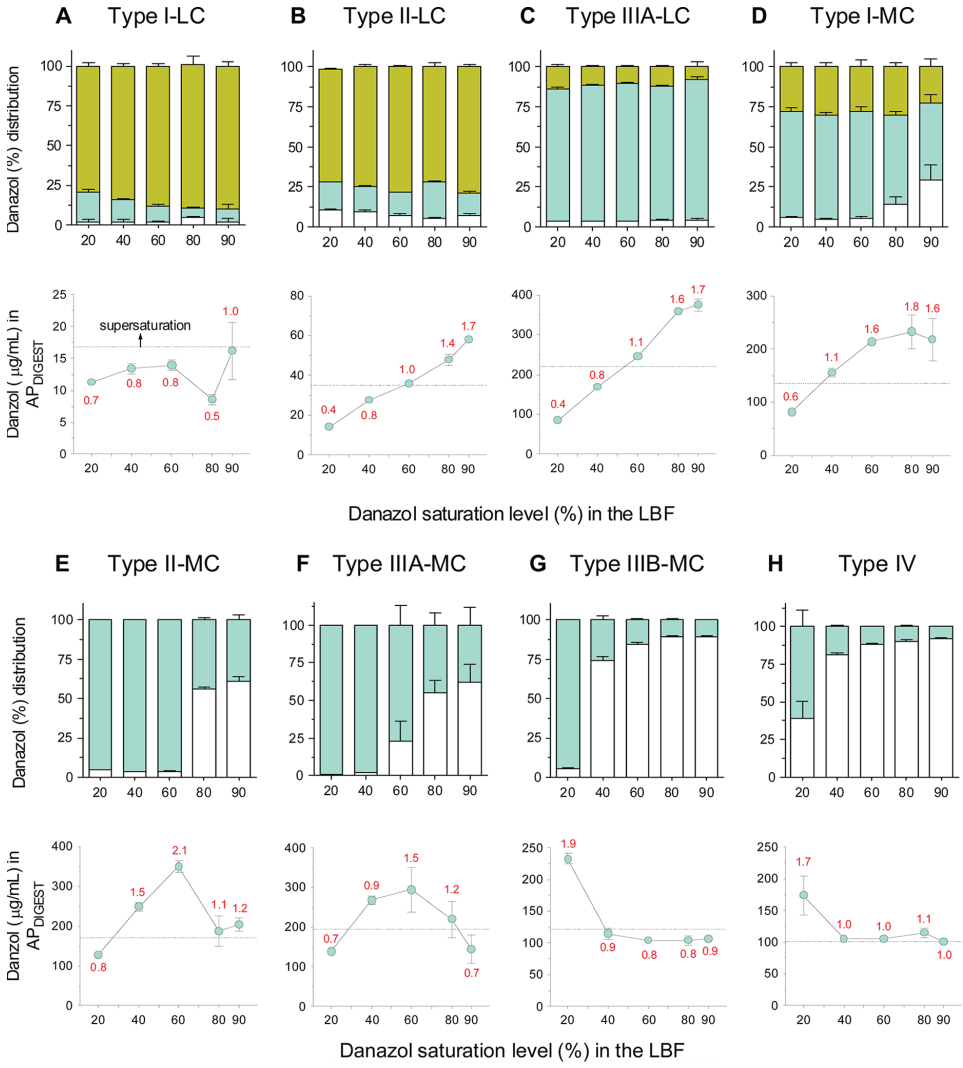

Figure 4. Effect of increasing danazol saturation level in eight lipid-based formulations (LBFs) on performance following 30 min of *in vitro* digestion. Upper panels for each LBF type show the distribution of drug across partially digested oil phase (dark shaded bars), colloidal aqueous phase, AP_{DIGEST} (lightly shaded bars), and pellet phase (white bars). Lower panels plot danazol concentrations in the colloidal AP_{DIGEST} as a function of danazol saturation level in the formulation. The horizontal dotted line denotes danazol solubility in the drug-free AP_{DIGEST}, hence concentrations above this line are supersaturated. Numerical values in red indicate the supersaturation ratio (SR) at 30 min (eq 2). Values are expressed as means (n = 3) ± SD. The formulation compositions can be found in Table 1.

across all eight investigated LBFs was 375 μg/mL and was obtained using the type IIIA-LC formulation at a drug load of ∼17 mg/g (vertical dotted line in Figure 5), which was equivalent to 90% saturation in this LBF. Interestingly, at a similar absolute dose (16.3 mg/g), the type II-MC formulation achieved a similar AP_{DIGEST} concentration although in this case the degree of saturation in the formulation was 60%. The type IIIA-MC LBF also achieved its highest AP_{DIGEST} concentration at 60% saturation although, because of drug precipitation, the absolute AP_{DIGEST} concentration in this case was lower than that achieved by the other LBFs.

DISCUSSION

The LFCS Consortium previously described[15] a series of eight lipid-based formulations (LBFs) that provide exemplar coverage of the LBF classes described in the Lipid Formulation Classification System[29] and suggested a set of defined experimental conditions under which *in vitro* digestion testing for LBFs might be conducted. These LBFs have been examined further here using the same experimental conditions but with additional focus on the impact of changing the concentration of bile salt and phospholipid employed in the *in vitro* digestion test,

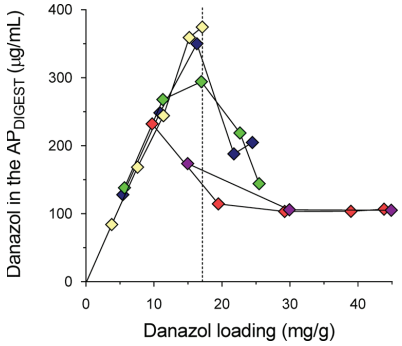

Figure 5. Comparison of solubilized danazol concentrations in the colloidal AP_{DIGEST} following 30 min *in vitro* digestion of selected lipid-based formulations (LBFs) plotted with respect to absolute drug loading (mg/g) in the formulation. Symbols represent various drug loads in type IIIA-LC (yellow symbols), type II-MC (blue symbols), type IIIA-MC (green symbols), type IIIB-MC (red symbols), and type IV (purple symbols) formulations. The vertical dotted line is at 17.1 mg/g and corresponds to the drug loading that gave rise to the highest concentration of danazol in the aqueous colloidal phase (AP_{DIGEST}).

and on increasing drug load (dose) in the formulation, on the potential to generate and sustain drug supersaturation during LBF digestion. The latter studies follow recent increased interest in the role of supersaturation in drug delivery[1,19,21,30−33] and specifically in drug absorption from LBFs.[18,19,30,34] LBFs are predisposed to the generation of supersaturation following GI events that cause the solubilization capacity of lipid formulations to decrease.[18] Depending on LBF composition, these processes include simple mixing and dilution with aqueous fluids in the stomach and small intestine,[26,29,35] and enzymatic processing of lipidic components by pancreatic lipases and esterases.[27,30,36,37] Since the concentrations of drug that are able to dissolve into free solution in the GI fluids are, for poorly water-soluble drugs, intrinsically low, the attainment of supersaturation and, in turn, higher concentrations of free drug in solution may provide an important driving force for absorption.[30] However, the selection of conditions and formulations that promote supersaturation must also be balanced against the risk of drug precipitation from the supersaturated state. The period over which supersaturation is maintained, prior to precipitation, is likely to be critical to LBF performance.

Bile Salt Concentration in *in Vitro* Digestion Tests Affects LBF Digestion, Fatty Acid Ionization, and Separation of Digestion Products. The effect of increasing bile salt (NaTDC) concentration on the performance of the eight exemplar LBFs during *in vitro* digestion experiments is reported in Figures 1 and 2. The NaTDC concentration was varied from 0 mM to 10 mM in order to explore the likely impact of differences in NaTDC concentration under typical conditions in the fasted small intestine.[38,39] The typical bile salt concentration in the small intestine is approximately 3−5 mM[38,39] but may be as high as 10 mM after administration of relatively small quantities of lipids.[40] The NaTDC concentration in the present study was therefore varied from 0 to 10 mM NaTDC in order to explore the likely impact of differences in NaTDC concentration on formulation digestion and solubilization properties.

Interestingly, for most LBFs, the impact of NaTDC concentration on lipid digestion was limited (Table 3), suggesting that small changes in NaTDC concentrations in *in vitro* digestion experiments are unlikely to have a dramatic effect on the extent of lipid digestion. Indeed, for all LBFs, except type II-LC and type IV, the quantities of fatty acid released in the complete absence of NaTDC were greater than 75% of the quantity observed at 10 mM NaTDC, and for all type III and for type II-MC LBFs, digestion of the formulation was essentially independent of NaTDC concentration.

For all LBFs, fatty acid ionization was incomplete at pH 6.5 (Table 3). This effect was more pronounced for the LBFs containing LC fatty acid when compared with MC fatty acid, and can be attributed to the fact that increasing the length of the alkyl chain increases hydrophobicity and, therefore, the tendency to self-associate.[41,42] Increasing NaTDC concentration did, however, increase the fraction of liberated fatty acid that was ionized at pH 6.5, and as expected, effects were again more pronounced for LBFs containing LC fatty acids (Table 3). As a result, analysis of titration data provided only an indication of the effect of NaTDC concentration on apparent digestion (Figure 1). This serves to confirm the difficulty in assessing lipid digestion using tests carried out at neutral (and biorelevant) pH unless back-titration correction protocols are included (as was the case here).

The differences in fatty acid ionization reflect differences in apparent pK_a under changing NaTDC conditions and likely result from differences in the molecular organization of fatty acids under the different experimental conditions: In the absence of NaTDC or under low NaTDC concentrations, lipid digestion results in the formation of a range of colloidal structures, many of which may show liquid crystalline character.[43−45] These self-assembled phase structures may limit proton access to the (ionizable) carboxylic acid headgroup of the fatty acid and therefore reduce apparent acidity (i.e., increase apparent pK_a).[41] In contrast, at higher NaTDC concentrations, and where formulations contain larger proportions of surfactant, phase transitions to micelle-rich systems are expected,[28,44] which in turn increase the ease of deprotonation[45] and the acidity of the fatty acid (i.e., decreasing apparent pK_a).

The digestion data also reveal that the physical presence of a phase-separated "oil" phase during the conduct of *in vitro* digestion tests does not necessarily indicate incomplete digestion. Indeed, in some cases, estimates of total formulation digestion (Table 3) suggested that the lipid components were completely hydrolyzed, yet an oil phase separated during centrifugation. In these examples, the oil phase consists of poorly solubilized lipophilic digestion products (monoglyceride and protonated fatty acids) rather than undigested formulation lipids. Typically these lipid phases floated during centrifugation, but in some cases, (e.g., the type II-MC in the absence of NaTDC), the lipid digestion products phase separated to the bottom of the centrifuge tube. The latter observation suggests that materials that are more dense than the digestion media (and therefore "pellet" on centrifugation) may not always reflect precipitated drug, but may also include phase-separated lipid (containing dissolved drug).

Regardless of the density of lipid phases produced, differences in NaTDC concentrations subsequently dictate changes to lipid distribution patterns. For example, increased NaTDC concentrations promote transfer of lipid digestion products from the phase-separated lipidic phases to the solubilized aqueous phase. In contrast to previous assumptions, however, this may not result from a difference in the extent of digestion, but instead may reflect increased fatty acid ionization, lower affinity for the oil phase, and improved solubilization of lipid digestion products in the colloidal aqueous phase formed on digestion (AP_{DIGEST}).

Under the standard *in vitro* digestion conditions (3 mM NaTDC), type I-LC, II-LC, and IV formulations were only partially digested (Table 3). For the lipid-containing formulations (i.e., I-LC and II-LC), this reflects saturation of NaTDC/phospholipid micelles in the AP_{DIGEST} with digestion products resulting in accumulation of poorly water-soluble fatty acid and monoglyceride molecules at the oil:water interface and ultimately blockade of enzyme access to the substrate.[38,46−51] Increasing NaTDC concentration increased the capacity of the AP_{DIGEST} to solubilize digestion products, promoting the digestion of type I-LC and II-LC formulations (Table 3). The increase in LBF digestion, however, was relatively low, suggesting that the high lipid content of these formulations (i.e., 65% w/w LC lipid) resulted in saturation of the bile salt/phospholipid phase even at the higher NaTDC concentrations (thereby preventing further increases in lipid digestion). In contrast to the situation with LC lipid containing formulations, digestion of MC lipids results in the production of more polar digestion products (capric acid, monocaprylin), and these more polar materials are more readily solubilized in the AP_{DIGEST}, thereby promoting ongoing digestion. The importance of the solubilization capacity of the AP_{DIGEST} in facilitating ongoing digestion also dictates that formulations that promote the solubilization of their own digestion products (e.g., those containing higher concentrations of surfactant) are ultimately more effectively digested—at least *in vitro*. From the perspective of the LFCS classification scheme, type I-LC and II-LC LBFs are therefore likely to be only partially digested during *in vitro* digestion tests, whereas the lower lipid/higher surfactant content in type IIIA/IIIB formulations (LC or MC) ensures complete/near-complete digestion under the same experimental conditions.

Bile Salt (NaTDC) Concentration in *in Vitro* Digestion Tests Has a Moderate Effect on Drug Solubilization Patterns. The impact of changing NaTDC concentration (and therefore lipid digestion/solubilization) on the solubilization of danazol following digestion of the eight LBFs is described in Figure 2. For the most lipophilic LBFs (i.e., the LC formulations and type I-MC formulation) drug solubilization patterns closely followed the changing distribution of lipids from the oil phase to the AP_{DIGEST} as NaTDC concentrations increased.

In many instances, the presence of an oil phase postcentrifugation resulted in reduced drug precipitation, presumably due to the effective reservoir capability of the oil phase for danazol. Conditions that favored drug (and lipid) solubilization in the AP_{DIGEST} include the presence of formulation surfactant or the addition of higher NaTDC. Indeed, increased digestion of the highly lipophilic LBFs (i.e., type I-MC and I-LC) with increasing NaTDC promoted drug partitioning from the depleting lipid phase into the AP_{DIGEST}. At the same time, enrichment of NaTDC/phospholipid micelles with lipid digestion products led to an increase in drug solubility in the AP_{DIGEST}. These measures enhanced drug solubilization in the AP_{DIGEST}, reduced sequestration in the oil phase, and maintained a low likelihood of drug precipitation. Drug precipitation from the highly lipophilic LBFs was also mitigated by the comparatively lower drug loadings in these formulations (since drug solubility in simple lipids is typically lower than that in surfactants or cosolvents) when compared to the more hydrophilic type III and IV LBFs.

In contrast, the presence of formulation-derived surfactant and/or increasing NaTDC concentrations was associated with an increased risk of drug precipitation from the more polar type II-MC and IIIA-MC formulations. This was likely a function of the higher drug loadings in MC formulations (discussed further in the next section) and a shift from the lipid-rich colloids produced by digestion of MC lipids under low surfactant/NaTDC conditions to more highly dispersed and solubilized micelle and mixed-micelle phases at higher NaTDC and in the presence of higher concentrations of surfactant.[28] As a result, the use of higher NaTDC concentrations can lead to lower drug solubilities in the AP_{DIGEST}. Interestingly, however, at NaTDC concentrations >5 mM, the degree of drug precipitation from the type IIIA-MC formulation was reduced ($p < 0.05$). Some evidence of a similar trend was apparent for the type II-MC formulation, however these differences were not significant. This decrease in precipitation could not be attributed to a decrease in supersaturation since, as described above, increasing NaTDC tended to decrease the solubilization capacity of the digested formulations (i.e., the solubility in the AP_{DIGEST}). As such, the additional NaTDC may have helped stabilize danazol in a supersaturated state. In general, however, NaTDC effects on either digestion or drug solubilization across the LFCS formulations were modest provided NaTDC concentrations were ≥3 mM. This further justifies the choice of 3 mM bile salt as a baseline condition for *in vitro* testing in the LFCS Consortium.[15]

Drug Saturation Level (Drug Loading) in LBFs Strongly Affects Drug Solubilization in Digested MC Formulations but Not LC Formulations. Increasing danazol dose had no significant effect on the digestion of LBFs investigated here (Figure 3). This finding is contrary to some recent work,[52,53] but is in line with prevailing observations in our laboratory where neutral drugs appear to show negligible capacity to affect LBF digestibility (other than simple displacement of a proportion of the lipid content of the formulation on a weight for weight basis, thereby decreasing the amount of available lipid substrate and therefore the absolute quantity of liberated fatty acid).

The effect of increasing drug load in the LBFs on subsequent performance during *in vitro* digestion is summarized in Figure 4. Target drug loadings were based on the saturation level in the respective LBFs and are depicted as the % of the equilibrium solubility in the formulation. For all the LC formulations, little precipitation was evident under all saturation levels, reinforcing the robustness of LC LBFs under a number of conditions. However, drug solubility in all the LC formulations was lower than in the equivalent MC formulations (Table 1), and the lower absolute drug load in these formulations also lowered the potential for drug precipitation. Nonetheless, the data suggest that for drugs where an appropriate dose may be dissolved in a LC LBF, relatively high degrees of drug saturation in the formulation may be tolerated before approaching conditions under which precipitation is likely.

The progressive increase in drug concentrations with increasing drug load in type II-LC and IIIA-LC formulations ultimately resulted in drug concentrations that were supersaturated relative to drug solubility in the equivalent blank digest. Similar patterns were observed for the type I-MC, II-MC, and IIIA-MC formulations, although in this case, a tipping point was ultimately reached where the increasing drug loading (and therefore supersaturation) stimulated drug precipitation. The saturation level required to simulate this precipitation was highly dependent on LBF type, and generally decreased with increasing hydrophilicity of the formulation.

Compared with LC formulations, the greater susceptibility of the MC and type IV formulations to drug precipitation during digestion can be attributed to a combination of factors that lead to higher degrees of supersaturation and, therefore, a higher precipitation risk. First, drug solubility in the MC formulations

and the lipid-free type IV formulation is higher than that of the LC formulations (Table 1), and therefore, the mass of drug in the MC and type IV formulations was higher at each % saturation level. Second, the physical properties of the type II-MC, IIIA/IIIB-MC, and IV formulations change rapidly during the *in vitro* tests and to a greater extent than the LC equivalents. More specifically, the hydrophilic components of the type IIIA/IIIB and IV LBFs (i.e., the high HLB surfactant and cosolvent containing formulations) are expected to be soluble on dispersion,[18,19,35,36,54] resulting in a rapid drop in solubilizing power as they are diluted. Digestion of MC digestion products is also rapid and results in the generation of MC digestion products that are more polar and less able to swell endogenous bile salt/phospholipid micelles when compared to LC lipids and LC lipid digestion products.[28] Collectively, the reduction in solubilizing power on dispersion and digestion, coupled to the relatively high drug loading in the MC and type IV formulations, results in the generation of a high degree of supersaturation and, therefore, an enhanced likelihood of precipitation. In comparison, the highly lipophilic type I-MC, I-LC, and II-LC LBFs were only partially digested and retained a fraction of the incorporated drug within a phase-separated lipid reservoir. The lipid reservoir limited the maximum concentration of drug within the colloidal AP_{DIGEST}, and in turn minimized the extent of supersaturation and drug precipitation.

The Maximum Degree of Supersaturation (SR^M) Dictates the Propensity for Drug Precipitation from LBFs during *in Vitro* Testing. Analysis of the data generated during the NaTDC study (Figure 2) and the drug saturation study (Figure 4) reveal general trends in precipitation behavior. Thus, increasing drug loading and decreasing solubility in the colloidal structures formed by LBF digestion appeared to promote the likelihood of precipitation, in line with previous suggestions.[18]

The ratio between the measured drug concentration in the AP_{DIGEST} and drug solubility in the AP_{DIGEST} provides an indication of the degree to which this phase is supersaturated with drug at any time (the supersaturation ratio; SR). To capture the maximum attainable degree of supersaturation and, therefore, the principle driving force toward precipitation, a maximum supersaturation ratio SR^M was calculated (eq 3) using the ratio of the maximum drug concentration that could be attained in the AP_{DIGEST} at 30 min in the absence of drug precipitation (AP_{MAX}) and drug solubility in the AP_{DIGEST} at the same time point.

A 3-dimensional plot (Figure 6) captures the relationship between SR^M (plotted on the *z*-axis) and the drug solubility in the AP_{DIGEST} (plotted on the *x*-axis) and AP_{MAX} (plotted on the *y*-axis) parameters (an example calculation of SR^M is provided in the figure legend). Thus, SR^M is directly proportional to the drug loading in the formulation, as evidenced by the positive gradient along the $z-y$ axis with increasing AP_{MAX}. Similarly, SR^M is (inversely) proportional to drug solubility in AP_{DIGEST}, as evidenced by the negative gradient along the $z-x$ axis with increasing solubility. Figure 6 also illustrates that much greater increases in SR^M (and therefore much greater risks of precipitation) are attained when drug loading is increased in LBFs that show reduced solubilization capacity postdigestion (e.g., type IV) when compared to similar increases in dose in a LBF with higher postdigestion solubilization capacity (e.g., type IIIA-LC).

The response profile in Figure 6 also presents the supersaturation potential of five of the eight investigated LBFs (i.e., type IIIA-LC, II-MC, IIIA-MC, IIIB-MC, and IV) overlaid with

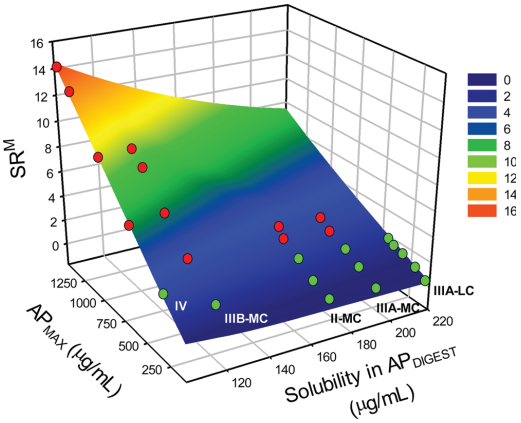

Figure 6. A 3-dimensional surface plot to illustrate the relationship between SR^M, AP_{MAX}, and drug solubility in the AP_{DIGEST}. SR^M is the maximum degree of supersaturation that is attained following 30 min *in vitro* digestion of a lipid-based formulation, and is calculated using eq 3 from the ratio between AP_{MAX} and drug solubility in the AP_{DIGEST}. Example calculation of SR^M: At 20% and 80% saturation, the type IV LBF contains 13.1 mg/g and 52.4 mg/g of danazol, respectively. AP_{MAX} values (i.e., drug loading divided by test volume) are, therefore, 0.33 mg/mL (13.1 mg divided by 40 mL) at 20% saturation and 1.31 mg/mL at 80% saturation. Danazol solubility in the type IV formulation AP_{DIGEST} is 0.10 mg/mL. The respective SR^M values (AP_{MAX} divided by solubility in the AP_{DIGEST}) are therefore 3.3 at the 20% saturation and 13.1 at the 80% saturation. With increasing AP_{MAX} (i.e., drug loading in the formulation), there is a proportional increase in SR^M. The dependency between SR^M and AP_{MAX} increases with decreasing drug solubility in the AP_{DIGEST}. The relationships for the type IIIA-LC, II-MC, IIIA-MC, IIIB-MC, and IV formulations are shown. Data points (filled circles) reflect the results shown in Figure 4 for five different drug loadings in each formulation, with circles in green describing low drug precipitation (<50%) and those in red describing high (>50%) precipitation.

the *in vitro* performance (precipitation/solubilization) data for each of the LBFs at the five different saturation levels examined. In this case the red-filled circles symbolize conditions where >50% precipitation is evident at 30 min digestion and green-filled circles <50% precipitation. Interestingly, across all the studies conducted here, and across a range of different LBFs, a relationship between SR^M and the propensity for precipitation was apparent. As such, increases in SR^M led to an increased likelihood of precipitation and a decrease in the % dose that was solubilized at 30 min. This relationship is illustrated in Figure 6 where the green circles (indicating low-level drug precipitation) are associated with low SR^M values. Closer examination of the data also reveals a threshold SR^M value for the appearance of drug precipitation in the digestion test, regardless of LBF type. This is better depicted in Figure 7A, where a marked decrease in % dose solubilized (due to precipitation) is evident above SR^M values of ~2.5. This critical SR^M value was consistent for all the formulations where no phase-separated lipid phase was formed (data for type I-LC, II-LC, and I-MC LBFs have been omitted from the current discussion since the undigested oil phase provides an improved (and stable) drug reservoir that limits supersaturation in the AP_{DIGEST} and ultimately protects against precipitation). The lack of formulation dependency in Figure 7A therefore suggests that the fate of the drug is controlled primarily by the supersaturation ratio obtained on LBF digestion.

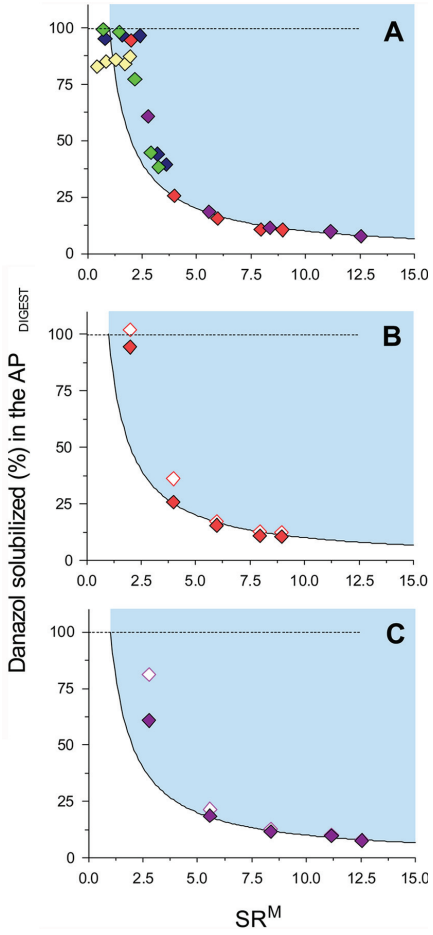

Figure 7. The relationships identified between the % of danazol dose solubilized in the aqueous colloidal phase (AP_{DIGEST}) after *in vitro* digestion with respect to SR^M. Symbols represent the type IIIA-LC (yellow symbols), type II-MC (blue symbols), type IIIA-MC (green symbols), type IIIB-MC (red symbols), and type IV (purple symbols) formulations. In A, individual data points for each formulation type correspond to performance at different danazol saturation levels in the formulations assessed following 30 min digestion. In B and C, the percentage of danazol solubilized at 15 min (15 min, open symbols; 30 min, closed symbols) is also shown for type IIIB-MC and IV formulations respectively. SR^M is calculated from eq 3. The solid line is the equilibrium curve, i.e., SR = 1, and follows a power law model. Points above the curve indicate evidence of supersaturation during *in vitro* digestion. The horizontal dashed line represents complete solubilization of drug in the AP_{DIGEST}.

Mechanistically, increasing supersaturation ratio shifts the system to a position further from the equilibrium position (i.e., the solubility of the crystalline drug). This leads to greater thermodynamic instability and increased potential for nucleation and crystallization of the supersaturated solution.[18,49−51] The vertical distance between the dashed horizontal line in Figure 7A and the solid curved line represents the thermodynamic driving force for drug precipitation from digested LBFs since the position of the solid line at each SR^M value equals the % of the dose that would remain in solution should the system reach equilibrium (i.e., the equilibrium drug solubility in the AP_{DIGEST}; SR = 1). This equilibrium curve decays according to a power law function ($y = 100x^{-1}$) since 50% of the drug would remain in solution on precipitation to the equilibrium solubility point for a system starting at $SR^M = 2$, 25% would remain in solution for a system starting from $SR^M = 4$, etc. The overall shape therefore captures the greater thermodynamic instability in the digested LBFs with increasing SR^M (moving from left to right). Furthermore, the degree to which the experimental data points (diamond symbols) are displaced vertically above the equilibrium curve captures the kinetic stability or metastability of the system, with points that lie above the equilibrium curve (i.e., within the blue shaded area) being supersaturated at 30 min and therefore capable of greater metastability compared to those that lie on the equilibrium curve.

In the case of the type IIIA−LC formulation (yellow symbols; Figure 7), the lower drug solubility in the formulation resulted in insufficient drug loading to allow generation of high SR^M values (>2.5) and as such precipitation was low at all possible drug loads. It appears likely that this will be the case for many LC formulations, and that lower drug loads will inherently protect against precipitation (although this will also limit the practical drug dose). Note that digestion of the type IIIA-LC formulation also resulted in the formation of a small quantity of phase-separated oil. The presence of this competing oil phase dictated that AP_{DIGEST} drug concentrations in Figure 7 were somewhat lower than 100%.

SR^M values for type II-MC and IIIA-MC (dark blue and green symbols respectively, Figure 7) were below 2.5 when the drug load in the formulation was below 80% of the saturated solubility in the formulation. Under these circumstances, the majority of drug remained solubilized (and supersaturated) after initiation of digestion (as indicated by vertical displacement of the data point from the equilibrium curve). However, higher drug loadings led to SR^M values >2.5 and less effective stabilization of supersaturation. This pattern was also evident for the type IIIB-MC (red symbols) and IV (purple symbols) LBFs, consistent with a generalized model of increasing supersaturation resulting in a greater driving force for nucleation and crystallization and therefore enhanced precipitation.[21,55−57] In addition to increases in the extent of precipitation, the rate of drug precipitation also increased with increasing SR^M. This is apparent in the data describing drug precipitation at 15 versus 30 min postdigestion. These data are shown in Figure 7B,C for type IIIB-MC and type IV formulations respectively, since these were the LBFs most prone to precipitation. Comparison of the data at 15 min (open symbols) and 30 min postdigestion (closed symbols) confirms that, for high SR^M, maximum precipitation had occurred by 15 min, whereas at lower SR^M, precipitation continued from 15 to 30 min. Decreasing SR^M therefore increased the kinetic stability of the supersaturated colloidal species.

CONCLUSIONS

Increasing the bile salt (NaTDC) concentration employed in *in vitro* digestion tests increased the digestion of lipophilic type I and II LBFs, but had little impact on the degree of digestion of other LBF types (type IIIA/IIIB) since these were rapidly digested, even in the absence of bile salt. Drug solubilization patterns were also moderately affected by bile salt concentration, and increases in bile salt concentration resulted in increases in

drug concentration in the AP_{DIGEST}. However, the effects of bile salt on digestion/drug solubilization patterns were limited above a 3 mM concentration, confirming this to be a suitable concentration for use in *in vitro* digestion testing. In contrast, increasing LBF drug load had a very significant impact on formulation performance *in vitro*. Stimulation of digestion of LBFs led in all cases to the generation of supersaturation, and this was enhanced by formulations with higher drug loads. Supersaturation was effectively maintained in the case of LC LBFs, and drug remained solubilized for extended time periods. In contrast, increasing drug load (and therefore increasing initial supersaturation on dispersion/digestion) resulted in greater precipitation from MC lipid containing formulations and the type IV LBFs. Across the formulations, the potential for drug solubilization versus precipitation was well predicted by assessment of the theoretical maximum supersaturation ratio attained on digestion (SR^M), and a threshold SR^M of ~2.5 appeared to provide the limit above which precipitation occurred in all cases. Lipid digestion tests in conjunction with the SR^M precipitation limit therefore provides useful new tools with which to calculate the maximum tolerated drug load for a candidate LBF. Finally, it is also apparent that increasing supersaturation may provide a driver for enhanced absorption (via increases in thermodynamic activity).[19] Further studies are therefore necessary to determine whether *in vitro* descriptors of formulation performance such as SR^M capture the *in vivo* threshold at which the thermodynamic consequences of supersaturation favor drug precipitation over drug absorption.

■ ASSOCIATED CONTENT

Supporting Information

Additional figures including *in vitro* digestion of the digestion medium in the absence of lipid formulation and effect of danazol drug loading on *in vitro* digestion of eight lipid-based formulations. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Williams, H. D.; Trevaskis, N. L.; Charman, S. A.; Shanker, R. M.; Charman, W. N.; Pouton, C. W.; Porter, C. J. H. Strategies to Address Low Drug Solubility in Discovery and Development. *Pharmacol. Rev.* **2012**, in press.

(2) Porter, C. J. H.; Trevaskis, N. L.; Charman, W. N. Lipids and lipid-based formulations: optimizing the oral delivery of lipophilic drugs. *Nat. Rev. Drug Discovery* **2007**, *6* (3), 231−248.

(3) Hauss, D. J. *Enhancing the Bioavailability of Poorly Water-Soluble Drugs*; Informa Healthcare: New York, 2007.

(4) Porter, C. J. H.; Pouton, C. W.; Cuine, J. F.; Charman, W. N. Enhancing intestinal drug solubilisation using lipid-based delivery systems. *Adv. Drug Delivery Rev.* **2008**, *60* (6), 673−691.

(5) Constantinides, P. P.; Wasan, K. M. Lipid formulation strategies for enhancing intestinal transport and absorption of P-glycoprotein (P-gp) substrate drugs: in vitro/in vivo case studies. *J. Pharm. Sci.* **2007**, *96* (2), 235−248.

(6) Lindmark, T.; Kimura, Y.; Artursson, P. Absorption enhancement through intracellular regulation of tight junction permeability by medium chain fatty acids in Caco-2 cells. *J. Pharmacol. Exp. Ther.* **1998**, *284* (1), 362−369.

(7) Goole, J.; Lindley, D. J.; Roth, W.; Carl, S. M.; Amighi, K.; Kauffmann, J. M.; Knipp, G. T. The effects of excipients on transporter mediated absorption. *Int. J. Pharm.* **2010**, *393* (1−2), 17−31.

(8) Patel, J. P.; Brocks, D. R. The effect of oral lipids and circulating lipoproteins on the metabolism of drugs. *Expert Opin. Drug Metab. Toxicol.* **2009**, *5* (11), 1385−98.

(9) Trevaskis, N. L.; Porter, C. J. H.; Charman, W. N. An examination of the interplay between enterocyte-based metabolism and lymphatic drug transport in the rat. *Drug Metab. Dispos.* **2006**, *34* (5), 729−733.

(10) Trevaskis, N. L.; Charman, W. N.; Porter, C. J. H. Lipid-based delivery systems and intestinal lymphatic drug transport: a mechanistic update. *Adv. Drug Delivery Rev.* **2008**, *60* (6), 702−716.

(11) O'Driscoll, C. M. Lipid-based formulations for intestinal lymphatic delivery. [Review] [85 refs]. *Eur. J. Pharm. Sci.* **2002**, *15* (5), 405−415.

(12) Pouton, C. W.; Porter, C. J. H. Formulation of lipid-based delivery systems for oral administration: Materials, methods and strategies. *Adv. Drug Delivery Rev.* **2008**, *60* (6), 625−637.

(13) Jannin, V.; Musakhanian, J.; Marchaud, D. Approaches for the development of solid and semi-solid lipid-based formulations. *Adv. Drug Delivery Rev.* **2008**, *60* (6), 734−746.

(14) Pouton, C. W. Lipid formulations for oral administration of drugs: non-emulsifying, self-emulsifying and "self-microemulsifying" drug delivery systems. *Eur. J. Pharm. Sci.* **2000**, *11*, S93−S98.

(15) Williams, H. D.; Sassene, P.; Kleberg, K.; Bakala N'Goma, J. C.; Calderone, M.; Jannin, V.; Igonin, A.; Partheil, A.; Marchaud, D.; Jule, E.; Vertommen, J.; Maio, M.; Blundell, R.; Benameur, H.; Carriere, F.; Mullertz, A.; Porter, C. J. H.; Pouton, C. W. Toward the establishment of standardized in vitro tests for lipid-based formulations: 1) Method parameterization and comparison of in vitro digestion profiles across a range of representative formulations. *J. Pharm. Sci.* **2012**, *101* (9), 3360−3380.

(16) Bakatselou, V.; Oppenheim, R. C.; Dressman, J. B. Solubilization and wetting effects of bile salts on the dissolution of steroids. *Pharm. Res.* **1991**, *8* (12), 1461−1469.

(17) Bergstrom, C. A. S.; Wassvik, C. M.; Johansson, K.; Hubatsch, I. Poorly soluble marketed drugs display solvation limited solubility. *J. Med. Chem.* **2007**, *50* (23), 5858−5862.

(18) Anby, M. U.; Williams, H. D.; McIntosh, M.; Benameur, H.; Edwards, G. A.; Pouton, C. W.; Porter, C. J. H. Lipid digestion as a trigger for supersaturation: In vitro and in vivo evaluation of the utility of polymeric precipitation inhibitors in self emulsifying drug delivery systems. *Mol. Pharmaceutics* **2012**, in press.

(19) Gao, P.; Akrami, A.; Alvarez, F.; Hu, J.; Li, L.; Ma, C.; Surapaneni, S. Characterization and Optimization of AMG 517 Supersaturatable Self-Emulsifying Drug Delivery System (S-SEDDS) for Improved Oral Absorption. *J. Pharm. Sci.* **2009**, *98* (2), 516−528.

(20) Warren, D. B.; Benameur, H.; Porter, C. J. H.; Pouton, C. W. Using polymeric precipitation inhibitors to improve the absorption of poorly water-soluble drugs: A mechanistic basis for utility. [Review]. *J. Drug Targeting* **2010**, *18* (10), 704−731.

(21) Brouwers, J.; Brewster, M. E.; Augustijns, P. Supersaturating Drug Delivery Systems: The Answer to Solubility-Limited Oral Bioavailability? *J. Pharm. Sci.* **2009**, *98* (8), 2549−2572.

(22) Fernandez, S.; Jannin, V.; Rodier, J. D.; Ritter, N.; Mahler, B.; Carriere, F. Comparative study on digestive lipase activities on the self

emulsifying excipient Labrasol (R), medium chain glycerides and PEG esters. *Biochim. Biophys. Acta, Mol. Cell Biol. Lipids* **2007**, *1771* (5), 633−640.
(23) Fernandez, S.; Rodier, J. D.; Ritter, N.; Mahler, B.; Demarne, F.; Carriere, F.; Jannin, V. Lipolysis of the semi-solid self-emulsifying excipient Gelucire (R) 44/14 by digestive lipases. *Biochim. Biophys. Acta, Mol. Cell Biol. Lipids* **2008**, *1781* (8), 367−375.
(24) Sek, L.; Porter, C. J. H.; Kaukonen, A. M.; Charman, W. N. Evaluation of the in-vitro digestion profiles of long and medium chain glycerides and the phase behaviour of their lipolytic products. *J. Pharm. Pharmacol.* **2002**, *54* (1), 29−41.
(25) Li, Y.; McClements, D. J. New Mathematical Model for Interpreting pH-Stat Digestion Profiles: Impact of Lipid Droplet Characteristics on in Vitro Digestibility. *J. Agric. Food Chem.* **2010**, *58* (13), 8085−8092.
(26) Cuine, J. F.; Charman, W. N.; Pouton, C. W.; Edwards, G. A.; Porter, C. J. H. Increasing the proportional content of surfactant (Cremophor EL) relative to lipid in self-emulsifying lipid-based formulations of danazol reduces oral bioavailability in beagle dogs. *Pharm. Res.* **2007**, *24* (4), 748−757.
(27) Fernandez, S.; Chevrier, S.; Ritter, N.; Mahler, B.; Demarne, F.; Carriere, F.; Jannin, V. In Vitro Gastrointestinal Lipolysis of Four Formulations of Piroxicam and Cinnarizine with the Self Emulsifying Excipients Labrasol (R) and Gelucire (R) 44/14. *Pharm. Res.* **2009**, *26* (8), 1901−1910.
(28) Kossena, G. A.; Boyd, B. J.; Porter, C. J. H.; Charman, W. N. Separation and characterization of the colloidal phases produced on digestion of common formulation lipids and assessment of their impact on the apparent solubility of selected poorly water-soluble drugs. *J. Pharm. Sci.* **2003**, *92* (3), 634−648.
(29) Pouton, C. W. Formulation of poorly water-soluble drugs for oral administration: Physicochemical and physiological issues and the lipid formulation classification system. *Eur. J. Pharm. Sci.* **2006**, *29* (3−4), 278−287.
(30) Porter, C. J. H.; Anby, M. U.; Warren, D. B.; Williams, H. D.; Benameur, H.; Pouton, C. W. Lipid based formulations: Exploring the link between in vitro supersaturation and in vivo exposure. *Gattefosse Bull.* **2011**, submitted.
(31) Friesen, D. T.; Shanker, R.; Crew, M.; Smithey, D. T.; Curatolo, W. J.; Nightingale, J. A. S. Hydroxypropyl Methylcellulose Acetate Succinate-Based Spray-Dried Dispersions: An Overview. *Mol. Pharmaceutics* **2008**, *5* (6), 1003−1019.
(32) Van Speybroeck, M.; Mols, R.; Mellaerts, R.; Do Thi, T.; Martens, J. A.; Van Humbeeck, J.; Annaert, P.; Van den Mooter, G.; Augustijns, P. Combined use of ordered mesoporous silica and precipitation inhibitors for improved oral absorption of the poorly soluble weak base itraconazole. *Eur. J. Pharm. Biopharm.* **2010**, *75* (3), 354−365.
(33) Guzman, H. R.; Tawa, M.; Zhang, Z.; Ratanabanangkoon, P.; Shaw, P.; Gardner, C. R.; Chen, H.; Moreau, J. P.; Almarsson, O.; Remenar, J. F. Combined use of crystalline salt forms and precipitation inhibitors to improve oral absorption of celecoxib from solid oral formulations. *J. Pharm. Sci.* **2007**, *96* (10), 2686−2702.
(34) Kataoka, M.; Sugano, K.; da Costa Mathews, C.; Wong, J. W.; Jones, K. L.; Masaoka, Y.; Sakuma, S.; Yamashita, S. Application of dissolution/permeation system for evaluation of formulation effect on oral absorption of poorly water-soluble drugs in drug development. *Pharm. Res.* **2012**, *29* (6), 1485−1494.
(35) Mohsin, K.; Long, M. A.; Pouton, C. W. Design of Lipid-Based Formulations for Oral Administration of Poorly Water-Soluble Drugs: Precipitation of Drug after Dispersion of Formulations in Aqueous Solution. *J. Pharm. Sci.* **2009**, *98* (10), 3582−3595.
(36) Cuine, J. F.; McEvoy, C. L.; Charman, W. N.; Pouton, C. W.; Edwards, G. A.; Benameur, H.; Porter, C. J. H. Evaluation of the impact of surfactant digestion on the bioavailability of danazol after oral administration of lipidic self-emulsifying formulations to dogs. *J. Pharm. Sci.* **2008**, *97* (2), 995−1012.
(37) Kaukonen, A. M.; Boyd, B. J.; Porter, C. J. H.; Charman, W. N. Drug solubilization behavior during in vitro digestion of simple triglyceride lipid solution formulations. *Pharm. Res.* **2004**, *21* (2), 245−253.
(38) Armand, M.; Borel, P.; Pasquier, B.; Dubois, C.; Senft, M.; Andre, M.; Peyrot, J.; Salducci, J.; Lairon, D. Physicochemical characteristics of emulsions during fat digestion in human stomach and duodenum. *Am. J. Physiol.* **1996**, *271* (1), G172−G183.
(39) Lindahl, A.; Ungell, A. L.; Knutson, L.; Lennernas, H. Characterization of fluids from the stomach and proximal jejunum in men and women. *Pharm. Res.* **1997**, *14* (4), 497−502.
(40) Kossena, G. A.; Charman, W. N.; Wilson, C. G.; O'Mahony, B.; Lindsay, B.; Hempenstall, J. M.; Davison, C. L.; Crowley, P. J.; Porter, C. J. H. Low dose lipid formulations: Effects on gastric emptying and biliary secretion. *Pharm. Res.* **2007**, *24* (11), 2084−2096.
(41) Cistola, D. P.; Hamilton, J. A.; Jackson, D.; Small, D. M. Ionization and phase-behavior of fatty-acids in water - application of the gibbs phase rule. *Biochemistry* **1988**, *27* (6), 1881−1888.
(42) Kanicky, J. R.; Shah, D. O. Effect of degree, type, and position of unsaturation on the pK(a) of long-chain fatty acids. *J. Colloid Interface Sci.* **2002**, *256* (1), 201−207.
(43) Warren, D. B.; Anby, M. U.; Hawley, A.; Boyd, B. J. Real Time Evolution of Liquid Crystalline Nanostructure during the Digestion of Formulation Lipids Using Synchrotron Small-Angle X-ray Scattering. *Langmuir* **2011**, *27* (15), 9528−9534.
(44) Salentinig, S.; Sagalowicz, L.; Leser, M. E.; Tedeschi, C.; Glatter, O. Transitions in the internal structure of lipid droplets during fat digestion. *Soft Matter* **2011**, *7* (2), 650−661.
(45) Salentinig, S.; Sagalowicz, L.; Glatter, O. Self-Assembled Structures and pK(a) Value of Oleic Acid in Systems of Biological Relevance. *Langmuir* **2010**, *26* (14), 11670−11679.
(46) Mullertz, A.; Fatouros, D. G.; Smith, J. R.; Vertzoni, M.; Reppas, C. Insights into Intermediate Phases of Human Intestinal Fluids Visualized by Atomic Force Microscopy and Cryo-Transmission Electron Microscopy ex Vivo. *Mol. Pharmaceutics* **2012**, *9* (2), 237−247.
(47) Reis, P.; Holmberg, K.; Miller, R.; Kragel, J.; Grigoriev, D. O.; Leser, M. E.; Watzke, H. J. Competition between lipases and monoglycerides at interfaces. *Langmuir* **2008**, *24* (14), 7400−7407.
(48) Reis, P.; Holmberg, K.; Miller, R.; Leser, M. E.; Raab, T.; Watzke, H. J. Lipase reaction at interfaces as self-limiting processes. *C. R. Chim.* **2009**, *12* (1−2), 163−170.
(49) Reis, P.; Holmberg, K.; Watzke, H.; Leser, M. E.; Miller, R. Lipases at interfaces: A review. *Adv. Colloid Interface Sci.* **2009**, *147−48*, 237−250.
(50) Reis, P.; Miller, R.; Leser, M.; Watzke, H.; Fainerman, V. B.; Holmberg, K. Adsorption of polar lipids at the water-oil interface. *Langmuir* **2008**, *24* (11), 5781−5786.
(51) Reis, P. M.; Raab, T. W.; Chuat, J. Y.; Leser, M. E.; Miller, R.; Watzke, H. J.; Holmberg, K. Influence of Surfactants on Lipase Fat Digestion in a Model Gastro-intestinal System. *Food Biophys.* **2008**, *3* (4), 370−381.
(52) Arnold, Y. E.; Imanidis, G.; Kuentz, M. Study of drug concentration effects on in vitro lipolysis kinetics in medium-chain triglycerides by considering oil viscosity and surface tension. *Eur. J. Pharm. Sci.* **2011**, *44* (3), 351−358.
(53) Thomas, N.; Mullertz, A.; Graf, A.; Rades, T. Influence of Lipid Composition and Drug Load on the In Vitro Performance of Self-Nanoemulsifying Drug Delivery Systems. *J. Pharm. Sci.* **2012**, *101* (5), 1721−1731.
(54) Chiang, P. C.; Thompson, D. C.; Ghosh, S.; Heitmeier, M. R. A formulation-enabled preclinical efficacy assessment of a farnesoid X receptor agonist, GW4064, in hamsters and cynomolgus monkeys. *J. Pharm. Sci.* **2011**, *100* (11), 4722−4733.
(55) Turnbull, D.; Fisher, J. C. Rate of nucleation in condensed systems. *J. Chem. Phys.* **1949**, *17* (1), 71−73.
(56) Lindfors, L.; Forssen, S.; Westergren, J.; Olsson, U. Nucleation and crystal growth in supersaturated solutions of a model drug. *J. Colloid Interface Sci.* **2008**, *325* (2), 404−413.
(57) James, P. F. Kinetics of crystal nucleation in silicate-glasses. *J. Non-Cryst. Solids* **1985**, *73* (1−3), 517−540.