MONASH University

Oral lipid-based drug delivery: Inhibiting and eliminating digestion to improve absorption of poorly soluble drugs.

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Copyright notice

This thesis is dedicated to my grandmother, Lily Harte,
who passed away while I was studying in Australia.
My education began with you.

Take hold of the shaft of the pen.
Subscribe to the first step taken
from a justified line
Into the margin.

Seamus Heaney
The first gloss (1984)

Contents

Abstract

This thesis has explored the utility of novel lipid-based formulations (LBF) to enhance the oral bioavailability of model poorly water-soluble drugs (PWSD) and examined the role of digestive processes on formulation performance. Particular focus was directed to the use of formulation excipients to inhibit or eliminate digestion in order to control drug solubilisation, supersaturation, precipitation and ultimately, *in vivo* absorption.

Understanding that rapid digestion can be detrimental to formulation performance, these studies were designed to develop formulations that could slow lipid digestion. A series of thirty formulations were developed to assess the impact of surfactant PEGylation on digestion of co-formulated medium chain triglycerides. Lipid digestion rates exhibited a surfactant PEG molecular weight dependency with the most inhibitory formulations named 'stealth' LBFs. It was established that digestion inhibition was a function of reduced lipase adsorption at the oil:water interface rather than changes in dispersion particle size. Interestingly, the presence of labile ester bonds within a PEGylated surfactant also impacted on the stealth properties of LBFs, with digestible surfactants requiring a PEG Mw of ~1800 g/mol and non-digestible ether-based surfactants ~800 g/mol to shield the triglycerides.

Assessment of formulations using an *in vitro* lipolysis models showed that stealth LBFs maintained drug solubilisation at a higher level and reduced supersaturation in comparison to digestible counterparts. This trend was also reflected *in vivo*, where the relative bioavailability of a model co-formulation PWSD (cinnarizine) increased after administration in two stealth LBFs in comparison to analogous digestible (non-stealth) formulations.

Subsequent studies with long chain triglycerides (LCT) revealed that digestion inhibition followed broadly the same pattern as that for medium chain triglycerides, with digestion rates also showing a surfactant PEG Mw dependency. In this case the fold reduction in digestion for LCT was lower due to long chain lipids having inherently lower digestion rates under the *in vitro* conditions employed. Drug loaded stealth LCT formulations also maintained higher solubilisation during lipolysis testing when compared to digestible counterparts and this was ultimately reflected in greater exposure after oral administration in rats. The increase in bioavailability seen for stealth LCT formulations, however, was lower than that observed for similar MCT formulations and for both LCT and MCT formulations increases in bioavailability were not apparent in dogs when compared to their digestible counterparts. The lack of effects in dogs may reflect altered *in vivo* conditions across species or the requirement for the stealth PEG layer to self assemble *in vivo*.

As controlling digestion yielded variable results, in the final studies, a series of non-digestible LBF were developed to eliminate the need for *in vivo* processing of the formulations. Non-digestible lipids were thus incorporated into formulations that spontaneously formed nanoemulsions on dispersion. Oleic Acid ND-LBFs showed the highest solubilisation capacity for the model drug, while the analogous alkene and fatty

alcohol-based formulations had considerably lower solubilisation capacity. Dynamic digestion experiments showed that all formulations maintained drug solubilisation and supersaturation throughout the *in vitro* digestion experiment, and formulations were not digested. Administration of these matched non-digestible formulations *in vivo*, however, showed that formulation performance differed dependent on the co-formulated lipid, with formulations containing octadecene outperforming all others. Comparison of changes to *in vivo* exposure with lipid LogD yielded a positive correlation suggesting that lipid absorption from the formulation (where lipids with higher LogDs may be absorbed more quickly) could be a driver for enhanced drug absorption.

In summary, the studies in this thesis contribute to a better understanding of the impact of digestion of the lipids in LBF on the solubilisation and absorption of a co-formulated PWSD. More specifically the work exemplifies the concept that excipients can alter the rate and extent of lipid digestion and that in some cases slowing or eliminating digestion can be beneficial for PWSD delivery.

Publications during enrolment

This thesis is a compilation of the following manuscripts:

Chapter 1:

O.M. Feeney, M.F. Crum, C.L. McEvoy, N.L. Trevaskis, H.D. Williams, C.W. Pouton, W.N. Charman, C.A.S. Bergstrom, C.J.H. Porter. "50 years of lipid-based formulations: provenance, progress and future perspectives." Advanced Drug Delivery Reviews (2016)

Chapter 3:

O.M. Feeney, H.D. Williams, C.W. Pouton, C.J.H. Porter. "Stealth Lipid-based Formulations: Poly(ethylene glycol) Mediated Digestion Inhibition Improves Oral Bioavailability for a Model Poorly Water-soluble Drug." Journal of Controlled Release,192 (2014) 219–227

Chapter 4:

O.M. Feeney, C.L. McEvoy, M.U. Anby, J.R.S. Dandrieux, T. Whittem, C.W. Pouton and C.J.H. Porter "Competition at the oil:water interface during *in vivo* self-assembly poses a significant challenge to the utility of 'stealth' lipid-based formulations for oral drug delivery." Manuscript in preparation.

Chapter 5:

O.M. Feeney, D.A. Patterson, D.H.S. Brundel, C.W. Pouton, C.J.H. Porter. "Eliminating digestion; the potential utility of non-digestible lipid-based formulations for oral drug delivery" Manuscript in preparation.

During my PhD I also made significant, but more moderate, contributions to the following manuscripts in the area of lipid-based drug delivery. However, they are not included in this thesis as they are outside the immediate aims of the studies described herein. These manuscripts are appended at the end of this thesis.

M. U. Anby, T-H. Nguyen, Y. Y. Yeap, O.M. Feeney, H. D. Williams, H. Benameur, C. W. Pouton, C. J. H. Porter. "An *in vitro* digestion test that reflects rat intestinal conditions to probe the importance of formulation digestion vs first pass metabolism in danazol bioavailability from lipid-based formulations" Molecular Pharmaceutics (2014) 11 (11) 4069-4083

M.U. Anby, H.D. Williams, O.M. Feeney, G.A. Edwards, H. Benameur, C.W. Pouton, C.J.H. Porter. "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". Pharmaceutical Research, 31 (2014) 1536-1332

C.L. McEvoy, N.L. Trevaskis, O.M. Feeney, G.A. Edwards, M.E. Perlman, C.M. Ambler, C. J.H. Porter. "Correlating *in vitro* solubilisation and supersaturation profiles of the CETP inhibitor CP-532,623 with *in vivo* exposure for long and medium chain lipid-based formulations" Molecular Pharmaceutics (2017)

Thesis including published works general declaration

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 two original papers published in peer reviewed journals and two manuscripts in preparation for submission. The core theme of the thesis is the examination of the utility of slowing or eliminating digestion on the absorption of poorly water-soluble drugs from lipid-based formulations. 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.

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 chapters one, 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.

Chapter	Publication Title	Status	Nature and % of student contribution	Co-author name(s) Nature and % of Co-author's contribution*	Co-author(s), Monash student Y/N*
1	50 years of lipid-based formulations: provenance, progress and future perspectives	Published	50%. Planning and conducting literature review. Coordination of co-author contributions. Data evaluation, IVIVC generation. Drafting & revision of manuscript. Generation of illustrations/diagrams.	[a]Matthew Crum 2.5% [a]Claire McEvoy 5% [b]Natalie Trevaskis 2.5% [a]Hywel Williams 2.5% [b]Colin Pouton 2.5% [c]William Charman 2.5% [c]Christel Bergstrom 2.5% [a,b,c,d,e]Chris Porter 30%	*No*
3	'Stealth' Lipid-based Formulations: Poly(ethylene glycol) Mediated Digestion Inhibition Improves Oral Bioavailability of a Model Poorly Water-soluble Drug	Published	60%. Experimental design Conducting work Data interpretation Drafting/revision of manuscript.	[b,e]Hywel Williams 5% [b,e]Colin Pouton 5% [a,b,c,d,e] Chris Porter 30%	*No*
Nature of contribution - key [a] Experimental design, [b] Conducting work/data interpretation, [c] Manuscript draft, [d] Literature review, [e] Manuscript review					

I have renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Student signature: **Date:** 01/10/2019

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student and co-authors' contributions to this work.

Main Supervisor signature: **Date:** 01/10/2019

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To the friends and family we left in Ireland, I'm sorry about our sudden drop off the face of the planet, intermittent contact, missed chances to celebrate the happy occasions, and the absence of our support during some very tough times. Your encouragement to do this has meant a lot and the *'C*ead míle fáilte' when we do return home makes it feel like we've never been away. Thank you so much for that.

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To my parents; I'm so lucky you taught me the value of education, determination and a sense of humour. You've never pressured or pushed me, but used the "as long as you tried your best" tactic, which means that I will forever be striving to try harder. The sacrifices you've made for the benefit of five children are monumental. It will take a lifetime to repay you. That's something I look forward to.

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Finally, to Jonny. We've already had some epic adventures, but I think this may have been the toughest yet. On occasion, it's been a lot less fun than when that chemical toilet leaked all over my rucksack, but it's also been an exhilarating adventure into the unknown. I owe that entirely to you. In all our endeavours you've been my catalyst and my compass. Thank you for unwavering support, unpaid loans and unconditional love. Because of you, I am an infinitely better version of myself.
Mo ghrá go deo.

Communications

O. M. Feeney, H. D. Williams, C. W. Pouton, C. J. H. Porter, “Stealth nanoemulsions evade intestinal lipolysis and improve drug absorption after oral administration”. ARC Centre for Bio-Nano Science (CBNS) Annual Workshop, December 2 - 4 (2015). Cumberland Lorne Resort, Lorne, VIC, Australia

O. M. Feeney, C. L. McEvoy, M. U. Anby, C. W. Pouton, and C. J.H. Porter, “‘Stealth’ Lipid-Based Formulations; Examining the utility of digestion modulating formulations in rats and dogs”. AAPS Annual Meeting and Exposition, October 25th – 29th (2015). The AAPS Journal: T3202, Orlando Convention Center, Orlando, Florida, USA.

O. M. Feeney, D. A. Paterson, D. H.S. Brundel, C. W. Pouton, and C. J.H. Porter, “Non-Digestible Lipid-Based Formulations; Investigating the impact of eliminating digestion on the absorption of a poorly water-soluble drug”. AAPS Annual Meeting and Exposition, October 25th – 29th (2015). The AAPS Journal: R6105, Orlando Convention Center, Orlando, Florida, USA.

O. M. Feeney, H. D. Williams, C. W. Pouton, C. J. H. Porter, “Stealth nanoemulsions evade intestinal lipolysis and improve drug absorption after oral administration”. 5th FIP Pharmaceutical Sciences World Congress, April 13 - 16 (2014) Abstract1 94. Melbourne Convention and Exhibition Centre, Melbourne, Australia.

O. M. Feeney, H. D. Williams, C. W. Pouton, C. J. H. Porter, “'Stealth’ Lipid-Based Formulations: Poly(ethylene glycol) Mediated Digestion Inhibition Improves Oral Bioavailability of a Model Poorly Water-soluble Drug”. AAPS Annual Meeting and Exposition, November 1st – 6th (2014). The AAPS Journal: T2071, San Diego Convention Center, San Diego, California, USA.

O. M. Feeney, H. D. Williams, C. W. Pouton, C. J. H. Porter, “Polymeric (Stealth) nanoemulsions evade intestinal lipolysis and improve drug absorption after oral administration”. Victorian Polymer Workshop, June 26 - 27 (2014). Monash University, Melbourne, Australia.

O. M. Feeney, H. D. Williams, C. W. Pouton, C. J. H. Porter, “Stealth lipid-based formulations: Poly(ethylene glycol) mediated digestion modulation improves oral bioavailability for a model poorly water-soluble drug”. 8th Annual Postgraduate Symposium, November 13 (2013) Abstract 19. Monash University, Melbourne, Australia.

O. M. Feeney, H. D. Williams, C. W. Pouton, C. J. H. Porter, “Controlled Digestion; Enhancing the Utility of Lipid-based Drug Delivery Systems”, 40th Annual Meeting & Exposition of the Controlled Release Society (CRS), July 21 – 24 (2013) Abstract 836. Honolulu, Hawaii, USA.

O. M. Feeney, H. D. Williams, C. W. Pouton, C. J. H. Porter, “PEGylated surfactants inhibit the digestion of lipid-based formulations in a PEG molecular weight dependent manner”, APSA-ASCEPT annual conference, December 2 – 5 (2012) Abstract 148. Sydney Convention Centre, Sydney, Australia.

O. M. Feeney, H. D. Williams, C. W. Pouton, C. J. H. Porter, “Digestion modulating formulations as a platform to enhance the utility of lipid-based drug delivery systems”, 9th biennial GPEN Meeting, November 28 – December 1 (2012), P-TH-23. Monash University, Melbourne, Australia.

O. M. Feeney, H. D. Williams, C. W. Pouton, C. J. H. Porter, “Using PEGylated surfactants to control the digestion of lipid-based formulations”, Inaugural Drug Delivery Australia (AUS-CRS) Meeting, November 26 – 27 (2012) Abstract P20. Monash University, Melbourne, Australia

Abbreviations

% CV	% coefficient of variation	LCFA	long-chain fatty acid
4-BPB	bromophenyl boronic acid	LCT	long-chain triglyceride
ABT	1-aminobenzodiazole	LFCS	lipid formulation classification system
ACN	acetonitrile	LLQ	lower limit of quantification
AUC	area under the curve	Log D	Log of the octanol/water distribution coefficient (at set pH)
BA	bioavailability	LogP	Log of the octanol/water partition coefficient
BCRP	breast cancer resistant protein	m	metre
BCS	biopharmaceutics classification system	M	molar
bOP	branched octyl phenol	MC	medium-chain
BS	bile salt	MCT	medium-chain triglyceride
Ch	cholesterol	mg	milligram
CIN	cinnarizine	MG	monoglyceride
cm	centimetre	min	minute
C_{max}	peak plasma concentration	mL	millilitre
CMC	critical micellar concentration	mm	millimetre
CO	castor oil	mM	millimolar
CYP	cytochrome P450	mol	mole
DAN	danazol	MO	monoolein
DG	diglyceride	Mw	molecular weight
EtOH	ethanol	NaTDC	sodium taurodeoxycholate
FA	fatty acids	ng	nanogram
FFB	fenofibrate	nm	nanometer
g	gram	NMR	nuclear magnetic resonance
GI	gastrointestinal	o/w	oil in water
h	hour	OA	oleic acid
HCO	hydrogenated castor oil	OEt	oleyl ether
HLB	hydrophilic-lipophilic balance	PC	phosphatidylcholine
HPLC	high performance liquid chromatography	PEG	poly(ethylene) glycol
IS	internal standard	P-gp	P-glycoprotein
kg	kilogram	PL	Phospholipid
L	litre	PPI	polymeric precipitation inhibitors
LBDDS	lipid-based drug delivery system	PWSD	poorly water-soluble drugs
LBF	lipid-based formulations	rpm	revolutions per minute
LC	long-chain	s	seconds

SAXS	small-angle X-ray scattering
SD	standard deviation
SEDDS	self-emulsifying drug delivery system
SEIF	simulated endogenous intestinal fluid
SEM	standard error of the mean
SEs	stearyl ester
SEt	stearyl ether
S^M	maximal supersaturation ratio
SMEDDS	self-microemulsifying drug delivery system
SNEDDS	self-nanoemulsifying drug delivery system
S^R	supersaturation ratio
sSEDDS	supersaturable self-emulsifying drug delivery systems
$t_{1/2}$	half-life
TBU	tributyrin units
TG	triglyceride
T_{max}	time of occurrence of peak plasma concentration
UPLC MS/MS	ultra performance liquid chromatography-mass spectrometry
UV	ultraviolet
UWL	unstirred water layer
v/v	volume in volume
w/o	water in oil
w/v	weight in volume
w/w	weight in weight
x g	relative centrifugal force
µg	microgram
µL	microlitre
µm	micrometre
µM	micromolar
µmol	micromole

Chapter 1. Introduction

50 Years of oral lipid-based formulations: provenance, progress and future perspectives

Orlagh M. Feeney[a,b], Matthew F. Crum[a], Claire L. McEvoy[a], Natalie L. Trevaskis[a], Hywel D. Williams[c], Colin W. Pouton[a], William N. Charman[a], Christel A. S. Bergström[d] and Christopher J.H. Porter[a,b]

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Advanced Drug Delivery Reviews (2016)

A significant proportion of this introduction has been published [1], however, as the scope of the review was broader than the context of this thesis, only pertinent excerpts have been integrated into this introduction. The review in full is appended at the end of this thesis and the methods used to generate the *in vitro-in vivo* correlations in sections six and seven of the review have been included in chapter two of this thesis.

This introductory chapter provides background on the oral delivery of poorly water-soluble drugs, lipid absorption in the GIT, the impact of lipids on PWSD absorption, lipid formulation strategies for oral delivery of PWSD and the use of PEGylation to reduce protein binding and its potential application in lipid-based drug delivery.

1.1. General Introduction

1.1.1. Statement of the problem

The global pharmaceutical market is projected to be worth over 1.3 trillion USD by 2020[2], with a significant portion of the market share occupied by oral formulations[3]. The popularity of the oral route can be attributed to administrative convenience, safety and cost-effectiveness[4, 5]. In spite of considerable efforts to reduce physicochemical liabilities, and to design-in reasonable 'developability' characteristics in prospective drug candidates, discovery programs continue to identify drugs with low water solubility, limited cellular permeability and high metabolic clearance - properties that are expected to reduce oral bioavailability.

Of the limiting factors to oral drug delivery described above, low water solubility is perhaps the most amenable to resolution based on the use of enabling formulation approaches[6, 7]. For poorly water-soluble drugs (PWSD) where low aqueous solubility limits absorption, several formulation technologies have been developed and applied to support increases in dissolution rate and/or apparent solubility in the gastrointestinal tract (GIT). These include particle size reduction and nanomilling, salt formation, isolation as a cocrystal or high energy polymorph, the generation of solid dispersions, and formulation in lipid-based formulations (LBFs)[8-10]. This thesis is focussed on the design of LBF for oral administration.

One of the major challenges to LBF performance is the precipitation of PWSD during formulation processing in the gastrointestinal tract. While some approaches have attempted to control digestion-mediated drug precipitation through manipulation of formulation components or addition of precipitation inhibitors, there have been few studies to evaluate the impact of delaying lipid digestion on the solubilisation (and subsequent precipitation) of PWSD.

Lipid digestion in the GIT is catalysed by the lipase superfamily of interfacially active enzymes[11]. Nonspecific adsorption of the inactive lipase/co-lipase proteins to the surface of an emulsified oil droplet results in a conformational change in the enzyme to the active form. The interfacial activation of pancreatic lipase therefore renders the lipolysis reaction highly sensitive to changes at the oil:water interface[11, 12].

'Stealth' drug delivery vehicles comprising a poly ethylene glycol (PEG) steric stabilisation layer have been extensively utilised in parenteral drug delivery to enhance plasma circulation times and to promote drug accumulation at sites of hypervascularisation such as tumours or inflamed tissues[13-15]. The 'stealth' effect of these drug delivery systems can be, in part, attributed to their polymeric PEG coatings that prevent nonspecific protein adsorption (opsonisation) and therefore reduce recognition and clearance from the blood by the mononuclear phagocyte system (MPS).

Understanding that digestion is driven by interfacial adsorption of enzyme, the initial hypothesis for this thesis was that controlling the digestion rate of lipids via the use of PEGylated surfactants, thereby generating 'stealth' LBFs, would delay the onset of drug precipitation, prolong drug solubilisation and improve bioavailability. In the course of the studies to address this hypothesis, a PEGylated surface was indeed shown to reduce lipid digestion *in vitro* and digestion inhibition found to be a function of PEG chain length and the chemical structure of the surfactant used. Administration of these formulations to rats resulted in an improvement in oral bioavailability when compared to digestible formulations.

Subsequent studies, however, revealed that the enhancements in bioavailability provided by 'stealth" LBFs were variable and were not, for example, replicated in beagle dogs when animals were administered encapsulated formulations rather than pre-dispersed emulsions. The working hypothesis to explain this phenomena was that in the dog studies, the need for in situ self emulsification and assembly of the PEG layer rendered the formulations more susceptive to interaction with endogenous materials that breached the integrity of the stealth PEG layer. Further studies examining formulation self-assembly suggested that administration of encapsulated formulations was likely to reduce the digestion inhibiting capability of the PEGylated surface.

As digestion inhibition yielded variable results, a second series of LBF was developed where the lipids formulated were not digestible. The hypothesis for these studies was that a self nanoemulsifying formulation that did not undergo luminal processing would be better able to stably solubilise a PWSD *in vivo*. This was shown to be the case and LBF containing the non-digestible lipid 1-octadecene resulted in robust bioavailability after oral administration.

Together, the studies provide the groundwork for a new approach to LBF development where non-traditional lipids and surfactants may be used to enhance the utility of LBF.

1.1.2. Factors affecting the oral bioavailability of poorly water-soluble drugs

1.1.2.1. Patient compliance and food effects

The administration of oral dosage forms (tablets, capsules, suspensions and solutions) is relatively facile when compared to parenteral formulations and therefore typically the preferred route of administration. Nonetheless drug effectiveness remains dictated by the level of patient compliance with the dosing regimen[16-18]. Consistent with this suggestion, intra- and inter-subject variability following oral drug administration is significant, even under rigid clinical trial conditions[19]. This variability is exaggerated when administering poorly water-soluble drugs, where slow or limited in-vivo dissolution commonly leads to inconsistent absorption.

One of the simplest means to enhance the exposure of PWSD is coadministration with food. The ability of lipids to enhance the absorption of PWSD was first reported in the context of co-administration with fats in food[20] and most PWSD show significant positive food effects when co-administered with a meal rich in fat[20-23]. This approach, although effective, is also inherently variable, as factors such as culture, gender, age and health status all play major roles in dictating the type and quantity of food consumed as part of a meal. Food effects can also induce reduced gastric emptying rates, increased gastric pH, adsorption of drug to food, competitive inhibition of transporter mediated uptake, chelation of food-borne metal ions and suppression of metabolic pathways[19, 24, 25]. Realising the potential benefits of co-administration with lipids in food, but the variability associated with meals stimulated the development of LBF as a more clinically reproducible means of harnessing the benefits of positive food effects.

1.1.2.2. Physiochemical properties of the administered drug

In order for a drug to be absorbed after oral administration, a number of physical and chemical prerequisites must be met. Assuming the drug is stable in the intestinal lumen, is not subject to enterocyte based metabolism and is not a substrate for efflux pumps, the most important properties for absorption are drug solubility and permeability across the gastrointestinal membrane[26, 27].

Drug solubility in intestinal fluids is dictated by the hydrophilicity and solid state properties of the compound, which in turn are influenced by the drug's physicochemical characteristics.

Poorly water-soluble drugs are typically slowly dissolving drugs. The rate of dissolution as described by Noyes and Whitney[25, 28] , is directly proportional to drug solubility and the surface area available for dissolution.

$$\frac{dM}{dt} = \frac{DA}{h}(C_s - C_t) \qquad \text{(Equation 1)}$$

Where:

dM / dt is the rate of mass transfer

D is the diffusion coefficient

A is the surface area of the drug

h is the static boundary layer

C_s is the saturation solubility of the drug

C_t is the concentration of the drug at time

Due to inherently slow dissolution rates, the free dissolved concentration of a PSWD (i.e. available for absorption in the GIT) is usually low. Where the dissolution rate of the drug is slower than that of the gastrointestinal transit time, the drug may not be adequately solubilised in the appropriate area of the GIT, missing the optimal absorption window and resulting in a therapeutically ineffective dosage form.

Common approaches to improving drug solubility have included prodrug strategies, crystallisation strategies (alternative polymorphs, co-crystallisation, amorphous forms), pH control via salt formation, complexing agents (e.g. cyclodextrins) and particle size reduction[4, 9]. Other solubilisation strategies include the use of co-solvents, surfactants and lipids in PWSD formulations. However, improving drug solubility alone cannot guarantee an improvement in systemic uptake. The absorption of a drug from the small intestine is also determined by the drug permeability across the gastrointestinal epithelium.

The difference in mass transfer into and out of the intestine (i.e. absorptive flux) is proportional to the concentration of the drug at the absorptive surface and intestinal permeability. Drug absorption is then dictated by the flux over the available surface area of the intestine. Drug solubility and permeability thus directly influence *in vivo* absorption[27].

Most drugs are absorbed via passive diffusion across the gastrointestinal epithelium[19]. This process is, also dependant on the physicochemical characteristics of the drug. Drug molecules that are small, lipophilic, with few rotatable bonds and limited polarity tend to diffuse rapidly or penetrate the lipidic domains of the cell membrane more effectively than larger, less rigid, hydrophilic or polar compounds.

The physicochemical characteristics of a drug candidate clearly impact both solubility and permeability, a fact that has been succinctly addressed by Lipinski's 'rule of five'[29, 30]. Typically used in early stage drug candidate screening, the 'rule of five' predicts that a compound with more than 5 hydrogen bond donors, 10 hydrogen bond acceptors, LogP greater than 5 and molecular weight greater than 500 will have poor bioavailability. When two or more of the rule of five criteria are exceeded, the drug may be too large, too pH dependant, or too lipophilic to facilitate rapid dissolution and absorption in the GIT.

Solubility and permeability also form the basis of the Biopharmaceutic drug Classification System (BCS, figure 1.1)[31]. Proposed by Amidon and co-workers in 1995, the BCS was established as a framework for identification of formulation independent drug absorption and biowaiver eligibility. Since then, the BCS use has expanded and it is now widely employed as a means of correlating *in vitro* drug dissolution with *in vivo* bioavailability.

Drug bioavailability standards are predicted based on four classes of drug, defined as; class I – high solubility-high permeability. Class II – low solubility-high permeability. Class III – high solubility-low permeability and class IV – low solubility-low permeability.

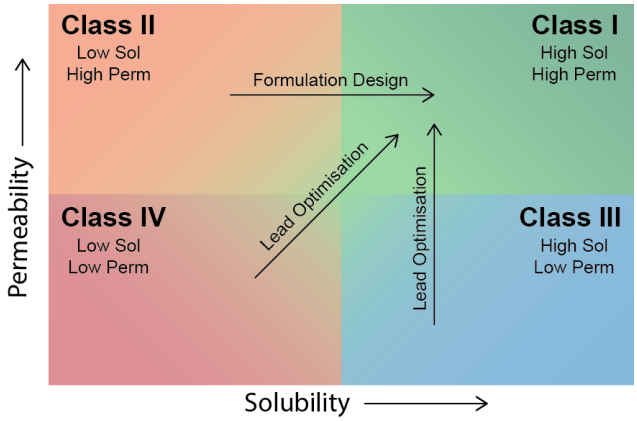


Figure 1.1 – A typical graphical description of the BCS. This project focuses on formulation design strategies for Class II model drugs[32]

Ideal drug candidates are both highly soluble and permeable, and are described as class I by BCS. Poorly water-soluble drugs fall into class II or class IV, depending on permeability. Notably, poorly water-soluble, hydrophobic drugs are not necessarily highly permeable. Poorly soluble and poorly permeable drugs (BCS class IV) thus require solubility and absorption enhancement to facilitate systemic uptake after oral administration and are particularly challenging to formulate[4]. For these molecules, increased absorption can be achieved either via modification of the drug itself (lead optimisation) or less frequently, through formulation strategies (co-administration with solubility and permeation enhancers[33, 34]). Realising the challenges associated with BCS class IV compounds, the current studies focus on drugs where solubility is the primary limitation to absorption (BCS class II).

1.1.2.3. Drug absorption pathway in the intestinal tract

Oral drug administration requires solubilised drug molecules to either pass through or between intestinal epithelial cells to reach the systemic circulation. The success of this approach, as described above, is intrinsically linked to the physicochemical characteristics of the drug[25].

Passive diffusion of molecules across the cell membrane (figure 1.2, 1C) is influenced by both the size and lipophilicity of the compound. The concentration of free drug and the diffusion coefficient both impact the flux and as a result, the extent of passive diffusion. Small molecules must have sufficient hydrophilic and lipophilic character to be soluble in the fluids in the intestinal lumen (hydrophilicity) and to be effectively absorbed across the apical and basolateral membranes (lipophilicity) to reach the systemic circulation[19, 25].

Carrier-mediated diffusion is primarily employed during the absorption of nutrients and may involve either passive diffusion down a concentration gradient or the active transport of molecules against a concentration gradient (figure 1.2 1b). Carrier-mediated diffusion is a saturable process and as a result, changes in drug concentration do not affect the transport rate once the system has been saturated.

Some xenobiotics utilise carrier-mediated diffusion as a means of absorption and this is reflected by their saturable absorption kinetics[19].

Paracellular drug transport does not involve penetration of the cell membrane and is reliant on the diffusion of small hydrophilic molecules through the tight junctions of the intestinal wall (figure 1.2 1A). The jejunal paracellular space has been estimated to be only 0.8 nm in diameter[19] The small surface area available for absorption this limits the likely extent of drug absorption via this route in the absence of permeation enhancers.

Uptake of macromolecules across the enterocyte is low and occurs via endocytosis, usually receptor-mediated endocytosis[19] (figure 1.2 1d). This process involves interaction with specific surface receptors on the cell which initiate transport across the apical membrane and subsequent systemic absorption. However, the likelihood of this process occurring enough to drive useful drug absorption is unknown.

Co-transport utilises the cell ion concentration gradient to drive transport of molecules across the cell membrane (figure 1.2 1e). Transport proteins couple molecules and ions for symport (transport in the same direction) or antiport (import of the molecule after export of an ion). Glucose uptake is moderated in this way[19].

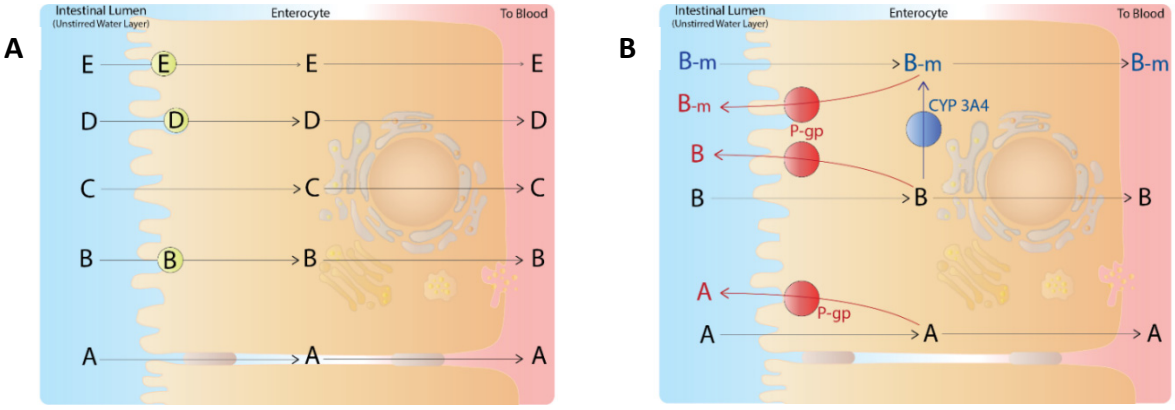


Figure 1.2 (**A**) Potential uptake mechanisms of drugs in the intestinal lumen. (A) paracellular transport, (B) carrier-mediated diffusion, (C) passive diffusion, (D) endocytic uptake, (E) co-transport. Adapted from[35]. (**B**) Pathways of drug elimination and metabolism in the intestinal lumen. (A) P-gp efflux (B) CYP metabolism coupled to P-gp. Adapted from[19, 35].

1.1.2.4. P-glycoprotein efflux

P-glycoprotein (P-gp) is an ATP-dependant efflux pump localised on the apical side of intestinal epithelial cells[19, 25, 35]. Substrates of P-gp are often hydrophobic and amphipathic and as such, P-gp can impact the absorption of poorly water-soluble drugs. P-gp efflux pumps are thought to mediate expulsion of drug from inside the cell back to the intestinal lumen (figure 1.2 2a).

The broad specificity of P-gp exists alongside other efflux pumps such as multidrug resistance-associated protein (MRP) and breast cancer resistance protein (BCRP), all of which were initially identified in the context of multidrug resistance (MDR) in cancer cells[19].

1.1.2.5. Cytochrome P450 oxidative metabolism in the small intestine

The intestinal cytochrome P450 family of mixed-function enzymes play an important role in the oxidative metabolism of poorly soluble drugs in the GIT[19]. Located in the endoplasmic reticulum, CYP450 enzymes exhibit broad substrate specificity. The cytochrome P450 3A4 (CYP3A4) is the most important enzyme subfamily in the GIT, despite comprising only 15 – 27% of total CYP450s and is responsible for metabolism of almost 50% of marketed drugs[36].

Inter-species differences in the expression of CYP enzyme isoforms also impacts the utility of *in vivo* models for prediction of drug absorption (Figure 1.2, table 1) and must be carefully considered when administering a drug which is a known substrate for these enzymes[36].

Table 1.1 Enzymes of the major drug-metabolising CYP family in humans, rat, and dog adapted from[36].

Family	Subfamily	Human	Rat	Dog
CYP1	A	1A1, 1A2	1A1, 1A2	1A1, 1A2
	B	1B1	1B1	1B1
CYP2	A	2A6, 2A7,	2A1, 2A2, 2A3	2A13, 2A25
	B	2B6, 2B7	2B1, 2B2, 2B3	2B11
	C	2C8, 2C9,	2C6, 2C7*, 2C11*,	2C21, 2C41
		2C18, 2C19	2C12*, 2C13*,	
	D	2D6, 2D7,	2D1, 2D2, 2D3,	2D15
	E	2E1	2E1	2E1
CYP3	A	3A4, 3A5,	3A1/3A23, 3A2*,	3A12, 3A26

*Gender difference. ‡Strain specific.

1.1.3. Understanding lipid processing in the gastrointestinal tract

1.1.3.1. Lipid dispersion, digestion and absorption

Endogenous lipid digestion and absorption pathways provide a highly dynamic and interactive conduit for drug delivery. Notably, dietary or formulation lipids (typically mixtures of glycerides), stimulate secretory processes in the GIT that profoundly alter the nature of the ingested lipid, resulting in altered GI conditions and significantly enhanced solvation capacity for the products of lipid digestion. In the context of drug delivery, these changes also (in the majority of cases) increase GI solvation capacity for a coadministered PWSD.

Although many of the studies detailing GI response to lipid ingestion have been undertaken under post prandial conditions[37, 38], and therefore under high lipid load, more recent studies have shown that lipid quantities of 2 g and lower are able to stimulate biliary secretion and elevate GI bile salt levels[39] as well as reduce gastric emptying[40]. Lipids and digestion products may also stimulate the ileal brake[41, 42], thereby extending residence time in the proximal small intestine, ensuring maximal exposure to absorptive pathways in the duodenum and jejunum.

Lipid absorption typically follows three main processes, dispersion, digestion (lipolysis) and uptake of more polar digestion products. During digestion in the human GIT, lingual and gastric lipases initiate hydrolysis in the mouth and stomach, liberating free fatty acid, and diglycerides which aid emulsification of the remaining triglycerides during gastric emptying[6]. This results in a coarse emulsion that reaches the antrum of the stomach where gastric churning and further emulsification reduce the lipid particle size to less than 0.5mm in diameter on presentation to the duodenum.

In the proximal (upper) small intestine, the presence of exogenous lipids stimulates the release of lipases. Simultaneously, biliary secretion of cholesterol, phospholipid (PL) and bile salt (BS) into the intestinal lumen results in the production of mixed colloidal species by solubilisation of lipid digestion products. Bile salts and phospholipid further emulsify triglyceride droplets, reducing particle size and increasing the surface area available to the enzyme.

Pancreatic lipase acts at the oil water interface with the aid of a one-to-one molar ratio of co-lipase as anchor[43, 44]. Triglycerides (TG) are cleaved in two steps at the sn-1 and sn-3 positions to produce 2-monoglyceride (MG) and free fatty acids (FA)[45]. 2-MG can undergo isomerisation to 1-MG which is again a substrate for pancreatic lipase, but this process *in vivo* is thought to be preceded by bile salt micelle solubilisation and absorption across the enterocyte[46, 47]. As a result of these processes, the *in-vitro* digestion of one mole of TG is expected to yield two moles of FA and one mole of 2–MG. FA and 2-MG are surface active by-products of digestion. Accumulation at the oil water interface can sterically impede lipase/co-lipase binding and quench the lipolysis reaction[48]. The presence of lipids in the upper GIT and subsequent release of digestion products triggers a slow-down in gastric emptying rate, presumably to allow maximal digestion and uptake of lipids in the duodenum [6]. The rate of lipid digestion is dependent on both the particle size of the dispersed droplet and the acyl chain length of the triglyceride. Smaller dispersions result in a larger surface area exposed to pancreatic lipases and consequently, a greater degree of lipolysis. After digestion, high concentrations of lipid digestion products (FA and MG) passively diffuse across the intestinal epithelium and are distributed to the portal blood (figure 1.3). At lower concentrations the absorption process is thought to be modulated by a saturable uptake mechanism[49].

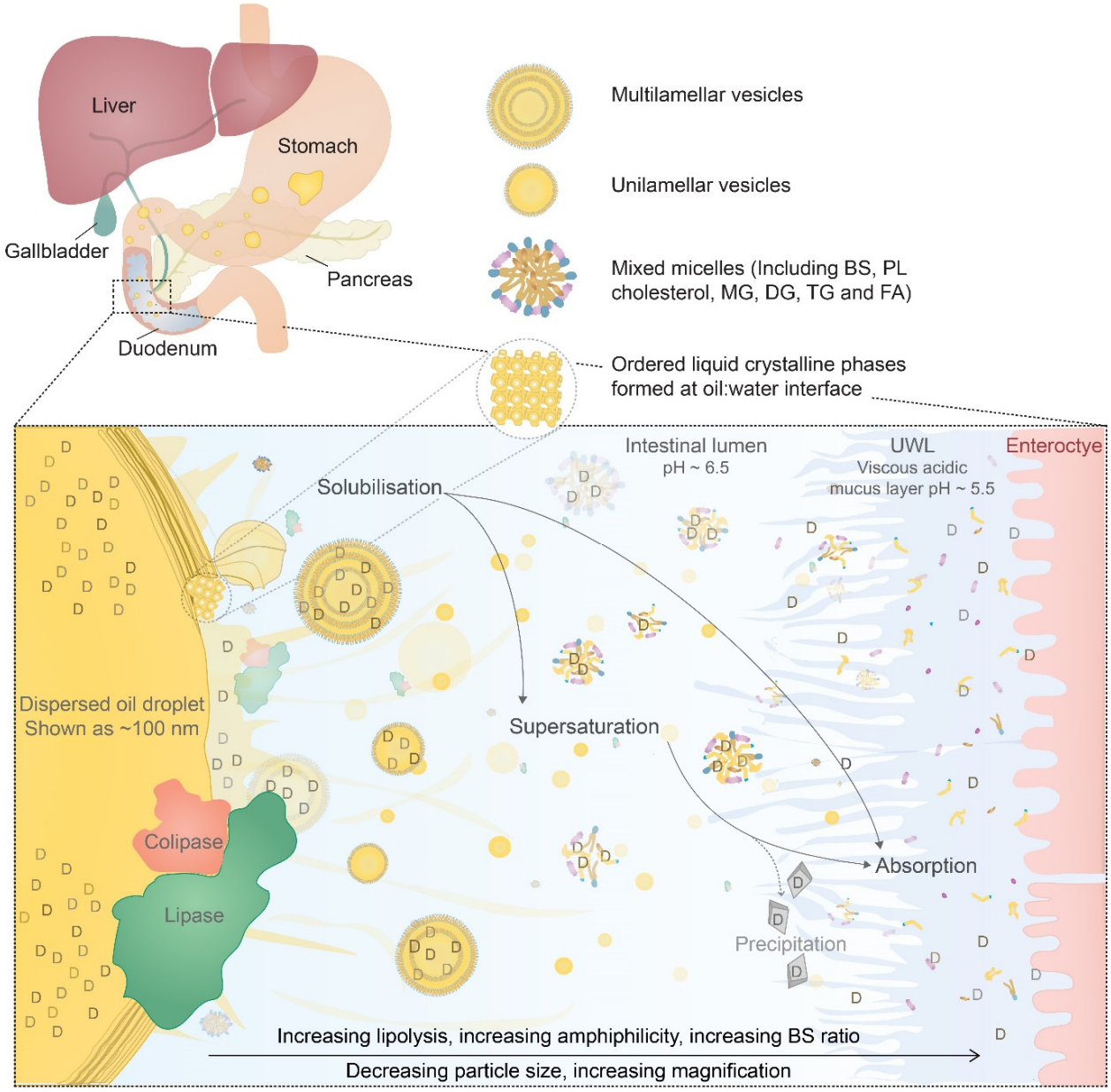


Figure 1.3: Formation of a continuum of lipid reservoirs during triglyceride dispersion and digestion in the small intestine. Lipid species range from large digesting oil droplets to liquid crystalline phases, multi- and unilamellar vesicles, mixed micelles and finally to monomolecular species that are in equilibrium with the colloidal reservoirs and are absorbed at the enterocyte surface. Solubilisation of coadministered PWSD within these lipidic microdomains supports apparent drug solubility within the GIT, avoids traditional dissolution and typically enhances absorption. Figure adapted from Rigler[50] and Porter[6]. The following abbreviations are used: bile salts (BS), phospholipids (PL), monoglyceride (MG), diglyceride (DG), triglyceride (TG), fatty acid (FA), unstirred water layer (UWL).

After absorption into the enterocyte, long chain FA and MG are re-esterified to form TG in the endoplasmic reticulum before incorporation into lipoproteins such as chylomicrons. These large colloidal structures cannot cross the endothelial barrier lining the intestinal vasculature and instead, enter the lymphatics, since lymphatic capillaries have much larger inter-endothelial gaps than blood vessels.

Intestinal lymph flows directly into thoracic lymph and from there into the systemic circulation, effectively bypassing first pass metabolism in the liver[6]. Shorter chain FA and MG are absorbed via more traditional pathways into the portal blood.

1.1.3.2. Lipases and their substrates

Digestion in the human GIT is catalysed by the lipase superfamily of interfacially active enzymes[51]. Of particular interest during gastrointestinal processing of lipidic formulations are; gastric lipase, human pancreatic lipase (HPL)/co-lipase, carboxyl ester hydrolase (CEH) and pancreatic lipase-related proteins (PLRP).

Lingual lipase is less likely to be encountered during administration of encapsulated dosage forms but has similar positional specificity to gastric lipase and will be considered in conjunction with gastric lipase[52].

The catalytic mechanism of serine hydrolase mediated lipid digestion involves adsorption of lipase at the oil:water interface, formation of an enzyme-substrate complex, release of lipolysis products and subsequent desorption[11]. Catalysis is activated at the interface where conformational rearrangement produces an active "open" form of enzyme[51, 53]. The open enzyme results in exposure of the active site serine which then interacts with the carbonyl atom of the ester substrate[51, 53]. The HPL co-lipase complex is the primary enzyme involved in dietary fat digestion and is preceded by hydrolysis in the stomach.

Human gastric and lingual lipase begin fat hydrolysis by releasing fatty acids from the sn-1 and sn-3 positions[54]. Gastric lipase also shows some limited specificity for sn-2 hydrolysis. Gastric lipolysis is not typically employed in fasted *in vitro* lipolysis models[55], since enzyme activity is thought to be low in the low pH of the fasted stomach.

Lipolysis in the stomach is also self-regulating, since the inability of unionised digestion products to disassociate from oil droplet at low pH prevents extensive ongoing enzyme binding[51]. Gastric lipase substrate specificity may also extend to the ester-based surfactants commonly used in SEDDS formulations[55, 56]. After passage to the duodenum, the primary catalyst for lipolysis is the HPL co-lipase complex. Specificity for the sn-1 and sn-3 positions results in release of two FA and 2-monoglyceride[51]. HPL can hydrolyse monomeric substrates in the sn-1 and sn-3 position but activity is dependent on the monoglyceride remaining at the interface. As the preferred substrate for HPL is tri- or di-glyceride, inclusion of these lipidic components in a SEDDS formulation will result in changes in performance on digestion.

Carboxyl ester hydrolase (CEH) is a non-specific esterase with a broad range of substrates such as acylglyerols, phospholipids, cholesterol esters and other esters[51, 55, 57]. Consisting of approximately 4% of the total proteins present in human pancreatic juice, CEH is the second most prevalent hydrolysing enzyme after HPL, comprising 10% of the total enzyme content[58, 59].

The non-specific activity of CEH, and optimal performance at pH 6.5 has been implicated in the hydrolysis of PEG-esters, Labrasol[58] and Gelucire[55]. These surfactants are widely used in lipid-based formulations[60]. It is therefore likely that other ester based surfactants are susceptible to CEH mediated hydrolysis in the GIT.

The final lipase anticipated to impact digestion of LBF is PLRP which is present at 0.3% - 0.5% of the total protein content of human pancreatic juice. PLRP exhibits activity up to pH 8 and is not present in porcine pancreatin[58]. PLRP is thought to hydrolyse Labrasol and may cause the degradation of other ester based formulation excipients[61].

1.1.4. The impact of lipids and lipid-based formulation components on intestinal drug absorption

Lipids and LBFs can mitigate the slow and variable dissolution of PWSDs in the GIT by presenting the drug in a pre-solubilised form. The presence of lipids and surfactants in pharmaceutical formulations also influences the route of absorption of the drug and can significantly impact eventual bioavailability[57].

1.1.4.1. LBF components influence drug solubilisation

As described previously, poorly water-soluble drugs are, according to the Noyes-Whitney equation, typically drugs with low dissolution rates. The administration of a pre-solubilised drug in a lipid vehicle circumvents this potentially rate-limiting dissolution step[57]. In the context of drug delivery, triglycerides are digested to diglycerides, monoglycerides and fatty acids by pancreatic lipases. Intercalation of these digestion products with biliary secretions generates a continuum of lipid reservoirs ranging from liquid crystalline phases at the oil:water interface, to multilamellar and smaller unilamellar vesicles, to mixed micellar species in bile salt rich areas of the GI fluids (Figure 1.3) [62, 63]. These lipid phases provide solubilising vehicles for poorly water-soluble lipid digestion products and are similarly able to solubilise PWSD. As dispersion and digestion proceeds, however, the changing nature of the colloids can result in changes in solvation capacity for PWSD, potentially resulting in supersaturation and precipitation of the drug as it transits the intestine. The complexity of the structural phases formed can be attributed to the unique physicochemical properties of hydrolysed lipid digestion products, molecules that are more amphiphilic than the parent triglyceride, but retain overall hydrophobicity. Hydration, swelling and self-assembly of these lipid digestion products results in the generation of liquid crystalline structures at the droplet interface and dispersion of these liquid crystal phases into intestinal fluids to form a range of structures, including lamellar, cubic and hexagonal phases, all of which have differing capacities to accommodate lipid digestion products or PWSD [38, 64].

Digestion products (and co-administered PWSD) are ultimately solubilised into mixed micellar systems composed of fatty acids, monoglycerides, phospholipid, bile salts and cholesterol. These small, highly dispersed colloids provide an effective transport shuttle for hydrophobic species across the viscous unstirred water layer (UWL) to the absorptive surface of the intestine [65, 66]. They also present a high surface area to promote free drug exchange between the solubilised reservoir and the GI environment [67]. Within the UWL, the slightly acidic environment results in protonation of solubilised fatty acid. This reduces fatty acid micellar

affinity, increases saturation and thermodynamic activity and drives absorption of monomolecular digestion products [68]. In cases where lipid absorption is faster than drug absorption, and particularly where PWSD affinity for triglyceride digestion products is high, partitioning and absorption of lipids from intestinal mixed micelles appears to reduce micellar solvation capacity. This in turn may have the potential to generate transient drug supersaturation and effectively couple drug and lipid absorption at the intestine [69].

1.1.4.2. Lipids slow gastrointestinal transit of drugs

The presence of lipid and lipid digestion products in the GI tract has been shown to slow the rate of gastric emptying[19, 70]. This in turn influences residence time in the proximal small intestine, ensuring maximal exposure to absorptive pathways in the duodenum. For poorly water-soluble drugs, the slow-down in transit facilitates the uptake of drugs in the upper GIT. Lipid chain length influences gastric emptying, and LCT have been observed to be more effective at retarding gastric emptying than shorter chain triglycerides[71]. Recent studies have shown that lipid quantities of 2 g and lower are able to stimulate biliary secretion and elevate GI bile salt levels [39] as well as reduce gastric emptying [40]. However, administration of a lipid-based formulation with high surfactant loads under fasted conditions may not influence gastric emptying as in some cases, lipid quantities may be too low for detection in the duodenal bulb[19].

1.1.4.3. Lipids and surfactants may alter drug intestinal permeability and efflux

Lipids, and many of the other common components of LBF (surfactants and cosolvents), have been described to impact intestinal permeability, both via changes to passive permeability and via inhibition of efflux transporters. These effects have been extensively reviewed [72-76], and are not repeated in detail here. Briefly, a range of lipids (most notably medium chain fatty acids and lysophospholipids), surfactants (including bile salts) and cosurfactants have been shown to increase passive paracellular permeability by opening tight junctions [77, 78], and to promote transcellular permeability by promoting membrane solubilisation and increasing membrane fluidity [74, 79]. Conversely, apparent permeability may be reduced in the presence of colloidal species due to the formation of a competitive sink for solubilised drug and a reduction in thermodynamic activity [80-83]. Reductions in effective permeability have also been reported in the presence of polyethylene glycol and propylene glycol cosolvents [84]. More recently, attention has focussed on the ability of a range of surfactants and some endogenous species (including bile salts) to inhibit the activity of efflux transporters including p-glycoprotein [85, 86], breast cancer resistance protein [87], multidrug resistance protein [88], and others. Almost all commonly employed surfactants have been suggested to show some inhibitory activity against efflux transporters, but perhaps the most compelling data has been generated with vitamin E TPGS and the Pluronic and Kolliphor surfactant families [83, 89-91]. Surfactants are thought to inhibit efflux via changes to the structure and/or fluidity of membrane lipid domains leading to alterations in membrane protein/transporter structure, or by changes to transporter expression. Notably, although an increasing number of studies show compelling inhibitory effects *in vitro*, exemplification of efflux transporter inhibition *in vivo* is less widespread and often complicated by parallel effects on solubilisation.

The effect of efflux transporters on *in vivo* absorption may also be limited in the presence of LBF by the attainment of luminal drug concentrations that are sufficiently high to saturate the transporter.

1.1.4.4. Lipids can alter first pass metabolism

Lipid effects on metabolism are less well described, although some evidence of lipid and surfactant-mediated inhibition of pre-systemic metabolism is apparent, and again appears to be mediated by effects on enzyme activity and expression [92, 93]. Lipids may also affect metabolism indirectly by changes to cellular and systemic drug distribution. For example, coadministration with lipids typically increases circulating lipoprotein levels, and for highly lipophilic drugs may increase drug association with plasma lipoproteins, reducing access to hepatic sites of metabolism. Drug abstraction into developing lipoproteins in the enterocyte has also been suggested to decrease enterocyte based metabolism [94]. However, effects on metabolism are hard to predict and increased lipoprotein association has been shown to both increase and decrease metabolism [95, 96]. Furthermore, whether the quantities of lipid present in a typical LBF are sufficient to alter plasma lipoprotein levels to the point where changes in drug disposition are practically important is unknown [94].

Pre-systemic drug metabolism is also avoided by drugs that are trafficked to the systemic circulation via the intestinal lymph – a process that is supported by coadministration with lipids. Long chain lipids that are absorbed from the GI lumen into the enterocyte are re-esterified to triglyceride in the endoplasmic reticulum and subsequently assembled into lymph lipoproteins. The physical size of lymph lipoproteins (100-1000nm) precludes diffusion across the continuous vascular endothelium and instead promotes selective uptake across the more permeable, and discontinuous, lymphatic endothelium. The intestinal lymphatics drain via the thoracic lymph directly into the systemic circulation and therefore circumvent the first pass metabolic events inherent in absorption via the portal blood. Drugs with high affinity for intestinal lymph lipoproteins typically have log D values greater than five and solubility in long chain triglycerides in excess of 50 mg/g [97]. These drugs may be solubilised in the apolar region of lipoproteins forming in the endoplasmic reticulum, and as a result, may be delivered to the systemic circulation via the lymphatic system[98, 99]. The dependency of this route on the lipophilicity of the compound or vehicle precludes lymphatic absorption as a routine mode of drug absorption, however, formulation approaches to ensure the drug is sequestered in a highly lipophilic environment have successfully influenced the extent of lymphatic drug transport and systemic absorption from oral dosage forms[99].

1.1.4.5. Nonionic surfactants can inhibit enterocyte efflux and metabolism

It has been observed that non-ionic surfactants have the capacity to inhibit CYP-mediated drug metabolism [91, 100, 101]. Effects appear to vary between test species, and whilst it is not fully understood how these excipients inhibit CYP metabolism, it has been observed that administration of non-ionic surfactants *in vivo* with a CYP substrate can result in increased drug absorption[100, 102]. Additionally, the CYP3A4 metabolic pathway shares many of the same substrates as P-gp.

The rate at which some of these substrates enter the enterocyte is thought to be controlled by P-gp, allowing CYP3A4 to remain unsaturated, and removing metabolites from the enterocyte to prevent competitive inhibition of the metabolic pathway[19, 35].

1.1.5. Lipid-based formulations; harnessing the potential of endogenous lipid digestion pathways for oral drug delivery

LBF have been investigated as a means to enhance oral drug absorption for many years[103, 104]. Indeed, lipid suspension and emulsion formulations of sulphonamides were described as early as the 1950s[105, 106]. It was probably not until the 1970s, however, that more detailed evaluations of the use of lipids to boost drug absorption were initiated[107-115]. Increasing application of soft gelatin capsule technologies further facilitated oral administration of undispersed LBF. The rationale for the initial exploration of LBF to promote the absorption of poorly water-soluble drugs was the realisation that many compounds of this type exhibit significant positive food effects. Thus, co-administration with lipids recruits (or at least partially recruits) the physiological events that are initiated by food administration to promote dietary lipid absorption. Lipid administration results in pancreatic and gallbladder secretions that initiate the process of lipid digestion and subsequent solubilisation of lipid digestion products in bile salt/phospholipid/cholesterol mixed micelles. Ultimately, this leads to the development of a range of colloidal particles in the GIT that serve to solubilise dietary lipids, but that also significantly enhance the solvation capacity of the GIT for co-administered drugs.

The importance of lipid digestion in the processing of LBF has led to the development of *in vitro* models of digestion that can be used to mimic the likely pathways of formulation processing in the GIT. This in turn has allowed examination of the potential fate of a co-solubilised drug during formulation digestion. The first reports of these models emerged in the late 1980s[116-121], and accelerated significantly in the 1990s and 2000s[57, 122-130]. These studies developed the hypothesis that for lipid formulations to be successful, drugs should remain in a solubilised state during formulation digestion and processing, as precipitation is likely to be detrimental to formulation performance. This concept was based on the expectation that drug precipitation during formulation processing would generate solid drug, and initiate the requirement for drug dissolution - a process that is typically slow for poorly water-soluble drugs (PWSD). For many (but not all) compounds this general hypothesis appears to hold and good *in vitro-in vivo* correlation has been reported between drug solubilisation during *in vitro* lipid digestion and systemic drug exposure after oral administration of some drugs[130-133].

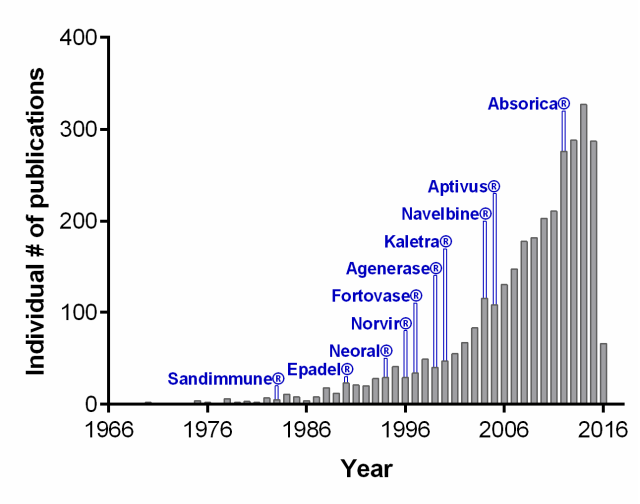


Figure 1.4 Growth in number of LBF/SEDDS publications in PubMed, with marketed LBFs overlaid at corresponding dates of release onto market. Search terms: ((((((poorly soluble) AND lipid formulation) OR self emulsifying) OR SEDDS) OR SMEDDS) OR SNEDDS).

Secondly, the perceived importance of the particle size of the dispersion formed on capsule rupture in the GIT, has driven the development of formulations that spontaneously emulsify to form lipid emulsions with particle sizes in the low nanometre size range. This hypothesis was propagated on the basis that endogenous lipid processing results in increasing degrees of lipid dispersion and solubilisation and that ultimately colloidal structures with small particle sizes are required to diffuse across the unstirred water layer (UWL) and present drug to the intestinal absorptive surface. As such formulations that are pre-dispersed to form small emulsion droplets (microemulsions, nanoemulsions), or that spontaneously emulsify on contact with GI fluids (self-emulsifying drug delivery systems or SEDDS) have been suggested to provide improved performance. The first examples of the potential pharmaceutical utility of SEDDS were described by Groves in the early 1970s [112, 134] and expanded in the 1980's by Pouton and colleagues [118, 135, 136]. The field subsequently accelerated (figure 1.4) with the clinical and commercial success of the Sandimmune and Neoral™ formulations of cyclosporine [137, 138], the properties of which led to increased focus on the particle size of the dispersion formed on capsule rupture as a possible indicator of *in vivo* LBF performance. A complex series of interactions take place in the GIT, however, including formulation digestion and interaction of digestion products with bile salt micelles, and these are likely to significantly change the nature of any SEDDS formulation. It therefore seems likely that the critical parameter in formulation assessment is not the nature of the initial dispersion, but the properties of the dispersion formed after interaction with biliary and pancreatic secretions that ultimately determine LBF performance.

1.1.5.1. Lipid formulation composition and classification

Realisation that many of the beneficial effects of co-administration of PWSD with food can be simulated by coadministration with a LBF drove a number of early studies that explored the use of simple dietary lipid-based suspensions or solutions [103, 109, 116, 117, 139], emulsions [107, 114, 140] and even non-digestible lipids to aid drug solubilisation [116, 117, 121, 141]. In most cases, these simple formulations significantly improved exposure when compared to oral administration of crystalline drug.

Although some of the earliest examples of LBFs are lipid suspensions, and the feasibility and performance of these materials *in vivo* is often reasonable[142, 143], suspension formulations pose significant challenges to robust material transfer and content uniformity, and may also be prone to stability issues due to Ostwald ripening[144]. Consequently, excipients in LBF are typically optimised to maximise the chance of complete drug solvation in the formulation. The least complex LBF comprise simple encapsulated solutions of drugs in oils and are typified by the many fat-soluble vitamin preparations. On capsule rupture in the GI fluids, these LBF are crudely emulsified by the shear associated with gastric emptying and GI segmentation, and digested by gastric and intestinal lipase enzymes to form more amphiphilic digestion products. These digestion products serve to stabilize the emulsions formed and are ultimately solubilised in bile salt/phospholipid/cholesterol micelles secreted in bile. The *in vivo* dispersion of these materials is therefore catalysed by the process of lipid digestion and is still significantly influenced by volume, intersubject variability and patient compliance.

To overcome these challenges, the first major step forward in the evolution of LBF was the development of self emulsifying drug delivery systems (SEDDS) formulations. SEDDS were adapted from the herbicide and pesticide industries where lipophilic actives have been formulated for many years as preconcentrates containing surfactants. These preconcentrates were shipped at lower cost (due to lower volumes) and then readily dispersed in situ to form a fine emulsion prior to spraying [112, 134].

The range of excipients employed to form self emulsifying LBF is wide, but is largely drawn from three broad categories of materials – lipids (the hydrophobic sink), surfactants (to aid emulsification/solubilisation) and cosolvents (to aid solvation/dispersion). The relative proportions of each of these materials dictate drug solubility, formulation dispersibility, transport and metabolic effects and also impact on formulation properties. Refinement of the SEDDS technology has subsequently resulted in formulations that disperse to generate colloids with smaller and smaller particle sizes, systems that were initially described as self microemulsifying formulations or SMEDDS on the basis that the dispersions generated were microemulsions[145]. In reality, whether the colloids so formed are thermodynamically stable (a requirement for definition as a microemulsion) is questionable in many cases[146, 147], and the 'micro'emulsion terminology is seemingly at odds with dispersions with dimensions in the nanometer size range. A recent study by Niederquell and Kuentz, for example, has shown that in an exemplar series of 20 dispersed SEDDS that the majority exhibited only kinetic stability and could not be accurately classified as 'microemulsions'[147]. I

In an attempt to simplify classification of LBF and to group formulations on the basis of their composition, Pouton introduced the lipid formulation classification system (LFCS) (Table 1) in 2000[148] and later updated the classification to expand the formulation groups[32]. The LFCS classifies LBF into four main types, based on the relative proportions of included lipids, surfactants and co-solvents (Table 1). Type I formulations are the simplest and comprise drug dissolved in triglyceride alone or in mixed glycerides. Type II formulations comprise combinations of glycerides and lipophilic surfactants (HLB <12) and are representative of some of the first SEDDS formulations that were described[136]. The original Type II formulations used polyethoxylated triglyceride-based surfactants (e.g. polyoxyethylene 25 glyceryl trioleate - Tagat TO). Similar, although not quite as efficient, emulsification behaviour is possible with polyoxyethylene 20 sorbitan trioleate (Tween 85). Type II LFCS formulations have been largely superseded by Type III formulations, not least because of the limited range of available lipophilic surfactants that promote self-emulsification of Type II formulations and that have been used in registered products. Type III formulations comprise mixtures of glyceride lipids, more hydrophilic surfactants (HLB >12), commonly also polyethoxylated glycerides, but with larger quantities of ethylene oxide, e.g. polyoxyethylene 35 castor oil (Kolliphor EL) or polyoxyethylene 40 hydrogenated castor oil (Kolliphor RH 40) and may also include cosolvents (e.g. PEG400, Transcutol or ethanol). Many SMEDDS or SNEDDS formulations are typical Type III formulations. Type III formulations are further stratified into Type IIIA, that contain larger proportions of lipids, and lower proportions of surfactant and cosolvent, and Type IIIB formulations that contain relatively limited amounts of glyceride lipid (<20%) and larger quantities of hydrophilic components. A classification of Type IV 'lipid' based formulations was introduced later in response to the increasing use of formulations that contain no traditional lipids[32]. Type IV formulations comprise only a combination of surfactants and cosolvents. The general properties of the different types of LBF are summarised in Table 1. In brief, the lipid rich Type I formulations require digestion to increase amphiphilicity and dispersion into intestinal fluids whereas Type II-IV contain sufficient surfactant to promote spontaneous dispersion. Progression from Type I-IV decreases triglyceride content and formulation susceptibility to digestion, and in general also leads to reductions in particle size of the resulting dispersion. For example, Type IV formulations typically disperse to form micellar solutions with particle sizes of 20 nm or below. Increasing quantities of surfactant and cosolvent in Type IIIB and Type IV formulations usually increases drug loading, since, with the exception of the most lipophilic drugs, the majority of PWSD are more soluble in surfactants and cosolvents than they are in glyceride lipids. The downside to the more hydrophilic Type IIIB and Type IV formulations is that inclusion of larger quantities of water-miscible components increases the risk of drug precipitation on dispersion of the formulation in the GI fluids.

Further amendments to the LFCS have been proposed to take into account the original classification system for lipids proposed by Small (rather than the combinations of lipidic excipients described by LFCS). These amendments were proposed in large part to better capture the properties of polar lipids that swell on contact with aqueous media and have markedly different properties to, for example, non-polar triglycerides[149] but that were grouped together as 'oils' in the LFCS.

Most recently, attention has switched to the classification of LBF based on *in vitro* performance, rather than solely on composition. This development was driven by the realisation that whilst excipient combinations that lead to useful self-emulsification are reasonably predicable, formulation performance for specific drugs is much more nuanced and the physicochemical properties of a drug alone are insufficient to inform *de novo* formulation design. As a result, preliminary formulation screening via *in vitro* dispersion and digestion testing is typically required to optimise LBF design. Work conducted under the Lipid Formulation Classification Scheme (LFCS) Consortium generated a large database describing the behaviour of Type I-IV formulations (containing a range of model drugs) during both dispersion in simulated GI fluids and on digestion under simulated intestinal conditions[150-155]. In a more high-throughput approach, the use of simplified *in vitro* digestion models may also allow ranking of formulation performance and has been recently explored by multiple groups[156-158]. Notably, however, in all cases *in vitro* dispersion/digestion testing is based on the assumption that drug precipitation from lipid formulations *in vitro* provides an indication of hindered performance *in vivo.* This is not always the case, and the lack of an absorptive sink *in vitro* may overestimate the precipitation rate of some model drugs.

Table 1.2: The LFCS adapted from Pouton *et al.* with permission[32, 148]

Excipients	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	-
Water insoluble surfactants *(HLB <12)*	-	20–60	-	-	0-20
Water soluble surfactants *(HLB >12)*	-	-	20–40	20–50	30-80
Hydrophilic cosolvents	-	-	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 and potential loss of solvent capacity	Significant phase changes and 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
Characteristics	Non-dispersing; requires digestion	SEDDS without water-soluble components	SEDDS/SMEDDS with water-soluble components	SMEDDS with water-soluble components and low oil content	Oil-free formulation based on surfactants and cosolvents
Advantages	GRAS status; simple; excellent capsule compatibility	Unlikely to lose solvent capacity on dispersion	Clear or almost clear dispersion; drug absorption without digestion	Clear dispersion; drug absorption without digestion	Good solvent capacity for many drugs; disperses to micellar solution
Disadvantages	Formulation has poor solvent capacity unless drug is highly lipophilic	Turbid o/w dispersion (particle size 0.25–2μm)	Possible loss of solvent capacity on dispersion; less easily digested	Likely loss of solvent capacity on dispersion	Loss of solvent capacity on dispersion; may not be digestible

1.1.5.2. Supersaturation and drug precipitation from lipid-based formulations

Coadministration of PWSD with lipids, or administration with a LBF typically enhances the overall solvation capacity of the GI fluids by creating additional, lipid swollen, colloidal species in which the solubility of a lipophilic PWSD is enhanced. However, to the best of our current understanding [68, 159], these colloidal species are not absorbed intact and drug absorption occurs from the free concentration of drug that is in rapid equilibrium with the solubilised colloidal reservoir. Whilst the total solvation capacity of the GI fluids is therefore enhanced by the presence of mixed bile salt-phospholipid-lipid digestion product micelles, in the absence of supersaturation, the free concentration of drug is expected to be no greater than the drug solubility in intestinal fluid. Where drug is present as a saturated solution in intestinal colloids the free drug concentration in equilibrium with the solubilised reservoir is approximately equal to the saturated solubility of drug in the absence of the solubilising species (essentially the aqueous solubility). Under normal circumstances, therefore, although drug solubilisation in intestinal colloids increases effective solubility, the (free) concentration of drug, i.e. the concentration that drives absorptive flux, is not increased significantly above the aqueous drug solubility. In light of this limitation, but realising that LBF (or food) typically provide for significant increases in drug absorption, recent work has explored the hypothesis that lipid formulations intrinsically generate supersaturation during GI processing [69, 128, 160, 161].

These studies suggest that for PWSD, there are three potentially complimentary routes by which supersaturation can be generated during LBF digestion. Firstly, solvation capacity is often lost during dispersion and digestion of drug loaded LBF [128, 152, 162]. Where this does not lead to immediate precipitation, supersaturation ensues. Secondly, absorption of lipid digestion products (that swell intestinal colloids and maintain drug solubilisation), also decreases drug solvation capacity and may lead to supersaturation in cases where lipid absorption is more rapid than drug absorption [69, 163]. Thirdly, for ionisable PWSD, and in particular ionisable weak bases, supersaturation is also generated via the pH gradient encountered during gastrointestinal transit [164-166]. Thus, higher drug solubility is typically attained in the low pH environment of the stomach and this drops on transition to the more neutral pH conditions in the small intestine before encountering the acidic unstirred water layer. Supersaturated systems formed via one or more of these mechanisms have higher thermodynamic activity (and therefore higher absorption potential) when compared to colloids containing drug at (or below) equilibrium solubility. However, transition from a high-energy (supersaturated) state to the equilibrium point is energetically favoured, and therefore supersaturation also inherently predisposes systems to precipitation in order to re-attain equilibrium solubility. These precipitation events must first overcome the activation energy associated with crystal nuclei formation. Where the activation energy is high, crystallisation cannot proceed and a supersaturated metastable state may be maintained for a sufficiently long period to support drug absorption.

In the context of supersaturation on LBF dispersion, the nature of the formulation and the drug load are major drivers of precipitation. Formulations containing high drug loads and high proportions of amphiphilic

excipients (surfactants and co-solvents) are most likely to result in rapid dissociation of water-soluble components, significant supersaturation and the greatest risk of drug precipitation [128, 152]. Conversely, in formulations comprising more lipophilic components, excipient dissociation is less likely on dispersion and precipitation is often delayed for extended periods of time. In this case, drug concentrations in the GI tract may be transiently elevated relative to apparent solubility in the GI fluids, leading to a metastable supersaturated state with higher thermodynamic activity (Figure 1.5B).

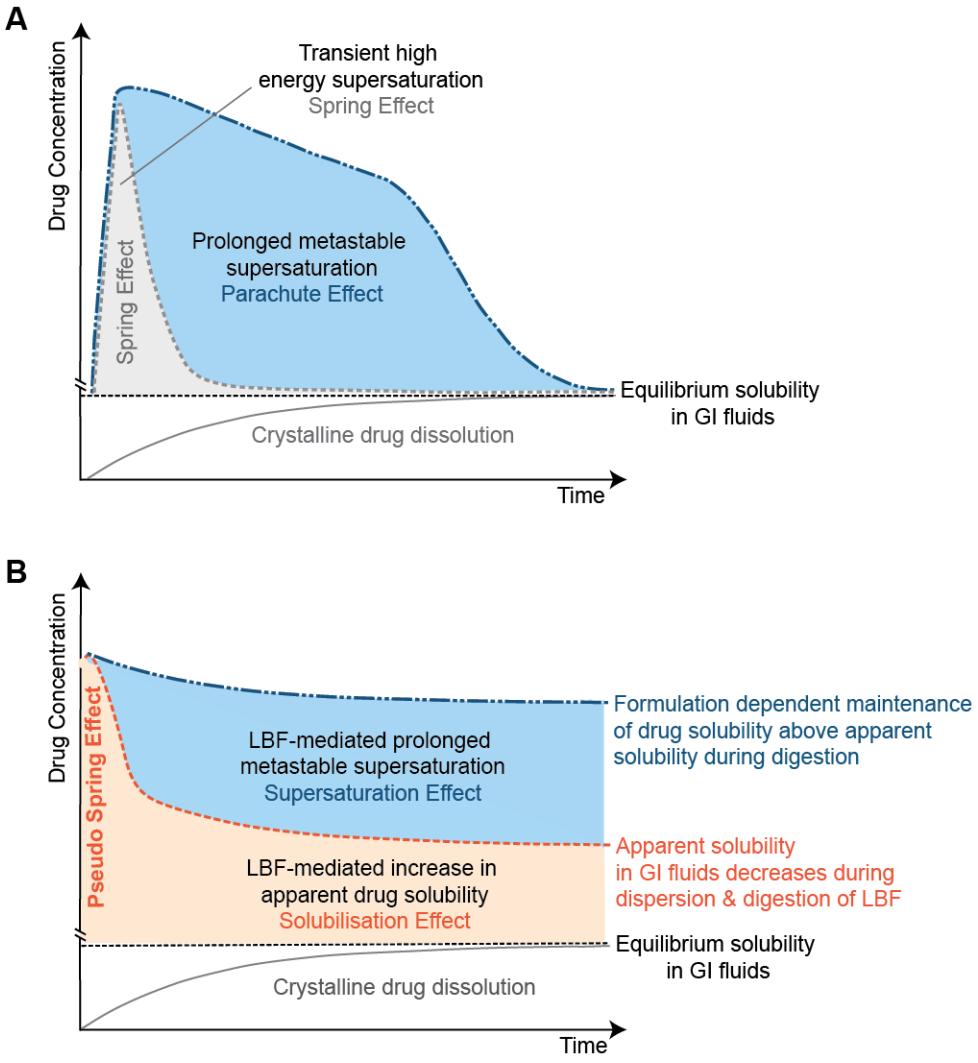


Figure 1.5: Pathways to drug supersaturation during LBF dispersion and digestion. (A) The spring and parachute effect typically observed from solvent-shift or pH-shift methods used to evaluate drug supersaturation and precipitation kinetics or after dissolution of amorphous solid dispersions. Adapted from Browers *et al.* [167]. (B) The solubilisation and supersaturation effect generated by LBFs during formulation dispersion and digestion, adapted from Anby *et al.* [128].

On exposure to lipolytic enzymes, LBF are once again challenged and for digestible components, dissociation of more amphiphilic lipolysis products will likely reduce the solvation capacity of the colloid further, increasing supersaturation. Under these circumstances, the acyl chain length of the lipid(s) employed in the formulation may have a significant impact on the likelihood of drug precipitation. Lipase mediated digestion

of long-chain lipids is slower than that of medium chain lipids [119, 168], and the long chain digestion products produced appear to more readily maintain solvation capacity. Conversely, digestion of medium chain triglycerides is rapid and produces more water-soluble digestion products, leading to higher supersaturation levels and faster rates of supersaturation with increased potential for drug precipitation. Drug solubility in medium chain triglycerides is also often higher than that in long chain triglycerides allowing for significantly higher initial drug loadings in the medium chain based LBF [169]. Together, the higher drug loading and rapid digestion-induced reduction in solvation capacity in formulations based on medium chain triglycerides increases the likelihood of drug supersaturation and precipitation. The incidence of danazol supersaturation, triggered by digestion of medium chain triglyceride LBFs, has been described recently by Anby *et al.* [128]. In these studies, drug solubilisation or precipitation was related to the degree of initial supersaturation stimulated by dispersion and digestion. Thus, supersaturation above a certain threshold (in this case concentrations approximately 3 fold higher than equilibrium drug solubility in the colloidal species formed) resulted in precipitation. The concept that increases in the degree of supersaturation are likely to drive increases in the potential for precipitation is in agreement with the fundamental principles of nucleation and the realisation that nucleation rate and consequent precipitation is dependent on the extent of supersaturation [167, 170]. Similar results (and similar threshold values) have subsequently been reported for a separate series of formulations of danazol, fenofibrate and tolfenamic acid [152], suggesting some degree of consistency in drug precipitation behavior from SEDDS *in vitro* across a range of drugs and a range of different formulations.

Recognising that drug supersaturation may be a crucial driver for absorption, efforts have also focused on developing formulation strategies to stabilise or prolong drug supersaturation during the dispersion and digestion of LBF. This is analogous to the “spring and parachute” mechanisms of supersaturation generation and stabilisation widely described in the polymer literature (figure 1.5A). In the case of a LBF, dispersion and digestion events that promote supersaturation drive either absorption or precipitation and form the ‘spring’, and polymeric formulation additives may be employed in an attempt to reduce drug precipitation (the “parachute”) [128, 171, 172]. However, the choice of polymeric precipitation inhibitor is not trivial and requires careful balance of hydrophobicity, lipophilicity and compatibility with the PWSD under investigation [173, 174].

In a somewhat related approach, others have sought to allow precipitation but to promote precipitation of the amorphous forms of a PWSD, in the expectation that re-dissolution of drug will be enhanced from the high energy solid [127, 163, 175, 176].

Finally, drug loaded lipophilic colloids must diffuse across the acidic environment of UWL in order to reach the absorptive membrane. Recent work has suggested that collapse of the colloidal structures in the acidic microenvironment of the UWL and stimulation of lipid absorption may result in drug supersaturation and promote drug absorption [69]. The process of absorption-triggered supersaturation has subsequently been modelled by Stillhart *et al.* [163]. In these studies the authors suggest that supersaturation may be achieved by

absorption of lipid digestion products but that concurrent drug absorption (in this case with fenofibrate as a model drug) may prevent attainment of high degrees of supersaturation and therefore prevent initiation of drug precipitation [163]. The latter studies illustrate that interpretation of supersaturation patterns using *in vitro* methodologies should ideally consider the role of absorption *in vivo*. Even in the presence of significant precipitation *in vitro*, drug absorption *in vivo* may reduce the drivers of precipitation and allow ongoing absorption, especially for highly permeable compounds.

1.1.6. In-vitro characterisation of lipid-based formulations

1.1.6.1. Drug solubility in lipid formulation preconcentrates

The solubilisation capacity of a SEDDS formulation is critical to practical utility, as the dose administered is contingent on adequate solubility in the neat formulation. Preparation of a stable formulation with sufficiently high drug loading that will elicit a biological response and/or deliver the intended oral dose must be approached with care. Drug loading must be below the equilibrium solubility of the drug in formulation to prevent crystallisation from a supersaturated state in the formulation preconcentrate.

Equilibrium solubility is assessed by preparation of a saturated solution of drug in formulation, followed by consecutive analyses until the variation from one analysis to another is less than 5%.

Drug loading in the tested formulation type will typically be no more that 80% of the measured solubility. This approach allows the formulator some degree of confidence in the intermediate stability of the proposed formulation[131, 177].

1.1.6.2. Dispersion properties on dilution with aqueous media

It can be argued that the Noyes-Whitney dissolution equation does not adequately describe *in vitro* performance of lipid formulations as the drug is administered in pre-solubilised form, however, the dispersion of lipid-based formulations in USP type II apparatus (rotating paddles) is often performed [131, 178]. During dispersion tests, a SEDDS is typically dispersed in biologically relevant media (simulated gastric/intestinal fluid) to assess the self emulsifying properties of the formulation and less frequently (but more importantly) the likelihood of drug precipitation on dilution. A critical determinant of dispersion performance therefore includes the concentration of solubilised drug following dispersion of the formulation. Poorly performing formulations are those that show evidence of drug precipitation as the formulation undergoes dilution. This phenomenon may be observed visually or quantified by separation of the solid precipitate from the solubilised drug in the aqueous phase. Maintenance of solubilisation capacity on dilution is important to the performance of a SEDDS formulation, but is not the only significant challenge to formulation viability.

1.1.6.3. Particle Size

The particle size of lipid-based drug delivery systems can vary from coarse Type I oil systems to microemulsified (<50nm) Type IV formulations[32]. Formulation particle size has been suggested to impact the bioavailability of a lipid-based formulation and in the case of cyclosporin A, the superior in-vivo performance of the Neoral™ formulation was in part attributed to a reduction in particle size of the reformulated product[35]. Lipolysis tends to reduce the particle size of orally administered digestible lipid-based formulations. However, for non-digestible formulations, the anticipation is that the formulation will not be hydrolysed, and therefore will not have the assitance of solubilising digestion products to increase emulsification of the formulation. As a result, a non-digestible SEDDS (ND-SEDDS) must form a fine dispersion on dilution as coarse emulsions would preclude efficient drug trafficking to the absorptive membrane and transfer from the centre of the droplet to the surface.

The required particle size of a non-digestible dispersion is expected to be less than 100nm. Drug transit from the centre of the dispersed droplet to the interface must be rapid. Concomitant to the smaller droplet size is an increase in surface area, potentially allowing increased diffusion of drug from the surface of the oil droplet to the absorption site.

1.1.6.4. Subjecting lipid-based formulations to an *in vitro* digestion challenge

The first reports of *in vitro* digestion models emerged in the late 1980s[116-121], and accelerated significantly in the 1990s and 2000s[57, 122-130].

In addition to maintaining solubility during dispersion, a lipid-based formulation must maintain solubilisation capacity during digestion (which will occur primarily in the upper small intestine). Lipid digesting conditions are mimicked *in vitro* by addition of a porcine pancreatic extract (containing pancreatic lipase) into a reaction vessel containing the lipid formulation dispersed in BS-lecithin mixed micelles[5, 6, 124, 179]. During digestion, one mole of TG is cleaved in two steps at the sn-1 and sn-3 position to produce one mole of 2- MG and two moles of free FA. The FA released reduces the pH of the reaction medium and is continuously monitored by a pH probe coupled to a pH stat system (figure 1.6). The FA is titrated by an autoburette which dispenses an equimolar quantity of sodium hydroxide to maintain steady pH and thus quantitate the rate and extent of digestion.

Throughout the digestion process, samples may be removed and centrifuged to separate the digestion products into discrete oil, aqueous micellar and pellet phases. The drug distribution between all three phases thus provides information on the propensity for precipitation during digestion and may give an indication of likely *in vivo* performance.

The outcomes from lipolysis experiments are contingent on a number of experimental parameters which must be tightly controlled, namely; buffer capacity, enzyme activity, BS/PL concentration and calcium

content. To ensure adequate sensitivity for FA-mediated pH changes, the buffering capacity of the reaction media must be low[180]. Increased buffer concentration may cause a decrease in sensitivity and impact the resultant digestion profile.

Enzyme activity of the pancreatin extract is expressed as TBU (tributryn units) where, under controlled conditions, 1TBU is equivalent to the enzymatic quantity required to liberate FA at a rate of 1 μmole per minute[181]. Enzyme activity is also dependant on the particle size of the oil droplet, therefore variation in activity measurement between instruments/methods is expected based on differences in mixing conditions[5, 182]. During lipid digestion, saturation of the enzyme will decrease the rate of lipolysis, however, pancreatic lipase is thought to be in excess *in vivo*[179]. Consequently *in vitro* lipolysis experiments involve addition of excess lipase to ensure rate limiting saturation does not occur and also mitigates the effects of batch-to-batch activity variability that can be expected from crude pancreatic extracts.

FA and 2-MG are surface active by-products of digestion. Accumulation at the oil water interface can sterically impede lipase/co-lipase binding and quench the lipolysis reaction[48]. BS/PL mixed micelles present in the upper small intestine will solubilise FA and 2-MG and therefore effectively remove these digestion products from the surface of the oil droplet and subsequently aid their absorption across the BBM. This natural process provides a continuum of unsaturated BS/PL mixed micelles and allows the digestion process to progress until all ingested lipid has been hydrolysed.

The concentration of bile salt and phospholipid during in-vitro experiments is fixed (usually at 3.0 – 5.0 mM and 0.75 – 1.2 mM respectively to mimic typical fasted conditions of the small intestine in humans). Under these conditions, it is possible that BS/PL mixed micelles become saturated with digestion products following an initially fast period of digestion to an extent that precludes ongoing digestion. Increasing bile salt concentration has therefore been shown to directly increase the extent of lipid digestion[180].

Coupled to this bile salt effect (i.e solubilisation of digestion products), the concentration of calcium will significantly influence the rate of lipolysis[179, 183]. Ionised calcium (Ca^{2+}) will form calcium soaps with FA, a process that effectively removes liberated FA from the oil:water interface and limits lipolysis inhibition. In addition to the effect of Ca^{2+} on digestion at the interface via removal of FA, Ca^{2+} is thought to form a catalytically more efficient complex with lipase and bile salts[181], increasing the observed lipolysis rate.

In vitro lipolysis tests thus provide a relatively simple representation of the complexity of intestinal digestion conditions and the outcomes from *in vitro* lipolysis experiments are contingent on a number of experimental parameters that must be tightly controlled to provide reproducible data. These include buffer capacity [180], enzyme activity [155, 156, 184], bile salt and phospholipid concentration [151, 180], stirring rate, calcium concentration [155, 179, 183] and pH. Relatively minor differences in the methods used for lipolysis studies can have a significant

impact on formulation performance *in vitro*. In the context of LBF development, variability in the *in vitro* methodology employed therefore often prohibits facile inter-laboratory comparison of data sets.

Efforts to standardise the *in vitro* lipolysis model were undertaken by a consortium of academics and industrialists (the Lipid Formulation Classification System (LFCS) Consortium) [185]. These studies led to the publication of a standard operating procedure for *in vitro* lipolysis testing. The present project has adopted these preliminary test conditions for lipolysis experiments.

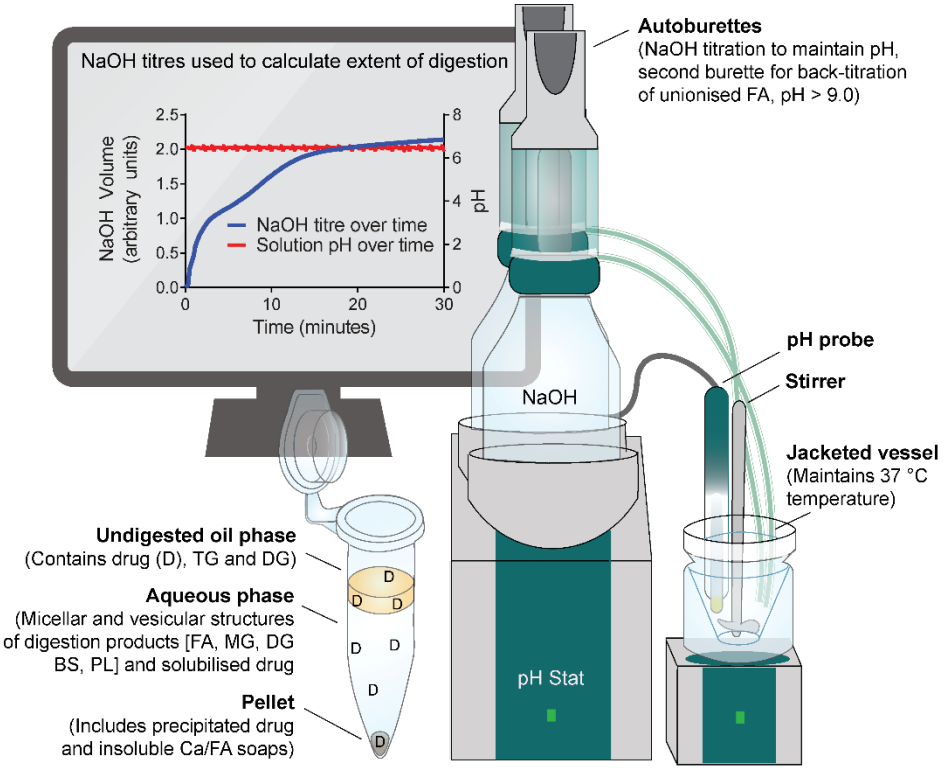


Figure 1.6 Experimental model for *in vitro* assessment of lipid-based formulations. Abbreviations used are; sodium hydroxide (NaOH), drug (D), triglyceride (TG), diglyceride (DG), fatty acid (FA), monoglyceride (MG), bile salt (BS), phospholipid (PL), calcium (Ca)[1].

1.1.7. Effects of non-ionic surfactants on lipid digestion

The development of digestion modulating SEDDS requires excipients that are capable of changing the rate of digestion. This is expected to involve changes at the oil:water interface. In the current studies this has been achieved using surface active excipients (surfactants).

Addition of surfactants to the lipolysis reaction medium is expected to have a significant impact on the oil-water interface and thus the rate and extent of digestion. Lipid digestion is dependent on the particle size of the substrate oil droplets[182, 186]. Emulsification typically increases with addition of surfactant[131, 177] and as a result, faster lipolysis rates might be expected for surfactants that produce no other effect than a reduction in particle size[11]. Conversely, surfactants have the potential to inhibit the lipolysis of lipids by acting as

competitive substrates for the enzyme or by displacing, or sterically blocking the HPL co-lipase complex from the surface of the lipid droplet[11].

Solomon *et al* investigated the inhibitory effects of non-ionic surfactants on the digestion of medium chain triglycerides[187]. Whilst the study assumed the inhibition of lipolysis by surfactants was detrimental to the performance of SEDDS formulations, the results are of particular use to this project. Lipolysis inhibition of MCT was observed in the presence of several non-ionic surfactants, including nonyphenol ethoxylates (NPE), lutensol AO, PEG alkyl ethers (Brij), and PEG alkyl esters.

MacGregor *et al* also observed the reversible inhibition of MCT lipolysis by Kolliphor RH40[180] and inhibitory effects of non-ionic surfactants on LCT digestion have also been reported. Thus, tween 80, CrRH40 and CrEL were observed to have significant inhibitory effect on olive oil digestion[59].

The consistent observation across all of the work described above is the presence of poly(ethylene glycol) (PEG) hydrophilic headgroups on each of the surfactants. This project proposes that developing increased understanding of how the PEG groups of different surfactants impact the digestion of triglycerides, particularly medium chain triglycerides, may facilitate better design of digestion modulating formulations.

1.1.8. The PEG-ylated micelle as an "anti-biofouling, digestion modulating" surface

Polyethylene glycol (PEG) has been extensively used as a surface modifier in systems that require low protein binding. A highly hydrated flexible mantle, neutral charge and uneven surface conferred by polyethylene glycol surface modification prevents or markedly reduces non-specific protein adsorption[188]. This has resulted in PEG-ylated surface applications as diverse as biosensors, contrast agents, chemical synthesis, stealth nanoparticles and drug delivery[189-194]. The anti-biofouling properties of these diverse systems are based on a recurring theme, that inhibition of protein binding is dependent on the chain length and surface density of the PEG.

PEG is extensively utilised as the hydrophilic moiety of non-ionic surfactants employed in LBF where the degree of PEG-ylation (i.e. the number of repeating PEG units) is expected to influence the hydrophile-lipophile balance, and consequently the dispersive characteristics of the surfactant[180, 187, 195]. However the use of PEG-ylated surfactants in lipid-based formulations to potentially create anti-biofouling/digestion resistant surfaces has not been well explored.

The hydrolysis of lipids in the GI tract is based on the adsorption of the lipase/co-lipase enzyme complex to the surface of the oil droplet i.e. non-specific protein adsorption. Prevention of digestion by disrupting the adsorption of lipase/co-lipase at the oil water interface could potentially be facilitated by use of PEG containing excipients.

Solomon et al have alluded to the impact of PEG-ylation on the digestion of medium chain triglycerides[187, 195]. Their work showed that the increase in HLB conferred by increasing PEG chain length of nonylphenol ethoxylates resulted in steric inhibition of pancreatic lipase/co-lipase activity. Whilst the authors advocated against the use of digestion inhibiting surfactants in lipid-based formulations of PWSD, their findings provide a reasonable basis for the development of digestion modulating formulations.

More recently, the ability of PEG-ylated surfactants to inhibit digestion of triglycerides has been explored by McClements et al and Gálvez-Ruiz et al with a view to improving the absorption of neutraceuticals, the development of functional foods and applicability to drug delivery systems[196-201]. Again, both groups have found that the presence of PEG-ylated surfactants in the digestion medium results in inhibition of digestion[196, 197]. The impact of PEG chain length has also been briefly investigated with Myrj stearyl ester surfactants[201]. These studies are summarised in table 1.3.

Table 1.3: Digestion modulating behaviour of surfactants in the literature

Surfactant(s):	**Impact on digestion**	**Impact on particle size**	**Reference**
Kolliphor EL	Increased	Reduced	131, 177
Bile salts, E500, SDS	Decreased		11
Nonylphenol ethoxylates	Decreased	Reduced	187, 195
Kolliphor RH40	Decreased (reversible)		180
Tween 80 Kolliphor RH40 Kolliphor EL	Decreased		59
Tween 20, Tween 80, Brij 35, DTAB, SDS	Decreased	Reduced	196
Lecithin, Pluronic F68	Decreased		197
Epikuron 145V, Pluronic F68	Decreased		198
Pluronic F68, Pluronic F127, Myrj 52, Myrj 59,	Decreased		199
Epikuron 145V, Pluronic F68	Decreased		200

The work in this thesis sought to further explore the impact of PEG-ylation on the digestion of medium-chain triglycerides, and in particular to explore the polymer characteristics which have been found to strongly influence the anti-biofouling properties of PEG surfaces, namely, PEG chain length and surface density. These properties will then be applied to the development of novel digestion-modulating formulations for the administration of poorly water-soluble drugs.

1.1.9. Non-digestible lipid-based formulations

The simplest approach to developing non-digestible lipid-based drug delivery system is to avoid hydrolysis of the formulation. Interestingly, despite digestible lipid solution and emulsion formulations evolving over the years to become more complex SEDDS and SNEDDS [118, 123, 136, 202], non-digestible LBFs have not been similarly developed. This potentially reflects the early use of poorly dispersible mineral and paraffin oil formulations that are unlikely to further emulsify *in vivo* and therefore performed poorly [116, 117, 121].

Single component lipid formulations of digestible triglycerides transform *in vivo* to more amphiphilic lipids. These digested lipids intercalate into bile salt micelles to form highly dispersed, solubilised vehicles that diffuse effectively across the intestinal unstirred water layer (regardless of the dispersibility of the initial formulation). In contrast, non-digestible lipids cannot incorporate into lipid digestion pathways and must therefore be pre-emulsified to form a fine colloidal dispersion in order to facilitate diffusion across the UWL and drug absorption. When formulated to generate highly dispersed micellar solutions, non-digestible lipids may well be highly effective, since formulations where digestion is inhibited avoid the variability and potential loss of solubilisation associated with lipid digestion [178, 203, 204].

Since many surfactants are esterified with PEG and since PEG esters may be substrates for digestive enzymes, non-ester containing surfactants are more appropriate for the design on non-digestible systems. Thus polyethylene glycol (PEG) alkyl ethers can replace ester analogues such as PEG stearate. Whilst PEG ethers are routinely used in topical pharmaceuticals and cosmetics, they have not (to this point) been widely used in oral SEDDS formulations[178].

Potential triglyceride replacements include; long chain fatty acids and alcohols, sucrose polyesters and very low HLB pluronic block copolymers, all of which either do not possess ester moieties, or in the case of sucrose polyesters, sterically hinder lipase mediated hydrolysis[205]. To date, application of these excipient types to lipid-based SEDDS has been limited.

The semi-fluorinated diblock alkane 1-perfluorohexyloctane (F6H8) has been assessed as a novel non-digestible oil in the presence of MCT[206]. Lipolysis was reduced by 20% in a 1:1 ratio of F6H8:MCT when compared to MCT alone. However, following *in vivo* assessment the authors concluded that the low solubilising capacity of F6H8 and the inability to increase bioavailability limited future use in SEDDS formulations. Interestingly, addition of a PEG-ylated surfactant (polysorbate 80, ester-based digestible surfactant) improved the in-vivo performance of the formulation.

1.2. Aims of research

The overarching hypothesis of this work was that inhibition (or elimination) of digestion provides a means to promote stable PWSD solubilisation in the GI tract and to enhance PWSD absorption.

The aim of this project was therefore to develop novel formulations that can control MCT and LCT digestion rates. This was achieved initially by systematically evaluating the influence of PEGylated non-ionic surfactants on the digestion of co-formulated lipids.

Studies then examined the influence of digestion-inhibiting or ‘stealth’ formulations on drug solubilisation, supersaturation and precipitation during *in vitro* digestion testing and ultimately explored whether any solubilisation advantage observed *in vitro* was translatable *in vivo* in both rat and dog models.

Lastly, these studies aimed to develop novel non-digestible lipid-based formulations capable of forming nanoemulsions on dilution. Formulations were assessed to see whether they were able to maintain drug solubilisation during *in vitro* dispersion/digestion testing and whether ND-LBF were able to enhance the absorption of PWSD *in vivo* in rats.

1.3. Thesis structure

This thesis is a compilation of two published manuscripts (Chapters 1 and 3), and two unpublished experimental chapters (Chapters 4 and 5). Detailed experimental methods have been extracted and compiled into Chapter 2. The thesis concludes with Chapter 6 which provides a summary and future perspectives. Appendices to this thesis are the two published papers and three papers on lipid-based drug delivery that I contributed to during my candidature but were outside the immediate scope of this thesis.

Chapter 2. General Methods

2.1. Materials

Danazol (pregna-2,4-dien-20-yno[2,3-d]isoxazol-17-ol) was supplied by Coral drugs PVT (New Delhi, India). Progesterone, cinnarizine, diazepam and 1-aminobenzotriazole (ABT) were from Sigma-Aldrich (St Louis, MO, USA). Captex® 355 (C_8 and C_{10} medium chain triglycerides - MCT) and Capmul® MCM were kindly donated by Abitec Corporation (Janesville, WI, USA) and was used as received. Etocas™ 5, 15, 35, 200 (PEGylated castor oils - CO), Croduret™ 7, 25, 40 (PEGylated hydrogenated castor oils - HCO), Myrj™ S8, 20, 40, 50, 100 (PEGylated stearic acids), Brij™ S2, 10, 20, 100, 200 (PEGylated stearyl alcohols), Brij™ O2, 3, 5, 10, 20 (PEGylated oleyl alcohols), Synperonic®L101 and Novol™ (Oleyl Alcohol) were donated by Croda International PLC (Yorkshire, England). Jeechem® CAH 16 (PEG 16 hydrogenated castor oil) and Jeechem® CA 25 (PEG 25 castor oil) were donated by Jeen® International Corporation (Fairfield, NJ, USA). Kolliphor® EL (PEG 35 castor oil) and RH40 (PEG 40 hydrogenated castor oil) were donated by BASF Corporation (Washington, NJ, USA). Nikkol® HCO 100 (PEG 100 hydrogenated castor oil) was kindly donated by Nikko Chemicals Co. Ltd. (Chuoku, Tokyo, Japan). Transcutol was provided by Gattefossé (Saint-Priest, France). Captisol was a kind gift from CyDex (now Ligand, La Jolla CA). Oleylamine (95%) was purchased from ABCR GmbH & Co KG (Mannheim, Germany). Soybean oil (C_{18} long chain triglycerides - LCT), Oleic Acid, 1-Octadecene, Triton™ X15, 165, 305, 705 (PEGylated branched octyl phenols), sodium taurodeoxycholate > 95% (NaTDC), porcine pancreatin (8 x USP specification activity) PEG 400, and 4-bromophenylboronic acid (4-BPB) were obtained 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). Sodium hydroxide 1.0 M, which was diluted to obtain 0.6 M and 0.2M NaOH titration solutions, 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. Hypergrade solvents were used for UPLCMS/MS analysis.

2.2. Choice of model poorly water-soluble drugs

2.2.1. Danazol

Danazol (17α-pregna-2,4-dien-20-ynol(2,3-d)-isoxazol-17-ol, molecular weight 337.46 g/mol) is a synthetic non-ester anabolic steroid[207]. Primarily used in the treatment of endometriosis, danazol acts as a gonadotropin inhibitor, reducing plasma levels of lutenizing hormone and follicle stimulating hormone[207]. Danazol is poorly water-soluble, moderately lipophilic and is an exemplar of a typical 'brickdust molecule'. Consequently, the oral bioavailability of danazol is low [208]. Danazol has therefore been extensively used as a model drug for investigating various formulations strategies (including lipid formulations) to improve oral bioavailability [22, 63, 128, 131, 150, 151, 178, 209, 210].

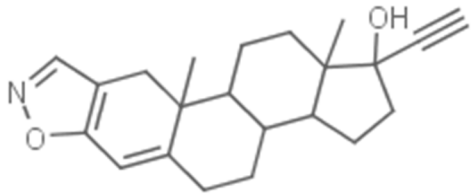
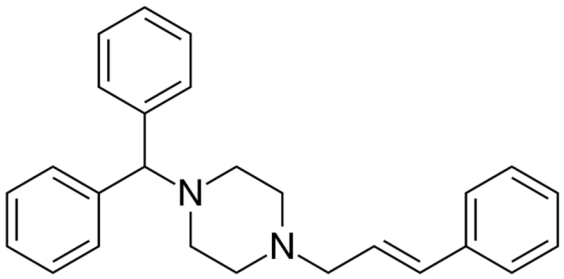


Figure 2.1 Chemical structure of Danazol [207]

2.2.2. Cinnarizine

Cinnarizine (1-benzhydryl-4-cinnamyl-piperazine) is a piperazine derivative with sedative, antihistamine, and calcium-channel blocking activity. It is clinically used in the symptomatic treatment of vestibular disorders as well as in the management of various peripheral and cerebral vascular disorders. Cinnarizine is a weak base (pK_{a1} = 1.95 [211], pK_{a2} = 7.47 [211]) with very low aqueous solubility and a high octanol/water partition coefficient (log P = 5.8 [212]). Cinnarizine has also been extensively used as a model basic drug for assessment of lipid-based formulations [56, 125, 206, 209, 211, 213-216,206].Cinnarizine has been selected as a second model PWSD as it is expected to be more soluble in fatty acids and will provide a robust challenge to ND-LBF.[150]



Fgure 2.2 Chemical structure of cinnarizine [211]

2.3. Formulation preparation

2.3.1. Stealth formulations

All 32 formulations were LFCS type II [32, 148], prepared as binary mixtures of medium or long chain triglyceride (Captex® 355, Captex® GTO) and surfactant (50/50 % w/w). The surfactants used are summarised in chapter 3, Table 3.1 and chapter 4 table 4.1. All lipids and surfactants were heated to 37 °C and mixed prior to use to ensure excipient homogeneity. Semisolid excipients (typically PEG Mw 800 and above) were heated to 60 °C prior to use. Formulations were vortexed for 30 s after preparation and equilibrated overnight at 37 °C before use. Formulations were visually inspected under cross polarised light (P. W. Allen and Co., London, UK) before use to ensure no phase separation.

The equilibrium solubility of danazol in each of the stealth LBFs was determined using previously described methodologies [217, 218]. An excess of danazol was accurately weighed to a glass screw cap vial with the required mass of formulation, vortexed and incubated at 37 °C, and equilibrated for 24 hours prior to assay. Equilibrium solubility was assessed in triplicate, undissolved drug was removed by centrifugation (30 min at a speed of 21,100 x g and temperature of 37 °C (Heraeus Fresco 21 microcentrifuge, Thermo Scientific, Osterode, Germany) and the supernatant transferred to a new vial before use. 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.

Danazol containing formulations had drug incorporated at a loading of 80% saturated solubility (based on measured values at equilibrium at 37 °C). Danazol was accurately weighed to a glass screw cap vial with the required mass of formulation, vortexted and equilibrated for 24 hours prior to use. Danazol content was confirmed by HPLC assay prior to formulation use.

2.3.2. Non-digestible formulations

All six formulations were LFCS type IIIb [32, 148] prepared with 27% lipid, 66% Surfactant blend, 7% Transcutol, detailed descriptions of the formulations prepared are in chapter 5, table 5.1. The surfactant blends used are a 65:35 ratio of low HLB:high HLB surfactant. The first series were prepared with hydrogenated castor oils HCO7 and HCO40 (also known as Kolliphor RH40), and Brij oleyl ethers OEt 3 and OEt 20 (Brij O20). All lipids and surfactants were heated to 37 °C and mixed prior to use to ensure excipient homogeneity. Formulations were prepared on a % mass basis, vortexed for 30 s after preparation and visually inspected under cross polarised light before use to ensure no phase separation. Formulations were then equilibrated overnight at 37 °C before use.

Cinnarizine equilibrium solubility was measured as in section 2.3.1, an excess of drug was added to prepared formulation and equilibrated overnight. Triplicate samples were removed and centrifuged at 21,000 x g and the supernatant assayed for drug concentrations by HPLC. In the case of cinnarizine, equilibrium solubility was attained within 48 hours.

Drug loaded formulations had cinnarizine incorporated at a loading of 80% saturated solubility (based on measured values at equilibrium at 37 °C). Cinnarizine was accurately weighed to a glass screw cap vial with the required mass of formulation, vortexed and equilibrated for 24 hours prior to assay. Drug content was confirmed by HPLC assay prior to formulation use.

2.3.3. Intravenous danazol formulation preparation for the dog studies in Appendix 4

Danazol IV formulations were prepared in a solution of 20 % (w/w) Sulphobutyl Ether ß-Cyclodextrin (Captisol®) in saline. Danazol was accurately weighed and transferred to a clean glass volumetric flask with the appropriate volume of 20% w/w Captisol® predissolved in saline added. The formulation was sonicated for one hour and allowed to equilibrate overnight. The entire formulation was then filtered through a 0.22 μm nylon membrane filter (Millex GV) to sterilise and used immediately. A portion of the filtrate was retained for HPLC assay to facilitate accurate quantitation of administered dose.

2.3.4. *In vitro* dispersion and digestion of formulations

In vitro dispersion and digestion were conducted under standard lipolysis (dog) conditions as reported by Williams *et al* [150] with adjustments to volume, enzyme activity and sample mass for rat *in vitro* lipolysis adapted from Anby *et al* [150].

Table 2.1. Conditions for *in vitro* experiments employing a standard (dog) and rat models of digestion. Table and digestion conditions adapted from Anby *et al* [Ref].

Digestion model (dilution/enzyme activity)	Dog (High / High)	Rat I (High / Low)	Rat II (Low / Low)
Mass Formulation [mg]	1000	125	425
Digestion medium	micelles	micelles	micelles
Pancreatic enzyme [mL]	4	0.010	0.040
Total volume [mL]	40	5	5
Sample volume [mL]	1	0.2	0.2
pH Probe	iUnitrode	Biotrode	Biotrode
Vessel Capacity [mL]	20 – 90 mL	5 – 70 mL	5 – 70 mL
Propeller Stirrer	25 mm, 3 propeller	20 mm, 4 propeller	20 mm, 4 propeller
Formulation dilution	40 (High)	40 (High)	12 (Low)
Enzyme activity	High	Low	Low
Equivalent to formulation dose [mg]	~1000	~30	~100

Briefly, dispersion and digestion experiments were conducted using a pH-stat apparatus (Metrohm® AG, Herisau, Switzerland), comprising a Titrando 802 propeller motor/804 Ti Stand combination, a glass pH electrode (iUnitrode or Biotrode) and two 800 Dosino dosing units coupled to 10 mL autoburets (Metrohm®). Tiamo 2.0 software (Metrohm®) was used for instrument control and data acquisition.

Formulations were weighed directly into a titration vessel with thermostat jacket (Metrohm®) and initially dispersed in 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, volumes as described in table 2.1) with continuous mixing at a speed of ~450 min^{-1}. The pH of the media was manually adjusted to pH 6.5 ±0.05 using NaOH or HCl as required. Digestion was initiated after 15 min of dispersion by addition of pancreatin extract. Pancreatin extract was prepared by the addition of 200 mg of porcine pancreatin (8 x USP) in 1 mL cold digestion buffer (200 mg/mL). The solution was mixed well and centrifuged at 2880 x g at a temperature of 4 °*C* for 15 min (Eppendorf 5804 R centrifuge, Eppendorf AG, Hamburg, Germany). Liberated fatty acids were titrated with sodium hydroxide to maintain pH at 6.5 (0.6 and 0.2 M were utilized for digests containing digestible and stealth formulations respectively, the lower NaOH molarity allowed finer control of pH in systems where limited digestion occured).

Digestion was monitored for 30 min for stealth formulations and 60 min for ND-LBF. Aliquots (sample volumes as described in table 2.1) were taken from the dispersion/digestion media throughout the experimental period at t = -10, 0, 5, 10, 20, and 30 min for the dog model and t = -10, -5, 0, 5 15 and 30 min for the rat models. Lipase inhibitor (4-BPB, 5 µL/mL of a 1.0 M solution in methanol) was added to each sample immediately after sampling to prevent further lipolysis. All samples were centrifuged at 37 °C for 10 min at 21000g (Heraeus Fresco 21microcentrifuge, Thermo Scientific, Langenselbold, Germany) in order to pellet any fatty acid/calcium soaps and pancreatin extract hat precipitated on dispersion and digestion. The digestion phases post-centrifugation were recovered according to the protocol described by Williams *et al*. [150].

Blank (drug free) formulations were used for determination of drug equilibrium solubility in the dispersed phase (AP_{DISP}) and digested phase (AP_{DIGEST}). These values were then utilised to calculate the maximum supersaturation ratio as described by Anby *et al*. [128].

To determine the total extent of digestion, back titrations were performed at the end of the digestion experiments (30 min) as previously described [150]. At the conclusion of the digestion period, 1 M sodium hydroxide was rapidly added to the reaction vessel to increase the pH to 9.0. The quantity of sodium hydroxide added was used to calculate the number of moles of unionized FA present. This was then used to calculate an ionisation ratio of ionised to total fatty acid; equation 2.1.

$$\text{Ionisation Ratio} = \frac{\text{mmol FA at 30min}}{\text{total mmol FA (ionised+unionised)}} \quad (1)$$

Lipolysis profiles were then adjusted for ionisation in order to more accurately estimate and plot the extent of digestion (% digestion) over time for each formulation.

Lipolysis is a dynamic process and ionisation of fatty acids is anticipated to vary over time as the quantity of fatty acids increase and the apparent pKa of the fatty acids change with the changing digestion environment.

Utilisation of ionisation ratios acquired at the end of the lipolysis experiment provides a maximum value of ionisation ratio, this method, while oversimplifying the lipolysis and fatty acid ionisation processes, allows for more accurate estimation of the extent of digestion than in systems where unionised fatty acids are not quantified.

$$\% \text{ Digestion} = \frac{\text{mmol FA}_{\text{(at time t)}} \text{ x } 100}{\text{ionisation ratio x Theoretical Max mmol FA}} \qquad (2)$$

All digestion experiments were corrected for fatty acids released from the digestion media in the absence of formulation.

Calculation of theoretical molar maxima of fatty acids was based on the assumption that 1 mole triglyceride will be hydrolysed to yield one mole of monoglyceride and two moles of fatty acid in the presence of pancreatic lipase. Triglyceride based surfactants were assumed to be subject to similar patterns of hydrolysis. Fatty acid based surfactants were expected to yield one mole of fatty acid per mole surfactant.

The fatty acid contribution of both lipid and surfactant (where applicable) was factored into the calculation of extent of digestion (% digestion).

In order to accurately reflect surfactant mediated changes on both the initial rate of lipolysis and extent of digestion, digestion modulation was expressed as area under the digestion curve and plotted against the molecular weight of the PEG surfactant headgroup. These digestion modulation plots were overlaid with the AUC for MCT digestion in the absence of surfactant (dashed line).

2.3.5. *In vitro* drug solubilisation during dynamic digestion experiments

Drug loaded formulations (80%) drug load, were dispersed as per blank formulations in 2.3.4 above. Digestion samples were centrifuged at 37 °C for 10 min at 21000g (Heraeus Fresco 21microcentrifuge, Thermo Scientific, Langenselbold, Germany) to pellet any drug that precipitated on dispersion and digestion. 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 acetonitrile prior to HPLC assay.

2.3.6. *In vivo* rat models

All surgical and experimental 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 series of one-way parallel studies in conscious fasted male Sprague Dawley rats (weights ranging from 260 – 330g). For surgical implantation of cannulas rats were anaesthetised via inhalation of isoflurane (5% v/v induction, 2.5% v/v maintenance; Abbott Laboratories, NSW, Australia) for the duration of the surgical procedure. Prior to incision, animals were administered a local anaesthetic 0.5% bupivacaine at the incision site. Surgery was conducted on a 37 *°C* heatpad to maintain body temperature and animal breathing and depth of anaesthesia were routinely monitored throughout surgery.

Cannulation of the carotid artery was performed in all animals. After hair removal and sterilisation with povidone iodine, a vertical midline incision approximately 0.5 cm above the clavicle was made. The carotid artery was isolated from surrounding muscle by blunt dissection. A sterile saline pre-filled cannula (polyethylene tubing, 0.96 mm o.d. x 0.58 mm i.d., Microtube Extrusions, Australia) was inserted at a depth of 2 – 2.5 cm into the right carotid artery. The cannula was secured with silk sutures and exteriorised to the nape of the neck before wound closure with silk suture.

Cannulation of the duodenum was performed in animals administered ND-LBF only. Immediately after carotid artery cannulation, animals had abdominal hair removal and surgical site sterilisation as described above. A lateral incision approximately 1 cm below the xyphoid process was made and the site was protected with warm saline soaked gauze. The duodenum was isolated, exteriorised and perforated approximately 1 cm below the pyloric sphincter with a 21G needle. A 1m polyethylene cannula (as described above) was heated to form a 1 cm U-shape and filled with sterile saline. The cannula was advanced approximately 0.5 cm into the duodenum and secured to the intestinal wall with cyanoacrylate glue. Once dry, the exteriorised duodenal section as carefully placed into the abdomen to ensure neither the intestine nor the cannula were occluded. The cannula was tunnelled to the nape of the neck before wound closure with silk suture.

Exteriorised cannulas were connected to a swivel tether system. Rats were then transferred to individual metabolic cages and allowed to recover overnight prior to dosing. Rats were also fasted for at least 12 h prior to and 5 h post dose. Drinking water was provided ad libitum.

For rats administered danazol, pre-dosing of the non-specific CYP inhibitor, 1-Aminobenzotriazole (ABT), was conducted 12-hours prior to dosing the lipid-based formulation. 1.2 mL of ABT 100mg/g (Sigma) was dosed via oral gavage to lightly anaesthetised rats. Rats were allowed to recover overnight prior to dosing of lipid formulations.

Oral doses were administered as predispersed formulations in a total of 0.5 g water with 28-30 mg of lipid-based formulation. Formulations were prepared 24 hours prior to study commencement and dosed within 5 minutes of dispersion.

Blood samples (250 μl) were obtained up to 5 h after oral administration of danazol and 16 hours after ID administration of cinnarizine. In all cases 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 Eppendorf tubes containing 10 IU sodium heparin and centrifuged for 5 min at 10,000g. A quantity of 125 μl of plasma was collected and stored at -20°C until assayed for drug content.

2.3.7. *In vivo* dog models

All surgical and experimental procedures were approved by the Melbourne University 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.

All experiments were conducted as a series of four-way crossover studies in fasted beagle dogs (12 – 21 Kg). Animals were fasted for at least 16 hours prior to dosing. Drinking water was provided ad libitum.

Formulations were prepared 24 hours prior to dosing. On the morning of dosing, formulations were hand filled into two 00 hard gelatin capsules (Capsugel). Formulations were administered to the back of the throat with a 50ml water flush post-dose.

IV Dosing for the studies described in appendix 4: Two cephalic vein catheters were inserted into each forelimb, one with a 50 cm extension attached. The catheter extension was pre-filled with the formulation prior to the infusion to remove air from the line and to take into consideration the void volume associated with the final dose administered. After placement of catheters (with sampling hub) the limbs were taped up with surgical bandage and a head-cone placed around each beagle's head to prevent chewing of the catheter during the sampling regimen. 10ml formulation was infused over 5 min at a rate of 2 ml/min.

Blood samples (3 mL) were obtained up to 24 h after formulation administration. In all cases catheters were flushed with 1 IU/ml sodium heparin saline solution after each sample to ensure patency. Collected blood samples were immediately centrifuged and transferred to 1.5 ml Eppendorf tubes A quantity of plasma was collected and stored at -20°C until assayed for drug content.

2.3.8. HPLC analysis

All HPLC analyses of *in vitro* samples were conducted using a Waters Alliance 2695/2697 separation module and Waters 486 tunable absorbance detector (Waters Instruments, Milford, MA). Fluoresence detection was carried out in series with UV detection on a Perkin Elmer LC-240 fluorescence detector (Beaconsfield, Buckinghamshire, England).

Danazol chromatographic separation was carried out on a Waters Symmetry C18 column (150 × 3.5 mm, 5 μm) coupled to a C18 security guard cartridge (4 × 2.0 mm, Phenomenex), the column was maintained at

ambient temperature. The injection volume was 50 µL and UV absorbance was monitored at 286 nm. The mobile phase consisted of methanol and water in a 75:25 v/v ratio at a flow rate of 1 mL/min. Total run time was 8 min.

Assay validation was conducted by multi-day matrix analysis of validation samples (n = 6 per group) of 0.5, 5, and 50 µg/mL danazol in blank aqueous phases. System precision, % RSD (n = 5) 2.4%; interassay precision, % RSD (n=18) 8.2%; intermediate precision, % RSD (n = 12) 7.6%; accuracy, (n = 24) 89.5 – 107.4%; recovery from spiked blank digesta, (n = 6) oil 93%, pellet 95.2%, aqueous phase 98.4%; linearity, R^2 0.999 – 1; specificity, no interfering peaks at RT of danazol.

Cinnarizine chromatographic separation was carried out on the same HPLC on an Agilent Eclipse XDB C18 column (50 × 4.6 mm, 3.5 µm, Agilent Technologies, Santa Clara, CA) coupled to a C18 security guard cartridge (4 × 2.0 mm, Phenomenex), the column was maintained at 40°C. The injection volume was 50 µL and UV absorbance was monitored at 254 nm while fluorescence detection was monitored at EX 249nm and EM 311nm. The mobile phase consisted of (A) 20mM $NH_4H_2PO_4$ pH 4.2 and (B) acetonitrile at a flow rate of 0.75 mL/min. Total run time was 3.5 min. UV and Fluorescence assay validations were conducted by multi-day matrix analysis of validation samples (n = 6 per group) of 0.5, 5, and 50 µg/mL cinnarizine in blank aqueous phases.

UV validation results: System precision, % RSD (n = 5) 3.4%; interassay precision, % RSD (n=18) 1.6%; intermediate precision, % RSD (n = 12) 3.6%; accuracy, (n = 24) 97.6 – 102.0%; recovery from spiked blank digesta, (n = 6) oil 94.1%, pellet 93.1%, aqueous phase 99.7%; linearity, R^2 0.999 – 1; specificity, no interfering peaks at RT of CIN.

Fluorescence validation results: System precision, % RSD (n = 5) 5.0%; interassay precision, % RSD (n=18) 3.0%; intermediate precision, % RSD (n = 12) 4.0%; accuracy, (n = 24) 99.7 – 102.3%; recovery from spiked blank digesta, (n = 6) oil 102.1%, pellet 97.2%, aqueous phase 102.1%; linearity, R^2 0.999 – 1; specificity, no interfering peaks at RT of CIN.

2.3.9. Plasma Analysis via UPLC MS/MS

All plasma analyses were performed on a Waters Xevo TQS UPLC MS/MS system (Waters, Milford, MA, USA).

Danazol chromatographic separation was conducted on a Kinetex phenyl hexyl column (50 × 2 mm, 2.7 µm) (Phenomenex, Torrence, CA), coupled to a Gemini C6-phenyl security guard cartridge (4 × 2.0 mm) (Phenomenex). The injection volume was 5 µL. The mobile phase consisted of 0.1% formic acid in Mili Q water (A) and 0.1% formic acid in acetonitrile (B). Gradient elution at a constant flow rate of 0.5 mL/min was performed as follows: 41% B linearly increasing to 55% B in 3.75 min. Under these conditions the retention times of danazol and internal standard (IS) progesterone were 2.1 and 1.9 min, respectively. Elution was immediately succeeded by a 1.25 min wash step. Total run time was 5 min including the wash step. The

MS/MS conditions were optimised as follows: source temperature, 150 ^{o}C; desolvation temperature, 200 oC; cone gas flow, 150 L/h, desolvation gas flow, 900 L/h; collision gas flow, 0.16 mL/min; cone voltage, 31 kV; capillary voltage, 3.68 kV; collision energy, 24 kV. Data acquisition and peak integration were performed using MassLynx software, version 4.1 (Waters).

Unknown danazol plasma concentrations were determined by interpolation from a weighted (1/X) calibration curve of danazol:IS peak response plotted as a function of danazol concentration. Assay validation was conducted by multi-day matrix analysis of validation samples (n = 6 per group) of 0.5, 5, and 125 ng/mL danazol in blank plasma with IS. System precision, % RSD (n = 5) 3.4%; interassay precision, % RSD (n=18) 11.5%; intermediate precision, % RSD (n = 18) 14.6%; accuracy, (n = 24) 81.3 – 126.4%; recovery, (n = 6) 79%; linearity, R^2 0.995 – 0.999; specificity, no interfering peaks in blank plasma extracts. The limit of quantitation was arbitrarily set at 0.5 ng/mL; the lowest concentration of the validated concentration range.

Cinnarizine was assayed on the same instrument using a Supelco Ascentis Express RP Amide 2.7µm, (50 x 2.1 mm) with a C18 security guard cartridge (4 × 2.0 mm, Phenomenex, Torrance, CA) at 40°C and mobile phase (A) Milli-Q water with 0.05% Formic acid and (B) Acetonitrile and 0.05% formic acid. The injection volume was 2 µL. Gradient elution at a constant flow rate of 0.5 mL/min was performed as follows: 41% B linearly increasing to 80% B in 2.5min. Under these conditions the retention times of cinnarizine and internal standard (IS) diazepam were 2.3 and 2.6 min, respectively. Elution was immediately succeeded by a 1.5 min wash step. Total run time was 4 min including the wash step.

The MS/MS conditions were optimised as follows: source temperature, 150 oC; desolvation temperature, 200 ^{o}C; cone gas flow, 150 L/h, desolvation gas flow, 900 L/h; collision gas flow, 0.16 mL/min; cone voltage, 6 kV; capillary voltage, 0.89 kV; collision energy, 18 kV. Data acquisition and peak integration were performed using MassLynx software, version 4.1 (Waters).

Unknown cinnarizine plasma concentrations were determined by interpolation from a weighted (1/X) calibration curve of cinnarizine:IS peak response plotted as a function of cinnarizine concentration. Assay validation was conducted by multi-day matrix analysis of validation samples (n = 6 per group) of 2, 20, and 200 ng/mL cinnarizine in blank plasma with IS. System precision, % RSD (n = 5) 4.2 %; interassay precision, % RSD (n=18) 14.5%; intermediate precision, % RSD (n = 18) 15.2%; accuracy, (n = 24) 83.3 – 131.4%; recovery, (n = 6) 84%; linearity, R^2 0.995 – 0.999; specificity, no interfering peaks in blank plasma extracts. The limit of quantitation was arbitrarily set at 2 ng/mL; the lowest concentration of the validated concentration range.

2.3.10. Particle Size Determination

Photon correlation spectroscopy (Malvern Nano-ZS zetasizer, Malvern Instruments, Worcestershire, U.K.) was used to determine the average particle size (average particle diameter in nm based on light scattering by volume), the % of each size population in the sample and the polydispersity index (PDI) of the dispersed

LBFs. Formulations were dispersed to match the concentrations obtained in lipolysis experiments, thus 0.1g formulation was dispersed in a total of 3.6 ml digestion media. Samples were stirred at 37*°C* for 15 min and maintained at 37*°C* throughout particle size analysis. Size data was plotted as the mean for three separate sample measurements (mean ± SD (n = 3).

2.3.11. Total bile acid analysis via enzyme cycling assay for values reported in Appendix 4

Total bile acid analysis was carried out on a Fluostar Optima plate reader (BMG Labtech, Ortenberg, Germany) with absorbance measured at 405nm. A commercial enzyme cycling kit (Vital Diagnostics Total Bile Acid Kit) was used for analysis of bile acids in canine gastric, intestinal and gall bladder samples. Glycodeoxycholate Na Salt was used as a bile acid standard. Bile samples were diluted 1 in 1000 or 1 in 500 dependent on the sample and literature values of bile acid concentration.

2.3.12. Statistical Data Analysis for *in vitro* studies

Graphpad Prism for windows (Version 6.0, Graphpad Software Inc., CA, USA) was used to statistically analyse differences in pairs of *in vitro* data using unpaired parametric t-tests. Data were expressed as the mean ± standard deviation (SD). A difference was considered statistically significant when $p \leq 0.05$.

2.3.13. Pharmacokinetic data analysis

PK data were plotted as drug concentration (ng/mL) vs. time (h) (normalised to a nominal dose of 1 mg/kg danazol or 10 mg/kg cinnarizine). Non-compartmental pharmacokinetic parameters were calculated using Phoenix™ 64 Software (WinNonlin® version 6.3, Pharsight Corporation, CA, USA). Graphpad Prism for windows (Version 6.0, Graphpad Software Inc., CA, USA) was used to statistically analyse differences in pairs of *in vivo* data using unpaired parametric t-tests while group analyses were performed using a one-way analysis of variance with Tukey's multiple comparison. All data represented are expressed as the mean ± standard error of mean (SEM). A difference was considered statistically significant when $p \leqslant 0.05$.

Validation results: Interassay precision, % RSD (n=30) 3.1%; intermediate precision, % RSD (n = 30) 3.6%; accuracy, (n = 30) 97.6 – 114.7%; linearity, weighted 1/X, R^2 0.997; Calibration range; 5.25 – 100.5 □M. Gastric and intestinal samples were diluted 200-fold and gallbladder samples diluted 2000-fold prior to analysis.

2.3.14. Data Extraction and analysis for systematic review of IVIVC of LBFs reported in Appendix 1

Data sets from previously published papers were digitised and reanalysed to generate IVICs for the review that forms part of chapter one. A detailed analysis of the data is outside the scope of this thesis but the methodologies used to generate the original figures are described herein.

All data was digitised from the published manuscripts using Engauge open source digitizing software (version 4.1). Supersaturation ratios were calculated from the digitised data using AUC values generated using GraphPad prism version 6.07. The potential for linear *in vitro in vivo* correlations were subsequently calculated using GraphPad prism version 6.07.

In all instances, the nomenclature of the x axes reflects the original nomenclature employed to describe the reported *in vitro* data. In some cases, therefore the resultant plots have different terms to describe PWSD solvation (% in solution, % dispersed, % drug in digestion aqueous phase (AP), digestion AP concentration, Supersaturation (S) during digestion).

The nomenclature of the formulations in each IVIVC plot was retained as published. Where full *in vitro* solubilisation profiles were available, data have been plotted as the AUC of the profile, calculated using the linear trapezoidal rule. Otherwise, data were plotted using reported solubilisation values at a fixed time point. *In vivo* AUC data was reported to tlast. Datasets that did not specify the time range used to calculate AUC do not have an AUC time range specified on the y axis.

Chapter 3. 'Stealth' Lipid-based Formulations: Poly(ethylene glycol) Mediated Digestion Inhibition Improves Oral Bioavailability of a Model Poorly Water-soluble Drug.

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3.1. Abstract

For over 20 years, stealth drug delivery has been synonymous with nanoparticulate formulations and intravenous dosing. The putative determinants of stealth in these applications are the molecular weight and packing density of a hydrophilic polymer (commonly poly(ethylene glycol) (PEG)) that forms a steric barrier at the surface of the nanoparticle. The current study examined the potential translation of the concepts learned from stealth technology after intravenous administration to oral drug delivery and specifically, to enhance drug exposure after administration of oral lipid-based formulations (LBFs) containing medium-chain triglycerides (MCT). MCT LBFs are rapidly digested in the gastrointestinal tract, typically resulting in losses in solubilisation capacity, supersaturation and drug precipitation. Here, non-ionic surfactants containing stealth PEG headgroups were incorporated into MCT LBFs in an attempt to attenuate digestion, reduce precipitation risk and enhance drug exposure. Stealth capabilities were assessed by measuring the degree of digestion inhibition that resulted from steric hindrance of enzyme access to the oil:water interface. Drug loaded LBFs were assessed for maintenance of solubilising capacity during *in vitro* digestion and evaluated *in vivo* in rats. The data suggest that the structural determinants of stealth LBFs mirror those of parenteral formulations i.e. the key factors are the molecular weight of the PEG in the surfactant headgroup and the packing density of the PEG chains at the interface. Interestingly, the data also show that the presence of labile ester bonds within a PEGylated surfactant also impact on the stealth properties of LBFs, with digestible surfactants requiring a PEG Mw of ~1800 g/mol and non-digestible ether-based surfactants ~800 g/mol to shield the lipidic cargo. *In vitro* evaluation of drug solubilisation during digestion showed stealth LBFs maintained drug solubilisation at or above 80% of drug load and reduced supersaturation in comparison to digestible counterparts. This trend was also reflected *in vivo*, where the relative bioavailability of drug after administration in two stealth LBFs increased to 120% and 182% in comparison to analogous digestible (non-stealth) formulations. The results of the current study indicate that self-assembled 'stealth' LBFs have potential as a novel means of improving LBF performance.

3.2. Introduction

Drug bioavailability from an oral formulation in the gastrointestinal tract (GIT) is heavily reliant on favourable physiochemical characteristics, including adequate solubility and permeability and resistance to metabolism. However, increasing numbers of new chemical entities (NCE) derived from e.g. combinatorial and high throughput screening processes do not meet these criteria [29] and as a result, attrition rates in early stage clinical development are rising [6, 9]. Lipid-based drug delivery systems are well established as a means to circumvent the low solubility issues associated with hydrophobic drugs [6, 177, 219]. The past twenty years have seen lipid-based formulations (LBFs) advance from simple one-excipient or binary systems to more complex multi-component self-emulsifying drug delivery systems (SEDDS) [60, 107, 136, 176, 216, 220]. Despite this progressive rise in popularity, however, LBFs occupy less than 4% of the oral market and the development of LBFs remains largely empirical [6].

One limitation to the wider use of medium-chain triglyceride (MCT) containing LBFs is the realisation that these formulations often exhibit a rapid loss in drug solubilisation capacity when subjected to digestion by pancreatic enzymes [128, 142, 209, 210, 221]. Under digesting conditions, there is a risk that dissolved drug will precipitate into a more slowly dissolving crystalline form, leading to reduced bioavailability. Judicious design of lipidic formulations is therefore required to generate formulations that are able to withstand the solubilisation challenges encountered on digestion.

Lipid digestion in the GIT is catalysed by the lipase superfamily of interfacially active enzymes [11]. Nonspecific adsorption of the inactive lipase/co-lipase enzyme complex to the surface of an emulsified oil droplet results in a conformational change in the enzyme to the active form. The interfacial activation of pancreatic lipase renders the lipolysis reaction highly sensitive to changes at the oil:water interface [11, 12]. The presence of commonly used polyethoxylated non-ionic surfactants has previously been reported to modify the rate of *in vitro* lipid digestion [59, 180, 200, 201, 203, 222], however, the available data are contradictory. Thus, increases in lipid digestion rates have been observed on surfactant addition to LBFs, and tentatively attributed to enhanced solubilisation of digestion products [203], whilst reduced digestion has also been reported and attributed to steric hindrance of lipase access to the oil:water interface [180, 200, 201, 222].

PEGylated (stealth) drug delivery vehicles comprising a polyethoxylated steric stabilisation layer have been extensively utilised in parenteral drug delivery to enhance plasma circulation times and to promote drug accumulation at sites of hypervascularisation such as tumours or inflamed tissues [13-15]. The stealth effect attributed to these drug delivery systems can be in part attributed to their polymeric PEG coatings that prevent nonspecific protein adsorption (opsonisation) and therefore reduce recognition and clearance from the blood by the mononuclear phagocyte system (MPS). PEGylated drug delivery systems typically exhibit prolonged circulation half-lives and improved accumulation at sites of increased vascular permeability when compared to their unmodified counterparts.

The aim of the current study was to examine the potential to translate the materials and concepts that have been successfully employed to enable parenteral stealth applications, to an advantageous role in oral drug delivery. This has been achieved via the systematic evaluation of a series of LBFs with varying degrees of PEG mediated steric stabilisation. Parenteral stealth formulations aim to evade recognition by the immune system through PEG-mediated prevention of opsonisation. Here, oral 'stealth' formulations are defined as formulations that use a hydrophilic polymeric interfacial layer to similarly prevent nonspecific protein binding, in this case the adsorption of pancreatic lipase-colipase to the surface of a lipid droplet. In this way, the proposed oral stealth LBF aim to evade lipolysis and in doing so prolong drug solubilisation in the GIT and improve drug absorption.

The development of self-assembled stealth LBFs was informed by the structural principles that govern effective stealth attributes in parenteral formulations, namely; surfactant PEG chain molecular weight (Mw) and PEG chain density [14, 15, 223-226]. Here we hypothesised that if oral stealth LBFs behaved in a similar fashion to their parenteral counterparts, digestion inhibition would correlate with the Mw of the surfactant PEG headgroup and the density of the PEG layer. The degree of PEG-mediated reduction in non-specific protein adsorption (as manifest by changes in lipid digestion) was assessed via *in vitro* lipolysis experiments. Changes in drug solubilisation and supersaturation were measured for selected formulations using danazol as a model drug, and these formulations were subsequently administered orally to male Sprague Dawley rats. Danazol bioavailability was compared after administration of the stealth formulations and structurally analogous formulations that were readily digested.

3.3. Methods

3.3.1. Materials and reagents

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) were from Sigma-Aldrich (St Louis, MO, USA). Captex® 355 (C_8 and C_{10} MCT) was donated by Abitec Corporation (Janesville, WI, USA) and was used as received. Etocas™ 5, 15, 35, 200 (PEGylated castor oils - CO), Croduret™ 7, 25, 40 (PEGylated hydrogenated castor oils - HCO), Myrj™ S8, 20, 40, 50, 100 (PEGylated stearic acids), Brij™ S2, 10, 20, 100, 200 (PEGylated stearyl alcohols) and Brij™ O2, 3, 5, 10, 20 (PEGylated oleyl alcohols) were kindly donated by Croda International PLC (Yorkshire, England). Jeechem® CAH 16 (PEG 16 hydrogenated castor oil) and Jeechem® CA 25 (PEG 25 castor oil) were donated by Jeen® International Corporation (Fairfield, NJ, USA). Kolliphor® EL (PEG 35 castor oil) and RH40 (PEG 40 hydrogenated castor oil) were donated by BASF Corporation (Washington, NJ, USA). Nikkol® HCO 100 (PEG 100 hydrogenated castor oil) was donated by Nikko Chemicals Co. Ltd. (Chuoku, Tokyo, Japan). Kolliphor® RH60 (PEG 60 hydrogenated castor oil) was purchased from Ingredients Plus (Notting Hill, VIC, Australia). Soybean oil (C_{18} long chain triglycerides - LCT), Triton™ X15, 165, 305, 705 (PEGylated branched octyl phenols), sodium taurodeoxycholate > 95% (NaTDC), porcine pancreatin (8 x USP specification activity) and 4-bromophenylboronic acid (4-BPB) were obtained 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). Sodium hydroxide 1.0 M, which was diluted to obtain 0.6 M and 0.2M NaOH titration solutions, 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. Hypergrade solvents were used for UPLCMS/MS analysis.

3.3.2. Formulation preparation

3.3.2.1. Blank formulations

All formulations were prepared as binary mixtures of MCT (Captex® 355) and surfactant (50/50 % w/w). The surfactants used are summarised in Table 1. All lipids and surfactants were heated to 37 °C and mixed prior to use to ensure excipient homogeneity. Semisolid excipients (typically surfactants with PEG Mw 800 and above) were heated to 50 °C prior to use. Formulations were vortexed for 30 s after preparation and equilibrated overnight at 37 °C before use.

3.3.2.2. Drug loaded formulations

The equilibrium solubility of danazol in each of the drug loaded LBFs was determined using previously described methodologies [217, 227]. Equilibrium solubility was assessed in triplicate and 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. Danazol containing formulations had drug incorporated at a loading of 80% saturated solubility (based on measured values at equilibrium at 37 °C, figure S6 supplementary material). Danazol was accurately weighed into a glass screw cap vial with the required mass of formulation, vortexed and equilibrated for 24 hours prior to assay. Danazol content was confirmed by HPLC assay prior to formulation use [128].

3.3.3. In vitro dispersion and digestion of formulations

3.3.3.1. Digestion conditions

For formulation screening experiments, *in vitro* dispersion and digestion were conducted as previously reported by the LFCS Consortium [150, 151] (Standard conditions Table 2). For experiments conducted on the formulations that were ultimately progressed into rat bioavailability studies, adjustments to volume, enzyme activity and sample mass were made to better reflect a rat model of *in vitro* lipolysis as previously described by Anby *et al* [156] (Rat conditions Table 3.2). *In vitro* dispersion was monitored for 15 min and digestion monitored for 30 min. Lipolysis curves were generated from titration of fatty acids with NaOH. As fatty acids liberated during digestion are likely to be partially ionised, titre values were corrected for the presence of unionised fatty acid by back-titration at pH 9 [61, 229]. Titre values were compared to the moles of fatty acid that were expected to be released from the formulation to yield a % digestion value. Details of the total extent of digestion calculations can be found in the supplementary material.

To accurately capture surfactant mediated changes to both the initial rate of lipolysis and the extent of digestion, the degree of 'digestion inhibition' was expressed as the area under the % digestion curve (% digestion.min). To compare relative changes in digestion in the presence of structurally related surfactants, these AUC values were plotted against the molecular weight of the surfactant PEG headgroup. These digestion inhibition plots were compared to the AUC for MCT digestion in the absence of surfactant to provide an indication of the relative change to lipolysis over the entire period of digestion.

3.3.3.2. Calculation of total extent of digestion

To determine the total extent of digestion, back titrations were performed at the end of the digestion experiments (30 min). At the conclusion of the digestion period, 1 M sodium hydroxide was rapidly added to the reaction vessel to increase the pH to 9.0. The quantity of sodium hydroxide added was used to calculate the number of moles of unionized FA present. This was then used to calculate an ionisation ratio of ionised to total fatty acid; equation 1.

$$\text{Ionisation Ratio} = \frac{\text{mmol FA at 30min}}{\text{total mmol FA (ionised+unionised)}} \quad (1)$$

Lipolysis profiles were then adjusted for ionisation in order to more accurately estimate and plot the extent of digestion (% digestion) over time for each formulation. Lipolysis is a dynamic process and ionisation of fatty acids is anticipated to vary over time as the quantity of fatty acids increase and the apparent pKa of the fatty acids changes with the changing digestion environment. Utilisation of ionisation ratios acquired at the end of the lipolysis experiment provides a maximum value of ionisation ratio, this method, while oversimplifying the lipolysis and fatty acid ionisation processes, allows for more accurate estimation of the extent of digestion than in systems where unionised fatty acids are not quantified.

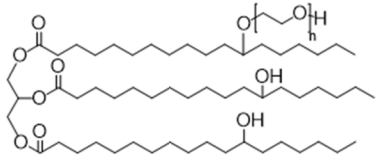
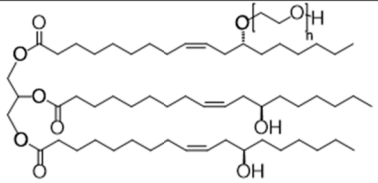
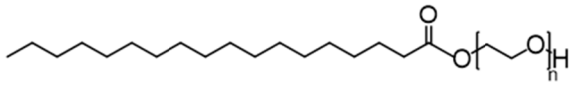
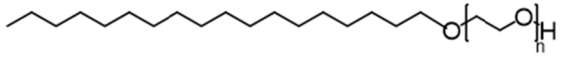
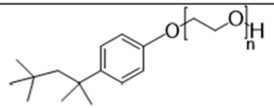
$$\%\ \text{Digestion} = \frac{\text{mmol FA}_{\text{(at time t)}}\ \text{x}\ 100}{\text{ionisation ratio x Theoretical Max mmol FA}} \quad (2)$$

All digestion experiments were corrected for fatty acids released from the digestion media in the absence of formulation. Calculation of theoretical molar maxima of fatty acids was based on the assumption that 1 mole triglyceride will be hydrolysed to yield one mole of monoglyceride and two moles of fatty acid in the presence of pancreatic lipase. Triglyceride based surfactants were assumed to be subject to similar patterns of hydrolysis. Fatty acid based surfactants were expected to yield one mole of fatty acid per mole surfactant. The fatty acid contribution of both lipid and surfactant (where applicable) was factored into the calculation of extent of digestion (% digestion).

3.3.3.3. HPLC-UV method parameters and validation

Digested samples were analysed on a Waters Alliance 2695 separation module and Waters 486 tunable absorbance detector (Waters Instruments, Milford, MA). Chromatographic separation was carried out on a Waters Symmetry C18 column (150 × 15 mm, 5 µm) coupled to a C18 security guard cartridge (4 × 2.0 mm, Phenomenex), the column was maintained at ambient temperature. The injection volume was 50 µL and UV absorbance was monitored at 286 nm. The mobile phase consisted of methanol and water in a 75:25 v/v ratio at a flow rate of 1 mL/min. Assay validation was conducted by multi-day matrix analysis of validation samples (n = 6 per group) of 0.5, 5, and 50 □g/mL danazol in blank aqueous phases. System precision, % RSD (n = 5) 2.4%; interassay precision, % RSD (n=18) 8.2%; intermediate precision, % RSD (n = 12) 7.6%; accuracy, (n = 24) 89.5 – 107.4%; recovery from spiked blank digesta, (n = 6) oil 93%, pellet 95.2%, aqueous phase 98.4%; linearity, R^2 0.999 – 1; specificity, no interfering peaks at RT of Danazol.

Table 3.1 Details of the six series of PEGylated surfactants studied during development of stealth LBFs.

PEGylated Surfactant Series (Trade name(s))	Surfactant General Structure	PEG Mw (g mol^{-1})	PEG Units [n] (approx.)	HLB*	Digestible***
Hydrogenated Castor Oils (HCO)** (Croduret™, Kolliphor®, Jeechem®, Nikkol®)		308[b]	7	4.9	Yes
		705[d]	16	8.6	
		1102[b]	25	10.8	
		1763[c]	40	13.0	
		2644[c]	60	14.7	
		4407[e]	100	16.5	
Castor Oils (CO)** (Etocas™, Kolliphor®, Jeechem®)		220[b]	5	3.8	Yes
		661[b]	15	8.3	
		1102[d]	25	10.8	
		1542[c]	35	12.5	
		1763[b]	40	13.1	
		8814[b]	200	18.1	
Stearyl Esters (SEs) (Myrj™S)		353[b]	8	11.1	Yes
		881[b]	20	15.1	
		1763[b]	40	17.2	
		2201[b]	50	17.7	
		4407[b]	100	18.8	
Stearyl Ethers (SEt) (Brij™S)		88[b]	2	5.0	No
		441[b]	10	12.4	
		881[b]	20	15.3	
		4407[b]	100	18.8	
		8814[b]	200	19.4	
Oleyl Ethers (OEt) (Brij™O)		88[b]	2	4.8	No
		132[b]	3	6.4	
		220[b]	5	8.8	
		441[a]	10	12.2	
		881[a]	20	15.2	
Branched octylphenols (bOP) (Triton™X)		198[a]	4.5	9.8	No
		419[a]	9.5	13.4	
		705[a]	16	15.5	
		1322[a]	30	17.3	
		2424[a]	55	18.4	

Supplier: [a] Sigma Aldrich, [b] Croda, [c] BASF, [d] Jeen, [e] Nikko

* HLB calculated from surfactant general structure and PEG Mw according to method proposed by Griffin [163].

** PEGylation of castor oil and hydrogenated castor oils results in a mixture of surfactants with PEG groups at any (or multiple) points on the triglyceride. The PEG unit values for these complex systems represents the number of moles of PEG reacted with one mole of triglyceride. The general structure is simplified.

*** Digestible surfactants are those containing an ester bond which may be labile to hydrolysis by gastrointestinal enzymes.

Table 3.2 Conditions for *in vitro* experiments employing standard and rat models of digestion. Table adapted from Anby *et al* [156].

Digestion model (dilution/enzyme activity)	**Standard (High / High)**	**Rat (High / Low)**
Mass Formulation [mg]	1000	125
Digestion medium	micelles	micelles
Pancreatic enzyme [mL]	4	0.010
Total volume [mL]	40	5
Sample volume [mL]	1	0.2
pH Probe	iUnitrode	Biotrode
Vessel Capacity [mL]	20 – 90 mL	5 – 70 mL
Propeller Stirrer	Titrando 802 (25 mm, 3 propeller)	Titrando 802 (20 mm, 4 propeller)
Formulation dilution	40 (High)	40 (High)
Enzyme activity	High	Low
Equivalent to formulation dose [mg]	~1000	~30

For drug solubilisation during digestion experiments, formulations were loaded at 80% of the saturated solubility of drug in the formulation (supplementary material, figure 3.10). Aliquots (sample volumes as described in table 2) were taken from the dispersion/digestion media throughout the experimental period at t = -10, -5, 0, 5 15 and 30 min relative to initiation of digestion. Lipase inhibitor (4-BPB, 5 µL/mL of a 1.0 M solution in methanol) was added to prevent further lipolysis. All samples were centrifuged at 37 °C for 10 min at 21,000g (Heraeus Fresco 21 microcentrifuge, Thermo Scientific, Langenselbold, Germany) in order to pellet drug that precipitated on dispersion and/or digestion. The digestion phases post-centrifugation were recovered according to previously reported protocols [150], and assayed for danazol content by HPLC using a validated HPLC method as previously described [128] (validation results detailed in supplementary material). Blank (drug free) formulations were also subjected to dispersion and digestion to obtain aqueous colloidal phases at the timepoints outlined above for determination of danazol equilibrium solubility in the dispersed phase (AP_{DISP}) and digested phase (AP_{DIGEST}). These values were then utilised to calculate the maximum supersaturation ratio as described by Anby *et al*. [128]. Graphpad Prism for windows (Version 6.0, Graphpad Software Inc., CA, USA) was used to statistically analyse differences in pairs of *in vitro* data using unpaired parametric t-tests. Data were expressed as the mean (n=3) ± standard deviation (SD). A difference was considered statistically significant when $p \leq 0.05$.

3.3.4. Oral bioavailability studies

3.3.4.1. Surgical and experimental procedures

All surgical and experimental procedures were approved by the Monash Institute of Pharmaceutical Sciences Animal Ethics Committee and were conducted in accordance with 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 fasted male Sprague Dawley rats (270 – 330g) maintained on a 12 h light/dark cycle. Rats were anaesthetised via inhalation of isoflurane (5% v/v induction, 2.5% v/v maintenance; Abbott Laboratories, NSW, Australia) for the duration of the surgical procedure. A cannula (polyethylene tubing of 0.96 mm o.d. x 0.58 mm i.d.) was inserted into the right carotid artery to allow serial blood collection. Cannulae were exteriorised to the back of the neck and were connected to a swivel tether system. Rats were transferred to individual metabolic cages and were fasted for at least 12 h prior to and 5 h post dose. Drinking water was provided ad libitum. The non-specific CYP inhibitor, 1-aminobenzotriazole (ABT), was pre-dosed 12-hours prior to administration of the LBF to avoid complications to data interpretation due to potential differences in first pass metabolism [156, 230]. 1.2 mL of 100 mg/g ABT was dosed via oral gavage to lightly anaesthetised rats. Rats were allowed to recover overnight prior to dosing of lipid formulations.

3.3.4.2. Formulation administration and sample collection

Rats were dosed with 30 mg of each of the oral lipidic formulations dispersed in 470 mg water and administered via oral gavage. Drug doses were 0.5, 1.1, 0.8 and 1.3 mg/kg for the HCO7, HCO40, CO5 and CO35 formulations respectively. After oral administration of the formulation, a 0.5 ml water flush was administered via a clean oral gavage needle. Blood samples (250 □l) were obtained at pre-dose, 15, 30, 45, 60, 90 min, 2, 3, 4 and 5 h after oral administration. In all cases, cannulae 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 sodium heparin and centrifuged for 5 min at 6,700 x g (Eppendorf minispin plus, Eppendorf AG, Hamburg, Germany). Plasma was collected and stored at -20 °C until assayed for danazol content.

3.3.5. Quantitation of danazol in plasma samples by UPLC-MS/MS

All plasma analyses were performed using a validated method on a Waters Xevo TQS UPLC MS/MS system (Waters, Milford, MA, USA). Plasma samples were analysed on a Waters Xevo TQS UPLC MS/MS system (Waters, Milford, MA, USA), autosampler temperature was 4 °C and column temperature was 25 °C. Chromatographic separation was conducted on a Kinetex phenyl hexyl column (50 × 2 mm, 2.7 µm) (Phenomenex, Torrence, CA), coupled to a Gemini C6-phenyl security guard cartridge (4 × 2.0 mm) (Phenomenex). The injection volume was 5 µL. The mobile phase consisted of 0.1% formic acid in Mili Q water (A) and 0.1% formic acid in acetonitrile (B). Gradient elution at a constant flow rate of 0.5 mL/min was performed as follows: 41% B linearly increasing to 55% B in 3.75 min.

Under these conditions the retention times of danazol and internal standard (IS) progesterone were 2.1 and 1.9 min, respectively. Elution was immediately succeeded by a 1.25 min wash step. Total run time was 5 min including the wash step. The MS/MS conditions were optimised as follows: source temperature, 150 ^{o}C; desolvation temperature, 200 ^{o}C; cone gas flow, 150 L/h, desolvation gas flow, 900 L/h; collision gas flow, 0.16 mL/min; cone voltage, 31 kV; capillary voltage, 3.68 kV; collision energy, 24 kV. Data acquisition and peak integration were performed using MassLynx software, version 4.1 (Waters).

Unknown plasma concentrations were determined by interpolation from a weighted (1/X) calibration curve of danazol:IS peak response plotted as a function of danazol concentration. Assay validation was conducted by multi-day matrix analysis of validation samples (n = 6 per group) of 0.5, 5, and 125 ng/mL danazol in blank plasma with IS. System precision, % RSD (n = 5) 3.4%; interassay precision, % RSD (n=18) 11.5%; intermediate precision, % RSD (n = 18) 14.6%; accuracy, (n = 24) 81.3 – 126.4%; recovery, (n = 6) 79%; linearity, R^2 0.995 – 0.999; specificity, no interfering peaks in blank plasma extracts. The limit of quantitation was arbitrarily set at 0.5 ng/mL; the lowest concentration of the validated concentration range.

3.3.6. Pharmacokinetic data analysis

The data were plotted as danazol concentration (ng/mL) vs. time (h) (normalised to a nominal dose of 1 mg/kg danazol). Non-compartmental pharmacokinetic parameters were calculated using Phoenix™ 64 Software (WinNonlin® version 6.3, Pharsight Corporation, CA, USA). Graphpad Prism for windows (Version 6.0, Graphpad Software Inc., CA, USA) was used to statistically analyse differences in pairs of *in vivo* data using unpaired parametric t-tests. All data represented are expressed as the mean ± standard error of mean (SEM). A difference was considered statistically significant when $p \leq 0.05$.

3.3.7. Particle Size Determination for Stealth LBF dispersions

Photon correlation spectroscopy (Malvern Nano-ZS zetasizer, Malvern Instruments, Worcestershire, U.K.) was used to determine the average particle size (average particle diameter in nm based on light scattering by volume), the % of each size population in the sample and the polydispersity index (PDI) of the self-emulsified LBFs. Formulations were dispersed to match the concentrations obtained in lipolysis experiments, thus 0.1g formulation was dispersed in a total of 3.6 ml digestion media. Samples were stirred at 37°*C* for 15 min and maintained at 37°*C* throughout particle size analysis. Size data was plotted as the mean for three separate sample measurements (mean ± SD (n = 3).

3.4. Results

3.4.1. In vitro evaluation

3.4.1.1. Stealth LBF development – effect of surfactant PEG Mw

To evaluate the impact of PEG molecular weight on the ability of non-ionic surfactants to modulate lipid digestion, a series of commercially available hydrogenated castor oil (HCO) surfactants of increasing PEG Mw (Table 1) were formulated with MCT and subjected to digestion experiments under standard lipolysis conditions. Figure 3.1 shows data for triglyceride alone and the series of HCO surfactants. The extent of digestion of MCT in the absence of surfactant was only 50% after 30 min (Figure 3.1 A). Addition of a HCO of PEG Mw 308 g/mol (approximately 7 PEG units, HCO 7) increased the initial rate of lipolysis, however the overall extent of digestion was slightly lower than that of MCT alone (Figure 3.1B). Further increasing the PEG Mw of the surfactant to 705 and 1102 g/mol (Figure 3.1 C, HCO 16 and Figure 3.1 D, HCO 25) had a marked impact on the rate and extent of *in vitro* lipolysis with the overall extent of digestion reducing to less than 20%. Maximal digestion inhibition (2% digestion) was equivalent to a 15-fold reduction in lipolysis, and was observed for the HCO 40 surfactant (Figure 3.1 E, PEG Mw 1763 g/mol, Kolliphor® RH40), a surfactant that has been previously used in more complex self-emulsifying formulations *in vitro* and *in vivo* [178, 231]. Further increasing the PEG Mw to 2644 g/mol (Figure 3.1 F, HCO 60, Kolliphor® RH60) resulted in a recovery in digestion to 6%, indicating that this surfactant was marginally less effective at preventing lipid digestion. When the surfactant hydrophilic headgroup was increased to a molecular weight of 4407 g/mol (Figure 3.1 G, HCO 100), the digestion inhibiting properties of the PEG headgroup were lost.

Plotting these lipolysis profiles as area under the lipolysis curve (% digestion.min) versus surfactant PEG Mw in Figure 3.1H, reveals a parabolic relationship between the Mw of the PEG groups in the surfactants and the rate and extent of lipolysis. The dotted line shows the degree of digestion of the MCT formulation alone (i.e. in the absence of surfactant). Formulations below the dotted line reduced the extent of lipid digestion. The formulations that occupy the minima of these digestion inhibition curves were considered to be the most effective "stealth" formulations, preventing adsorption of enzyme and thus lipolysis of the co-formulated triglyceride.

To gain a better understanding of the relationship between surfactant PEG Mw and LBF stealth properties, five further series of formulations comprising structurally diverse surfactants were screened using the same *in vitro* lipolysis assay. Figures 3.1 to 3.6 *in vitro* lipolysis and digestion inhibition curves for a range of ethoxylated castor oils (CO, Figure 3.2), stearyl esters (SEs, Figure 3.3), stearyl ethers (SEt, Figure 3.4), oleyl ethers (OEt, Figure S4) and branched octyl phenols (bOP, Figure 3.6).

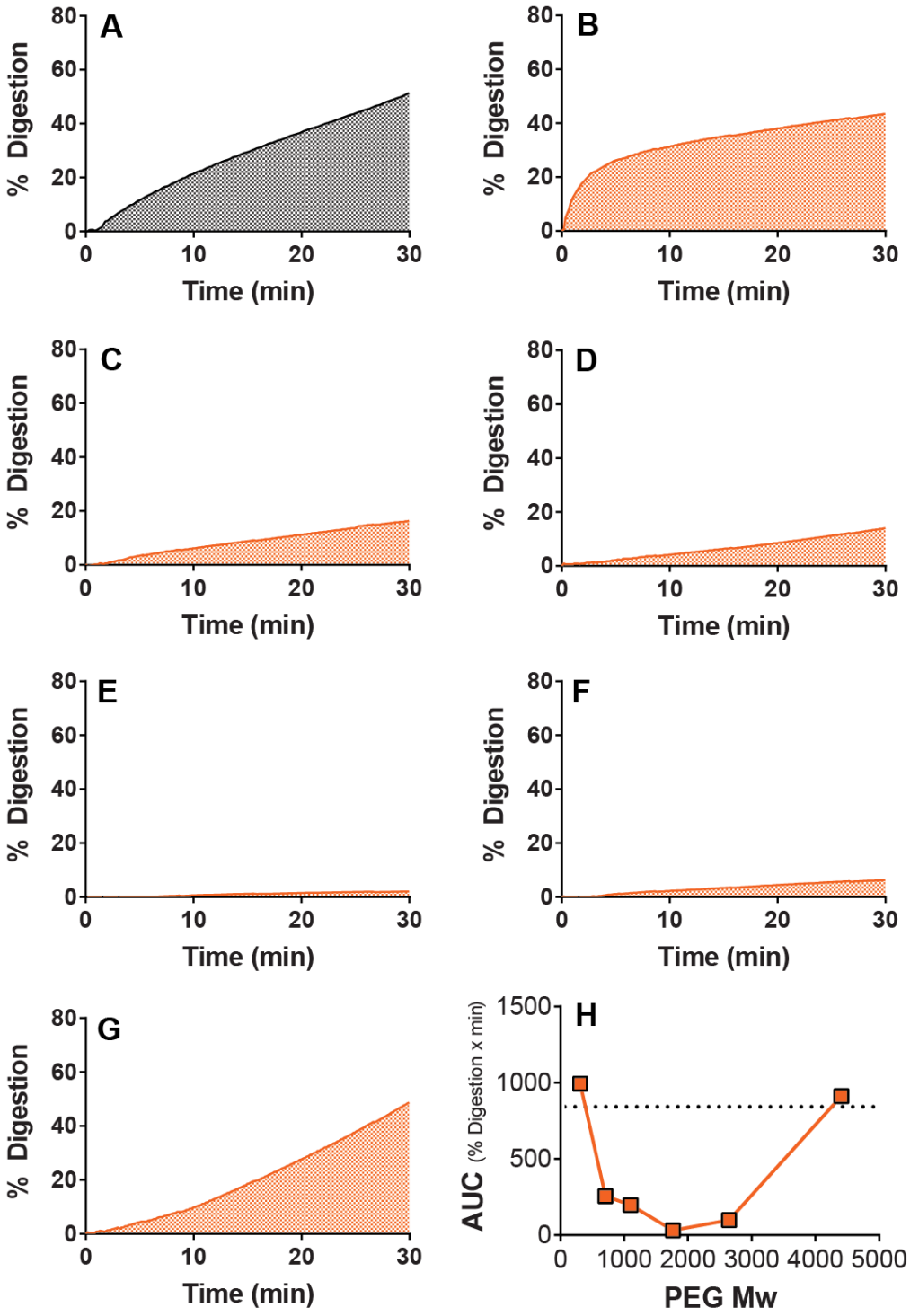


Figure 3.1. (A – G) *In vitro* lipolysis profiles for formulations comprising MCT and hydrogenated castor oil surfactants of increasing PEG Mw. Shaded areas represent the AUC calculated using the linear trapezoidal method and this was used to plot the digestion inhibition curve in H. (A) MCT alone, (B) HCO 7 (PEG Mw 308), (C) HCO 16 (PEG Mw 705), (D) HCO 25 (PEG Mw 1102), (E) HCO 40 (PEG Mw 1763), (F) HCO 60 (PEG Mw 2644), and (G) HCO 100 ((PEG Mw 4407). (H) Digestion inhibition curve for the hydrogenated castor oil surfactants plotting AUC (% digestion.min) against PEG Mw.

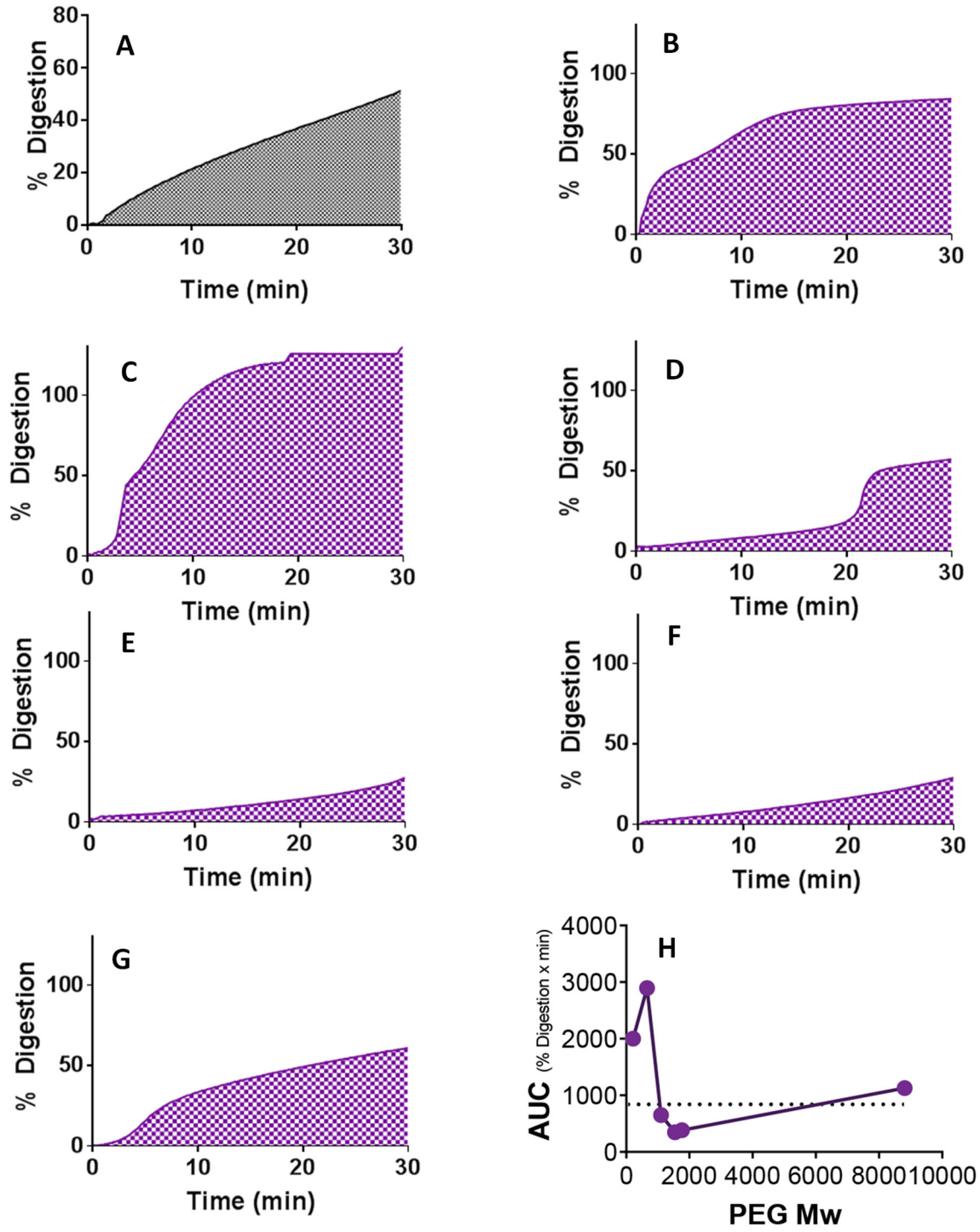


Figure 3.2. (A – G) *In vitro* lipolysis profiles for formulations comprising MCT and castor oil surfactants of increasing PEG Mw. Shaded areas represent the AUC calculated using the linear trapezoidal method and were used to plot the digestion modulation curve in H. (A) MCT alone, (B) CO 5 (PEG Mw 220), (C) CO 15 (PEG Mw 661), (D) CO 25 (PEG Mw 1102), (E) CO 35 (PEG Mw 1542), (F) CO 40 (PEG Mw 1763), and (G) CO 200 ((PEG Mw 8814). (H) Digestion modulation curve for castor oil surfactants plotting AUC (% digestion.min) against PEG Mw. The dashed line represents the AUC for MCT in the absence of surfactant.

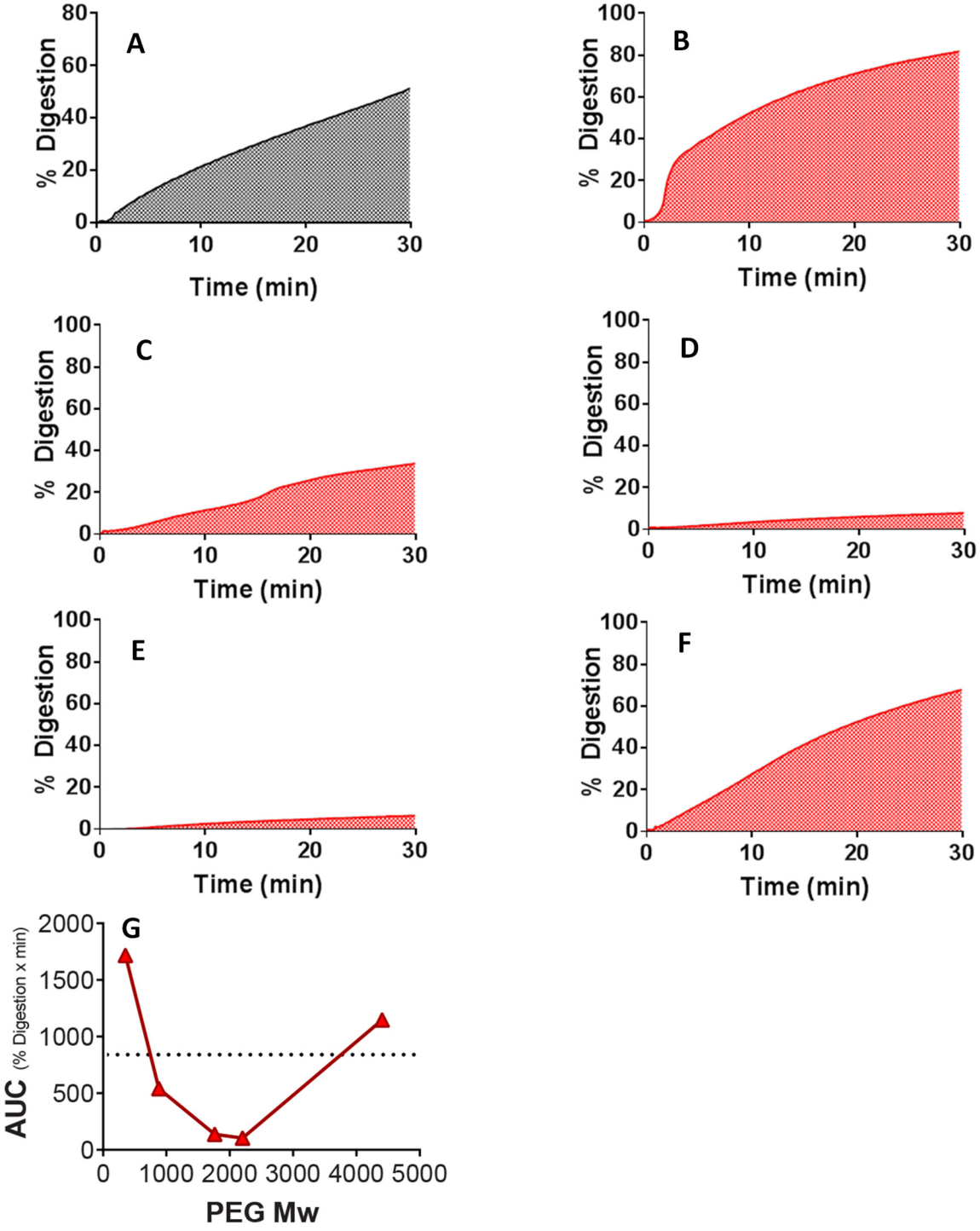


Figure 3.3. (A – F) *In vitro* lipolysis profiles for formulations comprising MCT and stearyl ester surfactants of increasing PEG Mw. Shaded areas represent the AUC calculated using the linear trapezoidal method and were used to plot the digestion modulation curve in G. (A) MCT alone, (B) SEs 8 (PEG Mw 353), (C) SEs 20 (PEG Mw 881), (D) SEs 40 (PEG Mw 1763), (E) SEs 50 (PEG Mw 2201) and (F) SEs 100 (PEG Mw 4407). (G) Digestion modulation curve for stearyl ester surfactants plotting AUC (% digestion.min) against PEG Mw. The dashed line represents the AUC for MCT in the absence of surfactant.

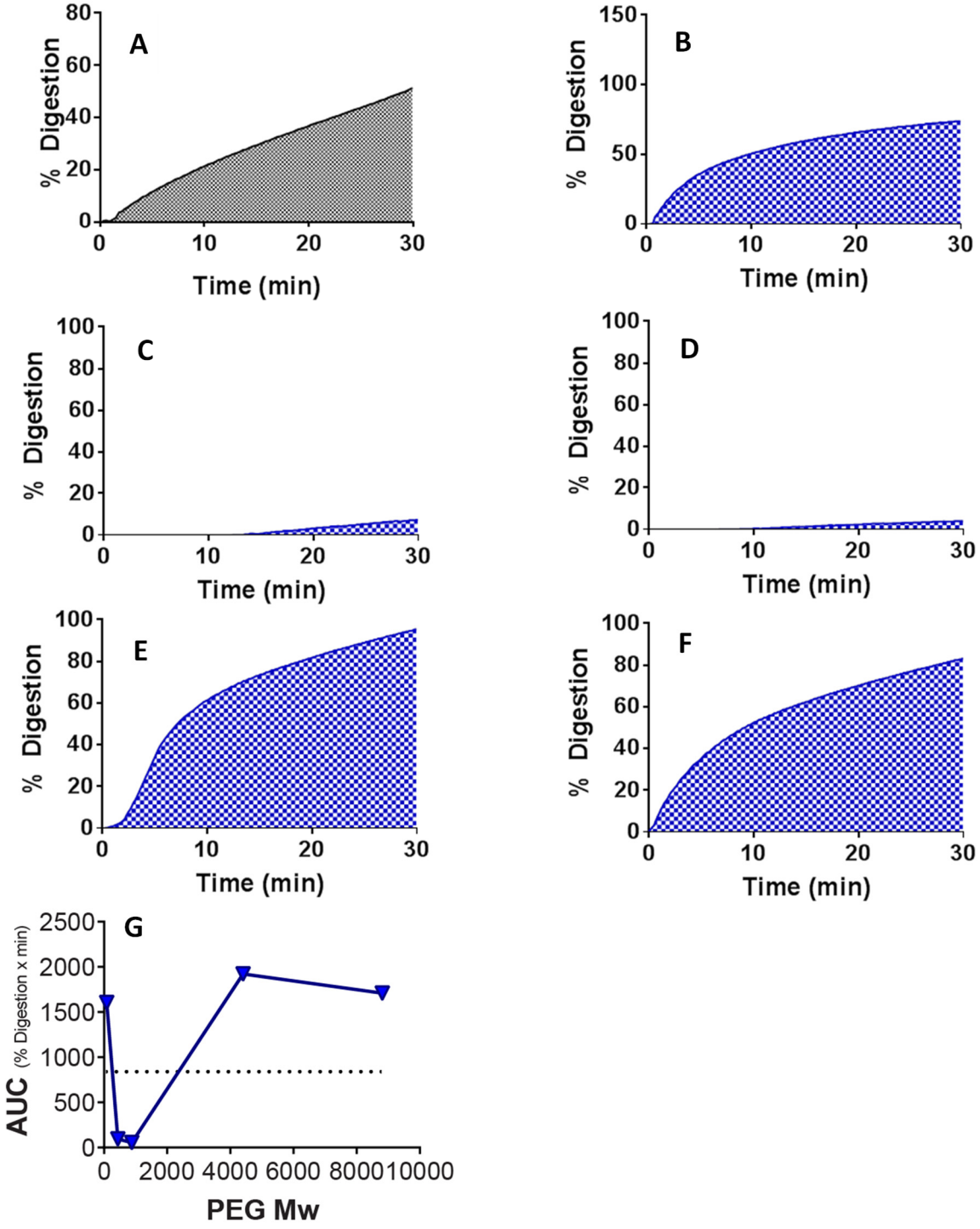


Figure 3.4. (A – F) *In vitro* lipolysis profiles for formulations comprising MCT and stearyl ether surfactants of increasing PEG Mw. Shaded areas represent the AUC calculated using the linear trapezoidal method and were used to plot the digestion modulation curve in G. (A) MCT alone, (B) SEt 2 (PEG Mw 88), (C) SEt 10 (PEG Mw 441), (D) SEt 20 (PEG Mw 881), (E) SEt 100 (PEG Mw 4407) and (F) SEt 200 (PEG Mw 8814). (G) Digestion modulation curve for stearyl ether surfactants plotting AUC (% digestion.min) against PEG Mw. The dashed line represents the AUC for MCT in the absence of surfactant.

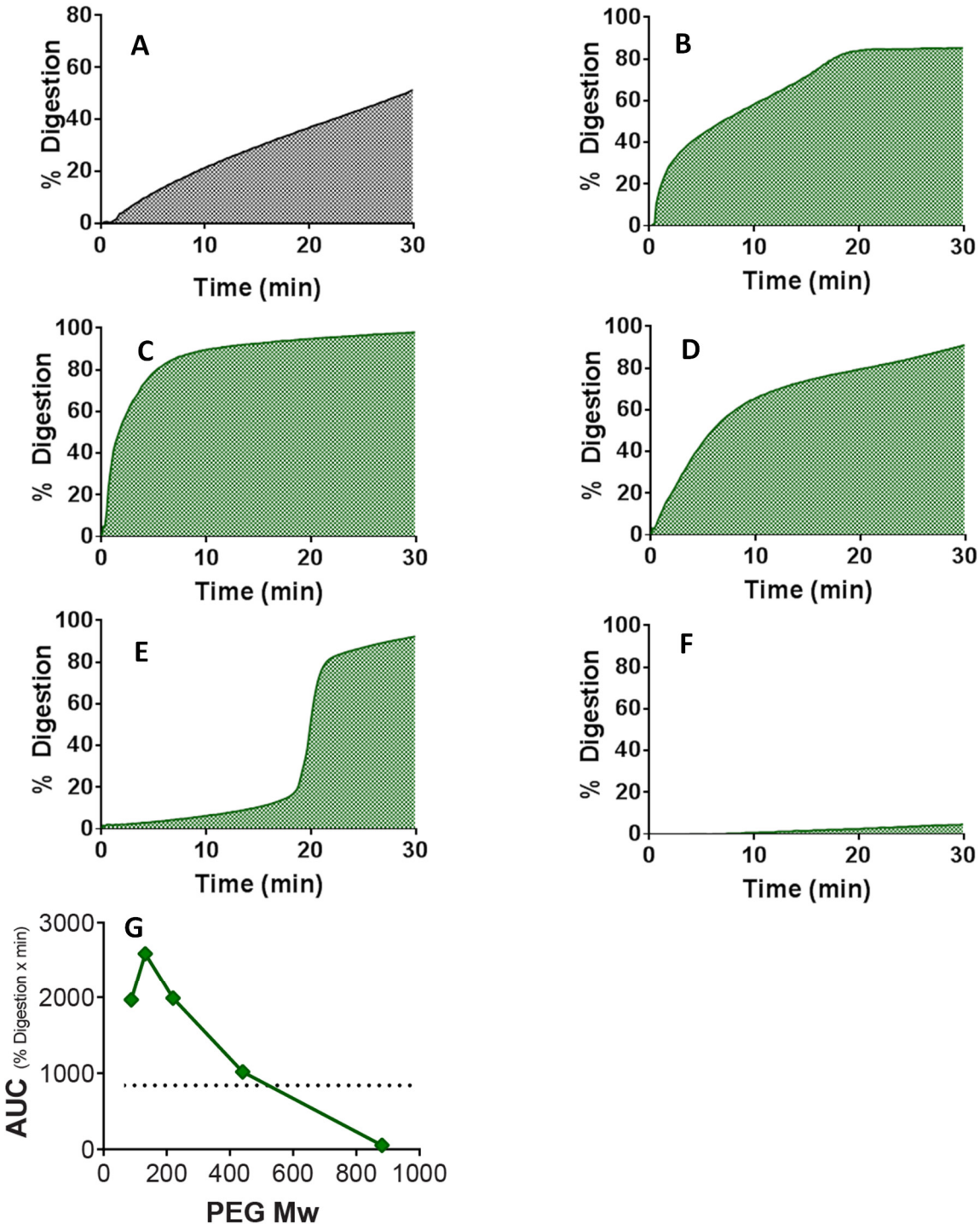


Figure 3.5. (A – F) *In vitro* lipolysis profiles for formulations comprising MCT and oleyl ether surfactants of increasing PEG Mw. Shaded areas represent the AUC calculated using the linear trapezoidal method and were used to plot the digestion modulation curve in G. (A) MCT alone, (B) OEt 2 (PEG Mw 88), (C) OEt 3 (PEG Mw 132), (D) OEt 5 (PEG Mw 220), (E) OEt 10 (PEG Mw 441) and (F) OEt 20 (PEG Mw 881). (G) Digestion modulation curve for oleyl ether surfactants plotting AUC (% digestion.min) against PEG Mw. The dashed line represents the AUC for MCT in the absence of surfactant.

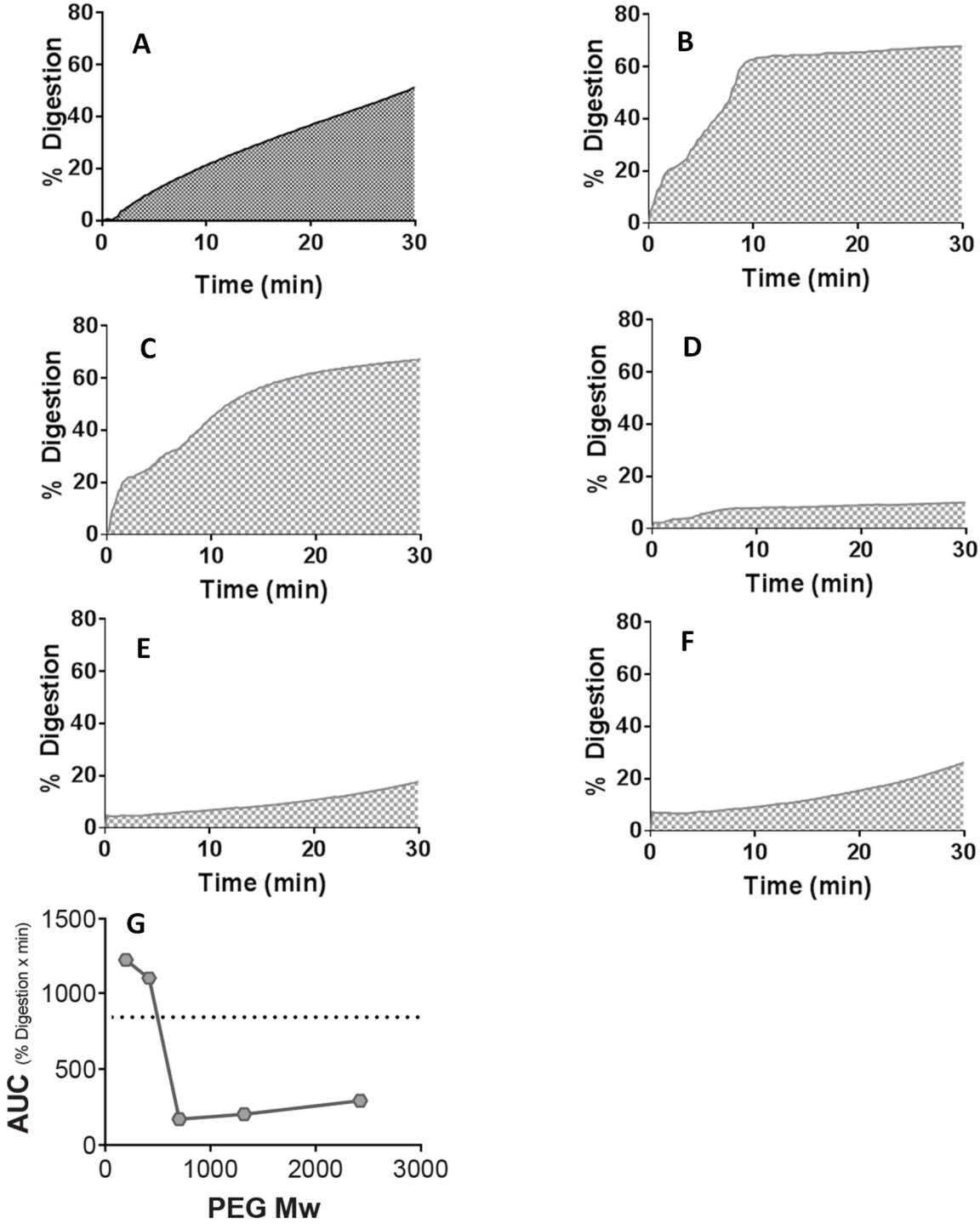


Figure 3.6. (A – F) *In vitro* lipolysis profiles for formulations comprising MCT and branched octyl phenol surfactants of increasing PEG Mw. Shaded areas represent the AUC calculated using the linear trapezoidal method and were used to plot the digestion modulation curve in G. (A) MCT alone, (B) bOP 4.5 (PEG Mw 198), (C) bOP 9.5 (PEG Mw 419), (D) bOP 16 (PEG Mw 705), (E) bOP 30 (PEG Mw 1322) and (F) bOP 55 (PEG Mw 2424). (G) Digestion modulation curve for branched octyl phenol surfactants plotting AUC (% digestion.min) against PEG Mw. The dashed line represents the AUC for MCT in the absence of surfactant.

All surfactant series showed a parabolic relationship between PEG Mw and the rate and extent of digestion with the exception of the oleyl ethers and branched octyl phenols. These two data sets were truncated to lower PEG Mw headgroups due to difficulty in sourcing a commercial supply of the high Mw PEG variants for each surfactant. Interestingly, the minimum rate of digestion obtained for the castor oil (CO, Figure 3.2E) formulation series corresponds to the Kolliphor® EL surfactant, which has been extensively employed in the development of self-emulsifying drug delivery systems and is well tolerated *in vivo* [128, 156, 178, 231]. As lipolysis rates are also influenced by surface area [11], the particle size of two series of stealth LBFs was measured by photon correlation spectroscopy to ensure that reductions in digestion rate were not attributable to a reduction in surface area available for enzyme interaction. Figure 3.7 shows the particle size distribution of the formulations containing the hydrogenated castor oil and castor oil series of surfactants respectively. The particle size data displays a similar parabolic relationship between surfactant PEG Mw and particle size, indicating that in general as the PEG Mw increases, the dispersed droplet size decreases (and therefore surface area increases, in contrast to the reduction in digestion). However, at low and very high PEG Mw the formulations become more polydisperse with multiple populations and the presence of large oil droplets.

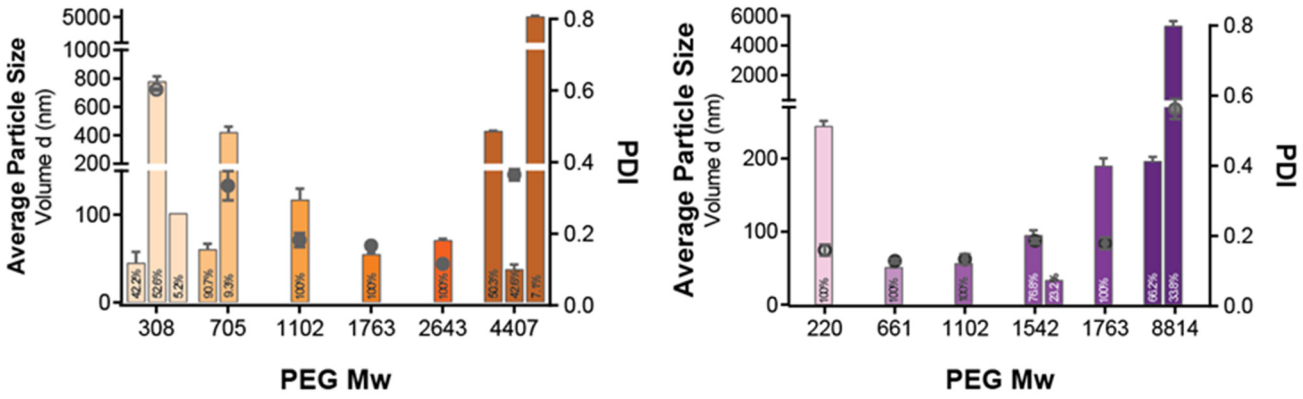


Figure 3.7. Average particle size (bars, left axis) and polydispersity index (dots right axis) for LBFs formulated from the series of PEGylated hydrogenated castor oil and castor oil surfactants. Each size population is plotted as a separate bar with % volume annotated inside the bar. [Mean ± SD (n = 3)].

3.4.1.2. Stealth LBF development – effect of surfactant digestibility

To further probe the structural determinants of stealth in regard to oral LBFs, digestion inhibition plots were separated into digestible (esters) and non-digestible (ether) groups.

A trend in optimum surfactant PEG Mw and digestion inhibition was apparent. Figure 3.8A shows overlaid digestion inhibition curves for the three series of digestible surfactants, namely; ethoxylated hydrogenated castor oils, ethoxylated castor oils and ethoxylated stearyl esters. Maximal digestion inhibition was achieved in a molecular weight range of ~ 1500 – 2000 g/mol. Figure 3.8B shows overlaid digestion inhibition curves

for the three series of non-digestible surfactants; ethoxylated stearyl ethers, ethoxylated oleyl ethers and ethoxylated branched octylphenol ethers.

The molecular weight range that resulted in maximal digestion inhibition was ~700 – 900 g/mol. Figure 3.8C provides summary data showing the optimal PEG Mw for digestible and non-digestible surfactant components of stealth LBFs.

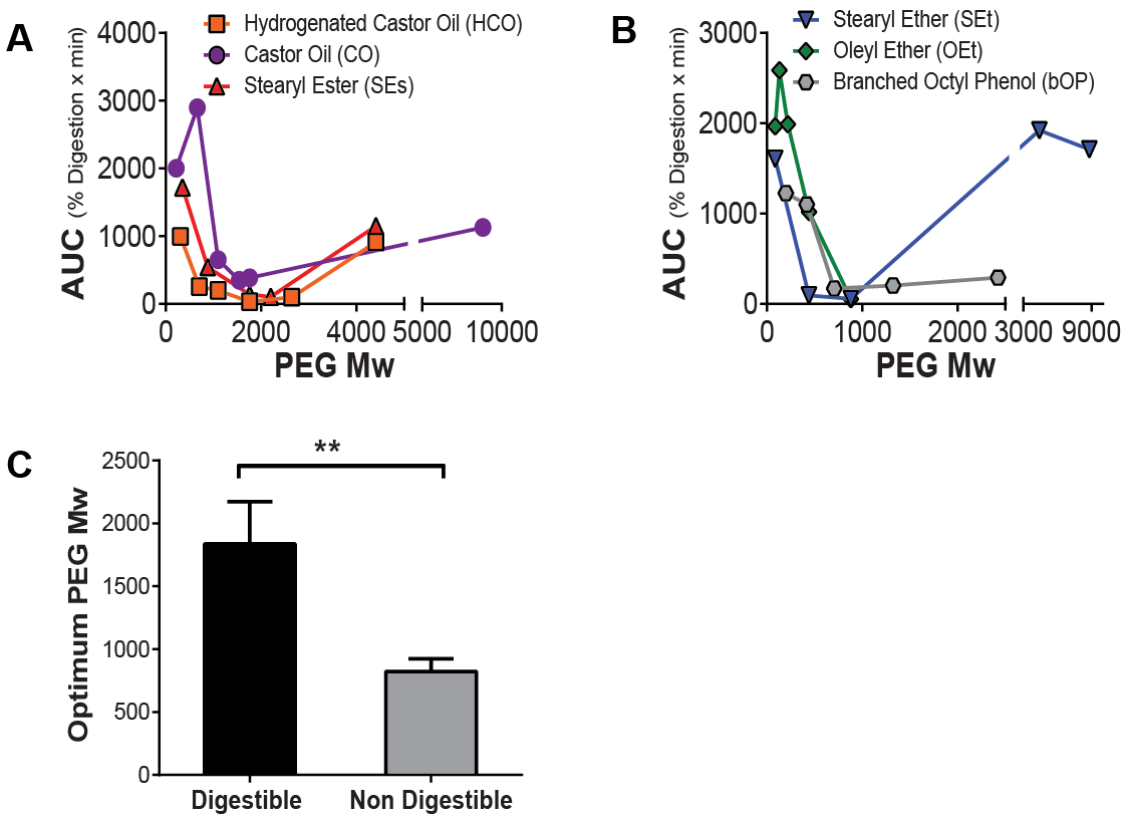


Figure 3.8. Overlaid digestion inhibition curves for MCT formulations comprising six series of PEGylated surfactant (A) digestible surfactants, (B) non-digestible surfactants, (C) Optimal PEG Mw for digestible (black bar) and non-digestible (grey bar) surfactant components of stealth lipid-based formulations.
**Statistically significant difference (P<0.05).

3.4.1.3. Stealth LBF development – effect of PEG chain density

Pairs of structurally analogous surfactants with the same PEG Mw were compared to evaluate the potential for structural differences in the surfactant hydrophobe to influence the digestion of co-formulated MCT. Figure 3.9 shows the impact of fatty acid chain saturation/linearity (and indirectly, PEG packing density) on digestion inhibition. The non-digestible ethoxylated stearyl and oleyl ethers (SEt, OEt) were compared at PEG molecular weights of 88, 441 and 881 g/mol. The lower molecular weight PEG surfactant formulations show a clear difference between the straight chain saturated stearyl ether and the unsaturated oleyl ether, with the former showing a greater digestion inhibition effect. When the surfactant PEG Mw was increased to 881 g/mol there were no discernible differences in digestion inhibition between formulations, however, this molecular weight also corresponded with maximal digestion inhibition for both formulations.

The effect of differences in the structure of the surfactant hydrophobe was also evaluated using pairs of digestible surfactants. Ethoxylated HCO and CO surfactants differ in fatty acid saturation in the hydrophobe chains, the former comprising an ethoxylated triglyceride based on 12-hydroxy stearic acid while the latter is based on a triglyceride comprising unsaturated ricinoleic acid. At a PEG Mw of 1102 and 1763 g/mol the saturated fatty acid HCO formulations were again more potent digestion modulators than the equivalent unsaturated CO formulation.

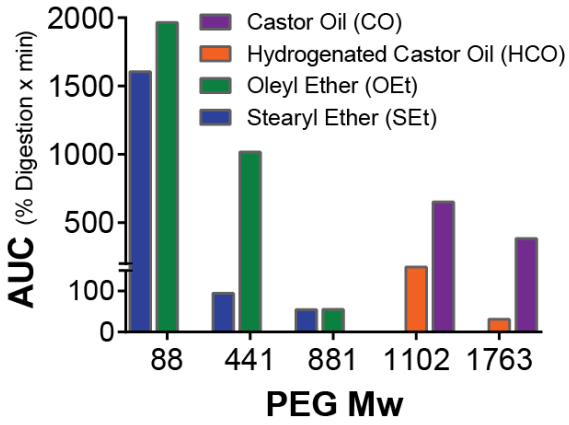


Figure 3.9. Comparison of digestion inhibition capability for matched pairs of formulations at equivalent PEG Mw. Formulations differ in fatty acid saturation on the surfactant hydrophobe moiety. Purple bars, PEGylated castor oils; orange bars, PEGylated hydrogenated castor oils; green bars, PEGylated oleyl ethers; blue bars, PEGylated stearyl ethers.

3.4.1.4. Impact of stealth on drug solubilisation in vitro

Figure 3.10 shows the equilibrium solubility of danazol in two series of formulations comprising PEGylated castor oils and hydrogenated castor oils; these surfactant series were selected as HCO 40 and CO 35 are well tolerated *in vivo*. Four formulations, one digestible formulation and one stealth formulation from each group (namely; HCO 7, HCO 40, CO 5 and CO 35) were selected for further *in vitro* analysis.

Drug solubilisation experiments were conducted based on the rat *in vitro* digestion model proposed by Anby *et al* (Table 3.2) and dynamic solubility data is plotted in figure 3.11 [156]. Formulations were loaded with danazol at 80% saturated solubility in the formulation. Figure 3.11A shows overlaid drug solubilisation and digestion profiles for the low molecular weight PEG surfactant (HCO 7) formulation. Under rat lipolysis conditions (where lipase activity is lower than the comparable conditions in the dog or human), this formulation maintains drug solubilisation for 15 min. At 30 min the formulation shows extensive precipitation which correlates to a concomitant increase in digestion. Maximal supersaturation ratios (SM) for this formulation during dispersion and digestion were 1.2 and 4.6 respectively. Overlaid solubilisation and digestion profiles are also plotted for the analogous stealth formulation HCO 40 (figure 3.11B).

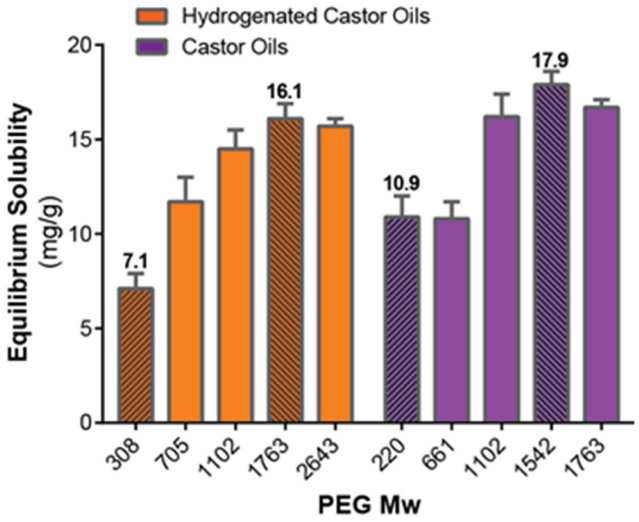


Figure 3.10. Danazol equilibrium solubility in LBFs formulated from the series of PEGylated hydrogenated castor oil surfactants (orange bars) and PEGylated castor oil surfactants (purple bars) [mean ± SD (n = 3)]. Darker shaded bars in both groups represent the formulations progressed for *in vitro* characterisation and *in vivo* administration. Equilibrium solubility values are annotated above the formulations.

The stealth formulation did not undergo digestion in the rat model of lipolysis (< 1%) and this was reflected in the maintenance of drug solubilisation and relatively unchanged maximal supersaturation ratios (SM) throughout the digestion period. Similar data for the castor oil surfactant based formulations was observed and is summarised in figure 3.11C and 3.11D. The digestible CO 5 formulation (figure 3.11C) shows a loss in drug solubilisation and increase in SM from 0.7 to 6.6 following digestion of the formulation. The stealth CO formulation (CO 35, figure 3.11D), however, shows little digestion of the formulation (< 1%) and results in sustained drug solubilisation and lower supersaturation.

3.4.2. In vivo evaluation of stealth LBFs

The mean plasma concentration versus time profiles for danazol following oral administration of stealth and equivalent digestible formulations are depicted in Figure 3.12. A summary of the pharmacokinetic parameters is provided in Table 3.3. To permit cross-comparison, pharmacokinetic data was normalised to a 1 mg/kg drug dose. The results of the *in vivo* study show that $AUC_{0-\infty}$ and C_{max} for danazol after administration of the stealth LBFs was greater than those obtained after administration of the corresponding digestible formulations. This trend was replicated for both hydrogenated castor oil and castor oil based formulations figures 3.12A and 3.12B respectively.

The relative bioavailability (defined as the ratio of $AUC_{0-\infty}$ for the stealth LBF and its structurally analogous digestible LBF) was 120% and 182% for the ethoxylated HCO and CO formulations respectively, illustrating that PEG mediated digestion inhibition (stealth) improved formulation performance and consequently danazol bioavailability. No significant differences in T_{max} were observed between the stealth formulations and the corresponding digestible formulations.

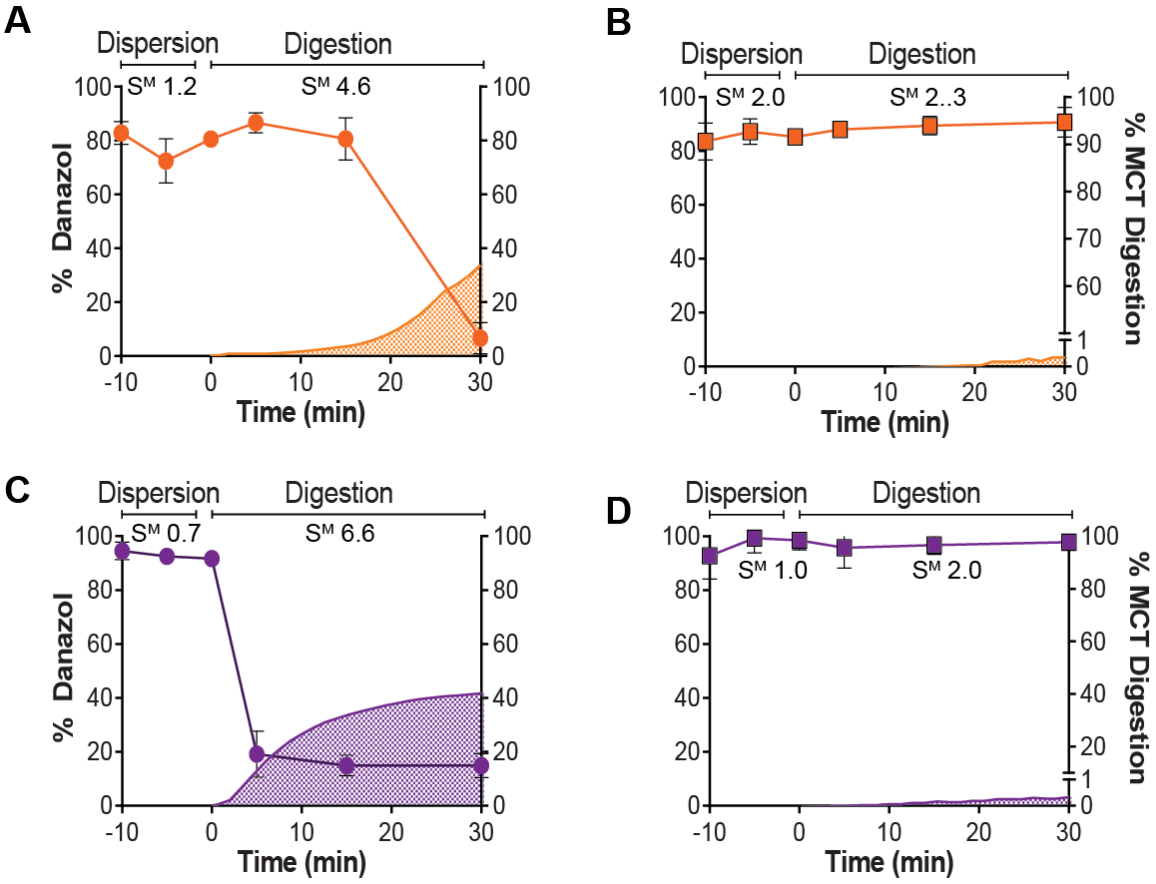


Figure 3.11. Danazol solubilisation profiles (filled circles, left Y axis) during dispersion and digestion of PEGylated hydrogenated castor oil (orange) and castor oil formulations (purple) (shaded areas represent lipolysis curves, right Y axis) [mean ± SD (n = 3)] using a rat model of digestion i.e. high dilution, low enzyme activity. (A) HCO 7, (B) HCO 40, (C) CO 5, (D) CO 35. Danazol was incorporated into all formulations at 80% saturated solubility.

Table 3.3. Pharmacokinetic parameters for danazol after oral administration of digestible and stealth formulations with danazol incorporated at 80% saturated solubility [mean ± SEM (n=3)] to fasted male sprague dawley rats.

	Digestible Formulations		**Stealth Formulations**	
	HCO 7	CO 5	HCO 40	CO 35
$AUC_{0-\infty}$ (ng.h/mL)[a]	169.0 ± 13.3	134.4 ± 18.2	202.2 ± 27.5	244.4 ± 67.4[b]
C_{max} (ng/mL)	86.5 ± 8.0	61.1 ± 5.8	139.8 ± 16.0	105.8 ± 3.4[b]
T_{max} (h)	0.6 ± 0.1	0.8 ± 0.1	0.6 ± 0.1	0.8 ± 0.0
$t_{1/2}$ (h)	1.4 ± 0.2	1.5 ± 0.2	1.4 ± 0.0	1.8 ± 0.6
Rel bioavailability (%)	N/A	N/A	119.6	181.8

[a]Data normalised to a 1 mg/kg danazol dose to facilitate comparison across differing drug doses.
[b]Statistically significant increase when compared to CO5 formulation, $p < 0.05$

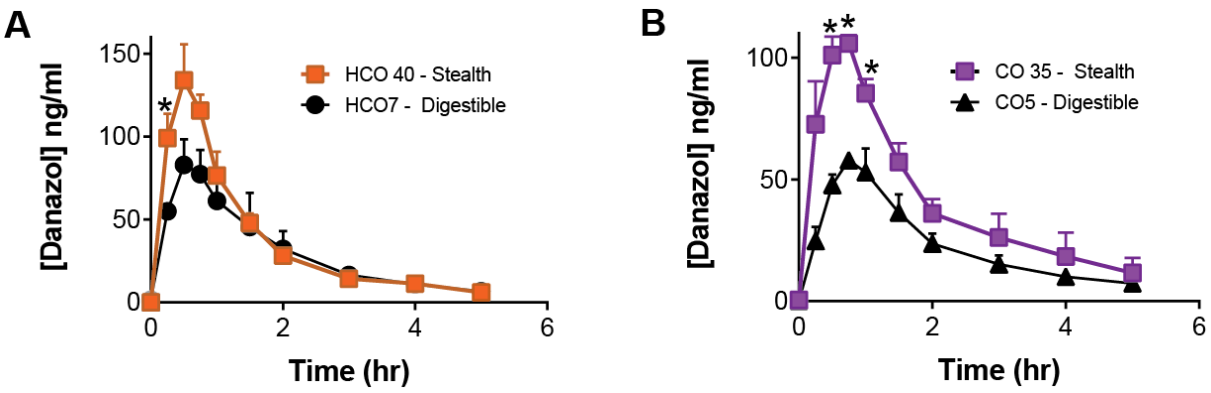


Figure 3.12. Mean danazol plasma concentration vs. time profiles for PEGylated hydrogenated castor oil formulations (A) and PEGylated castor oil formulations (B) after oral administration. Digestible formulations were HCO 7 and CO 5 (black circles and diamonds respectively) and stealth formulations were HCO 40 (orange squares) and CO 35 (purple squares). Data normalised to a 1 mg/kg dose of DAN, [mean ± SEM (n = 3)]. *Statistically significant difference ($P<0.05$).

3.5. Discussion

Increasing numbers of drug candidates emerging from lead optimisation programs have intrinsically low water solubility and high lipophilicity [9]. This has driven a need to develop enabling formulations that facilitate the oral delivery of poorly water-soluble drugs. Significant recent attention has focussed on the development of formulation approaches that enhance GIT solubility and dissolution rate [9]. Of these, lipid-based formulations (LBF) have grown in popularity and have been shown to markedly improve the oral bioavailability of a number of poorly water-soluble drugs [6, 9]. In spite of this, it is becoming increasingly apparent that digestion mediated changes to drug solubilisation can have a detrimental impact on the *in vivo* performance of LBF, particularly those containing medium-chain triglycerides (MCT). MCT are popular excipients in LBF since they typically allow higher drug doses to be dissolved in the formulation than their long chain counterparts.

Losses in drug solubilisation capacity during formulation digestion can lead to a transiently supersaturated state, which has the potential to promote drug absorption [128]. In instances where supersaturation is significant and/or prolonged, however, it may also promote precipitation. In some cases, the addition of polymeric inhibitors to prevent crystal seeding and reduce drug precipitation can recover these formulations and improve *in vivo* outcomes [128, 176, 232], but this is not always the case [128]. In contrast, prevention of digestion mediated losses in solubilisation capacity, through manipulation of the lipolysis process itself, has, to date, not been explored as an avenue to improved LBF performance *in vivo*. Lipolysis is an inherently surface sensitive reaction, and changes to the oil:water interface might be expected to alter the rate of digestion [11, 12]. Tan *et al*. have utilised the surface sensitivity of the digestion process to increase lipolysis rates, demonstrating that this in turn improves drug absorption from silica lipid nanoparticles [233, 234]. Conversely, the potential for non-ionic PEGylated surfactants to modulate or inhibit triglyceride digestion has been documented [11, 59, 200, 201, 203, 222] however, the utility of this inhibitory effect has not been explored in detail, nor translated into differences in *in vivo* bioavailability.

PEG is a neutral non-immunogenic polymer that is miscible with water and has high motility in aqueous media and a large hydrodynamic volume [235, 236]. These properties render the polymer resistant to protein adsorption. PEGylated interfaces find multiple biological applications where nonspecific protein adsorption is unwanted, most notably in nanomedicine where PEG surface coatings are used to prolong the circulation half-life of injectable nanomaterials through avoidance of opsonisation and phagocytosis. This PEG shielding effect was coined “stealth” by Allen and co-workers in 1991 [237]. The long-circulating performance of a stealth nanoparticle can be directly attributed to a combination of the molecular weight and surface density of the PEG mantle [14, 15, 223-226]. An understanding of these key structural determinants of stealth may provide a template for the development of improved LBFs after oral administration, since the adsorption of lipase to the droplet interface (and therefore the possibility of digestion mediated drug precipitation) is analogous to

the opsonisation of a nanoparticle in the systemic circulation. The prevalence of PEGylated non-ionic surfactants in lipid-based drug delivery provides a promising platform from which to develop stealth LBFs.

The main objective of the current study was therefore to develop 'stealth' oral lipid-based formulations from PEGylated surfactants and MCT, to evaluate the impact of these formulations on drug solubilisation and supersaturation *in vitro* and to assess whether this was manifest in improved *in vivo* drug absorption.

3.5.1. PEGylated surfactants as digestion modulators

3.5.1.1. Stealth properties are dependent on surfactant PEG Mw

Parenteral stealth formulations require a polymeric steric stabilisation layer of adequate size and surface coverage to prevent protein adsorption and to evade capture by the cells of the MPS. For example, Mori *et al*. described a relationship between PEG molecular weight and immunoliposome circulatory half-life in Balb/c mice [223] whilst Torchilin and co-workers demonstrated that long-circulating liposomes required optimised polymer-to-lipid ratios for adequate surface coverage and that this was directly dependent on the Mw of the PEG group [15, 224]. This putative PEG molecular weight/stealth relationship has since been extensively employed to guide the development of stealth nanoparticulate drug delivery vehicles.

More recently, it has been shown that PEGylated surfactants may change the rate and extent of lipolysis of homogenized emulsions in a PEG molecular weight dependant manner [201]. This data from Wulff-Perez and co-workers showed that the digestion of preformed sunflower oil emulsions containing different surfactants, two ethoxylated stearyl esters and two triblock co-polymers, was dependent on PEG Mw. However, the authors failed to find a comprehensive explanation for differences in digestion inhibition for two surfactants of equal PEG Mw.

The current studies applied the principles of stealth drug delivery to lipid-based formulation development by systematically investigating the impact of surfactant PEG Mw and packing density (hydrophobe structure) on the digestion of triglycerides. This data then informed the development of self-assembled stealth lipid-based formulations for oral administration of a poorly water-soluble drug. Figure 3.1 shows the direct impact of PEGylated surfactants on the digestion of MCT while figure 3.8 shows a parabolic relationship between PEG Mw and rate and the extent of digestion for six structurally diverse series of PEGylated surfactants. As the PEG Mw of the surfactants increased, the AUC of the digestion profiles (% digestion.min) decreased, suggesting that enzyme adsorption had been reduced, presumably via the generation of a polymeric steric barrier. Figure 3.7 shows that the reduction in digestion is not attributable to a reduction in surface area as the particle size of dispersed formulations decreased (and therefore surface area increased) with increasing PEG Mw.

As the PEG head group of the surfactant increased further and exceeded a critical maximum, the digestion inhibition effect was reduced. The latter effect may be attributable to an imbalance between the large motile hydrophilic head group and the smaller hydrophobic tail of the surfactant. At increasingly higher molecular

weights of the hydrophilic head group, the emulsifiers may become unstable at the surface of the lipid droplet and dissociate into the aqueous digestion environment, displaying at best, a transient inhibitory effect. This is consistent with the particle size data in figures 3.7 which show that surfactants with high Mw PEG headgroups were less efficient emulsifiers. These formulations dispersed to form multiple populations of droplets with very large (>5000 nm) phase separated oil droplets. Similar observations have been made in relation to the use of amphiphilic surface modifiers in liposome development [238].

3.5.1.2. Digestible surfactants require larger PEG Mw to impart stealth properties

Further examination of the digestion inhibition data in figures 3.1 to 3.6 reveals a statistically significant correlation ($P<0.05$) between the digestibility of the surfactant and the optimal surfactant PEG Mw for maximal digestion inhibition or "stealth" capability. The data in figure 3.8 show that as a rule of thumb, the digestible surfactants required a PEG Mw of ~1800 g/mol to shield the surfactant ester group and the co-formulated triglycerides from digestion. The non-digestible surfactants employed, however, did not possess labile ester bonds and as a result the PEG Mw required to sterically shield the MCT payload was significantly lower (approximately 800 g/mol).

3.5.1.3. The structure of the surfactant hydrophobe influences the extent of digestion inhibition

Parenteral stealth formulations have been developed based on optimising the quantity of polymer on the surface of the carrier (or the density of the PEG chains) [14, 15, 224, 238]. Accurately controlling this parameter for the development of self-assembled stealth LBFs is limited by the commercial availability of PEGylated surfactants where the hydrophobic moiety has been changed systematically. Despite these limitations, figure 3.9 provides some information as to the impact of the surfactant hydrophobe on PEG packing at the oil:water interface. Comparing pairs of formulations at constant surfactant PEG Mw but with differing degrees of saturation of the hydrophobe moiety, provides insight into the influence of surfactant packing at the droplet interface on digestion inhibition. Thus, unsaturated, non-linear (or 'kinked') surfactant hydrophobes (that presumably pack less efficiently at the interface, [239]) were less effective at reducing digestion than their straight chain counterparts. This trend was evident for simple non-digestible PEGylated fatty alcohols and more complex digestible PEGylated triglycerides.

3.5.1.4. Stealth LBFs improve drug solubilisation in vitro and drug absorption in vivo

Figure 3.11 shows the profound impact of stealth LBFs on the solubilisation of danazol during *in vitro* digestion. After a 30 min lipolysis period, the stealth formulations had been digested by less than 1%, and this was reflected in the maintenance of drug solubilisation at >80% of initial load. In contrast, the readily digestible, low Mw PEG equivalent formulations were rapidly digested and lost solubilisation capacity for the formulated drug. These results were also reflected in the maximal supersaturation ratios (S^M) generated for each formulation type, with stealth LBFs maintaining or marginally increasing S^M while digestible LBFs showed significant increases in S^M from dispersion to digestion. As has been previously reported [128, 151], S^M

values greater than 3 are typically associated with systems that are prone to precipitation and less likely to perform efficiently *in vivo*.

Finally, the plasma profiles in Figure 3.12 and the pharmacokinetic parameters presented in Table 3.3 reveal differences in *in vivo* exposure of danazol after oral administration of stealth and digestible formulations. Two series of surfactants were investigated, polyethoxylated hydrogenated castor oils and polyethoxylated castor oils. The trends between the two data sets were similar; generation of a self-assembled PEG mantle around the lipid droplet prevented rapid digestion and precipitation of the formulated drug. This in turn resulted in improved drug absorption where the increase in relative bioavailability of the stealth vs non-stealth formulations was 120% and 182 % for HCO and CO formulations respectively.

3.6. Conclusions

The current studies have explored the potential translation of concepts that have been successfully employed to enable parenteral stealth applications to oral drug delivery. The data suggest that similar to parenteral stealth formulations, the presence of a hydrophilic PEG mantle on the surface of a lipid droplet in the gastrointestinal tract is able to reduce protein binding (in this case, the binding of digestive enzymes). Furthermore the data suggest that the degree of stealth activity is dependent on the Mw of the PEG surfactant headgroup as well as the packing of the polymer at the surface of the oil droplet. Interestingly, the presence of hydrolysable groups within the surfactant structure affected the performance of the oral stealth LBFs, with digestible surfactants requiring larger Mw PEG headgroups to generate an effective stealth barrier.

Controlling the rate of formulation hydrolysis through the generation of a stealth PEG barrier also altered the propensity of a formulation to supersaturate on digestion, reducing the maximal supersaturation ratio (S^M) and thus reducing the risk of drug precipitation. Finally, *in vivo* administration of stealth and analogous digestible formulations to male SD rats resulted in increased exposure for groups dosed with stealth formulations, confirming that the prevention of rapid supersaturation and precipitation in the GIT is an effective strategy for improving the oral absorption of poorly water-soluble drugs. Danazol equilibrium solubility in formulations containing hydrogenated castor oil and castor oil based PEGylated surfactants

Chapter 4. Competition at the oil:water interface during in vivo self-assembly poses a significant challenge to the utility of 'stealth' lipid-based formulations for oral drug delivery

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4.1. Abstract

Increasing numbers of new drugs are poorly water-soluble, have slow dissolution in the GIT and low oral bioavailability. While enabling formulations such as lipid-based formulations (LBFs) can deliver PSWD in a molecularly dispersed form, rapid digestion of formulated triglycerides can result in drug supersaturation, precipitation and reduced bioavailability. Previous studies have shown that generation of a PEG ('stealth') mantle around the formulated MCT can reduce lipid digestion rates, maintain drug solubilisation after *in vitro* digestion and improve oral bioavailability in rats when compared to digestible counterparts. The current study examined if stealth LBFs of long-chain triglycerides (LCT) would provide an advantage in systems with inherently lower digestion rates. Stealth formulations were then further examined in a canine model to evaluate the utility of these formulations after *in vivo* self-assembly.

The data suggest that LCT stealth LBF behaviour mirrors MCT formulations i.e. stealth formulations of LCT reduced digestion, maintained drug solubilisation and improved bioavailability after administration of predispersed formulations to SD rats. Stealth formulations were progressed to studies in a dog model of *in vivo* self-assembly. Interestingly, after administration of stealth and digestible formulations to dogs in hard gelatin capsules, there was no apparent advantage to stealth LBFs. These results prompted further *in vitro* studies to examine the impact of surface active species present *in vivo* on formulation self-assembly. This data showed that the stealth effect can be attenuated in the presence of fatty acids, monoglycerides, and proteins (digestion rates increased from 2% to 20%) and may have contributed to formulation underperformance. However inter-subject variability may also have confounded the dog data as the model drug is highly metabolised.

The results of this study indicate that slowing the rates of digestion of long and medium chain lipids through the use of stealth LBFs is an effective strategy to improve oral absorption of poorly soluble drugs. However, performance is currently limited to administration of pre-assembled stealth species. Self-assembly of these formulations may be prone to disruption *in vivo* and competition at the oil:water interface contributed to the underperformance of stealth LBFs in dogs.

4.2. Introduction

Oral administration of medications remains the favoured dosing route for clinicians and patients alike, unequalled for both convenience and compliance. However, absorption after oral administration is dependent on favourable drug physicochemical characteristics [29]and adequate solubility in gastrointestinal (GI) fluids [1, 6]. As the proportion of hydrophobic new chemical entities (NCE) emerging from drug discovery programs grows, so too does the need for oral formulations which can enable the delivery of drugs with aqueous solubility limitations. Low water solubility typically precludes administration of solid drug, as dissolution of poorly water-soluble drugs (PWSD) in the absence of enabling technologies is likely to be rate limiting to absorption, leading to both low and variable bioavailability.

One strategy to improve oral absorption of PWSD involves the use of lipid-based formulations (LBF) to deliver lipophilic drugs in a pre-solubilised, molecularly dispersed form to the GI tract [1, 6, 60]. However, these drug delivery systems are usually subject to lipolysis in the intestinal lumen and the resultant digestion products can, in some cases, have lower solubilisation capacity for the formulated drug. This digestion mediated reduction in solubilisation capacity leads to supersaturation and can stimulate drug precipitation. This phenomenon is particularly evident in formulations composed of medium chain triglycerides (MCT). Drug solubility in MCT-based formulations is often higher than in equivalent long chain triglycerides (LCT) [169], but they are hydrolysed faster than LCT [119, 240] and their typical lipolytic products (caprylic and capric monoglycerides and fatty acids) are significantly more hydrophilic than their long chain counterparts. Taken together, these factors contribute to a larger difference between pre- and post-digestion drug solubilisation capacity and this is reflected in a greater tendency for MCT formulations to result in drug precipitation after digestion [128, 209, 210]. LBF development thus typically involves a trade-off between higher mg/g drug solubilisation capacity in MCT-based formulations but greater solubilisation capacity post-digestion in LCT-based digestion products.

Lipid digestion in the GI tract is catalysed primarily by pancreatic lipases [11, 241]. These water-soluble hydrolytic proteins adsorb onto aggregated water insoluble substrates with the enzymatic reaction occurring at the oil:water interface. Modifying the interface can lead to changes in lipase adsorption, as exemplified by Borgström and Erlanson who reported a reduction in digestion of lipid droplets coated in bile salt [44, 242]. In this case, however, co-lipase was absent and addition of this anchoring protein restored lipase activity. These seminal studies combined with more recent data describing the ability of non-ionic surfactants to modulate (increase or decrease) lipid digestion in the presence of the lipase/co-lipase enzyme complex [59, 180, 200, 201, 222], indicate that the rate and extent of triglyceride digestion may be manipulated by changing the interface presented to the enzyme complex. This provides a potentially novel means of controlling digestion, drug solubilisation patterns and ultimately, drug absorption.

In a previous study [204], we examined the relationship between surfactant-mediated changes to the oil droplet interface and the rate of digestion of co-formulated surfactant-MCT systems to develop 'stealth' LBFs. The term 'stealth' in drug delivery generally applies to IV nanoparticulate drug carriers which utilise a steric coating (typically poly(ethylene glycol), PEG) to prevent protein adsorption, recognition and clearance by the immune system [13, 15]. Stealth LBFs were developed using PEGylated surfactants to generate a self-assembled steric coating which reduced adsorption of the lipase/co-lipase enzyme complex to the surface of the oil droplet. The data showed a correlation between the PEG molecular weight (Mw) of the surfactant hydrophile and the rate and extent of MCT digestion, mirroring the behaviour of parenteral stealth formulations. The ability of these 'stealth' LBFs to modify solubilisation patterns of the model PWSD danazol after digestion was shown *in vitro* and subsequent *in vivo* studies demonstrated that stealth MCT formulations conferred an advantage over more digestible analogues after administration of predispersed formulations in SD rats. While these proof of concept studies were promising, the stealth behaviour of analogous LCT formulations, and the behaviour of other surface active excipients *in vivo* were unknown. The aim of the current study was therefore to explore in detail the performance of a range of self-assembled 'stealth" LBFs of long and medium chain triglycerides.

Firstly, we evaluated the impact of increasing triglyceride chain length from MCT to LCT on the digestion inhibition behaviour of stealth formulations. We then investigated the effect of LCT stealth LBFs on drug absorption in SD rats to establish whether inhibiting the digestion of long chain lipids could be advantageous in delivering poorly water-soluble drugs.

Secondly, we aimed to investigate why the digestion inhibition phenomenon associated with stealth LBFs was not observed for 'typical' self-emulsifying drug delivery systems (SEDDS) in contrast to binary mixtures of triglycerides and surfactants. This was achieved by evaluating the impact of common formulation excipients on the digestion inhibition capability of stealth LBFs *in vitro*. The working hypothesis of these studies was that the presence of surface active moieties which can compete for space at the oil:water interface may disrupt the assembly of a continuous PEGylated steric barrier.

Thirdly, a study was conducted to evaluate formulation performance after administration of undispersed LBF preconcentrates in gelatin capsules to beagle dogs, better reflecting the anticipated dosing conditions in man. This study was based on the hypothesis that the increased lipase activity in dog relative to rats [156], would lead to greater differences in bioavailability between digestible and stealth formulations. However these studies showed little benefit of the stealth effect across dosing groups. Finally, therefore the data generated in the dog studies prompted further *in vitro* studies to investigate the potential disruption of stealth LBF self-assembly *in vivo* by surface active "impurities" present in the GI tract.

4.3. Methods

4.3.1. Materials and reagents

Captex® 355 (C_8 and C_{10} MCT), Capmul® MCM (MC monoglyceride) and Captex® GTO ($C_{18:1}$ LCT) were kindly donated by Abitec Corporation (Janesville, WI, USA) and were used as received. Transcutol was a gift from Gattefossé (St Priest, France). Etocas™ 5, (PEGylated castor oil - CO) and Croduret™ 7, 25, 40 (PEGylated hydrogenated castor oils - HCO) were kindly donated by Croda International PLC (Yorkshire, England). Jeechem® CAH 16 (PEG 16 hydrogenated castor oil) was donated by Jeen® International Corporation (Fairfield, NJ, USA). Kolliphor® EL (PEG 35 castor oil) and RH40 (PEG 40 hydrogenated castor oil) were donated by BASF Corporation (Washington, NJ, USA). Nikkol® HCO 100 (PEG 100 hydrogenated castor oil) was donated by Nikko Chemicals Co. Ltd. (Chuoku, Tokyo, Japan). Hard gelatin capsules, size 00 were kindly donated by Capsugel (Morristown, NJ, USA). Kolliphor® RH60 (PEG 60 hydrogenated castor oil) was purchased from Ingredients Plus (Notting Hill, VIC, Australia). 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), sodium taurodeoxycholate > 95% (NaTDC), PEG400, absolute ethanol, porcine pancreatin (8 x USP specification activity) and 4-bromophenylboronic acid (4-BPB) were obtained 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). Sodium hydroxide 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. Hypergrade solvents were used for UPLCMS/MS analysis.

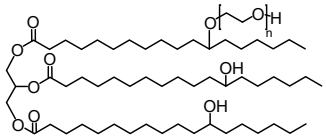
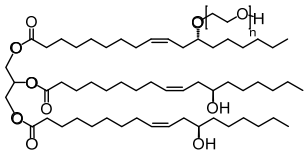
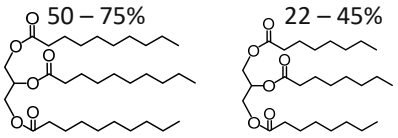
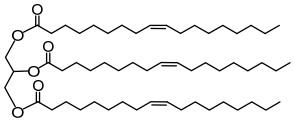
4.3.2. Formulation preparation

4.3.2.1. Blank formulations

All formulations were prepared as binary mixtures of MCT (Captex® 355) or LCT (Captex® GTO) and surfactant (50/50 % w/w) as previously described [204]. The details of the surfactants and lipids used are summarised in Table I.

Typical SEDDS formulations also utilise monoglycerides and cosolvents to aid rapid emulsification. To evaluate the impact these more hydrophilic excipients may have on the self-assembly and resultant stealth behaviour of LBFs, medium chain monoglyceride (namely, Capmul MCM) and cosolvents (PEG400, Ethanol and Transcutol) were incorporated at 20% w/w (200 mg) into the HCO:40 formulation. All formulations were prepared at 37 °C, mixed and equilibrated overnight before use.

Table 4.1: Details of the PEGylated surfactants and triglycerides used in the formulations studied

Excipient (Trade name(s))	**General Structure**	**PEG Mw (g mol^{-1})**	**Abbreviated name** (indicating approx. headgroup PEG Units [n])
Polyethoxylated Hydrogenated Castor Oils (HCO)** (Croduret™, Kolliphor®, Jeechem®, Nikkol®)		308[a§] 705[b] 1102[a] 1763[c§] 2644[c] 4407[d]	HCO 7 HCO16 HCO25 HCO 40 HCO 60 HCO 100
Polyethoxylated Castor Oils (CO)** (Etocas™, Kolliphor®)		220[a§] 1542[c§]	CO5 CO35
Medium Chain Triglyceride (MCT) (Captex® 355)		N/A[e]	N/A
Long Chain Triglyceride (LCT) (Captex® GTO)		N/A[e]	N/A

Supplier: [a] Croda, [b] Jeen, [c] BASF, [d]Nikko, [e]Abitec

* HLB calculated from surfactant general structure and PEG Mw as previously described [204].

** Surfactant general structure is simplified.

§ Surfactant components of formulations evaluated for *in vitro* drug distribution and *in vivo* absorption.

4.3.2.2. Drug loaded formulations

The equilibrium solubility of danazol in each of LBFs was determined as previously described [217]. Equilibrium solubility was typically reached after equilibration times of between 48 and 72 h. Danazol containing formulations were prepared at a loading of 80% saturated solubility (based on measured values at 37 °C), drug content was confirmed by HPLC assay prior to formulation use.

4.3.3. In vitro dispersion and digestion of formulations

4.3.3.1. Digestion conditions

In vitro dispersion and digestion studies were conducted using both a standard model and a rat model of *in vitro* lipolysis as previously described [150, 204]. Lipolysis experiments were preceded by a dispersion step (15min) with digestion monitored at pH 6.5 for 30 min. Back-titration at pH 9.0 was performed at the end of the experiment. Fatty acid titres were corrected with back-titration data to account for all fatty acids (ionised and unionised) present and these values were then used to calculate the extent of digestion (% digestion) as previously described [204].

Digestion inhibition plots were generated from the area under the % digestion curve (% digestion.min) in the presence and absence of surfactant and plotted against the PEG molecular weight of the surfactant. Inhibition AUCs were compared to the AUC for LCT and MCT digestion in the absence of surfactant to provide an indication of the relative change to lipolysis over the entire period of digestion.

To evaluate the impact of *in vivo* disruption of self-assembly, potential amphiphilic species likely to be present in the GI tract were added to the digestion media (namely; Capmul MCM, octanoic acid, gelatin, bovine serum albumin). 20% (w/w, i.e. 200 mg) of each surface active 'species was incorporated into the digestion media prior to initiating digestion. Changes to rate and extent of digestion were expressed as changes in AUC (% digestion.min).

Differences in the bile salt concentrations *in vivo* could also impact the self-assembly of stealth LBFs when formulation is released in the upper GI tract. To evaluate the impact of increased bile salt concentration on stealth properties, the bile salt content of the digestion media was increased from 4 mM to 8 mM and changes in digestion plotted as changes in % AUC (% digestion.min)

The impact of initiating MCT digestion followed by the addition of HCO40 surfactant was also investigated to mimic emulsification of MCT in the presence of surfactant and lipases. Digestion of MCT alone was initiated and after 30 seconds HCO40 dispersed in 2ml of digestion media was added to the digestion vessel at a rate of 4ml/min i.e. over 30 seconds. Changes to rate and extent of digestion relative to coformulated MCT and HCO40 were expressed as changes in AUC (% digestion.min).

In vitro drug solubilisation (digestion) experiments were carried out on drug loaded formulations as previously described [204]. Blank (drug free) formulations were also dispersed and digested to obtain aqueous colloidal phases for determination of danazol equilibrium solubility and were then used to calculate maximum supersaturation ratios S^M as described by Anby *et al* [128].

Graphpad Prism for windows (Version 6.0, Graphpad Software Inc., CA, USA) was used to statistically analyse differences in pairs of *in vitro* data using unpaired parametric t-tests. Data were expressed as the mean (n=3) ± standard deviation (SD). A difference was considered statistically significant when $p \leq 0.05$.

4.3.4. Oral bioavailability studies in Sprague Dawley rats

4.3.4.1. Surgical and experimental procedures

All surgical and experimental procedures were approved by the Monash Institute of Pharmaceutical Sciences Animal Ethics Committee and were conducted in accordance with the Australian and New Zealand Council for the Care of Animals in Research and Teaching guidelines. Experiments were conducted as previously described [204].

4.3.4.2. Formulation administration and sample collection

Since danazol has very high metabolism in rats, and because differences in first pass metabolism may obscure differences in absorption, experiments were conducted here in the presence of ABT to remove metabolism related variability. ABT pre-treated rats (1.2 mL of 100 mg/g ABT dosed via oral gavage) were dosed with 30 mg of each formulation dispersed in 470 mg water and administered via oral gavage. Samples were collected as previously described [204]. Drug doses were 0.9, and 1.6 mg/kg, for the HCO7 and HCO40 LCT formulations respectively.

4.3.5. Oral bioavailability studies in beagle dogs

4.3.5.1. Surgical and experimental procedures

All surgical and experimental procedures were approved by the Melbourne University 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.

4.3.5.2. Formulation administration and sample collection

Four MCT formulations with varying surfactant PEG Mw were evaluated in dogs to examine the impact of self-assembly of stealth LBFs *in vivo* and the potential impact of increased lipolysis in the dog on *in vivo* exposure. Treatments were based on two formulation types, digestible low Mw PEG surfactant-based formulations (HCO 7:MCT and CO 5:MCT) and stealth LBFs (HCO 40:MCT and CO 35:MCT). All formulations contained danazol at nominally 80% of saturated solubility in the formulation. The studies were conducted as a four-way crossover (7day washout period) in male beagle dogs (14- 20 kg). On the day prior to dosing, 700 mg of drug loaded formulation was accurately weighed into a 00 hard gelatin capsule, after which the capsule lid was replaced and sealed with ethanol. Each treatment was administered in two capsules (2 x 700 mg formulation) at 80% of nominal maximum drug loading with approximately 50 mL water. Actual doses were 8.0 mg Danazol (0.5 mg/kg) for HCO 7:MCT, 16.1 mg Danazol (1.0 mg/kg) for HCO 40:MCT, 12.2 mg Danazol (0.7 mg/kg) for CO 5:MCT and 18.7 mg Danazol (1.2 mg/kg) for CO 35:gMCT. The dogs were fasted for at least 12 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 throughout the study.

Blood samples (3 mL) were collected pre-dose and 15, 30, 45, 60, 90, min post dose and then at 2, 3, 4, 6, 8, and 10 h post dose via an indwelling catheter. Catheters were flushed with 1 IU/mL sodium heparin saline solution after each sample to ensure patency. Additional samples at 24, 28 and 32 h were obtained by individual venepuncture. Blood samples were deposited into tubes containing 5.4 mg EDTA. Plasma was separated within 2 h by centrifugation (1328g, 10 min) in an Eppendorf 5702 R/A-4-38 centrifuge, (Eppendorf AG, Hamburg, Germany) and stored at -80 °C until sample analysis.

4.3.6. Quantitation of danazol in plasma samples by UPLC-MS/MS

All plasma analyses were performed using a validated method on a Waters Xevo TQS UPLC MS/MS system (Waters, Milford, MA, USA) as previously described [204].

4.3.7. Pharmacokinetic data analysis

The data were plotted as danazol concentration (ng/mL) vs. time (h) (normalised to a nominal dose of 1 mg/kg danazol). Non-compartmental pharmacokinetic parameters were calculated using Phoenix™ 64 Software (WinNonlin® version 6.3, Pharsight Corporation, CA, USA). Graphpad Prism for windows (Version 6.0, Graphpad Software Inc., CA, USA) was used to statistically analyse differences in pairs of *in vivo* data using unpaired parametric t-tests. All data represented are expressed as the mean ± standard error of mean (SEM). A difference was considered statistically significant when $p \leq 0.05$.

4.4. Results

4.4.1. Impact of PEGylated surfactants on the digestion of long chain triglycerides

The potential for surfactants of increasing PEG molecular weight to change the digestion rate of LCT was investigated using a series of hydrogenated castor oil (HCO) based non-ionic surfactants (Table 1). Binary formulations were prepared from the surfactant and LCT and digested under standard lipolysis conditions. Titres were adjusted for fatty acid ionisation via back titration (inclusive of contributions from fatty acids liberated from the surfactant itself) and lipolysis curves were then plotted as % Digestion Vs Time as previously reported [204]. The extent of digestion of LCT in the absence of surfactant was only 24% after 30 min (Fig 1 A). The addition of a low molecular weight surfactant (HCO 7) significantly increased the initial rate and more than doubled the overall extent of digestion compared to LCT alone (Fig 1B). Further increasing the PEG Mw of the surfactant to 705 g/mol (Fig 1 C) marginally increased the rate and extent of *in vitro* lipolysis however, increasing the Mw of PEG in the surfactant headgroup to 1102 g/mol (Fig 1 D) resulted in a reduction in digestion to 7%, indicating that stearic hindrance effects were predominating. Maximal digestion inhibition (4% digestion) was observed for the HCO 40 formulation (PEG Mw of 1763 g/mol, Fig 1 E) consistent with data previously generated for MCT [204].

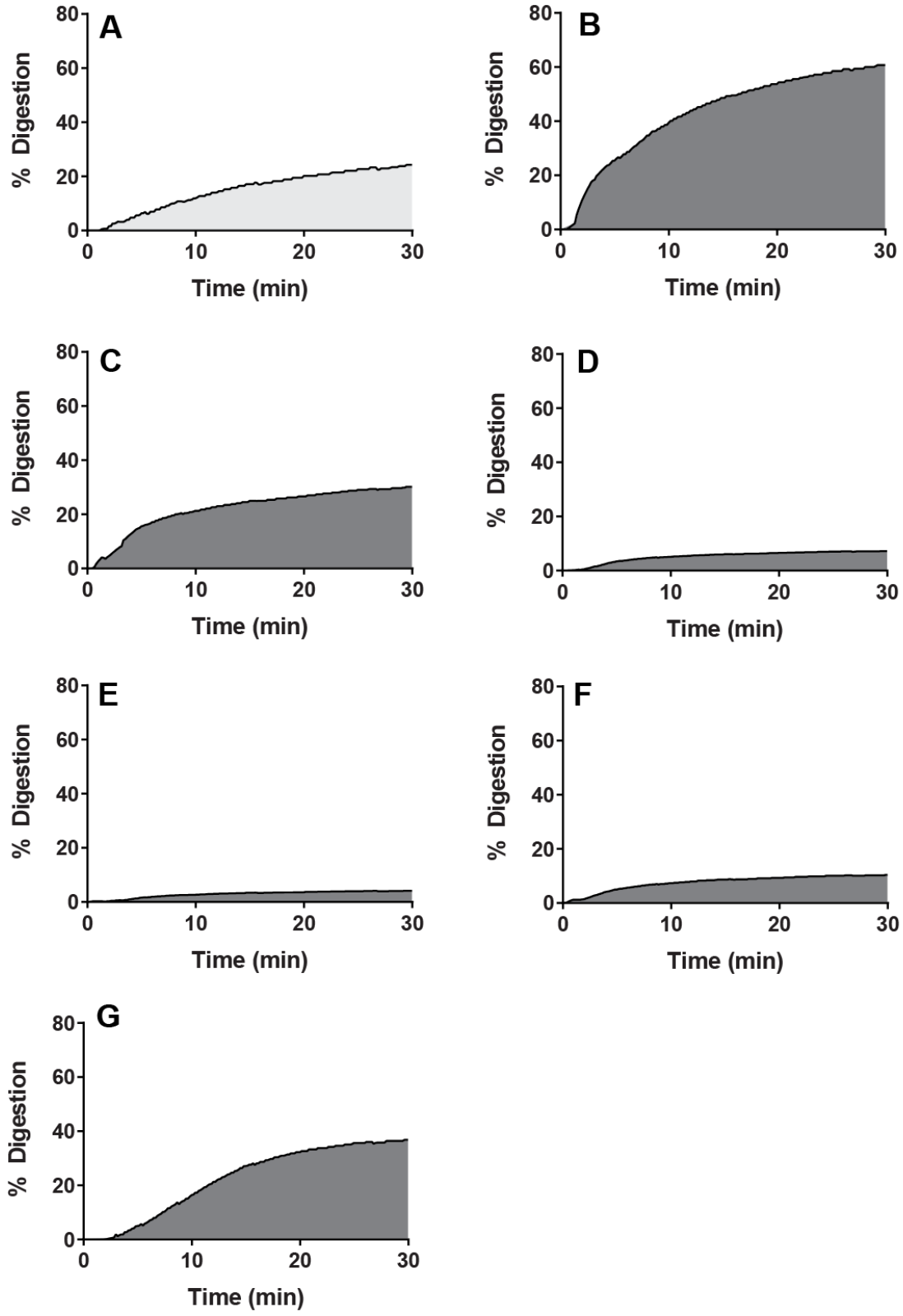


Figure 4.1. (A – G) *In vitro* lipolysis profiles for binary formulations of LCT and hydrogenated castor oil surfactants of increasing PEG Mw. Shaded areas represent the AUC calculated using the linear trapezoidal method used to plot the digestion inhibition curve in figure 4.2. (A) LCT alone, (B) HCO 7 (PEG Mw 308), (C) HCO 16 (PEG Mw 705), (D) HCO 25 (PEG Mw 1102), (E) HCO 40 (PEG Mw 1763), (F) HCO 60 (PEG Mw 2644), and (G) HCO 100 ((PEG Mw 4407).

Further increasing the PEG Mw of the surfactant headgroup did not result in a greater 'stealth' effect, (Fig 1F) and digestion inhibition capability was ultimately lost with the highest Mw PEG headgroup (Figure 4.1G). The AUC of these lipolysis profiles in Fig 4.1 were plotted against the PEG Mw of the surfactant headgroup (Fig 4.2A). The resultant parabolic relationship observed between the PEG Mw of the surfactant headgroup and AUC of lipolysis mirrors that previously observed for MCT (Fig 4.2B).

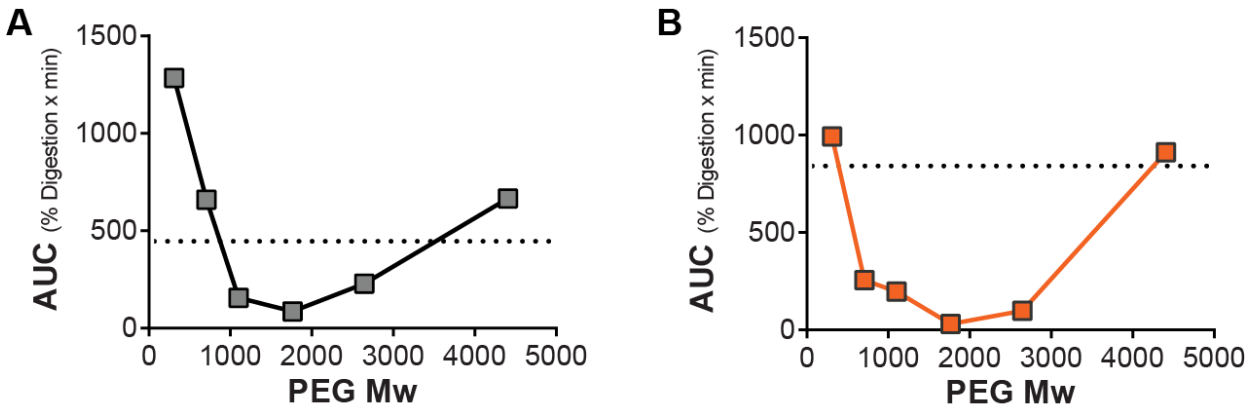


Figure 4.2. Comparison of digestion inhibition curves for the hydrogenated castor oil surfactants plotting lipolysis profile AUC (% digestion.min) against PEG Mw for (A) LCT and (B) MCT (reproduced from [204] with permission). The dotted line represents AUC of the triglyceride alone.

From Fig 4.2, it can be seen that the magnitude of the digestion inhibition or 'stealth' effect is greater for MCT systems (Fig 4.2B, parabola minimum relative to dotted line) with a 23-fold reduction in digestion for a stealth MCT formulation versus a 5-fold reduction in digestion of the analogous stealth LCT formulation (Fig 4.2A, parabola minimum relative to dotted line). This is due, in part, to the digestibility of the triglycerides themselves as LCT have been reported to digest at slower rates than MCT [168, 243]. The data in Fig 4.1A shows that the extent of digestion of LCT was 24% whereas MCT is approximately 47% digested under the same experimental conditions [204].

Two formulations (LCT:HCO7 and LCT:HCO40) were chosen to evaluate drug solubilisation capacity using a rat model of lipolysis and also to facilitate comparison to previously generated data with analogous MCT formulations [204]. LCT:HCO7 was chosen as an example of a digestible formulation and LCT:HCO40 as a non-digestible or stealth formulation based on the profiles in Fig 4.1.

Fig. 4.3A shows overlaid drug solubilisation and digestion profiles for the digestible (HCO 7) formulation. Under rat digestion conditions, this formulation maintains danazol solubility for 15 min but approximately 50% of the incorporated drug precipitated by 30 min. The onset of precipitation correlated with an increase in digestion (grey shaded area, right Y axis). The stealth formulation (Fig 3B, HCO40) was not digested (<1%) and maintained drug in solution throughout the experimental period. Drug supersaturation

maxima (S^M) increased from dispersion to digestion (1.4 and 2.8 respectively) for the digestible HCO7 formulation, whereas there was a lower relative change in S^M (0.9 and 1.6) for the stealth HCO40 formulation.

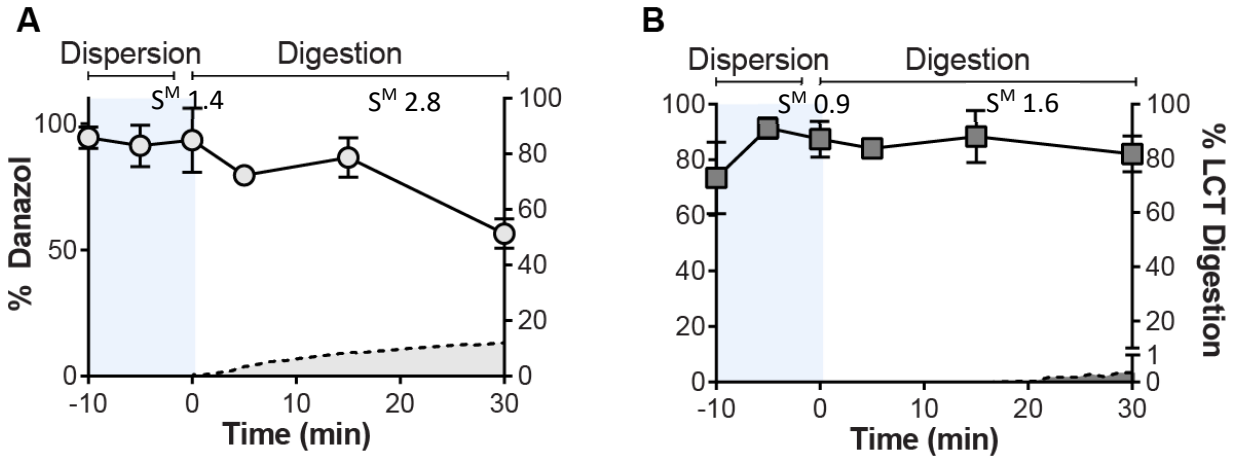


Figure 4.3. Danazol solubilisation profiles (filled symbols, left Y axis) during dispersion (blue shaded area) and digestion of PEGylated HCO formulations. Lipolysis is shown in the dotted line and shaded in grey, right Y axis) [mean ± SD (n =3)] and was generated using a rat model of digestion, i.e., under relatively high dilution conditions and low enzyme activity relative to traditional lipolysis conditions[156]. (A) HCO 7, (B) HCO 40. Danazol was incorporated into all formulations at 80% saturated solubility.

4.4.2. In vivo performance of LCT stealth formulations:

To investigate whether the stealth effect (and corresponding lack of precipitation) translated to improved drug absorption for LCT formulations, stealth and digestible HCO formulations were administered to fasted rats. The *in vivo* data show the same general trend for LCT and MCT formulations i.e. the stealth formulations resulted in slightly greater drug exposure than their digestible counterparts (Table 4.2, Fig 4.4).

Table 4.2. Pharmacokinetic parameters for danazol after oral administration of digestible and stealth formulations with danazol incorporated at 80% saturated solubility [mean ± SEM (n=3)] to fasted male Sprague dawley rats.

	LCT Formulations		**MCT Formulations**	
	HCO7:LCT	HCO40:LCT	HCO 7:MCT	HCO 40:MCT
$AUC_{0-\infty}$ (ng.h/mL)[a]	114.3 ± 12.6	209.2 ± 19.5[b]	169.0 ± 13.3	202.2 ± 27.5
C_{max} (ng/mL)	64.2 ± 8.9	69.8 ± 8.6	86.5 ± 8.0	139.8 ± 16.0
T_{max} (h)	0.5 ± 0.1	0.8 ± 0.2	0.6 ± 0.1	0.6 ± 0.1
$t_{1/2}$ (h)	1.0 ± 0.2	1.8 ± 0.1	1.4 ± 0.2	1.4 ± 0.0
Rel bioavailability (%)	N/A	183.0	N/A	119.6

[a]Data normalised to a 1 mg/kg danazol dose to facilitate comparison across differing drug doses
[b]Significant increase in exposure when compared to digestible LCT formulation

However, the plasma profile for the stealth LCT formulation (Fig 4A, LCT HCO40, grey squares) is markedly different to the stealth MCT formulation (Fig 4B, MCT HC040, orange squares). The pharmacokinetic parameters in table 4.2 show that while the MCT stealth formulation results in higher C_{max} and a corresponding increase in AUC, the LCT stealth formulation does not alter C_{max}. However, both t_{max} and $t_{1/2}$ are greater for the LCT stealth formulation than all other formulations, resulting in similar AUCs for both stealth formulations and a statistically significant increase in exposure from a stealth LCT formulation when compared to the digestible counterpart (figure 4.4C).

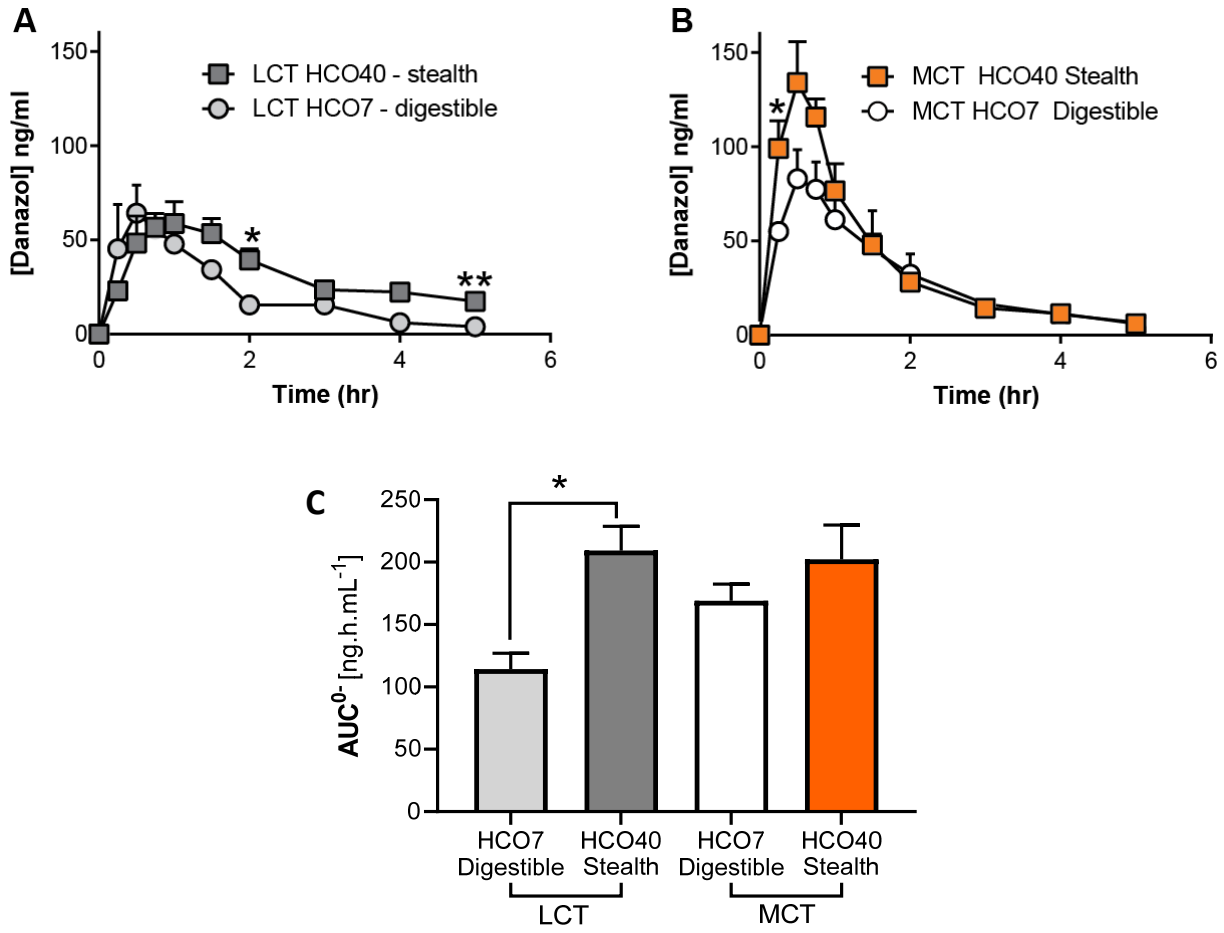


Figure 4.4. Mean danazol plasma concentration vs. time profiles for LCT (A) and MCT formulations (B, reproduced from [204] with permission) after oral administration. Digestible formulations comprised triglyceride and HCO 7 (circles) and stealth formulations were composed of triglyceride and HCO 40 (squares). (C) Plasma exposure expressed as AUC for each series of LCT and MCT formulations. Data were normalized to a 1 mg/kg danazol dose, [mean ± SEM (n=3)]. *Statistically significant difference ($p < 0.05$).

4.4.3. The impact of SEDDS excipients on stealth behaviour of MCT formulations

As the stealth or digestion modulating effect is not typically observed for self-emulsifying LBFs (SEDDS or SMEDDS), it was hypothesised that the other excipients commonly included in SEDDS formulations may impact on the digestion inhibition behaviour of PEGylated surfactants. In figure 4.5, the impact of adding medium chain monoglyceride (Fig 4.5C), PEG400 (Fig 4.5D), ethanol (Fig 4.5E) and Transcutol (Fig 4.5F) on the digestion profiles of MCT:HCO40 are presented. The addition of these commonly used SEDDS excipients significantly changed the lipolysis profiles. Notably addition of all the common alternative SEDDS excipients increased lipolysis above that of the stealth formulations ie reduced the surfactant stealth/digestion inhibition effect.

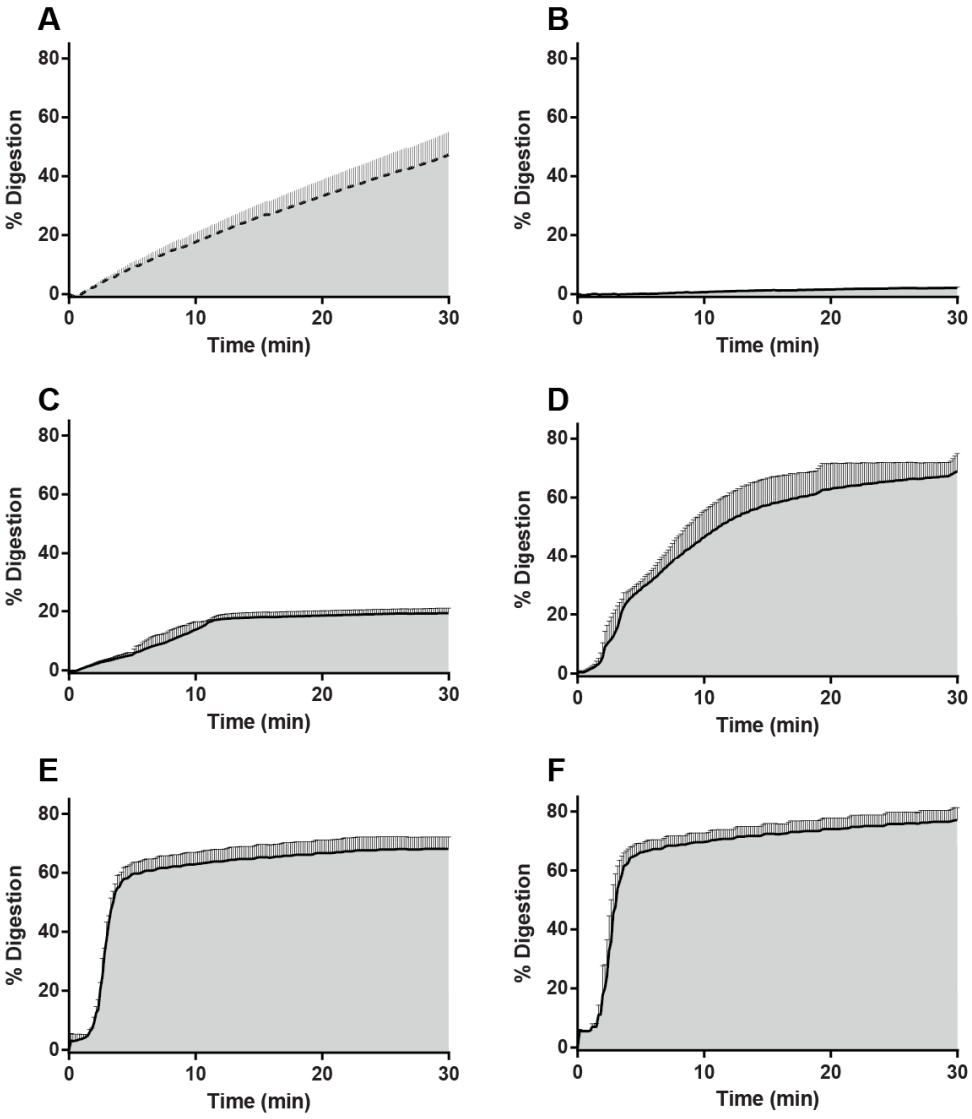


Figure 4.5. (A – F) *In vitro* lipolysis profiles for formulations of MCT and HCO40 with 20% (200 mg) common SEDDS excipients incorporated into the formulations [mean ± SD (n = 3)]. Shaded areas represent the AUC calculated using the linear trapezoidal method used to plot the bar chart in figure 4.6. (A) MCT alone, (B) HCO 40:MCT (C) HCO40:MCT + 200 mg Capmul MCM. (D) HCO40:MCT + 200 mg PEG400 (E) HCO40:MCT + 200 mg Ethanol (F) HCO40:MCT + 200 mg Transcutol.

Figure 4.6 shows the reversal of digestion inhibition observed after addition of excipients to the MCT:HCO40 stealth formulation. Addition of medium chain monoglyceride increased digestion of the formulation to a similar extent to that of MCT alone (dotted line). Addition of cosolvents PEG 400, Transcutol and ethanol to a stealth LBF, however, significantly increased both the rate and extent of formulation digestion even relative to that of triglyceride alone ($p < 0.0001$).

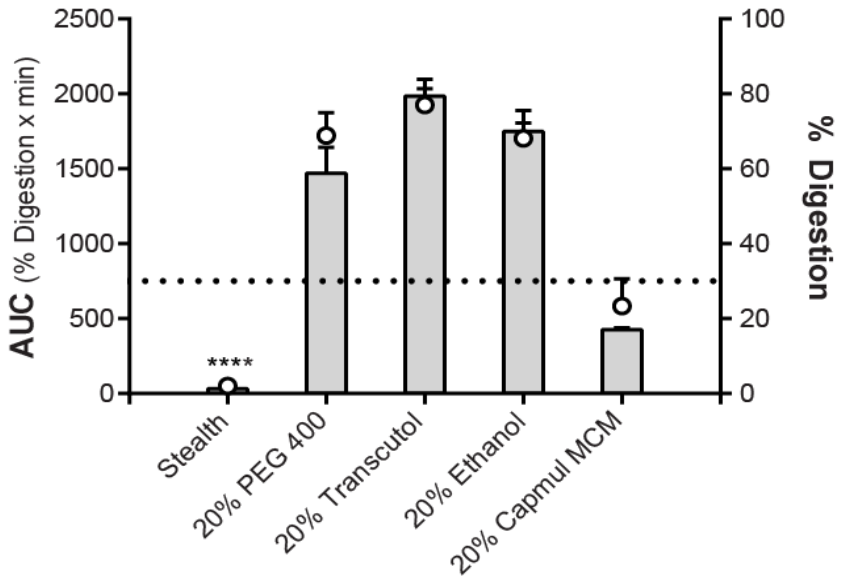


Figure 4.6. Reversal of stealth behaviour of MCT:HCO40 formulation after addition of 20% (w/w) surface active excipients. Changes in the AUC of the lipolysis curve (bars, left Y axis) Vs excipient [mean ± SD (n = 3)]. The change in % digestion is plotted as dot points, right Y axis. Dotted line, AUC of MCT digestion in the absence of surfactants. ****Statistically significant difference ($p < 0.0001$).

4.4.4. In vitro evaluation of drug solubilisation patterns in a standard model of stealth LBF digestion

The studies conducted with MCT and LCT show that stealth formulations administered orally to fasted rats did improve drug absorption when compared to their digestible analogues. These formulations were dispersed in water immediately preceding oral gavage, ensuring some self-assembly had occurred before administration. However, dosing conditions in man are anticipated to involve administration of neat LBF in a gelatin capsule. This mode of administration requires the self-assembly of formulations in the GIT. To better reflect this, studies were conducted in male beagle dogs administered MCT stealth LBF in hard gelatin capsules.

Prior to initiation of the *in vivo* studies, the drug solubilisation patterns after digestion were established using *in vitro* lipolysis conditions more representative of conditions in the dog. These are the conditions more commonly described in the literature and feature higher enzyme concentrations and larger dilutions that the conditions employed to represent rat conditions [156]. Figure 4.7 shows the drug solubilisation and formulation digestion patterns for digestible (HCO) and stealth (CO) MCT formulations.

The digestible formulations (Fig 4.7A and C) show significant drug precipitation as formulation digestion proceeds and this is reflected in higher supersaturation maxima (S^M), whereas the stealth formulations (Fig 4.7B and D) show better maintenance of drug solubilisation, reduced digestion rates and more moderate supersaturation values.

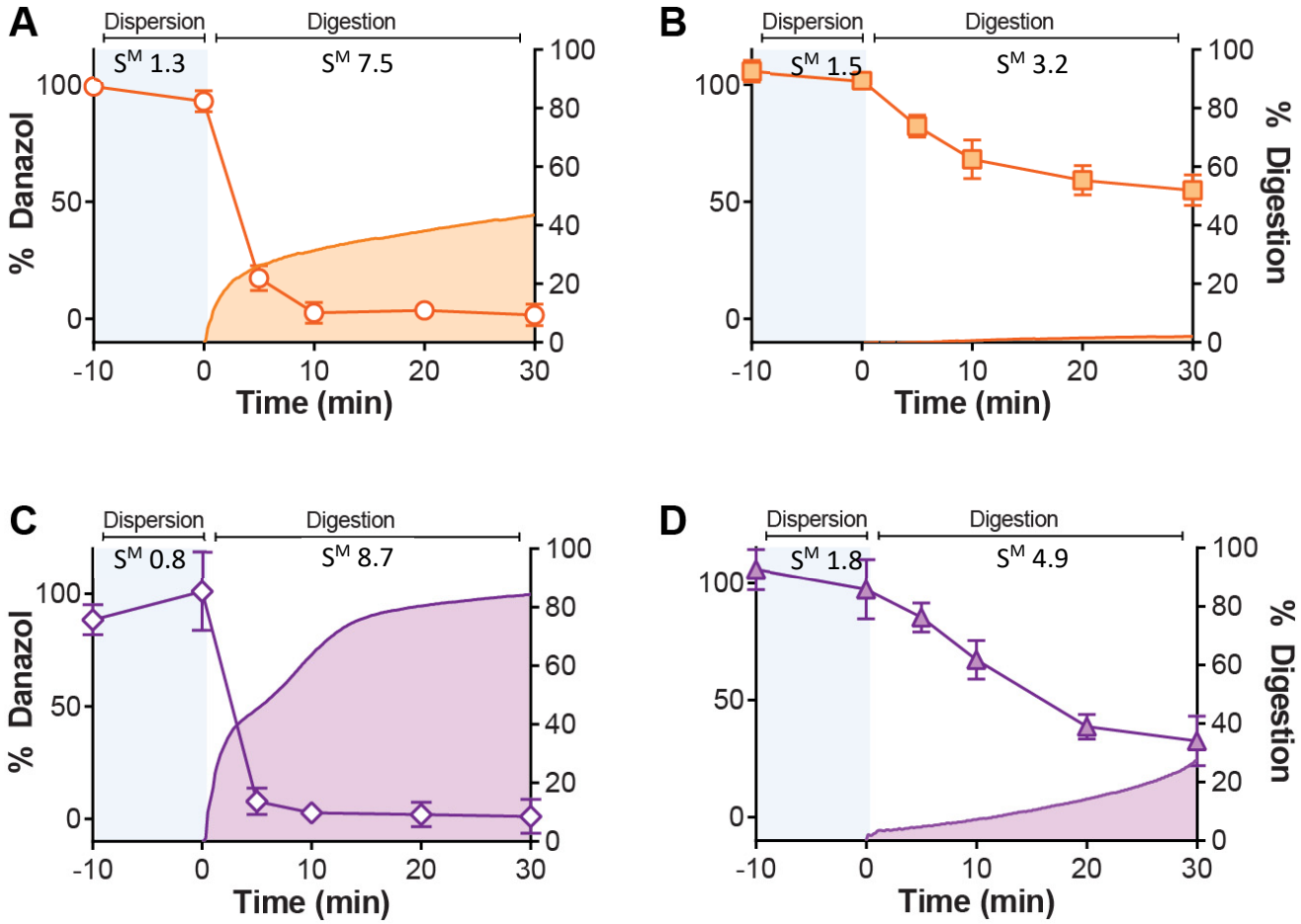


Figure 4.7. Danazol solubilisation profiles (symbols and line, left Y axis) during dispersion and digestion of PEGylated hydrogenated castor oil (HCO - orange) and castor oil formulations (CO-purple) (shaded areas represent lipolysis curves, right Y axis) [mean ± SD (n = 3)] using a dog model of digestion [156]. (A) HCO 7, (B) HCO 40, (C) CO 5, (D) CO 35. Danazol was incorporated into all formulations at 80% saturated solubility.

4.4.5. In vivo evaluation of the self-assembly capability of stealth LBFs after oral administration to dogs

The four formulations analysed *in vitro* in figure 4.7 were administered in hard gelatin capsules to fasted male beagle dogs. Figure 4.8 shows the mean plasma concentration versus time profiles for each of the four formulations after oral administration. Corresponding pharmacokinetic data is summarised in table 4.3. Figure 4.8A shows that for the HCO-based formulations, there was no discernible difference between the stealth LBF and analogous digestible formulation. Surprisingly, for the CO based formulations (fig 4.8B), the digestible formulation outperformed its stealth counterpart and the stealth CO35 formulation led to the lowest danazol absorption. Danazol absorption across the other three formulations was similar.

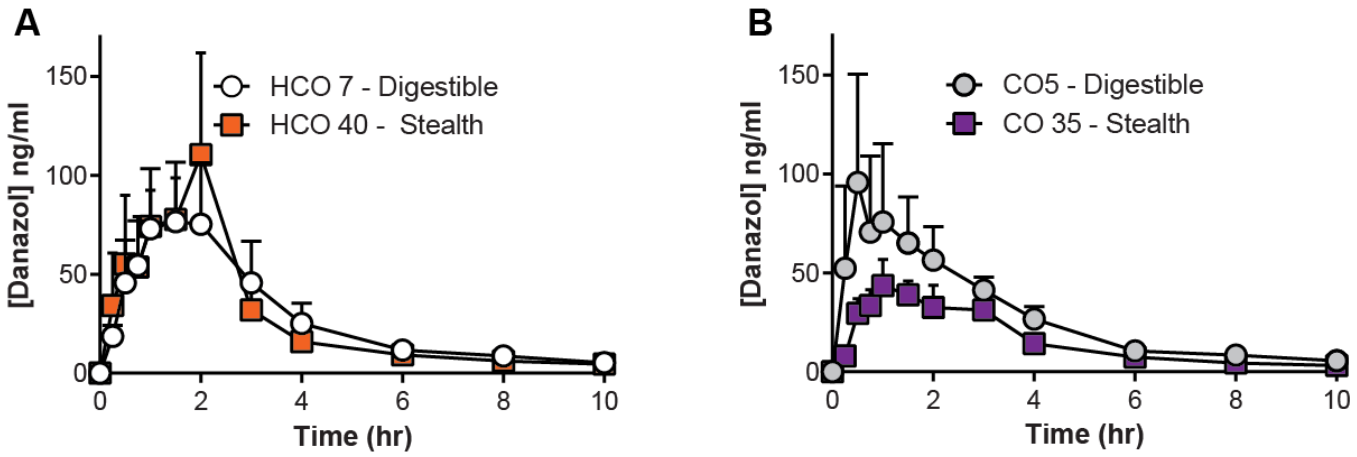


Figure 4.8. Mean danazol plasma concentration vs. time profiles for HCO (A) and CO (B) formulations after oral administration of encapsulated pre-concentrate formulations to male beagle dogs. Digestible formulations comprised MCT and HCO 7 or CO5 (circles) and stealth formulations were composed of MCT and HCO 40 or CO35 (squares). Data were normalized to a 1 mg/kg danazol dose, [mean ± SEM (n=4)].

Table 4.3. Pharmacokinetic parameters for danazol after oral administration of digestible and stealth formulations with danazol incorporated at 80% saturated solubility [mean ± SEM (n=3)] to fasted male beagle dogs.

	Digestible Formulations		**Stealth Formulations**	
	HCO 7	CO 5	HCO 40	CO 35
$AUC_{0-\infty}$ (ng.h/mL)[a]	326.5 ± 128.3	335.9 ± 107.9	341.0 ± 61.8	196.2 ± 38.5
C_{max} (ng/mL)	81.1 ± 30.1	115.2 ± 46.4	132.1 ± 49.4	53.3 ± 10.7
T_{max} (h)	1.5 ± 0.2	1.4 ± 0.6	1.4 ± 0.4	1.9 ± 0.4
$t_{1/2}$ (h)	4.6 ± 0.7	5.6 ± 0.8	6.2 ± 0.2	6.9 ± 1.0
Bioavailability (%)	35.5 ± 14.0	36.6 ± 11.7	37.1 ± 6.7	21.4 ± 4.2

[a]Data normalised to a 1 mg/kg danazol dose to facilitate comparison across differing drug doses.

[b]Bioavailability calculated from IV Danazol study conducted in the same cohort as reported in [244].

The data were complicated by apparently lower exposure from two animals regardless of formulation. Figure 4.9 shows the individual plasma versus time profiles for each dog. For dog three in particular, irrespective of formulation (and drug) dose, the plasma response was low in comparison to all other animals (Fig 4.9C).

The lack of a stealth effect in dogs may be attributable to a combination of factors, not least physiological differences in the animals leading to differential metabolic, digestive and absorption rates [156, 244]. However, this particular cohort of animals had been dosed with danazol SEDDS in studies conducted previously in our group, with absolute bioavailabilties ranging from 18% to 33% [244]. The bioavailability of all formulations dosed in these studies ranged from 21% to 37% (table 3) providing some indication that marked differences in metabolism were not apparent.

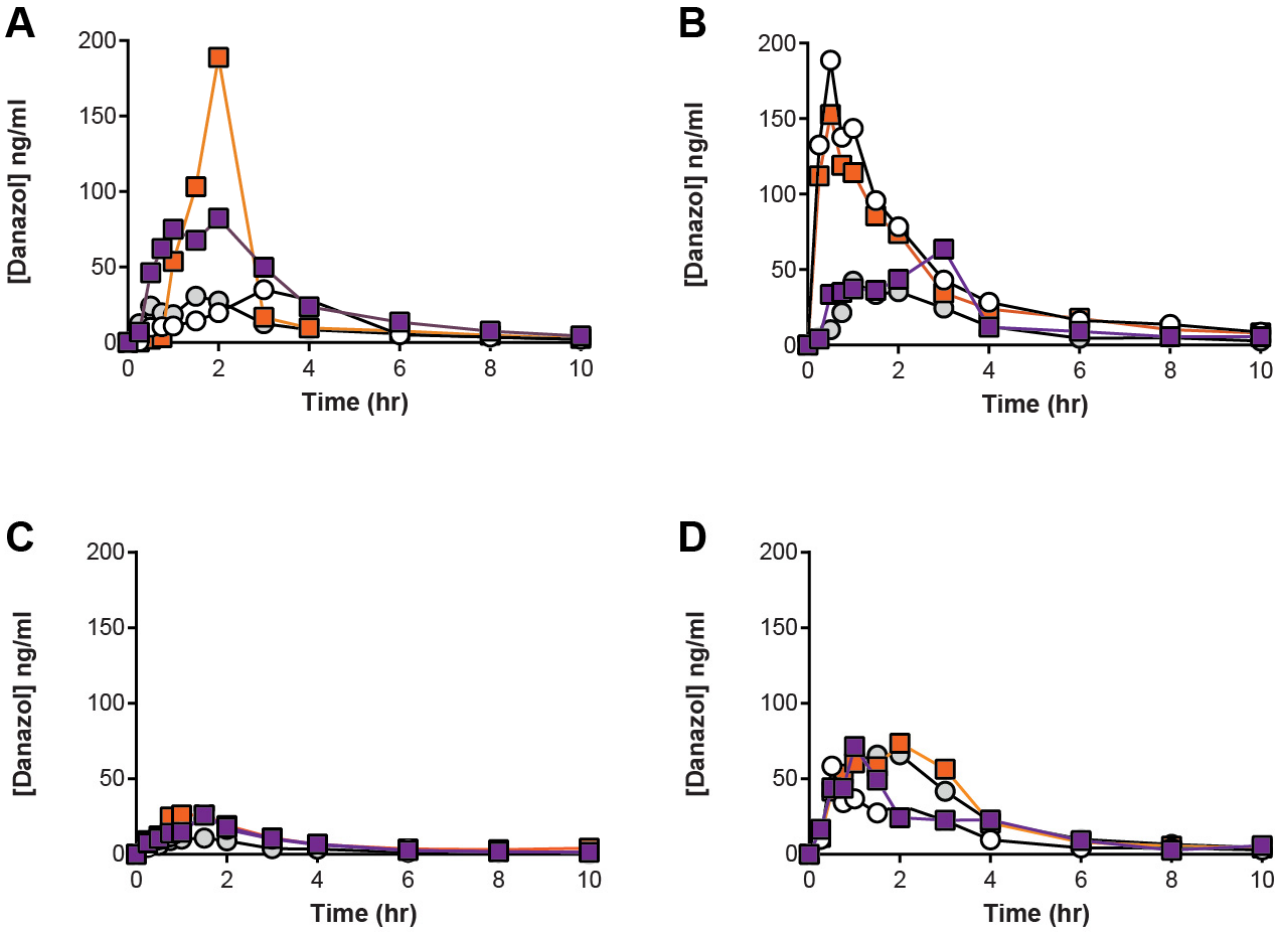


Figure 4.9. Individual danazol plasma concentration vs. time profiles for HCO7 (white circles), HCO40 (orange squares), CO5 (grey circles) and CO35 (purple squares) for individual animals. (A) dog 1 (B) dog 2, (C) dog 3 and (D) dog 4.

4.4.6. In vitro assessment of changes to stealth behaviour mediated by endogenous surface active species

Contrary to the data generated in rats, stealth LBFs did not outperform their digestible analogues after administration to dogs. The major difference between these studies was the requirement for the stealth formulations to self-assemble *in vivo* in the dog cohort. We hypothesise that from the time of capsule rupture to formulation self-emulsification, other surface active compounds may compete with the PEGylated surfactant for space at the interface, preventing adequate PEG coverage and facilitating enzyme access to the oil:water interface.

To further investigate how the stealth effect may be lost during *in vivo* self-assembly, a series of *in vitro* lipolysis experiments were carried out to evaluate the impact of endogenous surface active species (proteins, fatty acids, monoglycerides and bile salts) on formulation self-assembly and digestion modulation behaviour.

Figure 4.10 shows individual lipolysis profiles for the stealth HCO40 formulation and the same formulation after digestion in media containing 200 mg of each of the surface active moieties likely to be present in the stomach and/or upper small intestine of the dog during digestion. Figure 4.10B shows that the gelatin capsule itself can facilitate a greater extent of digestion (12%) relative to the stealth formulation (2%). Figure 4.10C also suggests that protein (BSA) adsorption to the emulsion interface in the stomach or intestinal lumen may also change digestibility, increasing digestion to a similar extent to that of gelatin (11%).

As the ingested formulation is self-emulsifying, some digestion of the triglyceride may occur in the stomach or upper GI tract. This in turn will release fatty acids and monoglycerides into the lumen which may also interact with the oil:water interface. To investigate this, medium chain fatty acid (octanoic acid, fig 4.10D) was added to the media and had a significant effect on the digestion of the formulation, with digestion increasing to approximately 40%. In the presence of the fatty acid, significant variability in the onset of digestion was observed, likely due to the presence of protonated and unprotonated fatty acid species in the lipolysis experiment. While back-titration accounts for some of this variability, the overall lipolysis rates were different. In a similar manner the presence of medium chain monoglycerides (figure 4.5C) increased the extent of digestion to 19%.

Inter subject variability in gallbladder secretion could also impact the self-assembly of stealth LBFs. For example, previous work in our group has shown that gallbladder bile concentrations in dogs may vary from 162 to 200 mM [244]. Digestion experiments were therefore performed in media containing 8 mM NaTDC (i.e. double the concentration of fasted lipolysis media) to examine the potential impact of increases in bile salt concentration. Figure 4.10E shows that the lipolysis rate increased with increasing bile salt concentration, with 21% of the available lipid digested at the end of the experimental period.

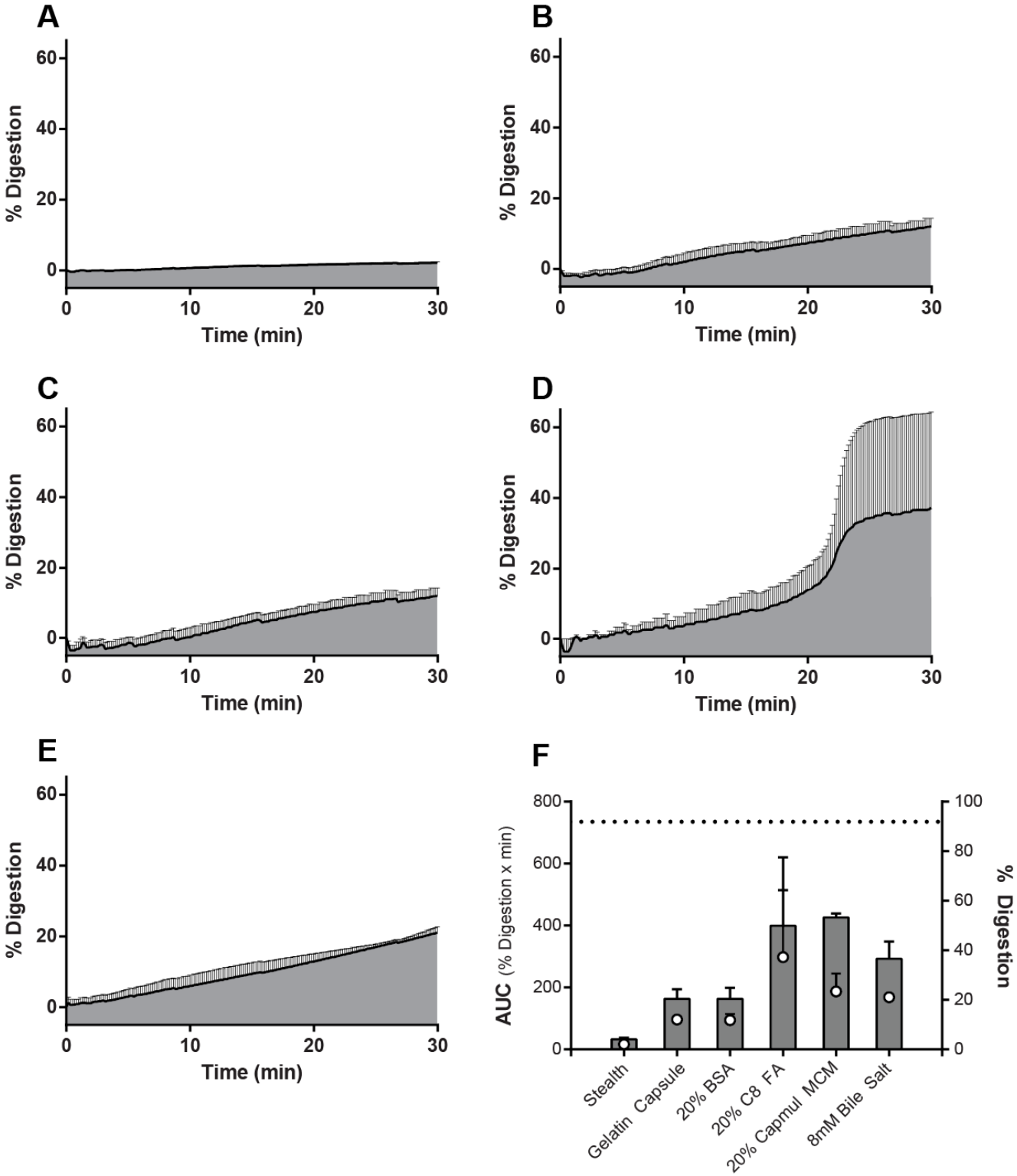


Figure 4.10. (A – E) *In vitro* lipolysis profiles for binary formulations of MCT and HCO40 in the presence of 20% (w/w) surface active species [mean ± SD (n = 3)]. Shaded areas represent the AUC calculated using the linear trapezoidal method used to plot the bar chart F. (A) HCO40:MCT Stealth formulation, (B) HCO 40:MCT + 200 mg gelatin (C) HCO40:MCT + 200 mg BSA (D) HCO40:MCT + 200 mg octanoic acid (E) HCO40:MCT + 8 mM NaTDC (F) Summarised lipolysis AUC (% digestion x min, bars, left Y axis) and extent of digestion data (dots, right Y axis). Dotted line, AUC of digestion of MCT in the absence of surfactant.

Taken together, these data show that proteins, lipolytic products and differences in endogenous bile salt concentrations can change the digestion behaviour of stealth LBFs. Figure 4.10F summarises the change in lipolysis for the stealth HCO formulation when dispersed and digested in the presence of gelatin, BSA, fatty acid, monoglyceride and increased bile salt concentrations. In all cases, however, the extent of MCT digestion was lower than that of MCT alone, indicating that the formulations retained some digestion inhibiting properties.

Formulation release from a capsule and self-emulsification in the canine stomach is likely to occur in the presence of lipases. This may negatively affect the stealth properties of the formulation if gastric lipase activity occurs during self-emulsification. To further investigate this, MCT digestion was initiated as described in section 2.3, ie. MCT alone was dispersed and digestion initiated by addition of pancreatic lipase. 2ml of HCO40 surfactant dispersed in lipolysis media was added to the media over 30 seconds (4ml/min) to allow some incorporation of lipase into the emulsified formulation.. This provides an indication of the potential impact of lipase access to the lipid interface prior to the assembly of an intact PEG barrier. Figure 4.11 shows how intercalation of the surfactant and lipid in the presence of lipase can result in complete loss of the stealth effect potentially hampering the performance of these formulations.

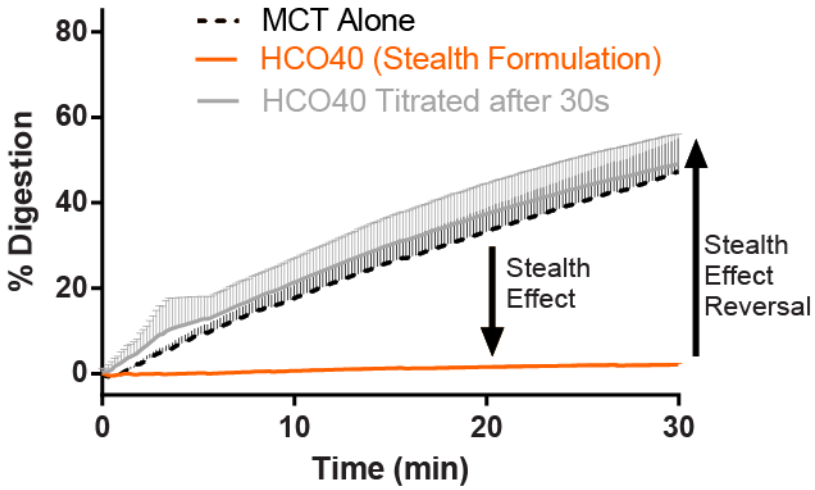


Figure 4.11. *In vitro* lipolysis profiles for MCT, MCT:HCO40 and MCT with HCO40 added after 30 seconds, [mean ± SD (n = 3)].

4.5. Discussion

Efforts to improve the oral absorption of poorly water-soluble drugs (PWSD) via the use of enabling formulation strategies are increasing as larger numbers of hydrophobic drugs emerge from drug development pipelines. These BCS class II drugs are typically hampered by slow dissolution from the crystalline form, low solubility in the GI fluids and even if drug can be forced into solution using a high energy dosage form, a propensity to precipitate in the gastrointestinal tract (GIT) [9].

Self-emulsifying drug delivery systems (SEDDS) are a well established means of improving bioavailability of BCS class II drugs [6]. These lipid-based formulations (LBFs) increase solubility and avoid traditional dissolution of PWSD in the GI lumen. LBFs also harness endogenous fat processing mechanisms to slingshot drug uptake by inducing supersaturation at the absorption site. This both increases absorption and often reduces the variability associated with food effects by facilitating fasted administration of lipophilic drugs [245, 246]. However, formulation performance is dependent on excipient composition and luminal processing. Dispersion, dilution and digestion of a LBF can result in a shift from stable solubilisation to significant losses in solubility, rapid supersaturation and drug precipitation. This is particularly true of medium chain triglyceride (MCT) based formulations, which are more rapidly digested than their long chain counterparts[168].

Recognising that drug supersaturation is a crucial driver for absorption [128], efforts have focused on developing formulation strategies to stabilise or prolong drug supersaturation after digestion while reducing the propensity for precipitation [128, 171, 247, 248]. Others have aimed to promote the generation of more soluble or amorphous forms of PWSDs . More recently, formulations have been developed to slow the digestion process, thereby delaying associated losses in formulation solubilisation and supersaturation and preventing precipitation of the drug prior to absorption[204].

Digestion is an interfacial reaction involving adsorption of lipases to the surface of an oil droplet and hydrolysis of triglyceride to monoglyceride and fatty acids. This yields lipolytic products with increased hydrophilicity that are more soluble in the GI fluids and more efficiently absorbed across the intestinal epithelium. The reduction in lipophilicity of the lipid components of the solubilised species in the GI fluids, however, is one of the contributing factors to losses in PWSD solubilisation capacity after digestion. By sterically shielding the interface of an oil droplet from lipase adsorption, the digestion process can be delayed, facilitating prolonged drug solubilisation *in vivo*. Polymer mediated steric hindrance of nonspecific protein adsorption in drug delivery has been termed ‘stealth’ and generally applies to parenteral nanoparticulate formulations with a hydrophilic, motile poly(ethylene glycol) (PEG) surface coating.

Stealth nanoparticles have longer plasma circulation times after parenteral administration than their uncoated counterparts due to their ability to decrease adsorption of plasma proteins and avoid clearance by the immune system. 'Stealth' LBFs apply a similar concept but utilise the polyethylene glycol headgroup of commonly used non-ionic surfactants to protect the triglyceride from digestion by lipases through a reduction in adsorption to the lipid droplet.

The current studies sought to further explore the robustness and utility of stealth LBFs for the administration of PWSD. Firstly we aimed to investigate whether the stealth effect could be beneficial to drug absorption from long chain triglyceride (LCT) formulations. Secondly, since previous studies suggested that typical SEDDS formulations did not show similar stealth behaviour to simple lipid-surfactant binary mixtures, we explored whether other SEDDS excipients, such as monoglycerides and cosolvents interfered with the stealth capabilities of these formulations. Finally these studies also aimed to establish whether stealth LBFs could efficiently self-assemble *in vivo* where there are likely to be other surface active species available for adsorption to the oil:water interface.

4.5.1. PEGylated surfactants as modulators of lipid digestion and drug absorption

4.5.1.1. The stealth effect is broadly applicable to both long and medium chain triglycerides

Previous studies from our group have shown that non-ionic surfactants inhibit the digestion of MCT in a PEG Mw dependent manner irrespective of the structure of the surfactant used [204]. This behaviour directly mirrors the reduced protein adsorption behaviour of stealth parenteral formulations [223]. However, the impact of systematically increasing surfactant PEG Mw on digestion of LCT was not explored. More recent data from the digestion of preformed sunflower oil emulsions with two non-ionic surfactants of differing PEG Mw by Wulff-Perez and coworkers provided some preliminary information on a Mw dependency of LCT digestion inhibition [201], however broader molecular weight ranges were not explored and the practical utility of this inhibition was not investigated.

The current studies therefore aimed to further expand the investigation of the impact of different surfactants on LCT digestion and the potential utility of reducing LCT digestion on drug absorption. Figure 4.1 A-G shows LCT digestion changes when co-formulated with hydrogenated castor oil surfactants of increasing PEG molecular weight. Plotting the lipolysis AUC (% digestion.min) for each formulation against PEG Mw (Fig 4.2A) reveals a similar parabolic relationship to that previously observed for MCT (Figure 4.2B). The most 'stealth' formulation i.e. the formulation with lowest rate and extent of digestion was evident with a surfactant with a PEG Mw of 1800 $g.mol^{-1}$, ie a PEG headgroup size of 40, directly mirroring the data generated for MCT. Interestingly, the magnitude of the stealth effect was lower for LCT than MCT i.e. the fold reduction in AUC of digestion for LCT was lower (5-fold) than that of MCT (24-fold).

This can be attributed in part to lower digestion rates for LCT versus MCT [168, 243]. In these studies, the extent of digestion of LCT (figure 4.1A) was approximately half that of MCT under the same lipolytic conditions [204]. To further investigate the utility of stealth LCT formulations we examined both *in vitro* drug solubilisation patterns and *in vivo* drug absorption for two LCT LBFs (HCO7:LCT - digestible and HCO40:LCT – stealth). The formulations were loaded with danazol at 80% saturated solubility. Figure 4.3A shows that a digestible LCT formulation (HCO7:LCT) resulted in some danazol precipitation as digestion progressed in a rat model of lipolysis, whereas the same formulation with a surfactant PEG Mw of 1800 $g.mol^{-1}$ (HCO40:LCT) significantly reduced digestion and this was reflected in increased drug solubilisation during the lipolysis experiment (figure 4.3B). After administration of the predispersed lipid formulations to rats, the plasma profiles in figure 4.4A and the pharmacokinetic parameters in table 4.2 show that the stealth LCT formulation outperformed its digestible counterpart. The previous data for MCT formulations is included to aid comparison. The trends between LCT and MCT were similar with the stealth formulations increasing relative bioavailability by 120% and 183% for MCT and LCT respectively.

These studies established that the applicability of the stealth effect can be broadened to both long and medium chain triglyceride formulations, with predispersed stealth formulations conferring an *in vivo* advantage over digestible formulations.

4.5.1.2. The stealth effect is attenuated in the presence of other surface active excipients

In vitro digestion inhibition by individual surfactants has previously been reported in both simple binary systems and in emulsions designed to probe the kinetics of lipase activity [11, 59, 180, 200, 201, 204, 222, 249]. However, this phenomenon is not widely observed in self-emulsifying LBFs ie SEDDS. One of the fundamental differences between common SEDDS formulation and the stealth LBFs described herein is the inclusion of additional amphiphilic species (monoglycerides and cosolvents) in SEDDS to aid rapid self-emulsification. The presence of these surface active excipients is likely to increase competition at the oil:water interface and may reduce the digestion inhibition capability of the stealth formulations. To probe this, 1g of HCO:40 MCT formulation was prepared with 20% (200 mg) of either Capmul MCM (medium chain monoglycerides) or 20% Transcutol, PEG400 or ethanol (common SEDDS cosolvents). These formulations were then dispersed and digested as previously described [204]. Figures 4.5A and 4.5B show the lipolysis profiles of MCT alone and in the presence of HCO40. The stealth effect was evident when only lipid and surfactant were present in the formulation. However on addition of amphiphilic or surface disrupting excipients such as monoglycerides and cosolvents, the stealth effect was attenuated (Figure 4.5C) or completely lost (figure 4.5D-F). Figure 4.6 more clearly shows the significant impact that cosolvents in particular can have on lipolysis rates. Recently, Zeeb and coworkers demonstrated that alcohols can disrupt protein stabilised nanoemulsions, reducing surface tension and particle size. Above concentrations of 10% the alcohols had a destabilising effect on the surface coating proteins inducing flocculation [250].

Ethanol has also been reported to permeabilise and cross lipid bilayers in molecular dynamics studies [251], while both propylene glycol and ethanol have been reported to act as cosurfactants at the surface of lyotropic liquid crystalline interfaces [252].

Realising that many SEDDS contain cosolvents, it is likely that the digestion inhibition phenomenon described here is not observed for many SEDDS formulations as the continuous PEGylated surface of the emulsifying droplet is likely to be disrupted by the presence of cosolvents at the interface.

4.5.2. In vivo self-assembly of stealth LBFs poses a significant challenge to utility

Stealth IV formulations are typically administered as a preformed PEGylated carrier [253]. While some systems involve multiple complex processes for formulation and encapsulation of the drug, others are formed by self-assembly to simplify preparation and drug encapsulation [254]. However, due to the nature of the administration route and the need for solution or solubilised formulations, self-assembly always occurs before intravenous administration. In contrast, for oral LBF, whether formulations are predispersed prior to administration or whether self emulsification happens in situ *in vivo*, is largely determined by animal model. Thus pre-dispersed formulations are often used in rodents to facilitate gavage, whereas in larger pre-clinical species (such as dogs) and in humans, a preconcentrate comprising drug dissolved in non-aqueous formulation components is typically filled into soft or sealed hard gelatin capsules. These encapsulated formulations then release the contents on capsule rupture *in vivo* and the colloidal dispersion self assembles in situ. This *in vivo* assembly/disassembly paradigm is unique to oral LBFs where efficient self-assembly and resistance to degradation in the GIT must be balanced with the ability to release drug at the enterocyte to facilitate absorption.

4.5.2.1. In vivo self-assembled stealth LBFs do not confer an advantage over digestible formulations

To investigate the *in vivo* utility of stealth LBFs after filling into capsules, digestible and stealth formulations were first evaluated *in vitro* using lipolysis conditions reflecting dog GI conditions. Formulations were then administered as the encapsulated preconcentrate to male beagle dogs. Figure 4.7 depicts the *in vitro* drug solubilisation patterns and lipolysis profiles for four MCT formulations in the presence of HCO and CO surfactants. The formulations that digested rapidly (figure 4.7A and 4.7C) resulted in a concomitant drop in drug solubilisation and high maximal supersaturation whereas the formulations that inhibited digestion (figure 4.7B and 4.7D) did not result in extensive drug precipitation. These data suggested that under the higher enzyme conditions more reflective of the dog GI tract, stealth formulations continued to outperform their digestible counterparts.

Despite the clear differences in formulation performance *in vitro,* however, the plasma versus time profiles in figure 4.8 and the pharmacokinetic data in table three show that there was no advantage to drug exposure after administration in a stealth formulation. Three of the administered formulations had similar

bioavailability (36 – 37%) while the stealth castor oil (CO) based formulation was the poorest performer *in vivo* (21% BA).

This data is in stark contrast to the *in vivo* data obtained from the same predispersed formulations in rats [204], although the individual plasma versus time profiles in the dogs varied significantly (figure 4.9), with low plasma drug concentrations from dog 3 in particular. Danazol is a CYP substrate and differences in individual metabolic activity may have confounded data interpretation for this study where suicide inhibition of CYPs (as employed in the rat studies) was not as feasible in dogs. All studies in rats were conducted after CYP inhibition with 1-ABT as data from our group has previously reported the significant impact of danazol metabolism on oral exposure in rats [156]. Variability in GI bile salt concentrations in dogs may also impact the stealth behaviour of these formulations and was therefore examined in a lipolysis experiment where the bile salt concentration was increased to 8 mM NaTDC, double that typically used in fasted media. Figure 4.10E and F shows that increased bile salt concentration did indeed increase the rate and extent of stealth formulation digestion, indicating that individual differences in gallbladder secretion could potetially have a detrimental impact on the stealth behaviour of these formulations.

4.5.3. Competitive processes at the oil:water interface during in vivo self-assembly of stealth LBFs likely hinder formulation performance

The poor relative performance of the stealth formulations *in vivo* may also be attributable to the potential for lipolysis to occur prior to effective self-assembly of the PEG-protected interface. Gastric lipases are present in the stomach, although activity is pH dependent [154, 255]. The gastric pH of dogs can vary significantly and in situations where the fasted gastric pH is higher, digestion in the stomach may be significant [256, 257]. The presence of gelatin in the lipolysis media may also impact on lipolysis if the gelatin adsorbs at the interface. Similarly, amphiphilic proteins that present in the stomach may adsorb to the surface of the oil droplet, since proteins have been used to stabilise food emulsions [250]. Initiation of digestion in the stomach will also generate fatty acids and these too might be expected to impact interfacial properties.

In order to approximate the impact of gastric lipolysis and the generation/presence of amphiphilic species on the *in vitro* self-assembly behaviour of MCT formulations, the HCO40:MCT formulation was subjected to lipolysis experiments in the presence and absence of these surface active species. However in these experiments, the amphiphiles were included in the lipolysis media rather than in the formulation. Figure 4.10 shows the lipolysis profile for the stealth formulation HCO:40 and the same formulation in the presence of gelatin (figure 4.10B), bovine serum albumin (figure 4.10C) and fatty acid (Figure 4.10D), all of which likely to be present in the stomach during self-assembly. The addition of all species to the digestion media attenuated the stealth effect and increased the overall rate and extent of hydrolysis. Despite this, however, lipolysis rates were below that of MCT alone indicating that the system maintained some digestion inhibition capability.

To further challenge the importance of the kinetics of self-assembly of stealth LBFS, a MCT formulation was initially emulsified in the presence of lipase (but absence of surfactant) and surfactant added shortly thereafter. To achieve this, a lipolysis experiment was conducted where lipase was added to MCT and surfactant titrated into the vessel over 30 seconds. This study aimed to evaluate how digestion of the triglyceride during self assembly/self emulsification of the lipid and surfactant impacted on digestion rates. Figure 4.11 shows that the stealth effect was completely reversed when surfactant was added in the 30 seconds after initiation of digestion. These data show that the surfactant is unlikely to inhibit lipase activity directly but rather forms a steric barrier between the oil:water interface and the lipolytic enzyme that needs to be pre-formed prior to enzyme challenge. Interestingly, the lipolysis rate matched that of MCT in the absence of surfactant, indicating that the delayed addition of HCO40 did not further increase lipolysis rates.

4.6. Conclusions

The current studies have examined in detail the applicability and utility of stealth lipid-based formulations for oral administration of danazol, a poorly water-soluble drug. The data suggest that the stealth effect i.e. PEG mediated reduction in protein (lipase) adsorption is evident for long and medium chain triglycerides coformulated with non-ionic surfactants. These formulations exhibit reduced lipid digestion in a surfactant PEG Mw dependent manner. Furthermore, these data also show that stealth formulations are better able to maintain drug solubilisation *in vitro* and improve drug absorption *in vivo* after administration of predispersed formulations to rats.

However the data also show that the stealth effect is sensitive to competition at the oil:water interface with the presence of amphiphilic excipients such as monoglycerides and cosolvents reversing the digestion inhibition effect. The challenges associated with *in vivo* self-assembly of these stealth formulations was also evidenced by a reduction in stealth behaviour when examined *in vitro* in the presence of amphiphilic species likely to be found *in vivo* and after oral administration in capsule form to beagle dogs where self emulsification occurs *in situ*. Together these data suggest that a pre-formed stealth barrier can improve oral drug absorption from digestible lipid-based formulations, however, initiation of digestion prior to completion of self emulsification (and formation of the steric barrier) *in vivo*, as is expected for an encapsulated pre-concentrate, may limit practical application.

Chapter 5. Eliminating digestion; the potential utility of non-digestible lipid-based formulations for oral drug delivery.

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5.1. Abstract

Poorly water-soluble drugs are often incorporated into lipid-based drug delivery systems, facilitating oral delivery of compounds with otherwise unfavourable physicochemical characteristics. In recent years, significant efforts have focused on better methods for evaluation of triglyceride-based formulation digestion, drug supersaturation and subsequent correlations to drug exposure. However, to date, there has been a lack of clear correlation between formulation performance during *in vitro* digestion testing and drug absorption *in vivo*. In these studies, we sought to examine whether other factors may influence drug absorption when the digestion rate and drug supersaturation were deliberately suppresed. We developed a series of non-digestible self microemulsifying formulations where lipid (and formulation) composition was tightly controlled in order to evaluate the impact of the co-formulated lipid on drug absorption. Six microemulsifying non-digestible lipid-based formulations based on oleic acid or structural analogues were evaluated for digestibility and *in vivo* drug absorption. None of the formulations developed were digestible, and all showed sustained drug solubilisation and low levels of supersaturation *in vitro*. When administered *in vivo* however, the formulations performed differently with formulations encompassing a high LogD lipid facilitating the greatest drug absorption.

5.2. Introduction

Oral drug delivery remains a preferred route of administration for patients as it is minimally invasive, easy to implement and in most cases, cost effective[1, 6]. For this reason, significant efforts have been devoted to the development of lipid-based formulations (LBF) to enable oral delivery of poorly water-soluble drugs (PWSD) emerging from drug development pipelines[7].

Traditionally, LBF development has used oils based on dietary lipids (tri- di- and monoglycerides of long or medium chain length)[258]. These lipids (and some surfactants) are substrates for digestive enzymes which hydrolyse ester bonds. Dispersion and digestion of these formulation components can alter drug solubilisation capacity as the formulation transits the gastrointestinal tract, in turn leading to drug supersaturation and if uncontrolled, precipitation[1, 128, 161, 259]. To account for this, concerted efforts have been made to develop consistent methodologies for formulation assessment *in vitro*, that have correlative power to *in vivo* formulation performance[161,150, 260]. In some cases IVIVC is possible, but in general, is largely dependent on the drug and formulation studied[1, 261].

In a recent review, Amara and colleagues raised the important question of how *in vitro* lipolysis methodologies represent patients with pathological enzyme insufficiencies (for example, Cystic Fibrosis, Pancreatitis and Hepatobiliary Cancer patients)[258]. In these populations, reliance on endogenous lipid processing to facilitate drug absorption is unlikely to deliver sufficient drug to the absorptive membrane, requiring a reversion to parenteral administration. In light of this, and the lack of correlative power for many current triglyceride based LBF formulations, we sought to develop non-digestible lipid-based formulations (ND-LBF) where formulation digestion and the consequent variability in drug solubilisation are no longer influencing factors in formulation performance. These systems may allow better examination of the mechanisms by which lipids influence drug absorption, and may also provide a drug delivery system that is less reliant on lipase activation and that therefore can deliver PWSD to patients with pancreatic insufficiencies.

Historically, non-digestible lipid formulations have been developed with poorly dispersible sucrose polyesters or mineral oils and typically perform poorly when compared to other oils or triglycerides[121, 262]. This is thought to reflect the fact that e.g. mineral oil is composed of non-absorbable (very) long chain (C40) alkanes that are poorly dispersible. These large lipid droplets (with associated low surface area per mass) likely act as a competitive sink/reservoir for PWSD in the GI lumen, restricting the release of free drug for absorption[263].

More recently, Holm and colleagues also investigated the utility of a novel non-digestible lipid-like excipient, a semi-fluorinated alkane (1-perflurohexyloctane, F6H8) as a component of medium-chain SEDDS[206]. The authors found that F6H8 was poorly dispersible, and that addition of surfactant to improve formulation dispersion had a limited impact on bioavailability. In these studies, medium chain triglycerides comprised

the lipid component of the formulation and the impact of F6H8 alone as a lipid replacement (rather than in combination with medium chain triglyceride) was not explored.

The current studies were therefore undertaken to investigate the potential for non-digestible, but absorbable, lipids to replace triglycerides in LBFs. We hypothesised that absorbable lipids were require to promote supersaturation as previous studies suggest that lipid absorption is a key driver of supersaturation and therefore thermodynamic activity for lipid formulations[69]. Formulations were also designed to form nanoemulsions on dispersion since further processing to generate the fine colloidal species typically generated during lipid digestion, would not occur.

Firstly we developed formulations that spontaneously formed nanoemulsions on dispersion by generating a LFCS Type III LBF with high surfactant load (66%). For this reason, the 'lipids" used had to have adequate solubilisation capacity for the model drug. Cinnarizine was chosen as it has high affinity for oleic acid and moderate solubility in other lipids. The lipid component of the formulation was then systematically replaced with an oleyl alcohol, oleyl amine, and 1-octadecene to investigate how the physicochemical properties of the lipid influence formulation dispersion and drug solubilisation.

Secondly, we aimed to investigate whether these formulations were indeed non-digestible and whether they were able to maintain drug solubilisation during an *in vitro* digestion challenge. Formulation digestion was assessed using *in vitro* lipolysis and the aqueous phase from the digestion experiments used to generate equilibrium solubility values to enable assessment of drug supersaturation during the dynamic digestion tests.

Finally, an *in vivo* study was conducted to evaluate formulation performance after intraduodenal administration of dispersed LBF to rats. As the formulations were controlled for composition, size, and initial supersaturation, we hypothesised that differences in drug uptake would reflect the physicochemical parameters (and subsequent absorption profile) of the incorporated non-digestible lipid.

5.3. Methods

5.3.1. Materials and reagents

Transcutol was a gift from Gattefossé (St Priest, France). Croduret™ 7 and 40 (PEGylated hydrogenated castor oils – abbreviated to HCO), Brij® O3 and O20 (PEGylated oleyl ethers) and Synperonic® L101 were kindly donated by Croda International PLC (Yorkshire, England). Cinnarizine (1-trans-Cinnamyl-4-diphenylmethylpiperazine), oleic acid (OA), oleyl alcohol (O-OH), 1-octadecene (O-ENE), oleylamine (O-NH2), porcine pancreatin (8 x USP specification activity), 4-bromophenylboronic acid (4-BPB) and diazepam (7-Chloro-1-methyl-5-phenyl-3H-1,4-benzodiazepin-2(1H)-one) were supplied by 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). Sodium hydroxide 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. Hypergrade solvents were used for UPLCMS/MS analysis.

5.3.2. Formulation preparation

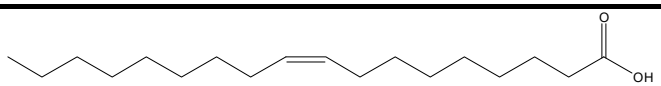
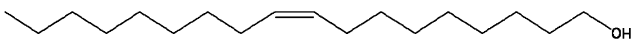
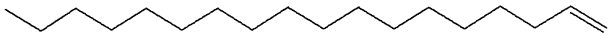
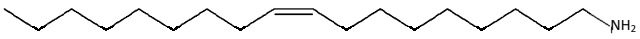
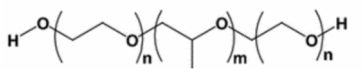
5.3.2.1. Blank formulations

Self emulsifying ND-LBFs were prepared from fatty acids or structural analogues (oleic acid, alcohol, alkene, amine and the low HLB surfactant synperonic 101). Formulations were prepared in batches using prepared surfactant blends and allowed to equilibrate overnight at 37^{o}C before use. All formulations were prepared in a 27:66:7 w/w ratio of lipid, surfactant blend and cosolvent (Transcutol). The surfactant blend was either a non-digestible ether based surfactant blend, Brij 03: Brij 020 in a 35:65 w/w ratio (Brij-NDSEDDs) or a partially digestible hydrogenated castor oil surfactant blend (HCO7:HCO40 in a 35:65 w/w ratio). Optimised formulations contained 27% (w/w) lipid, 66% surfactant blend and 7% cosolvent. The Synperonic formulation formed a gel that was poorly dispersed and could not be accurately sampled during *in vitro* testing. As a result, this formulation was not examined further. The oleylamine formulation stayed in liquid form for two days but phase separated after approximately three days and could not be redissolved. This formulation was also not used in further experiments.

All formulations were prepared at 37 °C, mixed and equilibrated overnight before use. The formulation composition, equilibrium solubility and details of lipids used are summarised in Table 5.1

Table 5.1: Details of the non-digestible LBFs tested

Formulation Components (% w/w)	Formulation Lipid				
	Oleic Acid	Oleyl Alcohol	Oleylamine	1-Octadecene	Synperonic L101
Lipid	27	27	27	27	27
Surfactant Blend (HCO7:HCO40, 35:65) **or** (BrijO3:BrijO20, 35:65)	66	66	66	66	66
Transcutol	7	7	7	7	7
Drug solubility (mg/g) Brij formulations HCO formulations	 84.82 ± 4.1 85.89 ± 1.7	 31.02 ± 1.7 29.34 ± 1.3	 28.66 ± 2.0 26.37 ± 0.9	 31.26 ± 1.1 25.45 ± 1.4	 29.00 ± 1.6 25.10 ± 1.1
LFCS Formulation Type[32, 148]	IIIb	IIIb	IIIb	IIIb	IIIb
Particle Size	17.7 ±2.1	23.2 ±1.8	Crystalline	35.3 ±6.4	Formed Gel

Lipid(s)	Structure	LogP	LogD (pH 5.5)
Oleic Acid		7.70[a] (assumed protonated)	6.57[a]
Oleyl alcohol		7.80[a]	7.47[a]
1-octadecene		9.81[a]	9.12[a]
Oleylamine		7.85[a]	3.94[a]
Synperonic L101	 n = 4, m = 59	-1.52[a]	-1.01[a]

[a]Predicted value, ACD/Labs retrieved from Royal Society of Chemistry Chemspider database

5.3.2.2. Drug loaded formulations

Cinnarizine was chosen as the model drug for these experiments as it exhibits high affinity for oleic acid and is soluble to an adequate extent in the examined formulations. The equilibrium solubility of cinnarizine in each of LBFs was determined as previously described. Equilibrium solubility was typically reached by 48 hours. Cinnarizine containing formulations were prepared at a loading of 80% saturated solubility (based on measured values at 37 °C), drug content was confirmed by HPLC assay prior to formulation use.

5.3.3. In vitro dispersion and digestion of formulations

5.3.3.1. Digestion conditions

In vitro dispersion and digestion were conducted using a rat model of *in vitro* lipolysis as previously described[156, 204]. Lipolysis experiments were preceded by a dispersion step (10min) with digestion monitored at pH 6.5 for 60 min. Sodium hydroxide titration volume was recorded using Tiamo 2.0 software.

In vitro drug solubilisation (digestion) experiments were carried out on drug loaded formulations as previously described[156, 204]. Blank (drug free) formulations were also dispersed and digested to obtain

aqueous colloidal phases for determination of cinnarizine equilibrium solubility and were then used to calculate maximum supersaturation ratios S^M as described by Anby *et al*[128].

Graphpad Prism for windows (Version 6.0, Graphpad Software Inc., CA, USA) was used to statistically analyse differences in pairs of *in vitro* data using unpaired parametric t-tests. Data were expressed as the mean (n=3) ± standard deviation (SD). A difference was considered statistically significant when $p \leq 0.05$.

5.3.4. Cinnarizine assay in formulations and dispersion/digestion samples

Cinnarizine HPLC analyses were conducted using a Water Alliance 2795 Separation Module and a Waters 2489 UV/Visible Detector, (Waters Alliance Instruments, Milford, Massachusetts, US) using an Agilent Eclipse XDB 3.5 µm C18 column (4.6 X 50mm) with a C18 security guard cartridge (4 × 2.0 mm, Phenomenex, Torrance, CA) at 40 °C and mobile phase (50%(v/v) acetonitrile and 50% 20 mM ammonium dihydrogen phosphate buffer (NH4H2PO4, pH 4.2) in Milli-Q water.

5.3.5. Particle Size Determination

Photon correlation spectroscopy (Malvern Nano-ZS zetasizer, Malvern Instruments, Worcestershire, U.K.) was used to determine the average particle size (average particle diameter in nm based on light scattering by volume), the % of each size population in the sample and the polydispersity index (PDI) of the self-emulsified LBFs. Briefly; 0.1g formulation was dispersed in a total of 3.6ml digestion media. Stirred at 37 °C for 15min and maintained at 37*°C* throughout particle size analysis.

5.3.6. Oral bioavailability studies in Sprague dawley rats

5.3.6.1. Surgical and experimental procedures

All surgical and experimental procedures were approved by the Monash Institute of Pharmaceutical Sciences Animal Ethics Committee and were conducted in accordance with 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 fasted male Sprague Dawley rats (260 – 330g) maintained on a 12 h light/dark cycle. Rats were anaesthetised via inhalation of isoflurane (5% v/v induction, 2.5% v/v maintenance; Abbott Laboratories, NSW, Australia) for the duration of the surgical procedure. A cannula (polyethylene tubing of 0.96 mm o.d. x 0.58 mm i.d.) was inserted into the right carotid artery to allow serial blood collection. A second cannula was inserted into the duodenum to facilitate formulation dosing. Cannulae were exteriorised to the back of the neck and were connected to a swivel tether system. Rats were transferred to individual metabolic cages and allowed to recover overnight prior to dosing. Rats were also fasted for at least 12 h prior to and 8 h post dose. Drinking water was provided ad libitum.

5.3.6.2. Formulation administration and sample collection

30 mg of dispersed LBF in water was administered by intraduodenal infusion to conscious male SD rats (1.5 ml/h for 2h). Blood samples were collected via an indwelling carotid cannula. Plasma samples were analysed on a validated UPLC-MS/MS method and PK parameters calculated using Phoenix WinNonlin.

5.3.7. Quantitation of Cinnarizine in plasma samples by UPLC-MS/MS

All plasma analyses were performed using a validated method on a Waters Xevo TQS UPLC MS/MS system (Waters, Milford, MA, USA) using a Supelco Ascentis Express RP Amide 2.7μm, (50 x 2.1 mm) with a C18 security guard cartridge (4 × 2.0 mm, Phenomenex, Torrance, CA) at 40°C and mobile phase (A Milli-Q water with 0.05% Formic acid and B Acetonitrile and 0.05% formic acid. The injection volume was 2 μL. Gradient elution at a constant flow rate of 0.5 mL/min was performed as follows: 41% B linearly increasing to 80% B in 2.5min. Under these conditions the retention times of cinnarizine and internal standard (IS) diazepam were 2.3 and 2.6 min, respectively. Elution was immediately succeeded by a 1.5 min wash step. Total run time was 4 min including the wash step.

The MS/MS conditions were optimised as follows: source temperature, 150 ^{o}C; desolvation temperature, 200 ^{o}C; cone gas flow, 150 L/h, desolvation gas flow, 900 L/h; collision gas flow, 0.16 mL/min; cone voltage, 6 kV; capillary voltage, 0.89 kV; collision energy, 18 kV. Data acquisition and peak integration were performed using MassLynx software, version 4.1 (Waters).

Unknown plasma concentrations were determined by interpolation from a weighted (1/X) calibration curve of Cinnarizine:IS peak response plotted as a function of cinnarizine concentration. Assay validation was conducted by multi-day matrix analysis of validation samples (n = 6 per group) of 2, 20, and 200 ng/mL cinnarizine in blank plasma with IS. System precision, % RSD (n = 5) 4.2 %; interassay precision, % RSD (n=18) 14.5%; intermediate precision, % RSD (n = 18) 15.2%; accuracy, (n = 24) 83.3 – 131.4%; recovery, (n = 6) 84%; linearity, R^2 0.995 – 0.999; specificity, no interfering peaks in blank plasma extracts. The limit of quantitation was arbitrarily set at 2 ng/mL; the lowest concentration of the validated concentration range.

5.3.8. Pharmacokinetic data analysis

The data were plotted as cinnarizine concentration (ng/mL) vs. time (h) (normalised to a nominal dose of 10 mg/kg cinnarizine). Non-compartmental pharmacokinetic parameters were calculated using Phoenix™ 64 Software (WinNonlin® version 6.3, Pharsight Corporation, CA, USA). Graphpad Prism for windows (Version 6.0, Graphpad Software Inc., CA, USA) was used to statistically analyse differences in pairs of *in vivo* data using unpaired parametric t-tests. All data represented are expressed as the mean ± standard error of mean (SEM). A difference was considered statistically significant when $p \leq 0.05$.

5.4. Results:

5.4.1. Non-digestible LBF development, impact of formulation components on particle size and drug solubility

To determine the utility of non-digestible lipid-based formulations (ND-LBF) for oral drug delivery, two formulation series were developed using oleic acid (O-COOH) or structrual analogues (oleyl alcohol (O-OH) and 1-octadecene (O-ene) as the non-digestible "lipid" component. To these lipids, a cosolvent (transcutol) was added as well as either a partially digestible surfactant mixture (PEGylated hydrogenated castor oils, HCO) or non-digestible surfactant mixture (PEGylated oleyl ethers, Brij) . These six rapidly self-emulsifying LFCS type III formulations[1] were dispersed in digestion media and the particle sizes measured. These data are reported in figure 5.1 (A) show that the Brij formulations formed systems smaller than 25nm, indicating a likely predominance of micellar species. Similarly, the HCO based formulations formed fine dispersions with both the oleic acid and 1-octadecene systems, figure 5.1 (B). The HCO oleyl alcohol formulation formed a larger dispersion of approximately 50 nm in diameter which may be due to the presence of larger vesicular species in the dispersed formulation. In all cases, however, formulations formed reasonably monodisperse nanoemulsions i.e. emulsions with a single size population (PDI less than 0.5) with average particle sizes less than 100 nm.

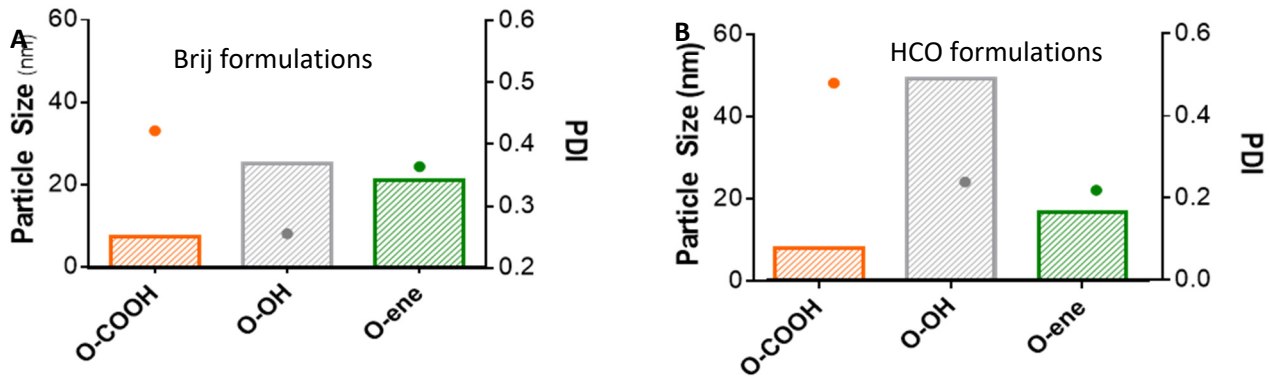


Figure 5.1. Dispersed particle sizes of (A) Brij® based ND-LBF and (B) HCO-based ND-LBF. Particle size is plotted on the left Y axis (Bars) while the polydispersity index (PDI) is plotted on the right Y axis, Circles. (Data represented as mean n = 2).

Cinnarizine is highly soluble in oleic acid[264], however it was unknown how cinnarizine solubility would be affected by formulation in non-digestible structural analogues of oleic acid, namely; oleyl alcohol and 1-octadecene. Figure 5.2 shows the equilibrium solubility of cinnarizine in lipid alone, confirming high affinity for oleic acid (greater than 200 mg/g) whereas the solubility of cinnarizine in both oleyl alcohol and 1-octadecene was ~10-fold lower. In the assembled formulation (containing lipids, surfactants and cosolvent) drug solubility dropped to 85 mg/g for the oleic acid Brij and HCO formulations, and increased marginally to approximately 30 mg/g for the oleyl alcohol and 1-octadecene formulations.

With the exception of oleic acid formulations, where cinnarizine shows clear affinity for the charged acid, drug solubility in each formulation series was similar to that in the non-digestible lipid alone, indicating that the drug solubility was predominantly attributable to the lipid in the formulation

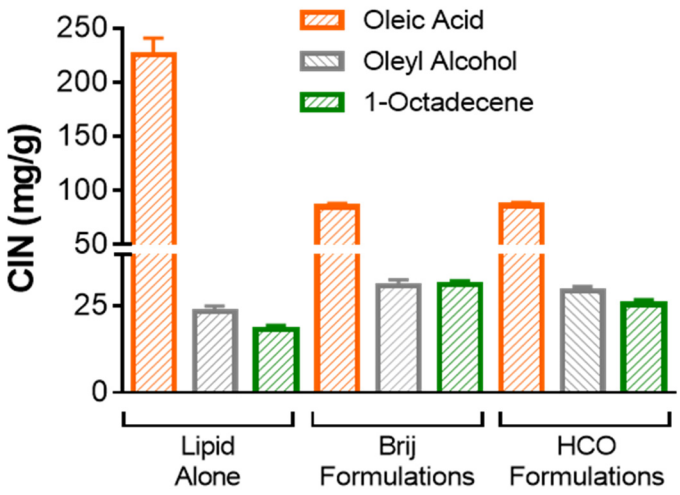


Figure 5.2. Solubility of cinnarizine in the non-digestible lipids (lipid alone) and the ND-LBF comprised of Brij surfactants and HCO surfactants. (Data represented as mean n = 3 ±SD)

5.4.2. Impact of formulation digestibility on drug solubilisation capacity and supersaturation in vitro

Having established that ND-LBF can form nanoemulsions and can adequately solubilise cinnarizine, dispersion and digestion experiments were conducted based on the rat *in vitro* digestion model[156, 204]. Formulations were loaded with cinnarizine at 80% saturated solubility. Figure 5.3 shows overlaid drug solubilisation and equilibrium solubility profiles for all six formulations. Under rat lipolysis conditions (where lipase activity is lower), there was no detectable titration of free fatty acids for any formulation, indicating that the formulations were non-digestible or that the digestion of HCO was not sufficient to be detected under the lipolysis conditions tested. The drop in solubilisation at five minutes is probably attributable to the addition of the porcine pancreatin mixture altering the physicochemical properties of the nanoemulsions rather than digestion (since no titration of NaOH occurred). In general, however, in all cases, drug solubilisation was maintained throughout dispersion (-10 to 0 min) and digestion (0 to 60 min). Interestingly, all formulations supersaturated on dilution, with maximal supersaturation ratios (SM) of 2, and this was maintained throughout the dispersion and digestion periods. Importantly, the supersaturation behaviour of all formulations was below the reported threshold for drug precipitation[128, 265], and all formulations were supersaturated to a similar extent, ensuring that differences in initial thermodynamic activity were unlikely to be a factor in any observed differences in absorption *in vivo*.

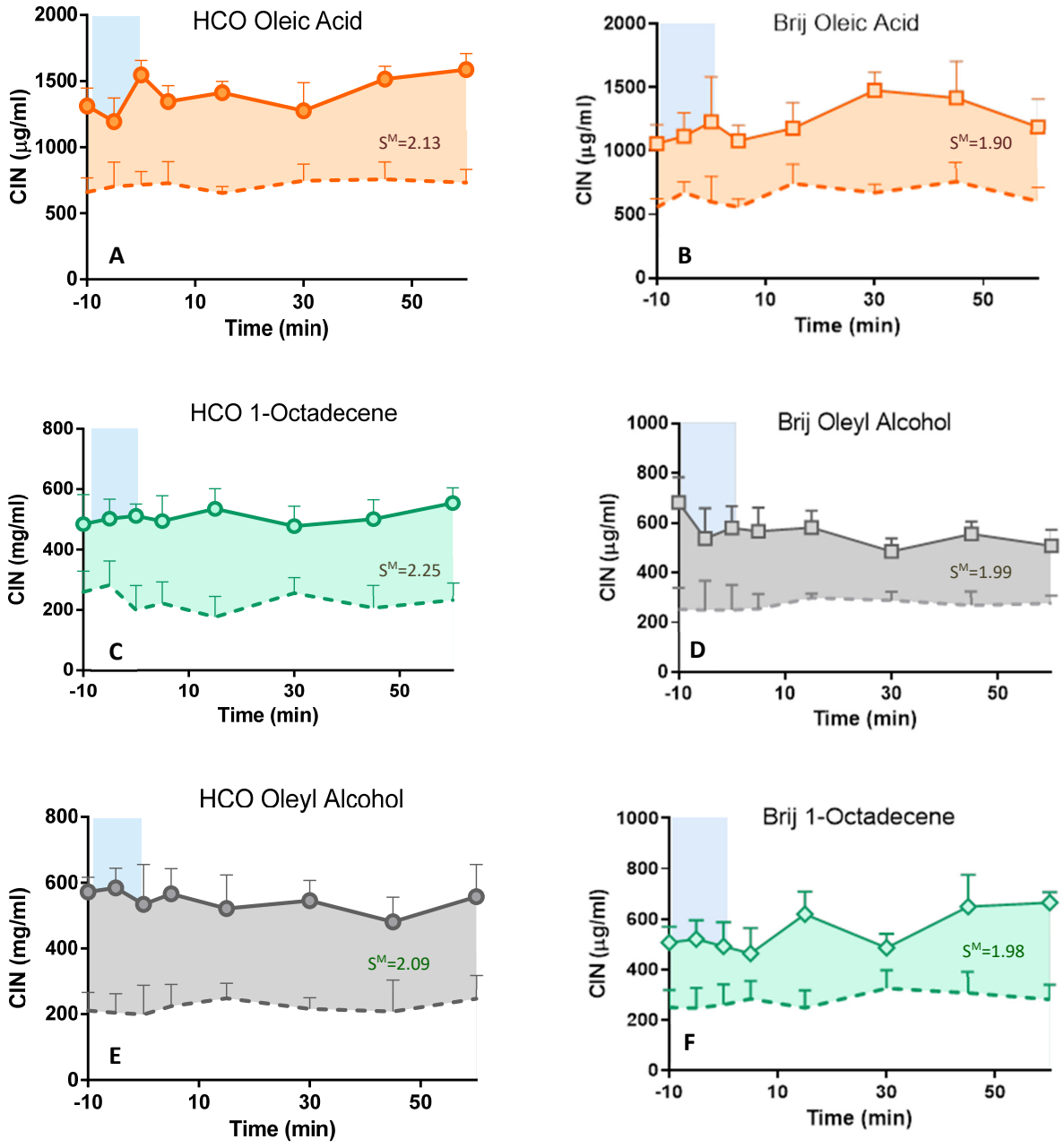


Figure 5.3. Comparison of equilibrium solubility, drug solubilisation and formulation supersaturation during digestion experiments for the series of non-digestible HCO formulations (A, C and E) and analogous data for non-digestible Brij formulations (B, D and F). The solid line and symbols represent the measured cinnarizine concentration in the aqueous phase during digestion experiments. The dotted line represents the equilibrium solubility of cinnarizine measured in the aqueous phase obtained from digested blank formulations. The maximal supersaturation ratio (S^M) is reported in the shaded area for each formulation, but was approximately 2 in all cases and over all timeframes. In all cases, addition of lipolytic enzymes did not stimulate NaOH titration, indicating that the formulations were non-digestible under these conditions (or that any fatty acids released from HCO digestion were not detectable in the systems used). (Data represented as mean n = 3 ± SD)

5.4.3. In vivo evaluation of ND-LBF performance

Understanding that non-digestible formulations must be sufficiently well dispersed to facilitate drug absorption, and that endogenous lipid processing is unlikely to play a significant role in the generation of nanoemulsions for ND-LBF, formulations were administered as dispersed emulsions via intraduodenal infusion over 2 hours. This mode of administration allowed direct administration of formulations of known particle size to the site of absorption.

Figure 5.4 shows the mean plasma concentration versus time profiles for cinnarizine following intra-duodenal infusion of dispersed ND-LBF. A summary of the pharmacokinetic parameters is provided in Table 5.2. To permit cross-comparison, pharmacokinetic data are normalised to a 10 mg/kg drug dose. The results of the *in vivo* study show that exposure of cinnarizine after administration of the non-digestible Brij 1-octadecene formulation was significantly greater than exposure obtained after administration of the oleyl alcohol containing formulations (figure 5.4A). Exposure from the 1-octadecene formulation was also greater than the oleic acid formulation, although this difference was not statistically significant.

Interestingly, the same trend was observed for formulations prepared with the partially digestible HCO surfactant blend (figure 5.4B), although in this case cinnarizine exposure after administration of the 1-octadecene formulation was significantly greater than both the oleic acid and oleyl alcohol formulations, $p < 0.05$. The octadecene HCO formulation also led to significantly greater cinnarizine exposure when compared to the equivalent octadecene Brij formulation. In contrast, surfactant type had little effect on drug absorption from the oleic acid and oleyl alcohol containing formulations.

In general, formulations containing the highest logD lipid, 1-octadecene, resulted in greater cinnarizine absorption than formulations containing oleic acid and oleyl alcohol. For the octadecene formulations, the non-digestible Brij surfactant based formulations resulted in lower cinnarizine absorption than the equivalent formulation comprised of hydrogenated castor oil surfactants which may be partially hydrolysed in the gastrointestinal tract.

Table 5.2. Comparative pharmacokinetic parameters for cinnarizine after ID administration of ND-LBF to fasted male Sprague dawley ratsa. Drug incorporated at 80% saturated solubility [mean ± SEM (n=3)]

	Brij Formulations			HCO Formulations		
Lipid Component	**Oleic Acid**	**Oleyl Alcohol**	**Octadecene**	**Oleic Acid**	**Oleyl Alcohol**	**Octadecene**
$AUC_{0-\infty}$ (ng.h/mL)	149.3 ± 7.6	134.4 ± 18.2	254.2 ± 37.3[b]	132.3 ± 7.6	142.3 ± 35.3	511.3 ± 116.7[c,d]
C_{max} (ng/mL)	52.1 ± 7.8	51.1 ± 5.8	74.3 ± 8.3	37.4 ± 0.9	46.8 ± 5.2	125.3 ± 82.8
T_{max} (h)	1.7 ± 0.2	1.5 ± 0.1	1.8 ± 0.1	1.8 ± 0.2	1.5 ± 0.5	1.8 ± 0.1
$t_{1/2}$ (h)	3.5 ± 0.3	4.1 ± 0.2	3.4 ± 0.6	3.6 ± 0.5	3.3 ± 0.0	3.8 ± 0.4

[a]Data normalised to a 10 mg/kg cinnarizine dose to facilitate comparison across differing drug doses.
[b]Statistically significant increase in Octadecene AUC when compared to Oleyl Alcohol formulation, $p < 0.05$
[c]Statistically significant increase in Octadecene AUC when compared to Oleic acid and Oleyl alcohol formulations, $p < 0.05$
[d]Statistically significant increase in HCO Octadecene AUC when compared to all Brj formulations, $p < 0.05$

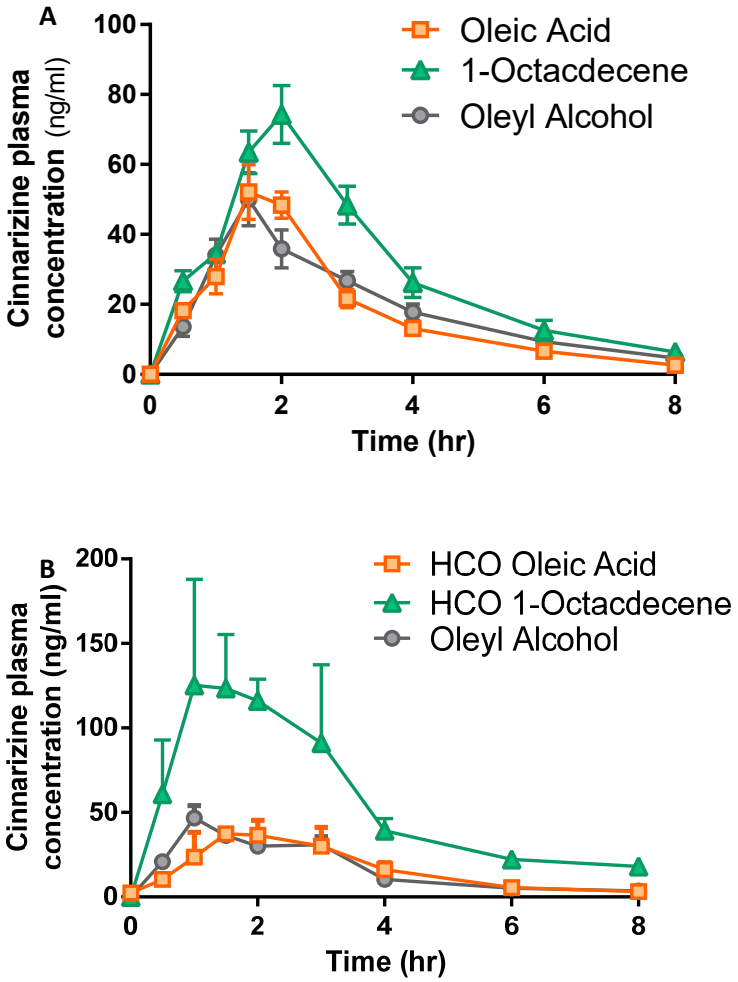


Figure 5.4. Mean cinnarizine plasma concentration vs. time profiles for Brij ND-LBF (A) and HCO ND-LBF (B) after ID administration. Data normalised to a 10 mg/kg dose of cinnarizine, [mean ± SEM (n = 3)].

Figure 5.5 shows a correlation of cinnarizine plasma exposure and the LogD of the formulation lipid at pH 5.5. This parameter was chosen (instead of Log P) as oleic acid is expected to exist in both protonated and unprotonated states at the unstirred water layer (where the pH is slightly acidic when compared to luminal pH). The data show a weak correlation between lipid LogD and drug absorption from ND-LBF.

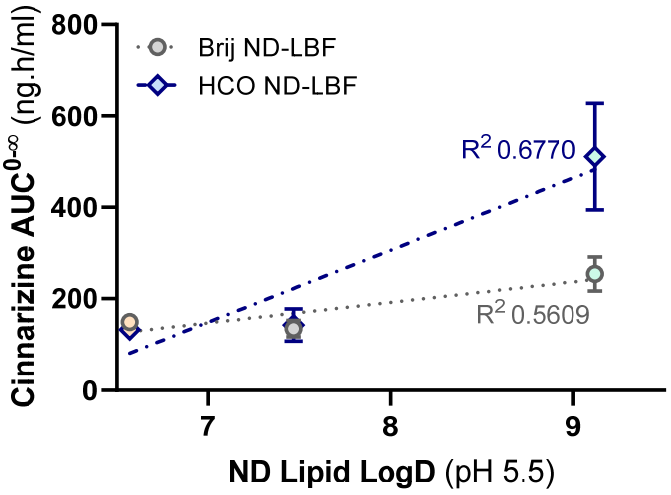


Figure **5.5**. Cinnarizine exposure vs. formulation lipid LogD for Brij ND-LBF (Grey circles) and HCO ND-LBF (Blue diamonds) after ID administration. Data normalised to a 10 mg/kg dose of cinnarizine, [mean ± SEM (n = 3)]. Data show a weak positive correlation between the LogD of the lipid and *in vivo* exposure.

5.5. Discussion

Lipid-based formulations have long been used to improve the oral bioavailability of BCS class II drugs by increasing both the dissolution rate and solubility of poorly water-soluble drugs in the GI tract[1]. LBF formulation performance is highly dependent on excipient composition and luminal processing, the former being controllable while the latter can lead to considerable intersubject variability. More recently, studies have shown that the propensity of LBFs to supersaturate during digestion may have a significant impact on bioavailability, with formulations that rapidly supersaturate above a critical threshold likely to result in drug precipitation *in vitro* potentially reducing *in vivo* exposure[1].

Previous studies in this thesis (Chapters 3 and 4) have shown that controlling or slowing lipid digestion may influence drug supersaturation rates and ultimately drug exposure *in vivo*[204]. Those studies used PEGylated surfactants to sterically hinder lipase access to formulated triglycerides and showed that the stealth characteristics of a formulation influenced exposure of danazol when administered in a medium-chain formulation to rats. However, subsequent studies (Chapter four) revealed that the absorption benefits flowing from the lipolysis control imparted by PEGylated surfactants was reversed when the formulations self-assembled in situ in larger animal species (dogs). This may be due to species differences, differential metabolism of the model drug or fouling of the PEG mantle during self-emulsification *in vivo*.

As strategies to slow or control digestion showed variable results, the current studies sought to deliberately engineer non-digestible lipid-based formulations (rather than using PEG to inhibit lipolysis) to enhance the delivery a model poorly water-soluble drug (cinnarizine). The studies performed sought first to determine whether a finely dispersed formulation could be prepared from non-digestible lipids, and secondly to evaluate formulation performance *in vitro* by quantifying solubilisation capacity and supersaturation during an *in vitro* digestion challeng teste. Finally, studies *in vivo* bioavailability studies were conducted in male SD rats to determine whether ND-LBFs could improve the oral absorption of cinnarizine.

5.5.1. Non-digestible LBFs form monodisperse nanoemulsions and solubilise a model drug to similar extent when compared to lipid alone.

In the absence of endogenous processing to reduce the particle size of an administered ND-LBF, formulations must form nanoemulsions on dispersion to facilitate efficient drug (and lipid) diffusion to the enterocyte (absorptive site) and subsequent rapid equilibration with the free concentration of drug in equilibrium with the solubilised reservoir in the oil droplet. These design principles were based on the hypothesis that larger, non-digestible lipid droplets would likely hinder both approach to the absorptive site and release of free drug from within the centre of a larger droplet. Early formulation development ruled out the use of the non-ionic block copolymer Synperonic L101 as a lipid replacement, as this formulation formed a viscous gel that could not be accurately sampled during dispersion and digestion. Likewise the use of oleylamine as a fatty acid analogue was not possible as the formulation developed insoluble lipid crystals over time (possibly due to a lack of miscibility of oleyl amine in the other formulation components) and could not be accurately sampled.

Of the remaining non-digestible lipids, figure 5.1 shows that all ND-LBFs formed nanoemulsions on dispersion in digestion media. The largest particle size for these formulations was 50 nm, suggesting the presence of some vesicular or emulsion species co-exisiting with primarily micellar species in the HCO-OH formulation. All other formulations were below 20 nm indicating that the species formed were primarily micellar.

Figure 5.2 provides information on both lipId and formulation drug solubility, where it can be seen that cinnarizine is considerably more soluble in oleic acid and oleic acid containing formulations. However the formulation solubility for the fatty acid analogues was in the same order of magnitude as the oleic acid formulations, this dictated that the impact of the nature of the lipid dosed could be systematically evaluated across formulations by dosing the same formulation mass.

5.5.2. Non-digestible LBFs are not hydrolysed during digestion, maintain drug solubilisation capacity and minimally supersaturate during in vitro dispersion and digestion tests

Figure 5.3 shows the solubilisation capacity of each formulation during *in vitro* digestion testing overlaid with the equilibrium solubility of cinnarizine in digested blank formulations. In all cases the formulations did not digest in the presence of porcine pancreatin extract, or the extent of digestion was too low to be detectable on the pH stat instrument. All formulations maintained drug solubilisation during the digestion assay, with minimal loss of solubility observed after addition of porcine pancreatin extract. In all cases, formulations were supersaturated (S^M = 2) on dispersion, with maximal supersaturation maintained at 2 throughout the 60 min digestion experiment indicating that ND-LBF were stable in the presence of lipases. Importantly, these systems had S^M values lower than those typically associated with precipitation potential.

5.5.3. Non-digestible LBFs comprising 1-octadecene outperform all other formulations after ID administration.

Finally, the plasma profiles in Figure 5.4 and the pharmacokinetic parameters presented in Table 2 reveal differences in *in vivo* exposure of cinnarizine after oral administration of ND-LBF formulations. Two series of surfactants were investigated, PEGylated oleyl ethers and PEGylated polyethoxylated hydrogenated castor oils, the former yielding a LBF that is comprised entirely of non-digestible components, while the latter potentially has hydrolysable fatty acids as the surfactant hydrophobic group. The trends between the two data sets were similar, with the octadecene containing formulation out performing the analogous oleic and acid and oleyl alcohol containing formulations. Furthermore, absorption from the HCO-1-octadecene formulation was significantly greater than the analogous Brij formulation, potentially indicating that some degree of digestion at the intestinal lumen may facilitate dissociation of micelles and promote absorption. Figure 5.5 shows that there is a weakly positive correlation between the LogD of the lipid formulated and cinnarizine exposure, with greatest AUC for the highest LogD lipid (1-octadecene) containing formulations. Interestingly this data shows that lipid absorption at the enterocyte may also influence drug uptake, which is in agreement with earlier work in our group [69] The differential uptake of cinnarizine in formulations with fatty acid, fatty alcohol or fatty alkene highlights the importance of evaluating the physicochemical characteristics of luminal formulation components as well as that of the incorporated drug.

This work also highlights that the *in vitro* model used was unable to discriminate between formulations due to a lack of absorptive sink. It is expected that uptake of drug *in vitro* by providing an absorptive membrane could significantly alter the performance of a PWSD[266], reducing supersaturation and precipitation potential. However an absorptive sink for a drug is also a viable sink for absorbable formulation components. Excipient uptake would be expected to reduce the solubilisation capacity of the aqueous phase under assay. Thereby increasing luminal supersaturation potential. As both drug and lipid absorption are expected to occur together, it is reasonable to assume that the competing processes of desaturation/supersaturation occur at the same time and that the dominant factor will be dependent on both the drug and formulation used.

5.6. Conclusions

In this work, we have examined the utility of non-digestible lipid-based formulations for oral administration of cinnarizine, a poorly water-soluble drug. The data show that fatty acids and fatty acid structural analogues are feasible substitutes for triglycerides in LBF. Furthermore, these data also show that ND-LBF are not digested, maintain drug solubilisation *in vitro*, show stable supersaturation and facilitate drug absorption *in vivo*. Interestingly, in the absence of differences in digestibility, solubilisation capacity, initial thermodynamic activity (supersaturation), particle size, or formulation components, the data suggest that the lipid used in a self-emulsifying formulation can significantly influence drug uptake, with greatest drug absorption from the formulation with the least polar, highest LogD lipid (octadecene).

These data are the first to show that the physicochemical properties of lipids included in LBF may be significant factors in oral drug absorption. The data also show that cinnarizine exposure was higher after administration of formulations containing a triglyceride-based surfactant blend, perhaps suggesting that some degree of luminal processing and formulation destabilisation is favourable for efficient uptake at the enterocyte.

These studies show that non-digestible formulations can have utility for the delivery of poorly water-soluble drugs, particularly in patient populations where pathological enzyme insufficiencies would preclude the administration of a conventional LBF that requires luminal processing for drug absorption. However, further evaluation of ND-LBF in an *in vitro* model with an absorptive sink is necessary to establish IVIC. Additionally, assessment of other non-traditional lipid components and evaluation of the link between physicochemical characteristics of the lipids formulated and exposure is warranted to further develop formulations where *in vivo* performance can be better controlled.

Chapter 6. Summary and future perspectives

This thesis has investigated the potential utility of inhibiting or eliminating digestion as a means to improve the oral absorption of poorly water-soluble drugs from lipid-based formulations. Notably, the role of formulation excipients in inhibiting digestion and influencing drug absorption has been examined in detail. The work contributes to a better understanding of the impact of common formulation excipients on the rate and extent of digestion of lipid-based formulations as well as the potential for lipid physicochemical parameters to influence the absorption of a PWSD.

Low and variable oral bioavailability of crystalline poorly water-soluble drugs (PSWD) poses a significant challenge to the clinical development of lipophilic drugs. As increasing numbers of these problematic compounds emerge from ligand binding, high throughput and combinatorial chemistry programs, the need for enabling technologies to provide a mechanism to bypass the slow and absorption-limiting dissolution rates of these drugs becomes more apparent. A range of approaches have been employed to improve the oral absorption of PWSD including changing the crystalline form, the generation of different salts, particle size reduction, formation of cyclodextrin complexes and administration of lipid-based formulations (LBFs). The work described in this thesis has focused on the development of novel LBFs to avoid or eliminate digestion and in so doing, to remove the risk of drug precipitation after lipolysis.

Typically, LBFs allow the delivery of a PWSD to the intestinal absorptive membrane in a molecularly dispersed form, circumventing the slow dissolution limitations of crystalline drug administration. Dispersion and digestion of lipids in the intestinal fluids is also expected to increase the drug solubilisation capacity of the intestinal tract. LBF development over the years has therefore been driven by the dogma that lipid digestion is required to optimise *in vivo* absorption. However, it is becoming increasingly apparent that correlating formulation digestion to drug solubilisation *in vitro* and ultimately to *in vivo* drug absorption is complex. Indeed, a wealth of information has emerged to show that as formulations, particularly those containing medium chain triglycerides (MCT) disperse and digest, the solubilisation capacity of LBFs can drop dramatically, inducing rapid drug supersaturation which results in drug precipitation. Recent efforts to either impede crystallisation or change the form of the precipitate after digestion have met with varied success.

The first series of experiments conducted in this thesis (chapter 3) explored the potential to reduce the extent of digestion of a MCT formulation thereby delaying or preventing digestion-mediated precipitation of a model PWSD. This was achieved by transferring knowledge of the effects of ‘stealth’ technology from parenteral to oral drug delivery. Thus previous work in our group and in others has reported that non-ionic surfactants containing PEG headgroups may inhibit lipid digestion. However the utility of this phenomenon for oral drug delivery has never been explored in detail or investigated *in vivo*. In parallel, over 20 years of literature in parenteral formulation development has comprehensively established PEG as an effective steric barrier to nonspecific protein adsorption. PEGylation of formulations as diverse as proteins, liposomes, dendrimers, micelles and solid lipid nanoparticles has been shown to result in the same outcome, ie

decreased protein adsorption, increased plasma circulation time, increased passive accumulation at sites of hypervascularisation and decreased renal clearance. The ability of these PEGylated formulations to evade recognition by the immune system and avoid renal filtration is termed the enhanced permeation and retention (EPR) effect and is attributed to the 'stealth' properties of PEG. Knowledge of the properties that predicated stealth behaviour in parenteral formulations, namely, PEG Mw and packing density, informed a series of studies here to investigate whether the same principles apply to lipase adsorption to the oil:water interface of a LBF. The data obtained from a series of lipolysis experiments show that the nonspecific adsorption of lipase to a lipid droplet can be disrupted by PEG and that this results in a change in the rate of lipolysis of co-formulated triglyceride. The reduction in digestion correlated with the Mw of the surfactant PEG headgroup and PEG packing density and was independent of the surfactant hydrophobe structure. The data mirror the stealth behaviour of many nanoparticle formulations. The particle size of the formulations was also investigated to provide assurance that digestion differences were not attributable to an increase in lipid droplet size which can reduce digestion rates. The data for two series of surfactants showed that the most digestion inhibiting formulations were also the most well dispersed formulations, negating a potential confounding effect on dispersion. The most 'stealth-like' LBFs were considered to be the formulations with the greatest reduction in lipolysis and these were taken into further *in vitro* studies where they were compared with an analogous digestible control formulation to examine their impact on drug solubilisation patterns. As anticipated, the reduction in formulation digestion resulted in the maintenance of drug solubilisation throughout the *in vitro* digestion experiment consistent with the expectation that rapid and uncontrolled digestion of LBF can result in supersaturation and drug precipitation. Notably very different effects were apparent from similar formulations which differed only in the Mw of PEG on the surfactant headgroup. Importantly, subsequent *in vivo* evaluation of stealth and comparative digestible formulations in Sprague Dawley rats showed that the stealth LBFs resulted in increased bioavailability of coformulated danazol as a model PWSD.

Chapter 4 subsequently provides a detailed investigation of the robustness and applicability of stealth LBFs as a viable alternative to conventional lipid-based formulations. Firstly, the effect of PEGylated surfactants on long chain triglyceride (LCT) digestion, drug solubilisation and *in vivo* absorption were evaluated for a single series of surfactants. As seen with the MCT formulations in chapter 3, the pattern of inhibition of digestion of LCT formulations exhibited a PEG Mw dependency. The most inhibiting or 'stealth' formulation was cremophor RH40 which was also the most digestion inhibiting surfactant in the stealth MCT formulation. However, the digestion inhibition effect of stealth LCT formulations was attenuated due to inherently lower digestion rates of LCT when compared to MCT. This was also reflected in reduced *in vitro* precipitation in both the stealth LCT formulation and digestible LCT formulations. As such the stealth LCT formulation resulted in only modest increases in bioavailability when compared to the MCT formulations.

In light of these findings, MCT (and not LCT) formulations were progressed to further studies. *In vivo* data generated to this point had been limited to the administration of predispersed formulations to rats via oral gavage. As stealth LBFs are more likely to dosed as neat encapsulated formulations, oral bioavailability studies were subsequently conducted where 1.4g of formulation was administered in two hard gelatin capsules to male beagle dogs. Since the pancreatic enzyme levels in dogs are higher than that in rats, the *in vitro* digestion studies were also repeated under dog-relevant conditions.

Again, the stealth formulations outperformed their readily digestible counterparts *in vitro*, maintaining drug solubilisation throughout the digestion period. However *in vivo* bioavailability studies failed to show an advantage of the stealth formulations in dogs. In an attempt to explain the differences in stealth behaviour between predispresed formulations in rats and self-assembled formulations in dogs, the potential for surface active species present in the dog stomach (proteins, fatty acids, monoglycerides and gelatin) to interfere with the self-assembly of stealth LBFs was evaluated *in vitro*. Addition of these species to the lipolysis media resulted in at least 10-fold increases in digestion of the stealth LBF and may have acted in concert with the formulation to attenuate the stealth effect in dogs. In an attempt to overcome these problems very rapidly self-emulsifying formulations were also explored with the aim of assembling the stealth barrier more rapidly and avoiding intercalation of the surface active impurities. It was hoped that this might be achieved by adding cosolvents to the formulation to increase the speed of dispersion. However, addition of three different cosolvents (PEG400, ethanol and transcutol) to the stealth formulation resulted in almost complete reversal of the stealth behaviour, and digestion of approximately 70% of the MCT. The data in Chapters 3 and 4 suggest that a pre-formed stealth barrier can improve oral drug absorption from pre-dispersed long and medium chain lipid-based formulations, but that the self-assembly of these systems *in vivo* (as is expected for typical self-emulsifying formulations) is fraught with challenges, limiting utility to pre-assembled dispersions, and potentially to preclinical studies in rats.

Understanding that stealth LBFs may have limited utility as typical encapsulated oral formulations, subsequent studies (chapter 5) focused on the development of non-digestible lipid-based formulations which would not undergo significant digestion-mediated changes to drug solubilisation. Historically, non-digestible lipids have been synonymous with non-absorbable lipids such as mineral oil and sucrose polyesters. These difficult to disperse lipids have been proven to be poor vehicles for oral drug delivery. In contrast, little attention had been focused to this point on absorbable, but non-digestible (or pre-digested) lipids. The potential of these systems was based on recent data in our group that had shown that fatty acid absorption from intestinal colloids may promote drug absorption by reducing the solubilisation capacity of the colloid, thereby supersaturating remaining drug. Studies in Chapter 5 therefore sought to capitalise on this potential through the development of a series of non-digestible self-emulsifying formulations (NDSEDDS) based on the pre-digested lipid oleic acid and two related structural analogues (oleyl alcohol and 1-octadecene) and Brij as a non-digestible surfactant. Formulations were developed to rapidly emulsify to

form colloids with small hydrodynamic radii to promote transfer to the absorptive surface and encourage rapid equilibration with the free fraction of drug available for absorption. Optimised formulations comprised 27% lipid, 66% surfactant and 7% cosolvent. Non-digestible formulations showed no change to drug solubilisation patterns during *in vitro* digestion challenge for all formulations tested.

Oral administration of these formulations showed that cinnarizine was absorbed from NDSEDDS formulations containing the ether based, non-digestible Brij surfactants. However, when the surfactant blend was changed to hydrogenated castor oils, surfactants that are triglyceride based and marginally digested (16% digestion), formulation performance drastically improved. Furthermore, cinnarizine absorption from NDSEDDS was greatest from formulations containing hydrogenated castor oils as surfactants and octadecene rather than oleic acid or oleyl alcohol as the included lipid. These data raise the possibility that in systems where digestion is limited or absent that drug absorption may correlate with the physicochemical properties of the lipid and be enhanced for systems containing nonpolar, high LogD lipid such as 1-octadecene. It is not clear at this time why octadecene is preferred although it is possible that the lack of polarity may promote rapid intestinal permeability and absorption. Furthermore, the utility of the hydrogenated castor oil surfactant-containing systems may also suggest that some (limited) degree of digestion is required, perhaps to perturb the interface and promote drug release.

In conclusion, the studies conducted in this thesis have shown that control or elimination of lipid digestion can improve oral bioavailability for BCS class II drugs which are likely to supersaturate and precipitate on digestion from conventional LBFs. While stealth LBFs showed promise as a novel platform, further work is required to improve or modify the *in vivo* self-assembly behaviour of these formulations. Likewise, drug absorption from non-digestible LBFs was effective but may suggest that a balance between GI stability and the ability to facilitate diffusion of free drug to the intestinal absorptive membrane is critical to the performance of ND-LBF.

During the course of the work presented here, the impact of formulation components on digestive and absorptive processes (and ultimately, on drug absorption) were established. However, there are a number of questions, that were beyond the scope of the current studies, that remain unanswered and form the basis of potential future work.

Firstly, and perhaps most obviously, during the development of these new series of formulations, a single model drug was used for the stealth formulations (danazol) and a for the ND-LBF (cinnarizine). Whether these formulations will improve absorption for other hydrophobic ‘brickdust’, lipophilic ‘greaseball’ or ionisable drugs is unknown and would be necessary to establish each formulation type as a viable drug delivery platform.

Secondly, during the development of the stealth LBFs in Chapter 3, initial attempts to correlate the extent of digestion *in vitro* with hydrophile-lipophile balance (HLB) (rather than surfactant structure and PEG molecular weight) did not yield appreciable trends. Subsequently, when the formulations were stratified to control for surfactant hydrophobic moiety and headgroup Mw, correlations did emerge. However, there were no cross comparisons between surfactant types. Similarly, after *in vivo* administration of ND-LBF in Chapter 5, the influence of the LogD of the formulated lipid was immediately apparent. These data highlight that the physicochemical characteristics of formulation components can be major influencing factors in formulation performance. In contrast surfactant and lipid structure are typically overlooked or described only with global properties such as HLB or medium- or long-chain. In this way the molecular specifics of the excipients are often overlooked and the impact of excipient absorption commonly ignored. Re-evaluating formulation performance based on the molecular properties of excipients may reveal examples of formulation components that are absorbable without being digested, and others that are digested but not absorbed. These may have very different effects on concurrent drug absorption but to this point are poorly explored and understood.

Thirdly, these studies employed a simplified *in vitro* digestion test to stratify formulation performance, which, in the absence of a gastric processing step and an absorptive sink, likely overestimated the digestion inhibition effect of stealth LBFs but was not sufficiently sensitive to effectively distinguish between the ND-SEDDS. Numerous other studies have established altered digestion tests, for example those with an absorptive sink and these may provide the opportunity for more detailed examination of formulation performance. For example, systems containing an immobilised recombinant lipase in conjunction with mucus coated CaCo-2 cell models. This approach may improve the predictive power of the *in vitro* model to evaluate the ND formulations developed here (and the different non-digestible lipids). However there are compromises that may limit data interpretation not least the shift from solubilised mammalian lipase *in vivo* to an immobilised bacterial lipase *in vitro*. The latter enzymes typically do not have a lid domain and have lower activity relative to pancreatic lipases. Nonetheless, the use of more complex *in vitro* systems may provide an opportunity to better understand the performance of non-digestible or digestion-inhibited LBF.

Finally, these studies utilised non-conventional surfactants and lipids. Some of these have not been used in registered products previously and therefore have unknow long term toxicity. Prior to human use, a detailed toxicological evaluation would therefore be required.

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Appendix 1

50 Years of oral lipid-based formulations: provenance, progress and future perspectives

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ABSTRACT

Lipid based formulations (LBF) provide well proven opportunities to enhance the oral absorption of drugs and drug candidates that sit close to, or beyond, the boundaries of Lipinski's 'rule-of-five' chemical space. Advantages in permeability, efflux and presystemic metabolism are evident; however, the primary benefit is in increases in dissolution and apparent intestinal solubility for lipophilic, poorly water soluble drugs. This review firstly details the inherent advantages of LBF, their general properties and classification, and provides a brief retrospective assessment of the development of LBF over the past fifty years. More detailed analysis of the ability of LBF to promote intestinal solubilisation, supersaturation and absorption is then provided alongside review of the methods employed to assess formulation performance. Critical review of the ability of simple dispersion and more complex *in vitro* digestion methods to predict formulation performance subsequently reveals marked differences in the correlative ability of *in vitro* tests, depending on the properties of the drug involved. Notably, for highly permeable low melting drugs e.g. fenofibrate, LBF appear to provide significant benefit in all cases, and sustained ongoing solubilisation may not be required. In other cases, and particularly for higher melting point drugs such as danazol, where re-dissolution of crystalline precipitate drug is likely to be slow, correlations with ongoing solubilisation and supersaturation are more evident. In spite of their potential benefits, one limitation to broader use of LBF is low drug solubility in the excipients employed to generate formulations. Techniques to increase drug lipophilicity and lipid solubility are therefore explored, and in particular those methods that provide for temporary enhancement including lipophilic ionic liquid and prodrug technologies. The transient nature of these lipophilicity increases enhances lipid solubility and LBF viability, but precludes enduring effects on receptor promiscuity and off target toxicity. Finally, recent efforts to generate solid LBF are briefly described as a means to circumvent the need to encapsulate in soft or hard gelatin capsules, although the latter remain popular with consumers and a proven means of LBF delivery.

Contents

Abbreviations: AP, Aqueous phase; API, Active pharmaceutical ingredient; b-r-o-5, Beyond rule-of-five; BS, Bile salt; DG, Diglyceride; FA, Fatty acid; GIT, Gastrointestinal tract; IL, Ionic liquid; LBF, Lipid based formulation; LCT, Long chain triglyceride; LFCS, Lipid formulation classification system; MCT, Medium chain triglyceride; MG, Monoglyceride; PL, Phospholipid; PWSD, Poorly water soluble drug; S, Supersaturation ratio; SEDDS, Self-emulsifying drug delivery system; SMEDDS, Self-microemulsifying drug delivery system; SNEDDS, Self-nanoemulsifying drug delivery; TG, Triglyceride; UWL, Unstirred water layer.

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1. Introduction

In spite of considerable efforts to reduce physicochemical liabilities, and to design-in reasonable 'developability' characteristics in prospective drug candidates, discovery programmes continue to identify drugs with low water solubility, limited cellular permeability and high metabolic clearance—properties that are expected to reduce oral bioavailability. The current theme issue is focused on the challenge of developing drug candidates with properties such as these and simplistically has been defined by reference to drugs that do not comply with the 'rule of 5' (r-o-5) suggested by Lipinski [1] *i.e.* 'beyond r-o-5' (b-r-o-5) drugs. In reviewing the available literature, however, it is apparent that most currently marketed drugs, even typical BCS class II/III/IV compounds with low solubility and/or low permeability, are largely r-o-5 compliant. This is especially the case if the requirement for compounds to violate two r-o-5 properties in order to sit within the b-r-o-5 chemical space is strictly applied. In the context of this article, therefore, much of the historical data that has been reviewed does not truly reflect the b-r-o-5 chemical space. Nonetheless, the approaches taken to address the solubility or permeability limitations of drugs that sit within, or close to the boundaries of r-o-5, are applicable to the increasing numbers of drug development candidates that are moving b-r-o-5.

Of the limiting factors to oral drug delivery described above, low water solubility is perhaps the most amenable to resolution based on the use of enabling formulation approaches [2]. In contrast, formulation approaches that markedly enhance intestinal permeability or reduce first pass metabolism, are much less common. Permeation enhancement for oral delivery has met with some moderate successes in early clinical development as described in a recent review by Aguirre et al. [3]. In the case of highly (first pass) metabolised compounds, strategies such as prodrugs, coadministration with inhibitors, or alternative routes of absorption, *e.g.* pulmonary, nasal and buccal administration are more commonly employed [4]. However, for many compounds with significant permeability or metabolic liabilities, parenteral administration is often required for efficient delivery.

For drugs where low aqueous solubility limits absorption, several formulation technologies have been developed and applied to support increases in dissolution rate and/or apparent solubility in the gastrointestinal tract (GIT). These include particle size reduction and nanomilling, salt formation, isolation as a cocrystal or high energy polymorph, the generation of solid dispersions, and formulation in lipid based formulations (LBFs). These approaches have been described in detail previously [5,6], and some are covered in the current theme issue [7,8]. This review focusses on the use of LBF to enhance exposure after oral administration. Whilst the major advantage of LBF, at least to this point, has been in increasing apparent gastrointestinal solubility, it is also becoming increasingly clear that they may provide advantages in permeability and, under some circumstances, in avoiding first pass metabolism.

LBF have been investigated as a means to enhance oral drug absorption for many years. Indeed, it is fitting that as the Journees Galeniques meeting (around which this theme issue is based), enters its 50th year, this retrospective analysis of the published literature shows examples of LBF development from at least 50 years ago [9,10]. Indeed, lipid suspension and emulsion formulations of sulphonamides were described as early as the 1950s [11,12]. It was probably not until the 1970s, however, that more detailed evaluations of the use of lipids to boost drug absorption were initiated [13–21]. Increasing application of soft gelatin capsule technologies further facilitated oral administration of undispersed LBF. The rationale for the initial exploration of LBF to promote the absorption of poorly water soluble drugs was the realisation that many compounds of this type exhibit significant positive food effects. Thus, coadministration with lipids recruits (or at least partially recruits) the physiological events that are initiated by food administration to promote dietary lipid absorption. Lipid administration results in pancreatic and gallbladder secretions that initiate the process of lipid digestion and subsequent solubilisation of lipid digestion products in bile salt/phospholipid/cholesterol mixed micelles. Ultimately, this leads to the development of a range of colloidal particles in the GIT that serve to solubilise dietary lipids, but that also significantly enhance the solvation capacity of the GIT for coadministered drugs.

The importance of lipid digestion in the processing of LBF has led to the development of *in vitro* models of digestion that can be used to mimic the likely pathways of formulation processing in the GIT. This in turn has allowed examination of the potential fate of a co-solubilised drug during formulation digestion. The first reports of these models emerged in the late 1980s [22–27], and accelerated significantly in the 1990s and 2000s [28–37]. These studies developed the hypothesis that for lipid formulations to be successful, drugs should remain in a solubilised state during formulation digestion and processing, as precipitation is likely to be detrimental to formulation performance. This concept was based on the expectation that drug precipitation during formulation processing would generate solid drug, and initiate the requirement for drug dissolution—a process that is typically slow for poorly water soluble drugs (PWSD). For many (but not all) compounds this general hypothesis appears to hold and good *in vitro–in vivo* correlation has been reported between drug solubilisation during *in vitro* lipid digestion and systemic drug exposure

after oral administration of some drugs [37–40]. This is discussed in more detail in Section 6.

Secondly, the perceived importance of the particle size of the dispersion formed on capsule rupture in the GIT, has driven the development of formulations that spontaneously emulsify to form lipid emulsions with particle sizes in the low nanometre size range. This hypothesis was propagated on the basis that endogenous lipid processing results in increasing degrees of lipid dispersion and solubilisation and that ultimately colloidal structures with small particle sizes are required to diffuse across the unstirred water layer (UWL) and present drug to the intestinal absorptive surface. Formulations that are pre-dispersed to form small emulsion droplets (microemulsions, nanoemulsions), or that spontaneously emulsify on contact with GI fluids (self-emulsifying drug delivery systems or SEDDS) have been suggested to provide improved performance. The complex series of interactions that take place in the GIT, however, including formulation digestion and interaction of digestion products with bile salt micelles, are likely to significantly change the nature of any SEDDS formulation. As such, it seems likely that the critical parameter in formulation assessment is not the nature of the initial dispersion, but rather the properties (including particle size) of the dispersion formed after interaction with biliary and pancreatic secretions that ultimately determine LBF performance.

To summarise, LBF continue to provide a robust option for delivery of drugs and drug candidates that do not conform to the r-o-5, as well as those that meet r-o-5 criteria, but still pose significant challenges with respect to formulation development. As experience with LBF grows, our understanding of the mechanisms by which they work, and therefore the design criteria required to optimise performance, also continues to evolve. This review provides an overview of the general composition, advantages, and basis for utility of LBF, and then provides more discussion of our evolving understanding of the relationship between solubilisation, supersaturation and absorption. We also discuss the role of drug lipophilicity in dictating the applicability of lipid formulations and the potential utility of lipophilic conjugates or complexes (for example; ionic liquids and lipophilic prodrugs) in enhancing the utility of LBF. Finally, we comment on the practicalities of LBF, including recent advances in solidification, in order to facilitate tabletting, and in the use of lipid multiparticulates.

2. Lipid formulation composition and classification

LBFs span a wide range of potential compositions and include diverse delivery systems ranging from lipidic subcutaneous depots, intravenous emulsions or liposomal formulations to topical creams or lotions. For the purposes of this review, discussion has been constrained to oral LBFs. The majority of these are ultimately filled into soft or sealed hard gelatin capsules for clinical and commercial application, but may also be dosed as the liquid fill material during preclinical development. Indeed, one of the significant advantages of LBF is that the same formulation can be scaled up from low volume liquid formulations gavaged to mice and rats, to encapsulated dose forms that can be hand filled into empty capsules for studies in dogs, through to soft or hard gelatin capsule products that can be manufactured on automated commercial filling lines. For an excellent overview of marketed products that employ LBF the interested reader is directed to the 2004 review by Strickley [41].

Physically, LBF are often liquids, but may also be solid or semi solid at room temperature when high melting lipids are employed or when lipids are adsorbed onto a carrier. They may also take the form of lipidic multiparticulates. Some examples of solid LBFs are described in more detail in Section 9.

Although some of the earliest examples of LBFs are lipid suspensions, and the feasibility and performance of these materials *in vivo* is often reasonable [42,43], suspension formulations pose additional challenges to robust material transfer and content uniformity in the finished product. These formulation types may also be prone to stability issues due to Ostwald ripening [44]. Consequently, excipients in LBF are typically optimised to maximise the chance of complete drug solvation in the formulation. For drugs with only limited lipid solubility this is not always possible and a range of approaches have been suggested to enhance lipophilicity and lipid solubility. This is discussed in more detail in Section 8.

The range of excipients employed to form LBF is wide, but is largely drawn from three broad categories of materials—lipids (the hydrophobic sink), surfactants (to aid emulsification/solubilisation) and cosolvents (to aid solvation/dispersion). The relative proportions of each of these materials dictate drug solubility, formulation dispersibility, transport and metabolic effects and also impact on formulation properties such as stability, capsule compatibility and viscosity. For a detailed overview of excipient properties and selection criteria, the interested reader is directed to ref. [45], and for previous reviews detailing LBF formulation design in the context of marketed formulations, self-emulsification and solubilisation capacity to ref. [41,46–53].

The least complex LBF comprise simple encapsulated solutions of drugs in oils and are typified by the many fat-soluble vitamin preparations. On capsule rupture in the GI fluids, these LBF are crudely emulsified by the shear associated with gastric emptying and GI segmentation, and digested by gastric and intestinal lipase enzymes to form more amphiphilic digestion products. These digestion products serve to stabilise the emulsions formed and are ultimately solubilised in bile salt/phospholipid/cholesterol micelles secreted in bile. The *in vivo* dispersion of these materials is therefore catalysed by the process of lipid digestion. In an attempt to circumvent the reliance on endogenous lipid processing for LBF dispersion, most contemporary LBF now contain surfactants and cosolvents. These materials are included to reduce interfacial tension to the point that emulsions are spontaneously formed on gentle mixing of the capsule content with the GI fluids (*i.e.* SEDDS formulations). Refinement of the SEDDS technology has subsequently resulted in formulations that disperse to generate colloids with smaller and smaller particle sizes, systems that were initially described as self-microemulsifying formulations or SMEDDS on the basis that the dispersions generated were microemulsions [54]. In reality, whether the colloids so formed are thermodynamically stable (a requirement for definition as a microemulsion) is questionable in many cases [55,56], and the 'micro'emulsion terminology is seemingly at odds with dispersions with dimensions in the nanometre size range. A recent study by Niederquell and Kuentz, for example, has shown in an exemplar series of 20 dispersed SEDDS, that the majority exhibited only kinetic stability and could not be accurately classified as 'microemulsions' [56]. In light of this, terminology based on particle size *e.g.* self-nanoemulsifying drug delivery systems (SNEDDS) has become increasingly popular. Alternatively, generic nomenclature that does not specifically refer to size (SEDDS) or terminology that prefixes the particle size of the dispersed formulation *e.g.* nanometre SEDDS (nSEDDS) or micrometre SEDDS (μSEDDS) may be more descriptive. For the purposes of this review, SEDDS, SMEDDS and SNEDDS nomenclature will be used where these were the terms employed by the original authors.

In an attempt to simplify classification of LBF and to group formulations on the basis of their composition, Pouton introduced the lipid formulation classification system (LFCS) (Table 1) in 2000 [47] and later updated the classification to expand the formulation groups [50]. The LFCS classifies LBF into four main types, based on the relative proportions of included lipids, surfactants and cosolvents (Table 1). Type I formulations are the simplest and comprise drug dissolved in triglyceride alone or in mixed glycerides. Type II formulations comprise combinations of glycerides and lipophilic surfactants (HLB <12) and are representative of some of the first SEDDS formulations that were described [57]. The original Type II formulations used polyethoxylated triglyceride-based surfactants (*e.g.* polyoxyethylene 25 glyceryl trioleate—Tagat TO). Similar, although not quite as efficient,

Table 1
The Lipid Formulation Classification System (LFCS) reproduced from Pouton et al. with permission [47,50].

Excipients	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	–
Water insoluble surfactants (*HLB* < 12)	–	20–60	–	–	0–20
Water soluble surfactants (*HLB* > 12)	–	–	20–40	20–50	30–80
Hydrophilic cosolvents	–	–	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 and potential loss of solvent capacity	Significant phase changes and 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
Characteristics	Non-dispersing; requires digestion	SEDDS without water-soluble components	SEDDS/SMEDDS with water-soluble components	SMEDDS with water-soluble components and low oil content	Oil-free formulation based on surfactants and cosolvents
Advantages	GRAS status; simple; excellent capsule compatibility	Unlikely to lose solvent capacity on dispersion	Clear or almost clear dispersion; drug absorption without digestion	Clear dispersion; drug absorption without digestion	Good solvent capacity for many drugs; disperses to micellar solution
Disadvantages	Formulation has poor solvent capacity unless drug is highly lipophilic	Turbid o/w dispersion (particle size 0.25–2 μm)	Possible loss of solvent capacity on dispersion; less easily digested	Likely loss of solvent capacity on dispersion	Loss of solvent capacity on dispersion; may not be digestible

emulsification behaviour is possible with polyoxyethylene 20 sorbitan trioleate (Tween 85). Type II LFCS formulations have been largely superseded by Type III formulations, not least because of the limited range of available lipophilic surfactants that promote self-emulsification of Type II formulations and that have been used in registered products. Type III formulations comprise mixtures of glyceride lipids, more hydrophilic surfactants (HLB >12), commonly also polyethoxylated glycerides, but with larger quantities of ethylene oxide, *e.g.* polyoxyethylene 35 castor oil (Kolliphor EL) or polyoxyethylene 40 hydrogenated castor oil (Kolliphor RH 40) and may also include cosolvents (*e.g.* PEG400, Transcutol or ethanol). Many SMEDDS or SNEDDS formulations are typical Type III formulations. Type III formulations are further stratified into Type IIIA, that contain larger proportions of lipids, and lower proportions of surfactant and cosolvent, and Type IIIB formulations that contain relatively limited amounts of glyceride lipid (<20%) and larger quantities of hydrophilic components. A classification of Type IV 'lipid' based formulations was introduced later in response to the increasing use of formulations that contain no traditional lipids [50]. Type IV formulations comprise only a combination of surfactants and cosolvents. The general properties of the different types of LBF are summarised in Table 1. In brief, the lipid rich Type I formulations require digestion to increase amphiphilicity and dispersion into intestinal fluids whereas Type II-IV contain sufficient surfactant to promote spontaneous dispersion. Progression from Type I-IV decreases triglyceride content and formulation susceptibility to digestion, and in general also leads to reductions in particle size of the resulting dispersion. For example, Type IV formulations typically disperse to form micellar solutions with particle sizes of 20 nm or below. Increasing quantities of surfactant and cosolvent in Type IIIB and Type IV formulations usually increases drug loading, since, with the exception of the most lipophilic drugs, the majority of PWSD are more soluble in surfactants and cosolvents than they are in glyceride lipids. The downside to the more hydrophilic Type IIIB and Type IV formulations is that inclusion of larger quantities of water-miscible components increases the risk of drug precipitation on dispersion of the formulation in the GI fluids.

Further amendments to the LFCS have been proposed to take into account the original classification system for lipids proposed by Small (rather than the combinations of lipidic excipients described by LFCS). These amendments were proposed in large part to better capture the properties of polar lipids that swell on contact with aqueous media and have markedly different properties to, for example, non-polar triglycerides [58] but that were grouped together as 'oils' in the LFCS.

Most recently, attention has switched to the classification of LBF based on *in vitro* performance, rather than solely on composition. This development was driven by the realisation that whilst excipient combinations that lead to useful self-emulsification are reasonably predicable, formulation performance for specific drugs is much more nuanced and the physicochemical properties of a drug alone are insufficient to inform *de novo* formulation design. As a result, preliminary formulation screening *via in vitro* dispersion and digestion testing is typically required to optimise LBF design. Work conducted under the Lipid Formulation Classification Scheme (LFCS) Consortium generated a large database describing the behaviour of Type I-IV formulations (containing a range of model drugs) during both dispersion in simulated GI fluids and on digestion under simulated intestinal conditions [59–64]. These data were then used to grade LBF based on *in vitro* performance in dispersion and digestion tests. In this lipid formulation—performance classification system (LF-PCS) [62], 'D' grade formulations were defined as those that resulted in rapid drug precipitation on formulation dispersion in model GI fluids. 'C′ grade formulations retained solvation capacity on dispersion but resulted in drug precipitation on initiation of digestion. 'B′ grade formulations retained solubilisation through dispersion and digestion challenges under normal conditions, but could be forced to precipitate under 'stressed' digestion conditions generated using *e.g.* high drug loads, increased dilution or longer time periods. 'A' grade formulations provided the most robust performance

and resisted precipitation under all dispersion and digestion challenges. In a more high-throughput approach, the use of simplified *in vitro* digestion models may also allow ranking of formulation performance and has been recently explored by multiple groups [65–67]. Notably, however, in all cases *in vitro* dispersion/digestion testing is based on the assumption that drug precipitation from lipid formulations *in vitro* provides an indication of hindered performance *in vivo*. This is not always the case, and the lack of an absorptive sink *in vitro* may overestimate the precipitation rate of some model drugs. Correlation between *in vivo* drug absorption and *in vitro* indicators of performance is discussed in more detail in Section 6.

3. Advantages of LBFs

LBF confer a range of biopharmaceutical, pharmaceutical and commercial advantages. Pharmaceutically, the ability to process LBF as solutions provides advantage for drugs with inherently low melting points (where solid dose forms may be impractical), for low dose compounds with potential content uniformity issues and for irritant and toxic compounds where dust control is a challenge. Commercially, LBF provide additional patient preference opportunities and in combination with a range of different finished dose forms (softgels, hard capsules or lipid multiparticulates) also provide a platform for evergreening and product life extension. The major advantages associated with the use of LBF, however, especially for b-r-o-5 compounds, are biopharmaceutical. These include changes to permeability, transit and metabolism, taste masking and for the great majority of reported applications, increases in intestinal solubility and avoidance of rate limiting dissolution. The latter are the major focal point of this review.

Lipids, and many of the other common components of LBF (surfactants and cosolvents), have been described to impact intestinal permeability, both *via* changes to passive permeability and *via* inhibition of efflux transporters. These effects have been extensively reviewed [68–72], and are not repeated in detail here. Briefly, a range of lipids (most notably medium chain fatty acids and lysophospholipids), surfactants (including bile salts) and cosurfactants have been shown to increase passive paracellular permeability by opening tight junctions [73,74], and to promote transcellular permeability by promoting membrane solubilisation and increasing membrane fluidity [70,75]. Conversely, apparent permeability may be reduced in the presence of colloidal species due to the formation of a competitive sink for solubilised drug and a reduction in thermodynamic activity [76–79]. Reductions in effective permeability have also been reported in the presence of polyethylene glycol and propylene glycol cosolvents [80]. More recently, attention has focussed on the ability of a range of surfactants and some endogenous species (including bile salts) to inhibit the activity of efflux transporters including p-glycoprotein [81,82], breast cancer resistance protein [83], multidrug resistance protein [84], and others. Almost all commonly employed surfactants have been suggested to show some inhibitory activity against efflux transporters, but perhaps the most compelling data has been generated with vitamin E TPGS and the Pluronic and Kolliphor surfactant families [79,85–87]. Surfactants are thought to inhibit efflux *via* changes to the structure and/or fluidity of membrane lipid domains leading to alterations in membrane protein/transporter structure, or by changes to transporter expression. Notably, although an increasing number of studies show compelling inhibitory effects *in vitro*, exemplification of efflux transporter inhibition *in vivo* is less widespread and often complicated by parallel effects on solubilisation. The effect of efflux transporters on *in vivo* absorption may also be limited in the presence of LBF by the attainment of luminal drug concentrations that are sufficiently high to saturate the transporter.

Lipid effects on metabolism are less well described, although some evidence of lipid and surfactant-mediated inhibition of presystemic metabolism is apparent, and again appears to be mediated by effects on enzyme activity and expression [88,89]. Lipids may also affect metabolism indirectly by changes to cellular and systemic drug distribution. For example, coadministration with lipids typically increases circulating lipoprotein levels, and for highly lipophilic drugs may increase drug association with plasma lipoproteins, reducing access to hepatic sites of metabolism. Drug abstraction into developing lipoproteins in the enterocyte has also been suggested to decrease enterocyte based metabolism [90]. However, effects on metabolism are hard to predict and increased lipoprotein association has been shown to both increase and decrease metabolism [91,92]. Furthermore, whether the quantities of lipid present in a typical LBF are sufficient to alter plasma lipoprotein levels to the point where changes in drug disposition are practically important is unknown [90].

Presystemic drug metabolism is also avoided by drugs that are trafficked to the systemic circulation *via* the intestinal lymph—a process that is supported by coadministration with lipids. Long chain lipids that are absorbed from the GI lumen into the enterocyte are re-esterified to triglyceride in the endoplasmic reticulum and subsequently assembled into lymph lipoproteins. The physical size of lymph lipoproteins (100–1000 nm) precludes diffusion across the continuous vascular endothelium and instead promotes selective uptake across the more permeable, and discontinuous, lymphatic endothelium. The intestinal lymphatics drain *via* the thoracic lymph directly into the systemic circulation and therefore circumvent the first pass metabolic events inherent in absorption *via* the portal blood. Drugs with high affinity for intestinal lymph lipoproteins typically have log D values greater than five and solubility in long chain triglycerides in excess of 50 mg/g [93], though exceptions have been reported [94]. These drugs may associate with lipoproteins in the enterocyte and can be delivered to the systemic circulation *via* the lymphatic system, resulting in reduced first pass metabolism [95,96]. The dependency of this absorption route on the presence of lipoproteins dictates that it is dependent on lipid re-esterification pathways, however, previous studies have shown that even a single capsule of lipid is able to support significant lymphatic transport in a dog [97]. Coadministration with lipids provides a source of lipids for lipoprotein assembly and this in turn provides the engine room for intestinal lymphatic lipid transport for drugs with inherent lipoprotein affinity. Oral lymphatic drug transport has been reviewed in more detail in a very recent review by Trevaskis et al. [98].

Finally, and perhaps most importantly, lipids and LBF significantly enhance the intestinal solubilisation of lipophilic PWSD. This increases exposure and in most cases also attenuates the large positive food effect commonly seen for PWSD after oral administration. These effects stem from integration of PWSD into the lipid digestion/absorption cascade and are described in greater detail in Sections 4 and 5.

4. Harnessing the potential of endogenous lipid digestion pathways

Endogenous lipid digestion and absorption pathways provide a highly dynamic and interactive conduit for drug delivery. Notably, dietary or formulation lipids (typically mixtures of glycerides), stimulate secretory processes in the GIT that profoundly alter the nature of the ingested lipid, resulting in altered GI conditions and significantly enhanced solvation capacity for the products of lipid digestion. In the context of drug delivery, these changes also (in the majority of cases) increase GI solvation capacity for a coadministered PWSD. Although many of the studies detailing GI response to lipid ingestion have been undertaken under post prandial conditions [99,100], and therefore under high lipid load, more recent studies have shown that lipid quantities of 2 g and lower are able to stimulate biliary secretion and elevate GI bile salt levels [101] as well as reduce gastric emptying [102]. Lipids and digestion products may also stimulate the ileal brake [103,104], thereby extending residence time in the proximal small intestine, ensuring maximal exposure to absorptive pathways in the duodenum and jejunum.

The biochemistry of lipid digestion has been described in detail on multiple occasions and will not be expanded on here. The interested reader is directed to the following reviews for a comprehensive overview of intestinal lipid absorption [2,105–109].

In the context of drug delivery, triglycerides are digested to diglycerides, monoglycerides and fatty acids by pancreatic lipases. Intercalation of these digestion products into colloidal biliary secretions generates a continuum of lipid reservoirs ranging from liquid crystalline phases at the oil:water interface, to multilamellar and smaller unilamellar vesicles, to mixed micellar species in bile salt rich areas of the GI fluids (Fig. 1) [108,110]. These lipid phases provide solubilising vehicles for poorly water-soluble lipid digestion products and are similarly able to solubilise PWSD. As dispersion and digestion proceeds, however, the changing nature of the colloids can result in changes in solvation capacity for PWSD, potentially resulting in supersaturation and precipitation of the drug as it transits the intestine. The complexity of the structural phases formed can be attributed to the unique physicochemical properties of hydrolysed lipid digestion products, molecules that are more amphiphilic than the parent triglyceride, but retain overall hydrophobicity. Hydration, swelling and self-assembly of these lipid digestion products results in the generation of liquid crystalline structures at the droplet interface and dispersion of these liquid crystal phases into intestinal fluids to form a range of structures, including lamellar, cubic and hexagonal phases, all of which have differing capacities to accommodate lipid digestion products or PWSD [100,111].

Evaluation of lipid phase behaviour *in vivo* is complex and is currently limited to *ex vivo* analysis. However, recent advances in computational models, microscopy and scattering techniques (particularly molecular dynamics (MD) simulations, atomic force microscopy (AFM), cryo-transmission electron microscopy (Cryo-TEM) and synchrotron small angle x-ray scattering (sSAXS)) are starting to provide much greater detail of the lipid nanostructures formed during digestion. Consistent with the general scheme described in Fig. 1, MD studies by Warren et al. have shown that as the water content of a LBF dispersion increases, the microstructure of the formulation changes from continuous phases, to reverse micelles to lamellar lipid dispersions

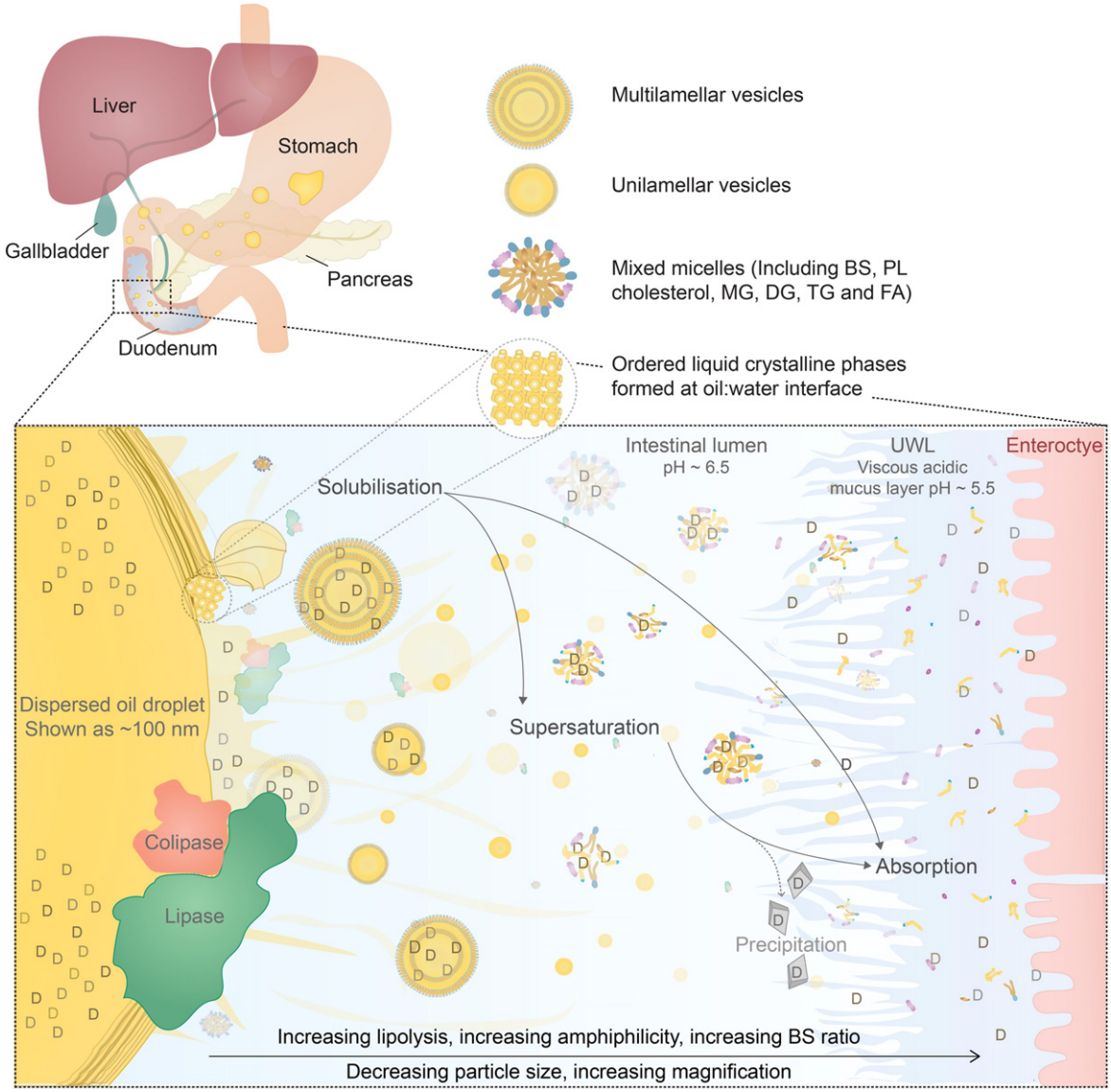

Fig. 1. Formation of a continuum of lipid reservoirs during triglyceride dispersion and digestion in the small intestine. Lipid species range from large digesting oil droplets to liquid crystalline phases, multi- and unilamellar vesicles, mixed micelles and finally to monomolecular species that are in equilibrium with the colloidal reservoirs and are absorbed at the enterocyte surface. Solubilisation of coadministered PWSD within these lipidic microdomains supports apparent drug solubility within the GIT, avoids traditional dissolution and typically enhances absorption. Figure adapted from Rigler et al. [112] and Porter et al. [2]. The following abbreviations are used: bile salts (BS), phospholipids (PL), monoglyceride (MG), diglyceride (DG), triglyceride (TG), fatty acid (FA), unstirred water layer (UWL).

with drugs situated at the interfaces of these structures [113]. Using TEM, Mullertz et al. have similarly shown that *ex vivo* post prandial human intestinal fluid (HIF) contains significant populations of micellar and vesicular species [114] and real-time SAXS studies of the *in vitro* digestion of milk have shown the transition of lipid structures from higher order liquid crystalline species to multi- and unilamellar vesicles in the presence of bile salts [115].

Digestion products (and coadministered PWSD) are ultimately solubilised into mixed micellar systems composed of fatty acids, monoglycerides, phospholipid, bile salts and cholesterol. These small, highly dispersed colloids provide an effective transport shuttle for hydrophobic species across the viscous unstirred water layer (UWL) to the absorptive surface of the intestine [116,117]. They also present a high surface area to promote free drug exchange between the solubilised reservoir and the GI environment [118]. Within the UWL, the slightly acidic environment results in protonation of solubilised fatty acid. This reduces fatty acid micellar affinity, increases saturation and thermodynamic activity and drives absorption of monomolecular digestion products [119]. In cases where lipid absorption is faster than drug absorption, and particularly where PWSD affinity for triglyceride digestion products is high, partitioning and absorption of lipids from intestinal mixed micelles appears to reduce micellar solvation capacity. This in turn may have the potential to generate transient drug supersaturation and effectively couple drug absorption to lipid absorption at the membrane [120].

Although a number of high profile papers have recently suggested the potential for absorption of oral particulates [121–123], evidence of absorption of intact lipid droplets including mixed micelles is less apparent. A recent study by Yeap et al. for example, revealed little difference in drug absorption from model colloids in the presence and absence of a range of inhibitors of putative lipid and particle uptake mechanisms. Subsequent studies examined drug absorption after administration in colloids with markedly different structure (vesicles *vs* micelles) but with matched thermodynamic activity and showed that drug absorption patterns correlated with thermodynamic activity and not structure [124]. The data are consistent with the suggestion that drugs that are solubilised in intestinal bile salt-lipid mixed micelles are absorbed *via* the free concentration that exists in rapid equilibrium with the solubilised reservoir, rather than *via* direct uptake of the colloid particle. These findings are in keeping with those reported by Shiau for the absorption of lipid digestion products [119].

To summarise, administration of PWSD in a LBF recruits a range of lipid processing pathways in the GIT that may be beneficial for drug absorption. These include enhanced initial GI solubilisation in intestinal colloids comprising mixtures of endogenous and exogenous solubilising components; improved transport across the UWL *via* micellar and mixed micellar transport shuttles; augmented absorption *via* increases in thermodynamic activity stimulated by lipid digestion and absorption, inhibition of intestinal efflux and potentially decreases in first pass metabolism (although these effects are largely driven by surfactants in LBF rather than lipids) and in some cases avoidance of first pass metabolism *via* stimulation of intestinal lymphatic transport [2,6,98].

A range of LBF have been employed in order to harness these advantages and their progression has, in large part, followed increases in our understanding of the mechanisms of lipid and drug absorption. These are described below.

5. LBF provenance; from solubilisation to self-emulsification to supersaturation

The ability of lipids to enhance the absorption of PWSD was first reported in the context of coadministration with lipids in food [125] and unsurprisingly, most PWSD show significant positive food effects [125–128]. However, coadministration with food as a means of enhancing drug exposure, whilst effective, is inherently variable as factors including culture, gender, age and health status all play major roles in dictating the type and quantity of food consumed as part of a meal. As a consequence, the potential to coadminister drugs with LBF formulations in order to match (and therefore circumvent) variability in food effects is highly appealing.

5.1. LBFs to improve PWSD solubilisation

To the best of our knowledge, the earliest published advances in translating the solubilisation advantage of the fed state to a formulated LBF were made by Bates and coworkers, whose seminal studies showed that emulsions comprising postprandial concentrations of bile salts and phospholipids significantly enhanced the solubilisation behaviour of griseofulvin *in vitro* [129]. The authors postulated that PWSD "may be absorbed by a mechanism involving preliminary solubilisation of the drug by bile salt micelles present in the small intestine" [10,129].

Realisation that many of the beneficial events of coadministration of PWSD with food could be simulated by coadministration with a LBF drove a number of early studies that explored the use of simple dietary lipid-based suspensions or solutions [9,15,22,23,130], emulsions [13,20,131] and even non-digestible lipids to aid drug solubilisation [22,23,27,132]. In most cases, these formulations significantly improved exposure when compared to oral administration of crystalline drug. Interestingly, despite digestible lipid solution and emulsion formulations evolving over the years to become more complex SEDDS and SNEDDS [24,29,57,133], non-digestible LBFs have not been similarly developed. This potentially reflects the early use of poorly dispersible mineral and paraffin oil formulations that are unlikely to further emulsify *in vivo* and therefore performed poorly [22,23,27]. Single component lipid formulations of digestible triglycerides transform *in vivo* to more amphiphilic lipids. These digested lipids intercalate into bile salt micelles to form highly dispersed, solubilised vehicles that diffuse effectively across the intestinal unstirred water layer (regardless of the dispersibility of the initial formulation). In contrast, non-digestible lipids cannot incorporate into lipid digestion pathways and must therefore be pre-emulsified to form a fine colloidal dispersion in order to facilitate diffusion across the UWL and drug absorption. When formulated to generate highly dispersed micellar solutions, however, non-digestible lipids may well be highly effective, since non digestible formulations or formulations where digestion is inhibited avoid the variability and potential loss of solubilisation associated with lipid digestion [134–136].

5.2. Transition to self-emulsifying LBFs

In vivo transformation of lipids from the bulk oil phase to nanostructured emulsions and finally micellar species signals the potential benefit of pre-dispersing LBF to promote progression through the intestinal processing pathway [10,16,17,116,129,137–139]. Although lipid emulsions can be effective delivery systems for PWSD [140], the stability, volume and patient compliance challenges of two-phase oral emulsion formulations limit commercial application. To overcome these challenges, the first major step forward in the evolution of LBF was the development of SEDDS formulations. SEDDS were adapted from the herbicide and pesticide industries where lipophilic actives have been formulated for many years as preconcentrates containing surfactants. These preconcentrates were shipped at lower cost (due to lower volumes) and then readily dispersed *in situ* to form a fine emulsion prior to spraying [18,141].

The first examples of the potential pharmaceutical utility of SEDDS were described by Groves in the early 1970s [18,141] and expanded in the 1980s by Pouton and colleagues [24,57,142]. The field subsequently accelerated (Fig. 2) with the clinical and commercial success of the Sandimmune and Neoral™ formulations of cyclosporine [41,143], the properties of which led to increased focus on the particle size of the dispersion formed on capsule rupture as a possible indicator of *in vivo* LBF performance.

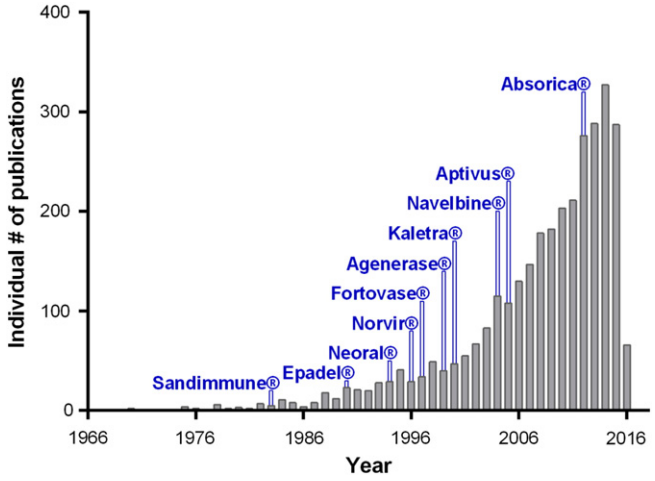

Fig. 2. Growth in number of LBF/SEDDS publications in PubMed, with marketed LBFs overlaid at corresponding dates of release onto market. Search terms: ((((oral) AND lipid formulation) OR SEDDS) OR SMEDDS) OR SNEDDS.

The Sandimmune Neoral formulation of cyclosporine was released in 1994 and was a reformulation of the original Sandimmune formulation, a relatively simple LBF that generated a crude lipid emulsion in the GIT on capsule rupture. Unlike the Sandimmune formulation, Neoral self-emulsified to form very small (sub-100 nm) droplets on capsule rupture and was arguably the first marketed SMEDDS formulation. Cyclosporine bioavailability was comparable or enhanced in the Neoral formulation, had the benefit of reduced food effects, reduced intersubject variability and was absorbed in liver transplant patients with disrupted biliary flow [54,144–149]. In many respects, Neoral has remained the 'gold standard' for self-emulsifying formulations ever since, although a causal link between particle size and *in vivo* bioavailability has never been proven and the relationship between the particle size of a dispersed lipid formulation and utility remains unclear. As described above, the action of lipolysis and the interaction of formulation components and lipid digestion products is likely to change the nature of any SEDDS formulation. Neoral contains digestible lipids and digestible surfactants [41] and might therefore be expected to undergo significant chemical and physical change *in situ*. Excipients contained within the formulation (or their digestion products) may also interact with drug transporters (or antitransporters) and metabolic enzymes. In the case of Neoral, Kolliphor RH40 is included in the formulation to generate finer emulsions, but has also been suggested to inhibit efflux transporters and metabolic enzymes [86]. The change in surfactant may also have improved solubilisation properties under conditions of intestinal digestion through changes in digestion rates or delaying drug precipitation. In this way, bioavailability may have been increased *via* mechanisms other than decreases in particle size (although effects on metabolism and efflux were specifically refuted by Choc et al., [150]). As such, direct correlation of the particle size of the dispersed Neoral formulation with its eventual utility is complex. However, it remains possible that the physicochemical properties of the formulation (including particle size) favourably impact downstream processing and in doing so, promote drug absorption.

In most cases, LBF digestion products have lower solvation capacities than the undigested parent formulation [35]. Where lipophilic drugs are predissolved in the undigested LBF, digestion therefore reduces solvation and increases drug saturation in the formulation. Ultimately, this may lead to drug supersaturation. This has two potential effects. Firstly, increasing saturation, and induction of supersaturation, may destabilise solubilised drug resulting in drug precipitation. Alternatively, increasing saturation will increase thermodynamic activity, effectively increasing the free concentration of drug in equilibrium with the solubilised reservoir and potentially increasing absorption. LBF development therefore involves a trade-off between maximising drug loading in the formulation, promoting moderate increases in saturation to drive increases in absorption and avoiding increases in supersaturation that are sufficient to promote drug precipitation. These aspects are discussed in more detail below.

5.3. Solubilisation versus supersaturation in LBF design

Coadministration of PWSD with lipids, or administration with a LBF typically enhances the overall solvation capacity of the GI fluids by creating additional, lipid swollen, colloidal species in which the solubility of a lipophilic PWSD is enhanced. However, to the best of our current understanding [119,124], these colloidal species are not absorbed intact and drug absorption occurs from the free concentration of drug that is in rapid equilibrium with the solubilised colloidal reservoir. Whilst the total solvation capacity of the GI fluids is therefore enhanced by the presence of mixed bile salt-phospholipid-lipid digestion product micelles, in the absence of supersaturation, the free concentration of drug is expected to be no greater than the drug solubility in intestinal fluid. Where drug is present as a saturated solution in intestinal colloids the free drug concentration in equilibrium with the solubilised reservoir is approximately equal to the saturated solubility of drug in the absence of the solubilising species (essentially the aqueous solubility). Under normal circumstances, therefore, although drug solubilisation in intestinal colloids increases effective solubility, the (free) concentration of drug, *i.e.* the concentration that drives absorptive flux, is not increased significantly above the aqueous drug solubility. In light of this limitation, but realising that LBF (or food) typically provide for significant increases in drug absorption, recent work has explored the hypothesis that lipid formulations intrinsically generate supersaturation during GI processing [35,120,151].

These studies suggest that for PWSD, there are three potentially complimentary routes by which supersaturation can be generated during LBF digestion. Firstly, solvation capacity is often lost during dispersion and digestion of drug loaded LBF [35,61,152]. Where this does not lead to immediate precipitation, supersaturation ensues. Secondly, absorption of lipid digestion products (that swell intestinal colloids and maintain drug solubilisation), also decreases drug solvation capacity and may lead to supersaturation in cases where lipid absorption is more rapid than drug absorption [120,153]. Thirdly, for ionisable PWSD, and in particular ionisable weak bases, supersaturation is also generated *via* the pH gradient encountered during gastrointestinal transit [154–156]. Thus, higher drug solubility is typically attained in the low pH environment of the stomach and this drops on transition to the more neutral pH conditions in the small intestine before encountering the acidic unstirred water layer. Supersaturated systems formed *via* one or more of these mechanisms have higher thermodynamic activity (and therefore higher absorption potential) when compared to colloids containing drug at (or below) equilibrium solubility. However, transition from a high-energy (supersaturated) state to the equilibrium point is energetically favoured, and therefore supersaturation also inherently predisposes systems to precipitation in order to re-attain equilibrium solubility. These precipitation events must first overcome the activation energy associated with crystal nuclei formation. Where the activation energy is high, crystallisation cannot proceed and a supersaturated metastable state may be maintained for a sufficiently long period to support drug absorption.

In the context of supersaturation on LBF dispersion, the nature of the formulation and the drug load are major drivers of precipitation. Formulations containing high drug loads and high proportions of amphiphilic excipients (surfactants and cosolvents) are most likely to result in rapid dissociation of water-soluble components, significant supersaturation and the greatest risk of drug precipitation [35,61]. Conversely, in formulations comprising more lipophilic components, excipient dissociation is less likely on dispersion and precipitation is often delayed for extended periods of time. In this case, drug concentrations in the GI tract may

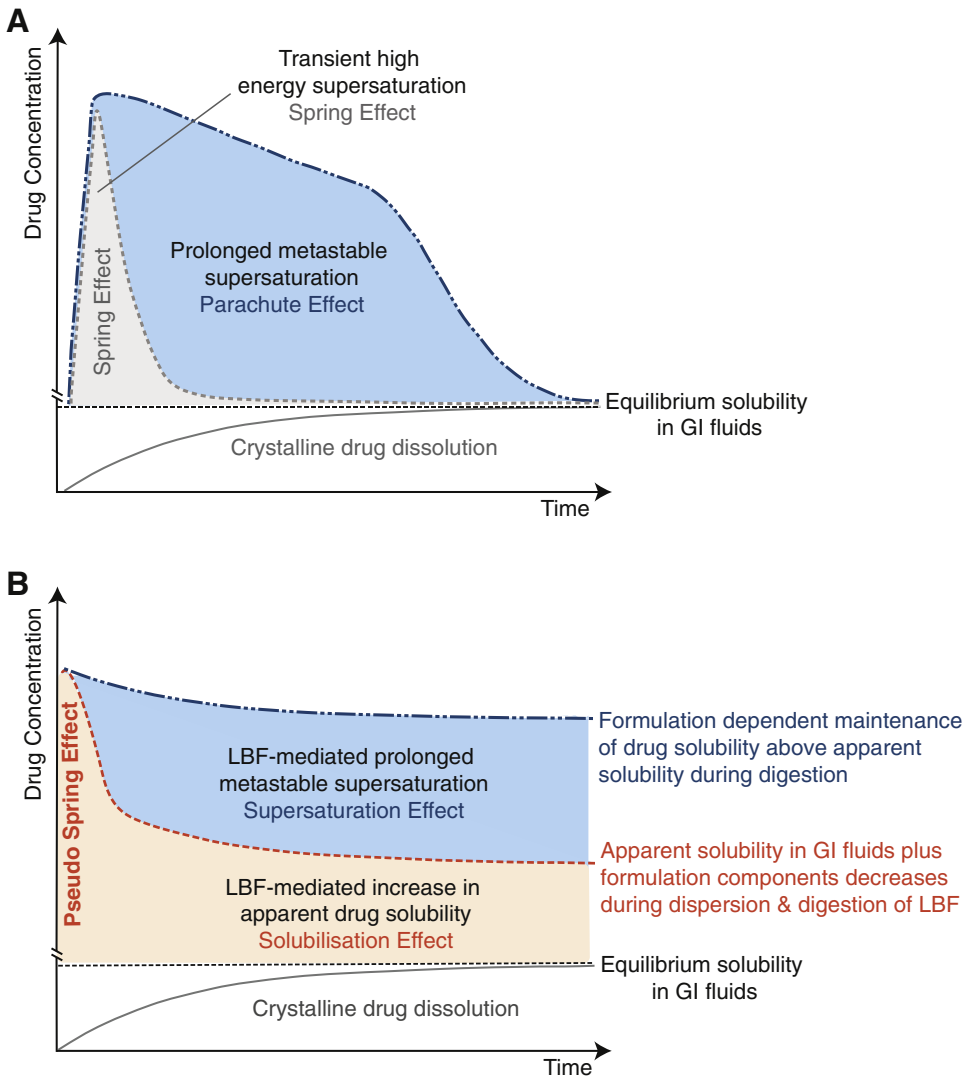

Fig. 3. Pathways to drug supersaturation during LBF dispersion and digestion. (A) The spring and parachute effect typically observed from solvent-shift or pH-shift methods used to evaluate drug supersaturation and precipitation kinetics or after dissolution of amorphous solid dispersions. Adapted from Browers et al. [157]. (B) The solubilisation and supersaturation effect generated by LBFs during formulation dispersion and digestion, adapted from Anby et al. [35].

be transiently elevated relative to apparent solubility in the GI fluids, leading to a metastable supersaturated state with higher thermodynamic activity (Fig. 3B).

On exposure to lipolytic enzymes, LBF are once again challenged and for digestible components, dissociation of more amphiphilic lipolysis products will likely reduce the solvation capacity of the colloid further, increasing supersaturation. Under these circumstances, the acyl chain length of the lipid(s) employed in the formulation may have a significant impact on the likelihood of drug precipitation. Lipase mediated digestion of long-chain lipids is slower than that of medium chain lipids [25,158], and the long chain digestion products produced appear to more readily maintain solvation capacity. Conversely, digestion of medium chain triglycerides is rapid and produces more water soluble digestion products, leading to higher supersaturation and increased potential for drug precipitation. Drug solubility in medium chain triglycerides is also often higher than that in long chain triglycerides allowing for significantly higher initial drug loadings in the medium chain based LBF [159]. Together, the higher drug loading and rapid digestion-induced reduction in solvation capacity in formulations based on medium chain triglycerides increases the likelihood of drug supersaturation and precipitation. The incidence of danazol supersaturation, triggered by digestion of medium chain triglyceride LBFs, has been described recently by Anby et al. [35]. In these studies, drug solubilisation or precipitation was related to the degree of initial supersaturation stimulated by dispersion and digestion. Thus, supersaturation above a certain threshold (in this case concentrations approximately 3 fold higher than equilibrium drug solubility in the colloidal species formed) resulted in precipitation. The concept that increases in the degree of supersaturation are likely to drive increases in the potential for precipitation is in agreement with the fundamental principles of nucleation and the realisation that nucleation rate and consequent precipitation is dependent on the extent of supersaturation [157,160]. Similar results (and similar threshold values) have subsequently been reported for a separate series of formulations of danazol, fenofibrate and tolfenamic acid [61], suggesting some degree of consistency in drug precipitation behaviour from SEDDS *in vitro* across a range of drugs and a range of different formulations.

Recognising that drug supersaturation may be a crucial driver for absorption, efforts have also focused on developing formulation strategies to stabilise or prolong drug supersaturation during the dispersion and digestion of LBF. This is analogous to the “spring and parachute” mechanisms of supersaturation generation and stabilisation widely described in the polymer literature (Fig. 3A). In the case of a LBF, dispersion and digestion events that promote supersaturation drive

either absorption or precipitation and form the 'spring', and polymeric formulation additives may be employed in an attempt to reduce drug precipitation (the "parachute") [35,161,162]. However, the choice of polymeric precipitation inhibitor is not trivial and requires careful balance of hydrophobicity, lipophilicity and compatibility with the PWSD under investigation [163,164].

In a somewhat related approach, formulations have also been developed that can slow or eliminate the digestion process, thereby attenuating decreases in solvation capacity, delaying initiation of supersaturation and reducing the drive towards drug precipitation [136,165]. Others have sought to allow precipitation but to promote precipitation of the amorphous forms of a PWSD, in the expectation that redissolution of drug will be enhanced from the high energy solid [34,153,166,167].

Finally, drug loaded lipophilic colloids must diffuse across the acidic environment of UWL in order to reach the absorptive membrane. Recent work has suggested that collapse of the colloidal structures in the acidic microenvironment of the UWL and stimulation of lipid absorption may result in drug supersaturation and promote drug absorption [120]. The process of absorption-triggered supersaturation has subsequently been modelled by Stillhart et al. [153]. In these studies the authors suggest that supersaturation may be achieved by absorption of lipid digestion products but that concurrent drug absorption (in this case with fenofibrate as a model drug) may prevent attainment of high degrees of supersaturation and therefore prevent initiation of drug precipitation [153]. The latter studies illustrate that interpretation of supersaturation patterns using *in vitro* methodologies should ideally consider the role of absorption *in vivo*. Even in the presence of significant precipitation *in vitro*, drug absorption *in vivo* may reduce the drivers of precipitation and allow ongoing absorption, especially for highly permeable compounds. This is described in more detail in Section 6 alongside a discussion of altered *in vitro* assessment models that include an absorption sink.

6. Progress in developing *in vitro in vivo* correlations for LBFs

The complexity of the relationship between formulation dispersion and digestion, and absorption of solubilised drug and lipids, has long complicated the establishment of robust *in vitro-in vivo* correlations (IVIVC) for LBF. Early attempts to develop formulation performance evaluation tools focussed on correlation of the particle size of the dispersed LBF to *in vivo* absorption. These studies postulated that the dispersed particle size of the formulation was critical to the generation of micellar phases of sufficiently small particle size and high surface area to facilitate rapid drug diffusion to, and absorption at, the luminal surface [24,141]. These correlations were stimulated in large part by the success of the Neoral formulation that dispersed to form sub 100 nm lipid droplets [41,143]. As described above, however, the relationship between particle size and utility is complex and the impact of particle size is much less evident in many other cases. For example, in a recent study correlating droplet size with oral bioavailability, whilst Nielsen et al. reported a trend towards faster absorption with a SNEDDS compared to a SEDDS formulation of probucol, the 10-fold reduction in particle size (45 and 458 nm for SNEDDS and SEDDS respectively) did not result in significant differences in bioavailability [168]. In this case digestion (rather than particle size) was suggested to be have a greater influence on drug solvation and bioavailability [168]. Poor correlations have also been described between dispersion particle size and *in vivo* exposure for LBF of danazol and halofantrine [136,169]. Nonetheless, despite a lack of consistent correlation between dispersion particle size and bioavailability, the generation of a refined, uniform emulsion after dispersion or digestion of a LBF is generally considered desirable since, at the very least, there is evidence that these systems can reduce inter- and intra-subject variability after oral administration [57,143,147,168].

6.1. Does in vitro LBF dispersion data correlate with in vivo absorption?

Dispersion testing using more formal dissolution testing apparatus (rather than simple assessment of particle size) is commonly used to evaluate formulation performance *in vitro*. Traditional USP-like dissolution methods or modifications thereof, are a useful means to evaluate the dispersion/self-assembly properties of self-emulsifying LBF, and also provides an assessment of the likelihood of drug precipitation on LBF dilution. Dispersion tests are generally conducted in physiologically relevant media (simulated gastric/intestinal fluid) using USP type II apparatus (rotating paddles) [37,38,135,170,171], or at lower volumes in a pH stat apparatus prior to lipolysis testing [40,61]. Although there are exceptions, maintenance of drug solubilisation on formulation dilution and emulsification appears to be an important driver of formulation performance. Thus, formulations that show evidence of drug precipitation on formulation dilution/dispersion appear more likely to result in poorer *in vivo* drug exposure [172]. The precipitation phenomenon may be observed visually or quantified by separation of the solid precipitate from solubilised drug in the aqueous phase of the dissolution/dispersion media [40,61]. Real time characterisation of drug solubilisation and precipitation during both dissolution and digestion is becoming increasingly popular and provides important information on both the solubilisation and precipitation kinetics of PWSD. Surface UV imaging, in-line raman spectroscopy and focused beam reflectance are some of the techniques that have been used by Kuentz and coworkers to generate real time drug solubilisation and precipitation data [36,151,152] and have been recently reviewed in the context of small scale dissolution and precipitation testing [173].

To evaluate the potential for IVIVC based on the available literature, Figs. 4, 6, 7, 8 and 9 have been generated for this review by digitization of published data using Engauge (version 4.1) open source digitising software. The potential for (usually) linear correlations were subsequently calculated using GraphPad prism version 6.07. In all instances, the nomenclature of the x axes reflects the original nomenclature employed to describe the reported *in vitro* data. In some cases, therefore the resultant plots have different terms to describe PWSD solvation (% in solution, % dispersed, % drug in digestion aqueous phase (AP), digestion AP concentration, Supersaturation (S) during digestion). The nomenclature of the formulations in each IVIVC plot has been retained as published. Where full *in vitro* solubilisation profiles were available, data have been plotted as the AUC of the profile, calculated using the linear trapezoidal rule. Otherwise, data were plotted using reported solubilisation values at a fixed time point. *In vivo* AUC data was reported to t_{last}. Datasets that did not specify the time range used to calculate AUC do not have an AUC time range specified on the y axis.

Do and colleagues utilised *in vitro* dispersion data to examine the performance of four fenofibrate LBFs and a micronized crystalline formulation and compared this data to *in vivo* exposure after administration to Wistar rats (Fig. 4A) [174]. In these studies, the authors reported that the utility of dispersion experiments extended only to ranking LBF above the micronized formulation but did not discriminate between LBF administered in the fasted or fed state. Consistent with their suggestion, re-plotting the area under the curve of the *in vitro* solubilisation data shows no correlation between *in vitro* solubilisation and *in vivo* absorption (Fig. 4A). Indeed, in this case, a slight trend towards reduced *in vivo* exposure with increasing *in vitro* solubilisation is apparent for the LBF. In another study with fenofibrate, Griffin and coworkers assessed *in vitro* dilution and dispersion behaviour for three LBFs and attempted to correlate with *in vivo* exposure in landrace pigs (Fig. 4B), [170]. In this instance, the dispersion/release tests again did not discriminate between formulations since no drug precipitation was observed from any of the formulations during dispersion testing, but in this case the data were consistent with a lack of significant differences in oral bioavailability *in vivo*. Replotting the reported data in Fig. 4B confirms the author's suggestions and shows similar performance from all three LBF. More encouragingly, Larsen and colleagues

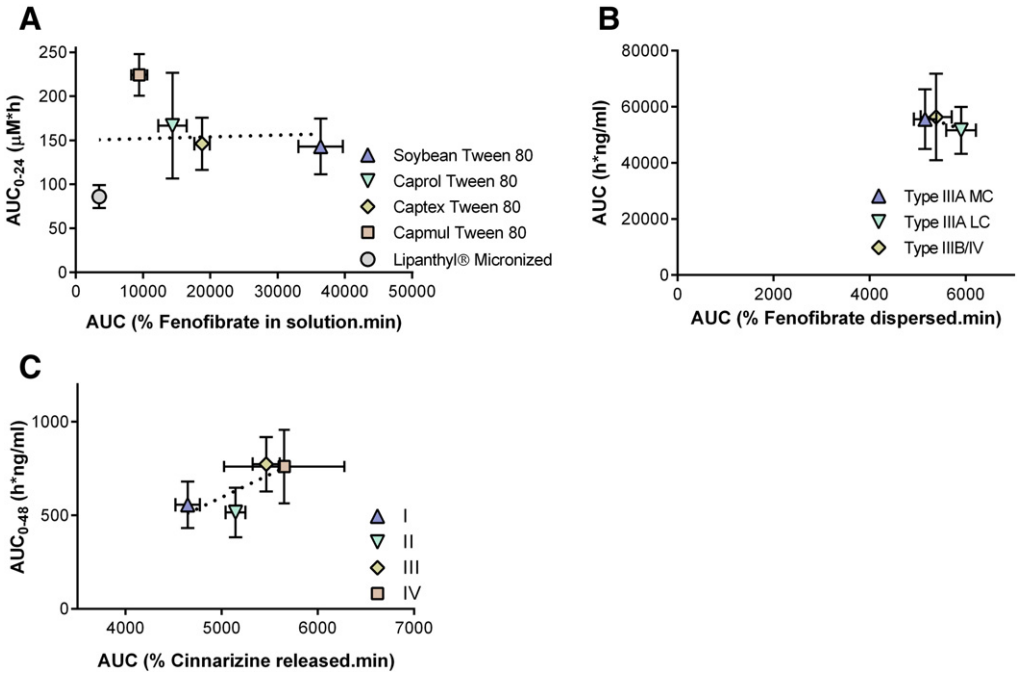

Fig. 4. *In vitro in vivo* correlation of drug absorption and drug solubilisation after formulation dispersion. (A) 'Dissolution' AUC (AUC of % fenofibrate 'in solution' (*i.e.* total fenofibrate in free solution plus solubilised in colloidal species) after 250 min) plotted against the AUC_{0-24h} of the plasma *Vs* time profiles after administration of four LBF formulations and one encapsulated crystalline formulation (Lipanthyl® micronized) to wistar rats. Data replotted from [174]. (B) 'Dispersion AUC' (AUC of % fenofibrate dispersed/released (*i.e.* total fenofibrate in free solution plus solubilised in colloidal species) after 120 min) plotted against the AUC of the plasma *Vs* time profiles after administration of three LBF formulations to landrace pigs. Data replotted from [170]. (C) 'Dispersion AUC' (AUC of % cinnarizine released (*i.e.* total cinnarizine in free solution plus solubilised in colloidal species) after 60 min) plotted against the AUC_{0-48h} of the plasma *Vs* time profiles after administration of four LBFs to Labrador dogs. R^2 0.64. Data replotted from [37].

reported a rank order correlation between solubilisation *in vitro* and *in vivo* absorption for the weak base cinnarizine from four LBFs [37]. These data are reproduced as reported in Fig. 4C, although the correlation of R^2 0.64 suggests only a moderate ability to discriminate between formulations based on dispersion data alone.

The studies outlined in Fig. 4 show that generation of IVIVC from dispersion data is not only a function of the drug investigated but is also a function of the formulations dosed. Notably, fenofibrate appears to benefit from most solubilisation strategies and to provide good exposure despite *in vitro* data that in some cases shows significant drug precipitation.

6.2. Does in vitro LBF digestion data correlate with in vivo absorption?

Maintenance of PWSD solubilisation on dispersion is expected to be important for the performance of a SEDDS formulation as precipitation prior to reaching the duodenum is likely to limit bioavailability for PWSD by reintroducing the need for dissolution from the solid state. However, it is not the only significant challenge to formulation viability, and drug precipitation may also occur on formulation digestion. Subjecting the formulation to an *in vitro* digestion challenge test has therefore become more common and allows some estimation of the GI disposition of drug after the lipid carrier has been hydrolysed.

In vitro digestion testing of LBF was pioneered in the 1980s and in general, lipolysis methods have not changed significantly since their inception by Reymond and Sucker and optimization by Alvarez and Stella [25,175,176]. In these models, *in vivo* lipid digestion conditions are mimicked *in vitro* by addition of a source of lipase enzymes (typically porcine pancreatin) into a temperature controlled reaction vessel containing the LBF dispersed in bile salt lecithin mixed micelles [30,177]. During lipid digestion, the fatty acid released reduces the pH of the reaction medium and is continuously monitored by a pH probe coupled to a pH stat system (Fig. 5). The fatty acid is titrated by autoburette against a known molarity of sodium hydroxide to both maintain pH and allow (indirect) quantitation of the rate and extent of digestion by assumption of stoichiometric titration.

In vitro lipolysis tests provide a relatively simple representation of the complexity of intestinal digestion conditions and the outcomes from *in vitro* lipolysis experiments are contingent on a number of experimental parameters that must be tightly controlled to provide reproducible data. These include buffer capacity [178], enzyme activity [64,65,176], bile salt and phospholipid concentration [60,178], stirring rate, calcium concentration [64,177,179] and pH. Relatively minor differences in the methods used for lipolysis studies can have a significant impact on formulation performance *in vitro*. In the context of LBF development, variability in the *in vitro* methodology employed therefore often prohibits facile inter-laboratory comparison of data sets.

To address this, attempts to standardise *in vitro* lipolysis procedures have been made to facilitate cross comparison of datasets between laboratories [59–64]. In parallel, the development of physiologically relevant media that better reflect the composition of human intestinal fluids remains an area of intensive research [180–182] and has been recently reviewed by Bergström and colleagues [183] and by Fuchs and Dressman [184].

The main advantage of *in vitro* lipolysis testing for analysis of drug-loaded LBF is that it allows estimation of drug distribution between the phases of a digesting formulation *i.e.* determination of the patterns of drug partitioning between an aqueous micellar phase containing bile salt, phospholipid, cholesterol and digested lipids (the 'aqueous phase' or AP); a solid precipitate (the 'pellet phase') or an undigested or partially digested oil phase (the 'oil phase') (Fig. 5).

Consistent with the rationale for formulation dispersion tests, increasing or maximising aqueous phase drug concentrations (and minimising drug precipitation) is expected to maximise the likelihood of robust drug absorption from LBF *in vivo*. It is increasingly apparent, however, that this is a conservative indicator. Thus, although formulations that provide for good post-digestion solubilisation almost always provide for good *in vivo* exposure, in some cases good drug absorption is possible from formulations where drug precipitation *in vitro* is

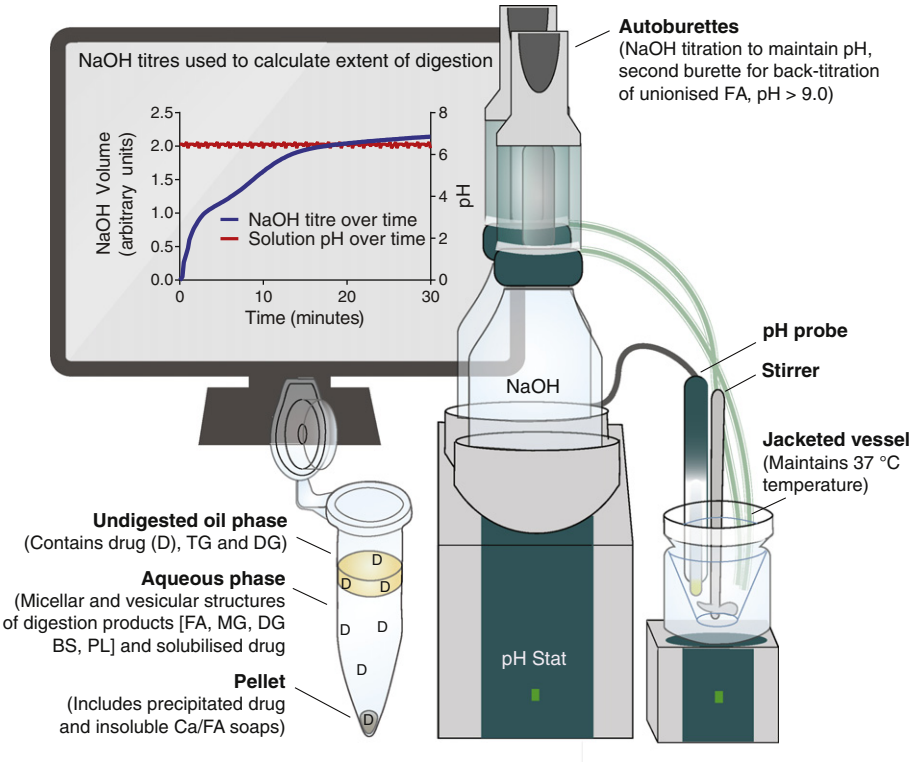

Fig. 5. Experimental model for *in vitro* assessment of lipid-based formulations. Abbreviations used are; sodium hydroxide (NaOH), drug (D), triglyceride (TG), diglyceride (DG), fatty acid (FA), monoglyceride (MG), bile salt (BS), phospholipid (PL), calcium (*Ca*).

significant. Digestion testing therefore provides a useful screening tool to identify formulations with a strong chance of good absorption *in vivo*—but may eliminate some formulation that might otherwise provide for useful absorption.

Analogous to the reanalyses presented in Fig. 4 for *in vitro* dispersion data, Fig. 6 shows a summary of published *in vitro–in vivo* correlation data using *in vitro* digestion tests.

From Fig. 6, it can be seen that IVIVC for differing LBFs is highly drug-dependent. Of the eight drugs examined four (50%) appear to show some degree of correlation (Fig. 6A, C, E and F) and four do not have strong correlations (Fig. 6B, D, G and H). Of those that show reasonable correlation between *in vivo* exposure and digestion AP solubilisation, two exhibit non-linear correlations (Fig. 6E and F) and two show clear linear correlation with the AP drug solubilisation after digestion (Fig. 6A and C). There is no one obvious physicochemical drug property (summarised in Table 2) that can be used to explain the likelihood of useful correlation, with the obvious exception of torcetrapib and its structural analogue CP-532,623 that as expected show similar correlation behaviours [40]. The two drugs that exhibit linear correlation (danazol and griseofulvin) are high melting point compounds with similar and relatively high logP. In contrast, dexamethasone which has a comparable melting point but lower logP appears to have formulation independent absorption [38,39] (possibly due to intrinsically higher aqueous solubility relative to dose). The logP of the drug alone clearly does not indicate the likelihood of IVIVC as danazol, cinnarizine and fenofibrate all have similar logP values but are starkly different performers *in vitro* and *in vivo* [37,38,170]. The data therefore suggest that simple physicochemical comparisons are not enough to deconvolute formulation performance *in vitro* and *in vivo*, although better correlations are apparent with poorly soluble, higher melting point drugs that presumably result in crystalline precipitates with slower rates of re-dissolution. Other physicochemical properties such as the glass-forming ability (*i.e.* the ability to form an amorphous state) may have an impact on the likelihood (or otherwise) of precipitation, the physical form of the precipitate (amorphous *vs* crystalline) and the redissolution kinetics of the PWSD in the lumen, but to this point have not been examined in detail in this context. To this end, early identification of PWSD glass forming potential through the use of computational models may allow more judicious selection of formulation approaches, and complementary *in silico* permeability modelling may help to further predict the behaviour of PWSD formulated in LBF. For an overview of the computational tools used to predict druggability, solubility, permeability and glass forming ability, the reader is referred to the review by Bergström and colleagues in this issue [186].

6.3. Do in vitro indicators of supersaturation improve IVIVC?

Although maintaining drug solubilisation, and reducing drug precipitation is a likely contributor to the absorption of PWSD from LBFs, the supersaturation events that precede precipitation are also important. Direct measurement of the free drug concentration (*i.e.* the fraction readily available for absorption) in a dynamic digestion environment is difficult as separation of free drug from solubilised drug is complex and takes finite time. As such the data obtained are unlikely to be able to track rapid changes in free and solubilised drug concentrations. A crude estimate of free concentration, however, may be made from measurement of the apparent solubility of the drug in a blank digested formulation and comparison of these values with the data obtained at the same timepoints during dynamic lipolysis experiments. This ratio provides an indication of the degree of saturation or supersaturation and therefore an indication of thermodynamic activity and free concentration. Supersaturation in the context of GI drug delivery, and with reference to LBF has been reviewed by both Bevernage and Williams respectively [187,188].

Despite increasing awareness of the importance of supersaturation in drug absorption from oral LBFs [7], relatively few *in vivo* studies have been published with accompanying correlations to

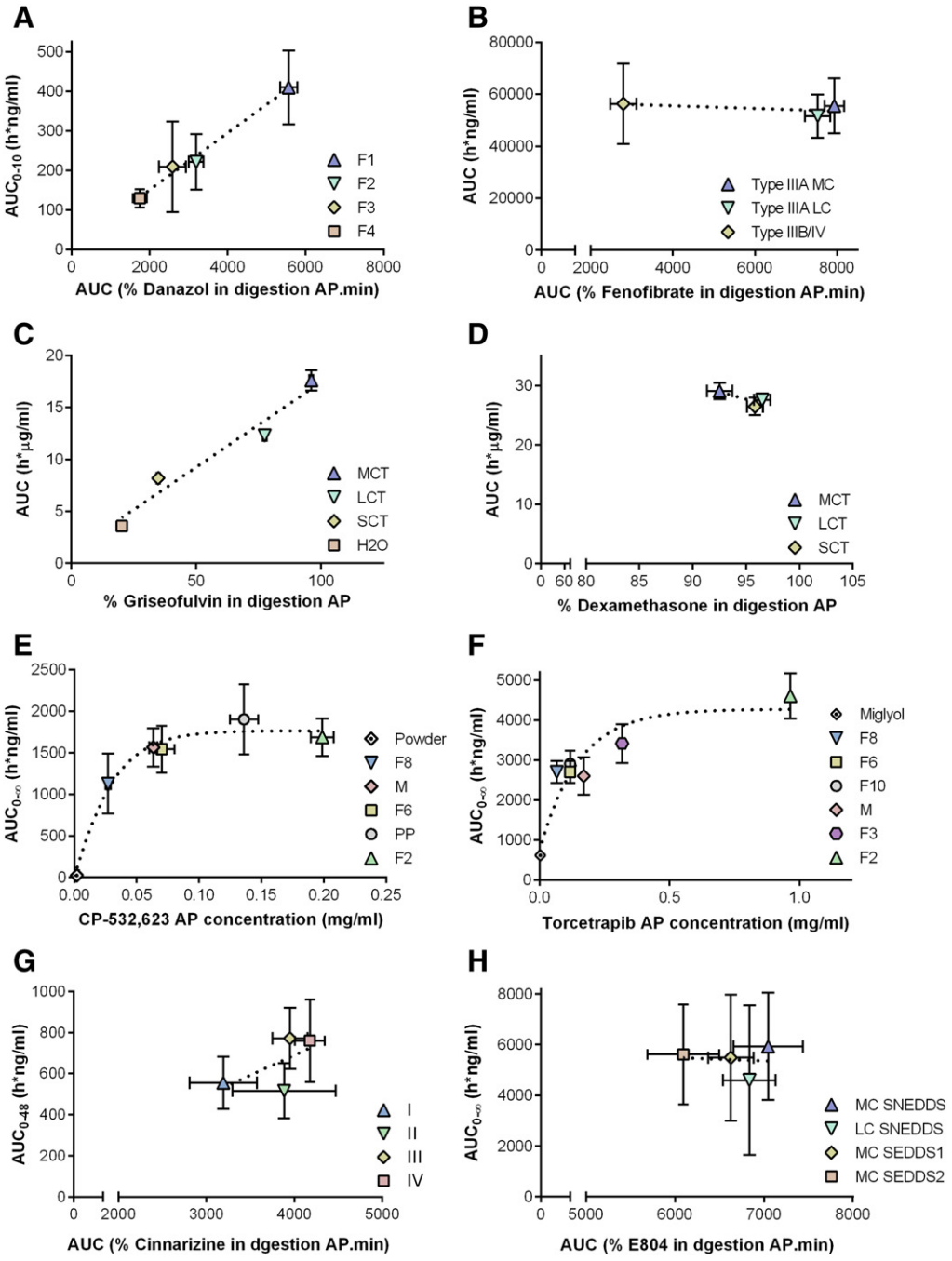

Fig. 6. *In vitro in vivo* correlation data of *in vivo* drug exposure and drug solubilisation after *in vitro* formulation digestion. (A) 'Digestion AP AUC' (AUC of % danazol in digestion AP after 60 min) plotted against the AUC_{0-10h} of the plasma *Vs* time profiles after administration of four LBF formulations to male beagle dogs, R^2 0.99. Data replotted from [38]. (B) 'Digestion AP AUC' (AUC of % fenofibrate in digestion AP over 80 min) plotted against the AUC of the plasma *Vs* time profiles after administration of three LBF formulations to landrace pigs, R^2 0.32. Data replotted from [170]. (C) % griseofulvin solubilised in digestion AP after 30 min *in vitro* digestion plotted against the AUC of the plasma *Vs* time profiles after administration of three LBF formulations and one suspension to male wistar rats, R^2 0.98. Data replotted from [39]. (D) % dexamethasone solubilised in digestion AP after 30 min *in vitro* digestion plotted against the AUC of the plasma *Vs* time profiles after administration of three LBF formulations to male wistar rats, R^2 0.68. Data replotted from [39]. (E) CP-532,623 concentration in digestion AP after 30 min *in vitro* digestion plotted against the $AUC_{0-\infty}$ of the plasma *Vs* time profiles after administration of five LBF formulations and a powder-in-capsule control to male beagle dogs. 4-PL curve fit (dotted line) shown as published, linear regression analysis ($R^2 = 0.60$, line not shown) was also conducted to allow comparison to other data in this figure. Data replotted from [40]. (F) Torcetrapib concentration in digestion AP after 30 min *in vitro* digestion plotted against the $AUC_{0-\infty}$ of the plasma *Vs* time profiles after administration of seven LBF formulations to male beagle dogs. 4-PL curve fit (dotted line) shown as published, linear regression analysis ($R^2 = 0.59$, line not shown) was also conducted to allow comparison to other data in this figure. Data replotted from [40]. (G) AUC of % cinnarizine solubilised in digestion AP after 60 min *in vitro* digestion plotted against the AUC_{0-48h} of the plasma *Vs* time profiles after administration of four LBF formulations to Labrador dogs, R^2 0.39. Data replotted from [37]. (H) AUC of % E804 solubilised in digestion AP over time plotted against the $AUC_{0-\infty}$ of the plasma *Vs* time profiles after administration of four LBF formulations to wistar rats, R^2 0.02. Data replotted from [185]. Data were extracted as described in Section 6.1.

supersaturation data during dispersion and digestion. This may be in part a function of the complexity of the measurement methods that are used to describe supersaturation during *in vitro* testing. Data analysis is also complicated by the potential to measure supersaturation at a fixed time point or to use an AUC measurement to estimate total supersaturation over a time period. Anby et al. first reported the impact of digestion-mediated supersaturation in relation to LBF and developed methodologies to quantify the degree of supersaturation over time [35]. In these studies, the AUC of drug solubilisation kinetics during digestion was divided by the AUC of drug apparent solubility in blank digested

Table 2
Physicochemical properties of the PWSD evaluated for IVIVC.

	Molecular weight	clogP[a]	clogD[a]	Melting point	Rule of 5 violations	Correlation[b]
Danazol	337.5	4.94	4.94 (pH 7.0)	225 °C	0	L
Fenofibrate	360.8	5.80	5.80 (pH 7.0)	81 °C	0	N
Griseofulvin	352.8	2.01	2.01 (pH 7.0)	219 °C	0	L
Dexamethasone	392.5	2.03	2.03 (pH 7.0)	262 °C	0	N
CP-532,623	598.5	6.77	6.77 (pH 7.0)	112 °C[c]	2	NL
Torcetrapib	600.5	6.64	6.64 (pH 7.0)	54 °C	2	NL
Cinnarizine	368.5	5.03	4.69 (pH 7.0)	118 °C	0	N
E804	365.4	1.65	1.65 (pH 7.0)	263 °C[d]	0	N

[a] clogP and clogD calculated values from ACD labs software V11.02, retrieved from SciFinder.
[b] Correlations classified as L—linear, NL—non-linear, N—no correlation.
[c] Mean (n = 3). Measured by hot-stage optical microscopy.
[d] Predicted melting point from US Environmental Protection Agency's EPISuite™ software. retrieved from RSC Chemspider.

formulation during the same time period and defined as the extent of supersaturation (S). This time resolved supersaturation ratio allows quick comparison of the precipitation potential of LBFs over the course of a dispersion/digestion experiment and is the method utilised to calculate supersaturation (S) in Figs. 7 and 8. However, this method requires *in vitro* dispersion and digestion data at multiple time points. In many published studies, full time-resolved profiles are not available and in these cases, supersaturation is typically calculated at a single time point by comparing the measured concentration in the aqueous phase at *e.g.* 30 min post digestion with equilibrium solubility in blank digested colloids at the same point. This method generates a supersaturation ratio defined as SR [60,189] to differentiate the calculation method from the time-resolved supersaturation (S) generated *via* AUC comparison as described above [35].

Gao and colleagues similarly described the development of supersaturable SEDDS (s-SEDDS) as early as 2003 [161,190]. In an approach analogous to the spring and parachute effect of prolonging metastable supersaturation, (as depicted in Fig. 3A), s-SEDDS formulations were prepared by incorporation of hydroxypropyl methyl cellulose (HPMC) as a polymeric precipitation inhibitor (PPI) into the SEDDS [190]. Formulation performance was evaluated using an *in vitro* precipitation (dispersion) test, where formulations were diluted in buffer at pH 2 to mimic release conditions in the stomach. Concentrations of the drug PNU-91,325 were defined as the total concentrations of both free drug and drug associated with the colloidal species formed on formulation dispersion. Addition of HPMC to the formulations retarded drug precipitation *in vitro* and subsequent *in vivo* administration of selected formulations showed that absorption from s-SEDDS was greater than from formulations that did not include a PPI. The IVIVC of the AUC of the reported *in vitro* precipitation (dispersion) data has been plotted against *in vivo* AUC in Fig. 7A using the methodology described in Section 6.1. Clear correlation between the dispersion AUC and *in vivo* absorption can be seen (R^2 0.90).

Interestingly, the authors reported the equilibrium solubility of the free drug in buffer, as well as dynamic drug precipitation data. These datasets allow calculation of a time resolved supersaturation ratio (achieved by calculating an AUC from the equilibrium solubility of free drug in solution (6 μg/ml) assuming that it remains unchanged for the duration of the three hour precipitation experiment). The resultant supersaturation ratios (S) are plotted against the reported *in vivo* AUCs in Fig. 7B. These data show the same trends as the solubilisation data in Fig. 7A since they are essentially the same data divided by a constant (the solubility AUC). Notably, unlike the supersaturation (S) or supersaturation ratio (SR) calculations described above, the methods employed by Gao et al. defined supersaturation relative to equilibrium solubility of drug in buffer alone, rather than drug solubility in the dispersed formulation. This leads to much larger values for the supersaturation ratio. Furthermore, the s-SEDDS and Tween formulations are likely to be digested *in vivo* leading to changes (potentially decreases) in solvation capacity. The combined effects of using equilibrium solubility in buffer and the absence of a digestion step, in this instance, probably overestimate absolute supersaturation, however the relative differences across formulations are likely to be similar. Subsequent studies by Gao et al. showed that the significant *in vitro* performance advantage of s-SEDDS extended to faster absorption of AMG 517 from formulations stabilised by PPIs, but in this case overall exposure in Cynomolgus monkeys was comparable to a conventional SEDDS [191].

In a similar manner to the studies by Gao et al., the supersaturation measures defined by Anby et al. [35] were employed to investigate the potential relationship between supersaturation during formulation digestion and drug absorption for lipid formulations containing PPIs. In this case the PPI significantly inhibited *in vitro* drug precipitation, thereby increasing supersaturation, but this did not lead to large changes in *in vivo* exposure in beagle dogs [35]. As such, no correlation was evident between supersaturation and absorption (Fig. 8A). In a more recent study using a rat model of digestion [136], and enhancing drug solubilisation by slowing MCT LBF digestion (therefore reducing supersaturation), improved drug solubilisation appeared to promote drug absorption after administration to Sprague Dawley rats. This led to a marginally negative correlation between drug exposure and supersaturation, Fig. 8B [136]. In contrast, Fig. 8C shows good correlation between

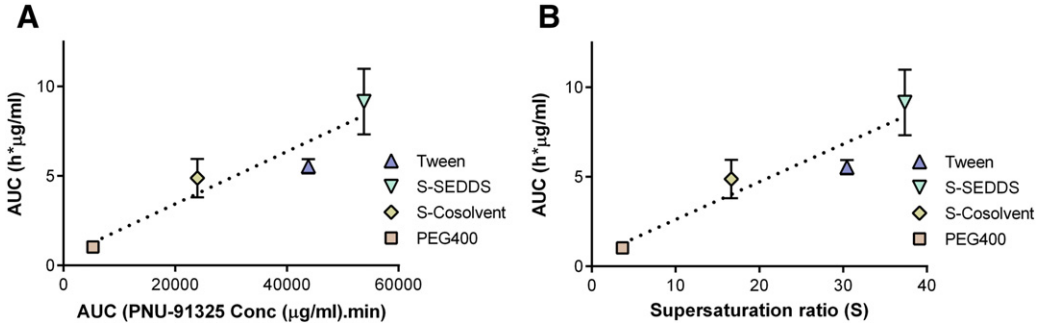

Fig. 7. IVIVC of *in vivo* exposure (y axis) of PNU-91325 with (A) AUC of *in vitro* drug concentration.min (R^2 0.90) and (B) *in vitro* supersaturation ratio (S) during *in vitro* precipitation tests (R^2 0.90). Supersaturation ratio (S) calculated using the method proposed by Anby [35]. Data recalculated and plotted from [190].

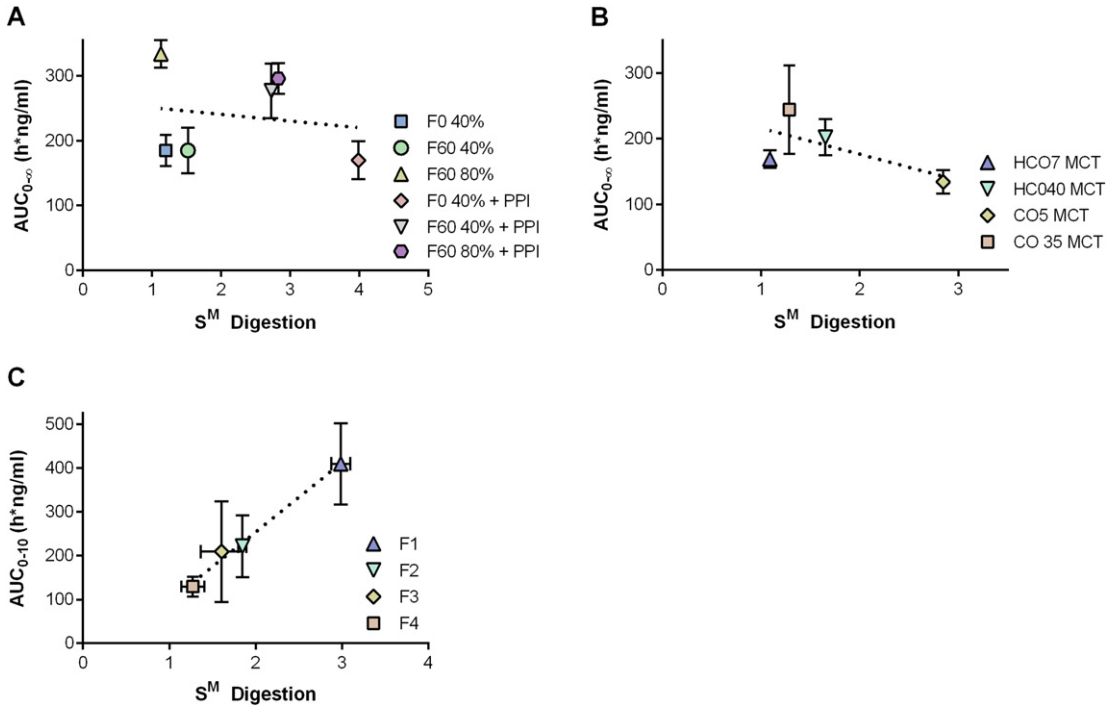

Fig. 8. IVIVC of danazol absorption and *in vitro* supersaturation (S) during digestion calculated using the method proposed by Anby et al. [35]. (A) Supersaturation ratio (S) in digestion AP plotted against the AUC of the plasma *Vs* time profiles after administration of four MCT LBF formulations to male beagle dogs. PPI = polymeric precipitation inhibitor. Data replotted from [35]. (B) Supersaturation ratio (S) in digestion AP (rat model) plotted against the AUC of the plasma *Vs* time profiles after administration of four MCT LBF formulations to Sprague Dawley rats. HCO = PEGylated hydrogenated castor oil surfactants. CO = PEGylated castor oil surfactants. Data replotted from [136]. (C) Supersaturation ratio (S) in digestion AP plotted against the AUC of the plasma *Vs* time profile after administration of four LCT LBF to male beagle dogs, R^2 0.99. LCT content of formulations decreases from F1 to F4. Data replotted from [38,192].

supersaturation during digestion and *in vivo* exposure for a series of long chain triglyceride based LBF administered to dogs. In this instance, the data from Cuine et al. was plotted against supersaturation data subsequently generated by Porter et al. [192]. A strong positive correlation of R^2 0.99 is evident for this data set. Interestingly, the three data sets in Fig. 8 have been generated from the same model drug (danazol) and from our research group, but do not appear to provide a consistent indication of utility. The data serve to underline the complexity of the relationship between digestion, supersaturation and drug absorption. It is evident that under some circumstances increases in saturation may promote absorption due to increased thermodynamic activity (Fig. 8C), whereas in others, increased supersaturation appears to reduce drug absorption, presumably since supersaturation is likely to be an indirect indicator of the likelihood of precipitation (Fig. 8B). Balancing the beneficial effects of supersaturation (increased thermodynamic activity) against the potentially negative effects (increased likelihood of precipitation) and identifying appropriate *in vitro* models to reflect these changes remains a challenge.

7. Recent developments in improving *in vitro* methods for LBFs

The disconnect in IVIVC that is evident in some cases (and exemplified above) has meant that efforts have redoubled to improve the physiological relevance of *in vitro* digestion tests. For example, gastric lipolysis is not typically addressed in the *in vitro* lipolysis model [37–39], and indeed, all the data shown in Figs. 6 and 8 was generated under simulated intestinal lipolysis conditions only. The development of gastric and intestinal lipolysis models may therefore provide a more comprehensive representation of the *in vivo* digestive environment [63,193–195]. To probe this possibility, Thomas and coworkers included gastric lipolysis in a recent evaluation of LBF of fenofibrate and attempted to correlate with *in vivo* exposure in Göttingen minipigs [189]. However, these data (Fig. 9) also failed to show a correlation between *in vitro* solubilisation and *in vivo* absorption. Three independent studies (Figs. 4B, 6B and 9) therefore suggest that fenofibrate exhibits robust *in vivo* absorption irrespective of formulation dosed, species used or whether dissolution or dispersion *in vitro* data is used to generate the IVIVC. In all cases the LBFs show improved absorption when compared to crystalline drug [170,174,189]. It seems likely that the lack of correlation in these studies reflects the choice of compound (fenofibrate), which appears to be well absorbed from most solubilised formulations (possibly due to very high permeability), rather than the intrinsic utility of the model evaluated.

The nature/form of the precipitate that is generated on LBF digestion may also impact on the likelihood of effective IVIVC. Thomas et al. recently reviewed the solid state characterisation techniques used to evaluate the nature of drug precipitates during *in vitro* digestion [196] and as such these will not be discussed in detail here. The most popular solid state characterisation techniques used in conjunction with *in vitro* digestion tests are summarised in Fig. 10. Precipitation of PWSD in the amorphous form might be expected to assist in drug redissolution

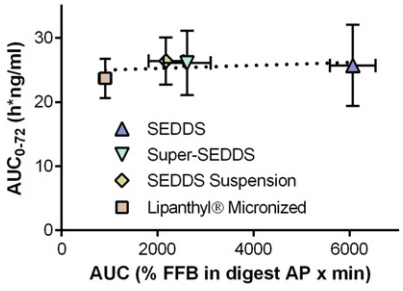

Fig. 9. *In vitro in vivo* correlation of drug absorption and *in vitro* digestion data. (A) AUC of % fenofibrate in digestion AP plotted against the AUC of the plasma *Vs* time profiles after administration of three LBF formulations and one encapsulated crystalline formulation (Lipanthyl® Micronized) to Göttingen Minipigs, R^2 0.12. Data replotted from [189]. Data were digitised and AUCs calculated as previously described.

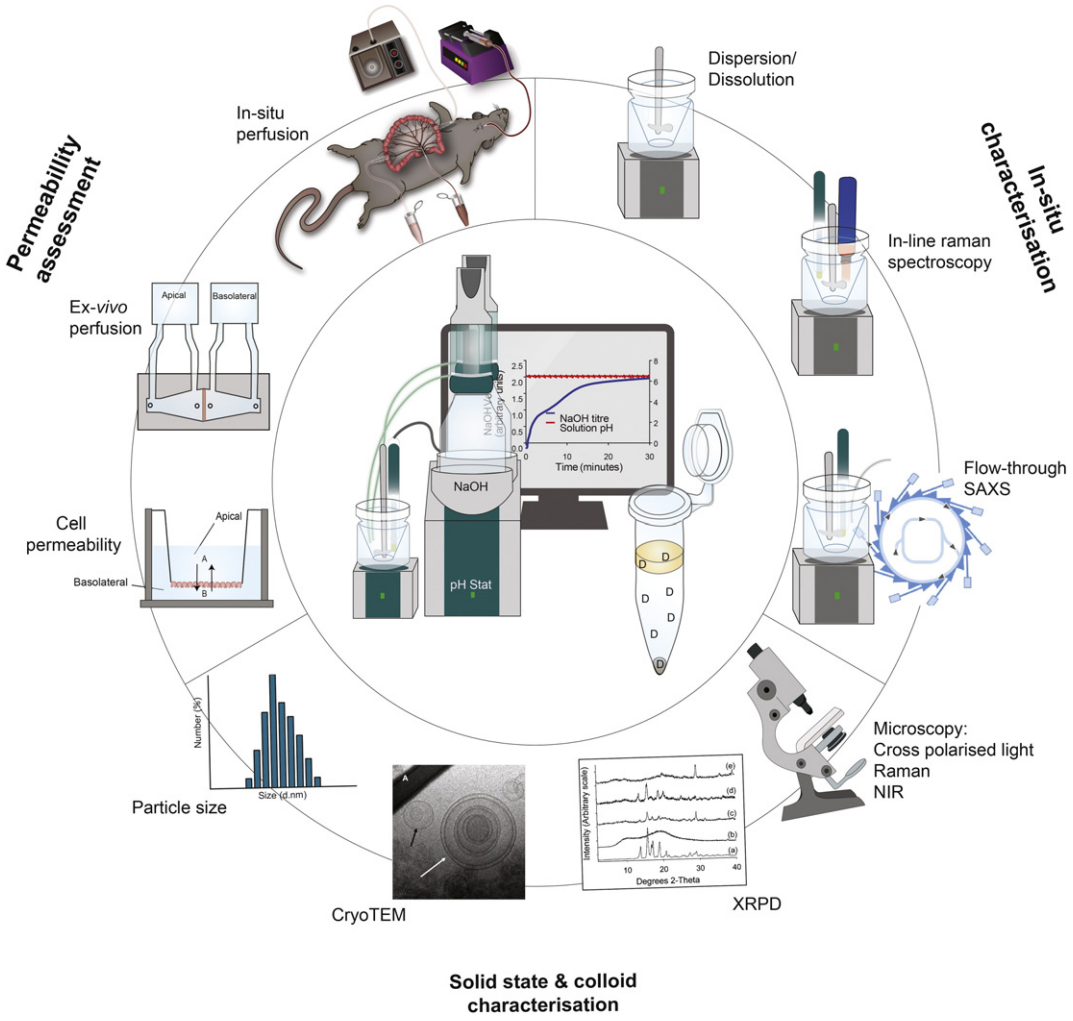

Fig. 10. Overview of the most prevalent characterisation techniques used in conjunction with *in vitro* lipolysis. In-line raman diagram adapted from [152], microscope adapted from Servier medical art, XPRD data for danazol reproduced from [35], cryo TEM of aspirated human intestinal fluid reproduced from [114], *in situ* perfusion diagram reproduced from [202]. Figures and data reproduced with permission.

when compared to precipitation in the crystalline form [167]. The addition of solid state property profiling of the precipitates formed during lipid digestion may therefore improve the quality of data interpretation in *in vitro* testing protocols [152,153,197]. To date, the formation of amorphous precipitates from dispersed or digested LBF has been reported most frequently for weakly basic drugs, possibly due to the potential to form amorphous complexes with fatty acid digestion products [34,167,198,199]. Amorphous precipitates have also been reported for drugs in formulations containing polymeric precipitation inhibitors [191,200]. For a recent review on the precipitation behaviour of PWSD in the presence of LBF the reader is referred to Khan et al. [201].

The species used for *in vivo* LBF evaluation may also have an impact on the applicability of the 'standard' *in vitro* lipolysis model. Rats are a convenient, economical and relatively high throughput *in vivo* model (when compared to dogs or pigs). For high cost drugs in particular, the use of a rat *in vivo* model may facilitate generation of early preclinical data without significant API outlay. An added benefit of this *in vivo* model is the potential for more facile inhibition of enzymes, transporters or efflux pumps to allow systematic evaluation of first pass metabolism or efflux on drug absorption from a LBF. *Ex vivo* or *in situ* analyses also tend to be conducted with excised rat tissue. In contrast, the majority of *in vitro* digestion experiments are run under conditions developed to reflect larger animals (dogs) or humans. To address this contradiction, an *in vitro* rat model of lipolysis which reflected the differences in volume and enzyme activity in the rat GIT was developed [65]. The model was subsequently used to evaluate the performance of a series of LBF in the presence and absence of 1-aminobenzotriazole (a CYP inhibitor) to investigate the impact of first pass metabolism on danazol absorption from LBFs. The data suggest that digestion in a rat is less efficient than in dog (and potentially in humans) and that danazol is very highly first pass metabolised, especially in the rat. An adaptation of the rat *in vitro* model with pH-stat control has since been utilised to explore IVIVC in rats for a series of digestion inhibiting LBFs [136]. In these studies, the mass of LBF administered to the rat was also scaled down to 30 mg per 300 g rat in order administer an approximately equal mg/kg formulation mass relative to dog (*i.e.* equivalent to 1500 mg formulation dosed to a 15 kg beagle). IVIVC of the adjusted rat *in vitro* digestion model and dose proportional *in vivo* model is presented in Fig. 8B.

7.1. Accounting for absorption in the in vitro digestion model

Perhaps most importantly, the IVIVC data described above rely on a closed system of *in vitro* lipolysis to generate solubility and supersaturation data that are then correlated with *in vivo* exposure. A limitation of these models is that they are unable to take into account the parallel process of drug (and lipid) absorption *in vivo*. As described above, lipid absorption might be expected to increase drug supersaturation in lipid swollen intestinal colloids (by reducing drug solvation capacity), whereas drug absorption is likely to reduce supersaturation. *In vitro*

tests that incorporate an adequate absorption sink might therefore be expected to better estimate both drug and digestion product absorption across the enterocyte [203].

Recognition of the limitation of closed *in vitro* systems is not confined to digestion testing. A far greater body of work has addressed the potential to integrate an absorption sink into *in vitro* dissolution apparatus to assess more traditional solid dose forms. In this case a number of different techniques have been employed to provide sink conditions. These include simple biphasic liquid dissolution models, transfer models, dialysis and filtration membrane systems and the coupling of dissolution models to Caco-2 monolayers and perfused intestinal segments in whole animals. These have recently been described in the excellent reviews by both McAllister and Kostewicz [204,205].

In the context of lipid-based formulations, a simplified biphasic liquid dissolution model utilising an aqueous (buffer) and an organic layer (1-octanol) has been employed by Pillay and Fassihi to evaluate release of nifedipine from a LBF [206]. In this case, the authors focused only on the release of drug from the formulation and chose not to investigate the impact of lipases on solubilisation and/or precipitation behaviour. There was also no *in vivo* data to allow correlation of the method with *in vivo* absorption. Shi and coworkers investigated a similar biphasic buffer–octanol system (where both the octanol and aqueous phases were stirred and coupled to a USP IV flow through cell) to evaluate the release of celecoxib from two LBFs [207]. The *in vitro* data were then compared to published clinical data to generate IVIVC. While the aqueous phase concentrations of celecoxib did not correlate with *in vivo* absorption, the AUC of drug concentration in the octanol phase (*i.e.* the 'absorption sink') showed rank order correlation to the clinical data (R^2 0.97). Unfortunately, the system lacked an *in vitro* digestion component and no attempts were made to generate or quantify supersaturation, which, in spite of the good correlation, may have better explained the comparatively poor *in vivo* absorption of, for example, a solution formulation of Tween 80 and ethanol. One significant drawback of biphasic systems for evaluation of LBF, however, is that the addition of media including solubilising species such as bile salt micelles and LBF derived surfactants to systems containing LBF components and digestion products is likely to cause some degree of emulsification at the octanol:water interface and may perturb efficient drug distribution between the two phases.

The addition of a more biologically relevant absorption sink to *in vitro* lipid digestion models is further complicated by the incompatibility of some simulated intestinal media, especially those containing bile salts and lipolysis enzymes, with many cultured cell monolayers [208]. Nonetheless, permeation studies of simple solutions of PWSD in buffers or SIF have been described and provide useful information as to the likelihood of dissolution, solubility or permeation limitations to *in vivo* exposure [209,210]. Kataoka et al., for example, evaluated the solubility-supersaturation-permeability paradigm for undigested SEDDS formulations of the permeable drug danazol and the poorly permeable drug pranlukast utilising a dissolution–permeation (D-P) model to predict absorption *in vivo*. Whilst acknowledging the limitation of the absence of lipolysis, and realising that the particle size of the undigested formulations was large in comparison to the colloids formed post digestion, the *in vitro* permeability studies did allow correlation between danazol absorption from the different LBFs in rats and absorption estimates from the D-P system. In contrast, pranlukast uptake appeared to be permeability limited, formulation independent and did not correlate to the D-P data [211].

These studies serve to reinforce the complexity in interpreting the data obtained from *in vitro* models such as the D-P model for drugs where absorption may be limited by solubility and/or permeability. Fig. 11 presents an adaptation of a figure from Ginski et al. [209] modified to incorporate the prospect of not only differentiation between drugs with low permeability, but also those that might have very high permeability. Thus, for drugs with low absorption and where absorption is dissolution or solubility limited, LBF are expected to enhance oral exposure when permeability is good (for example danazol [209]), but not when permeability is the primary limitation to absorption (as was the case with, for example, pranlukast [209]). Based on the published data for fenofibrate, however, it seems likely that further distinction might usefully be made between drugs with very high permeability, where LBF appear to enhance absorption, but do so in a relatively formulation independent manner. In this case even transient solubilisation appears to be able to drive significant membrane flux and absorption, and the rate of absorption may be sufficient to preclude *in vivo* precipitation. However, far more data is required to confirm this hypothesis and it remains possible that the data obtained for fenofibrate reflect differences in the drivers of precipitation *in vivo* when compared to the conditions employed in the *in vitro* digestion apparatus.

The GI mucous barrier may also play a role in PWSD absorption from LBF, but is rarely examined specifically [212]. Intestinal mucus forms a size, steric and molecular barrier to diffusion across the unstirred water layer and prevents rapid diffusion of protons away from the epithelial surface. Together these processes act to slow colloid transit towards the enterocyte, modulate exposure to bile salts and phospholipids and maintain the low pH of the UWL. The latter favours protonation of fatty acid digestion products and absorption of the unionised form of the lipid. Recent data shows that in spite of the potential permeability enhancing properties of some components of food, food ingestion actually enhances the barrier function of mucous (at least to the diffusion of model 200 nm nanoparticles), perhaps as part of a defence mechanism to avoid absorption of particulate contaminants in food. [213]. While the role of the GI mucosal barrier has been well explored in terms of nanoparticulate drug delivery [214–217], and has been extensively reviewed by Ensign and colleagues [218,219], the impact of GI mucus on cell-based permeability, stabilisation of drug supersaturation and UWL diffusion of PWSD solubilised in intestinal colloidal species, has been largely overlooked. In one of the few studies that have evaluated mucous effects, Preat and colleagues used Caco-2

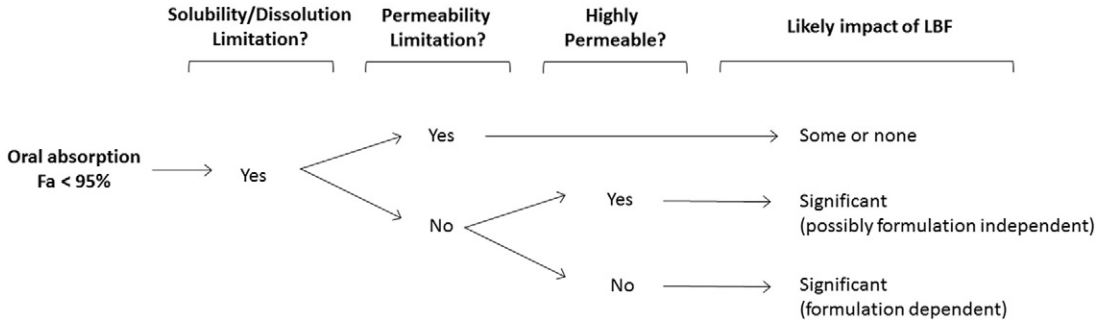

Fig. 11. Schematic presentation of the relationship between the limiting steps of oral absorption of poorly water-soluble drugs and the likelihood that LBF will promote absorption in a formulation dependent or independent manner.

cells and HT29-MTX/Caco-2 co-cultures (since HT29 cells secrete mucous) to evaluate the impact of mucus on the absorption of β-arteether (AE) from LBFs [220]. In this instance, the mucous layer appeared to reduce drug permeability slightly, however statistical significance was only reached for a tween 80 based SEDDS formulation, and not for a similar Cremophor based formulation. The more significant impact on absorption appeared to be lipid digestion and formulations that were partly resistant to *in vitro* lipolysis significantly increased the transport of AE across intestinal cell monolayers. Protection against lipolysis may have resulted from the use of PEGylated surfactants, which have been shown to inhibit lipase binding and lipid hydrolysis [136], but may also be attributable to the mucus penetrating properties of PEG [218,219]. A very recent study by Chang and McClements showed that fish oil droplets coated with a PEGylated surfactant (tween 80) were more stable in mucin than equivalent caseinate emulsions [221]. To better evaluate the role of mucous in drug absorption, Boegh and colleagues have established a caco-2/biosimilar mucus model as an alternative to co-culture for evaluation of protein and peptide absorption across cell monolayers [222,223] while Béduneau and coworkers have recently developed a tunable Caco-2/HT29-MTX co-culture model to better mimic human intestinal permeability [224]. In a subsequent study, Antoine et al. showed that the formation of a strongly adherent mucus barrier on the luminal side of the co-culture protected the cells from both bile salts and lipases when compared to a Caco-2 monoculture [225]. These studies exemplify the promise co-culture and *ex vivo* mucin models may hold as a more relevant *in vitro* model of the intestinal epithelium when compared to simple Caco-2 monolayers for the evaluation of LBF. Further work examining the potential impact of formulation excipients on the barrier properties of the GI mucosal layer could provide valuable insight into the impact of LBFs on both drug and formulation uptake. Cultured cell monolayer systems do, however, have one intrinsic drawback when assessing rates of drug absorption and that is the relatively low absorptive surface area when compared to the GIT.

In an effort to incorporate more physiologically relevant sink conditions (with an intact, higher surface area mucosal barrier) into the *in vitro* lipolysis model, Dahan and Hoffman investigated drug permeability (dexamethasone and griseofulvin) from digested lipid vehicles (long, medium and short-chain triglycerides) across 5 cm^2 *ex vivo* jejunal segments in a side-by-side modified Ussing chamber [39]. The formulations underwent dynamic *in vitro* digestion testing and at the end of lipolysis stage, the media was transferred to the donor (mucosal) cell of the chamber and drug accumulation in the acceptor (serosal) cell was monitored over 3 h. However, data from the *in vitro* digestion–*ex vivo* jejunal model did not correlate to the *in vivo* data. The authors concluded that for permeable, yet solubility limited drugs the use of short chain triglycerides (which enhanced permeation in the *ex vivo* model but not *in vivo*) were unlikely to translate to an *in vivo* advantage. Again, knowledge of the rate limiting step in PWSD absorption (solubility limited or permeability limited) was highlighted as a critical factor in determining the likelihood of formulation mediated changes being capable of changing *in vivo* exposure.

Potential limitations to excised *ex vivo* absorption models include the lack of intact nervous system, lack of blood flow, changes to transporter and enzyme function or expression and incompatibility with complex solvent systems. To address this, *in situ* absorption models may be used to allow measurement of absorption in a relatively intact *in vivo* system. The intestinal single pass perfusion method, for example, first described in 1958 [226], has long been used to explore intestinal drug absorption mechanisms. Despite the experimental challenges associated with the model, it remains one of the most highly used systems for investigation of intestinal drug absorption mechanisms and transporter/metabolism/efflux studies. Further information on the *in situ* perfusion model in rodents may be obtained from the very recent, and comprehensive review by Stappaerts et al. [227].

The robustness of the *in situ* perfusion model to physiological concentrations of bile, fatty acids and lipases renders it an ideal absorptive sink to couple to *in vitro* lipolysis experiments. In light of this, a coupled *in vitro* digestion–*in situ* perfusion model for the assessment of PWSD absorption from LBFs was recently developed [202]. Perfusion of a dynamic, digesting LBF through an externalised jejunum segment of a rat with direct measurement of drug flux into blood *via* mesenteric vein cannulation provides a unique opportunity to simultaneously examine the solubility-supersaturation-permeability paradigm in a relatively controlled system. Due to experimental complexities, the model is unsuitable for use as a high throughput screening tool, and is intended as a means to provide mechanistic understanding of drug absorption from LBFs. In these studies, Crum et al. report on the absorption of fenofibrate from three different LBFs. Despite the *in vitro* lipolysis portion of the model showing clear discrimination between supersaturation of the three LBFs, drug flux into the mesenteric vein was not significantly different between lipid formulations and all LBFs showed significantly greater flux than crystalline drug. This data is in agreement with the robust *in vivo* absorption data for fenofibrate in pigs, rats and minipigs that was summarised in Figs. 4, 6 and 9. The data lend support to the hypothesis that for highly permeable drugs such as fenofibrate, solubility (and/or dissolution) limited drug absorption from the crystalline state may be overcome through lipid formulation strategies, but that differences between formulations that may be seen using current *in vitro* methods are less apparent *in vivo* due to the high permeability and rapid absorption from the GIT.

8. Lipophilicity, lipid solubility and LBF utility—a role for deliberate increases in drug lipophilicity?

As described above and elsewhere, LBF are, in almost all cases where they can be employed, effective in increasing GI solubilisation and absorption of PWSD. The relative benefits of different LBF inevitably vary with excipient choice and drug properties, and recent efforts have been directed at fine tuning *in vitro* methods to better predict optimal formulations. In most cases where LBF performance is suboptimal, rapid drug precipitation from the dispersed or digested formulation is the likely cause (although even rapidly precipitating formulations can provide good drug exposure where permeability is high). Rapid drug precipitation on dispersion often results from the need to include large quantities of surfactant or cosolvent in the formulation in order to dissolve the target drug dose. This observation introduces perhaps the most significant limitation to the more widespread application of LBF as a means of enhancing oral absorption for PWSD—that of drug solubility in the formulation. In most cases, formulations where drug is dissolved in the formulation/capsule fill material are preferred and limitations to acceptable capsule size therefore limit the possible dose that can be administered. Drug suspensions in LBF have been successfully employed [52] and can provide for very significant increases in drug exposure [37]. Lipid solution formulations are therefore not an absolute prerequisite—but two phase systems provide additional challenges in material transfer, content uniformity, viscosity and *in vivo* reproducibility and where possible lipid solution formulations are typically preferred.

Drug solubility in the formulation is therefore a key determinant of the utility of LBF. Simplistically, the assumption is often made that PWSD with high logP are 'lipophilic' and therefore that solubility in the excipients often employed in LBF will be high. However, log P is a poor indicator of absolute lipophilicity [159,228], and instead provides only an indication of 'relative' lipophilicity compared to water. Indeed, there are many drugs where relative partitioning between octanol and water is high (*i.e.* high log P), but where absolute lipid solubility remains low. These compounds are indicative of the typical 'brick-dust' type of poorly water soluble drug, that is essentially 'everything-phobic', but where relative affinity for octanol over water is often high. In this case solubility in both water and lipids is commonly limited by

intermolecular forces in the solid state—a property indicated by high melting point. In contrast, 'grease ball-like' poorly water soluble drugs may have similar log Ps, but in this case have intrinsically higher lipid solubility (and usually lower melting point). The importance of melting point in dictating lipid solubility is reflected in recent computational models for predicting lipid solubility, the utility of which is markedly improved by including melting point [186,228].

It is apparent therefore that distinction needs to be made between PWSD that have high log P, low melting point and high lipid solubility—and that are ideally suited to LBF, and those that have high log P, high melting point and low lipid solubility—and are a significant challenge to formulate in LBF. This raises the question as to whether drug molecules might usefully be designed proactively to match an appropriate formulation strategy. For example, the development of analogues with higher lipid solubility. Clearly, this is unlikely to be a 'first-approach' and traditional lead optimisation programmes that seek to identify leads with reasonable water solubility and membrane permeability will be the first option. However, it becomes increasingly clear that for some targets, the likelihood of identifying a novel lead that is a traditional BCS class 1 compound is extremely low [186]. Under these circumstances, re-purposing a lead optimisation programme to identify poorly water soluble drugs that have good lipid solubility (rather than water solubility), and that might take advantage of the absorption benefits of LBF may be appropriate.

It must be acknowledged, however, that there are many arguments against this general concept and several excellent papers have shown that highly lipophilic drugs typically have higher receptor promiscuity and toxicity, and increase the potential for metabolic elimination and the likelihood of metabolic drug–drug interactions. As such the 'b-r-o-5 space' has trended towards tighter definition with the 'rule of 4' (molecular weight < 400, log P < 4) suggested by Gleeson [229], and even tighter requirements—the 'rule of 3' (molecular weight < 300, log P < 3) for fragments [230]. Others similarly caution against higher lipophilicity showing that compounds with log P > 3 and polar surface areas (PSA) < 75 A^2 are 2.5 times more likely to be toxic [231]. These correlative analyses cannot be disputed, but suffer from simple correlation with log P (or log D) as a global indicator of 'lipophilicity' and perpetuate the generic 'lipophilicity is bad' concept. In contrast, others suggest that log P may be too broad and that more specific properties (albeit properties that also drive increases in Log P), for example increasing aromaticity may be more responsible for poorer developability [232].

From a formulators perspective these analyses suggest the possibility of distinguishing molecular properties that drive increases in lipid solubility (and therefore applicability for LBF) from other indications of lipophilicity that may be associated with less effective development outcomes. For example, addition of aliphatic carbons may drive increases in lipophilicity that are not as highly correlated with changes in receptor promiscuity as increases in aromatic carbons but will likely increase lipid solubility. Similarly, additional carbon centres will increase lipophilicity, but where these are SP^3 carbons rather than SP^2, this may also reduce intermolecular packing and therefore melting point and in doing so increase lipid solubility. To the best of our knowledge, published examples of lead optimisation strategies to deliberately increase lipid solubility to increase developability *via* increases in lipid (rather an aqueous) solubility are not evident. But, if this could be achieved in a manner that does not significantly enhance toxic liability, it may be beneficial.

An alternate approach to achieving the same ends, but at the same time reducing potential toxicity and metabolic liabilities even further, is to develop or isolate drug leads with 'temporarily' high lipophilicity. Strategies to increase transient lipophilicity such as lipophilic prodrugs and salts are further described in the following sections.

8.1. Lipid prodrugs and LBF

Lipophilic prodrugs have been widely employed to enhance membrane permeability and in doing so to promote oral bioavailability for polar, hydrophilic drugs; often *via* masking charged carboxylic acid groups [233,234]. Similarly, alkyl and aryl esters have been employed to cap metabolic sites and reduce presystemic metabolism and to reduce GI irritation [233]. In almost all cases, the prodrug moiety reduces polarity and adds lipophilicity. Rarely is this performed specifically to enhance lipid solubility and promote incorporation into LBF—however, in most instances this is likely to be the case, and as such may have potential utility in enhancing access to the advantages in exposure that LBF provide for PWSD.

One recent study, has described the deliberate synthesis of a range of lipophilic prodrugs of a poorly water and lipid soluble thumb pocket 1 polymerase inhibitor (HCVNS5B) in order to promote solubility in SEDDS. In this case, a glycolic amide ester of the parent drug showed the most favourable lipid solubility (>100 mg/g), and also resulted in rapid *in vivo* hydrolysis to liberate parent drug from the prodrug [235]. Subsequent comparative *in vivo* bioavailability studies examined systemic drug exposure after oral administration of the prodrug in combination with a self-emulsifying LBF in comparison to parent drug, and showed good increases in exposure in rats for the prodrug, but lower exposure in dogs and monkeys. Relative exposure in rats and monkeys was consistent with rates of drug liberation in the presence of liver microsomes. In dogs, exposure was very low and the authors suggested that this may be an outlier species for prodrugs of this type. A similar approach was also taken by Bala and colleagues who generated fatty acid esters of SN38 (a camptothecin derivative) as a means of increasing solubility in lipidic vehicles [236], although in this case additional benefits in terms of enhancing intestinal permeability and potentially promoting intestinal lymphatic transport were also envisaged. Solubility of SN38 in a long chain triglyceride (soybean oil) was low but increased significantly for the prodrugs. This was particularly apparent with the undecanoate ester and a diundecanoate ester with increases in lipid solubility of >400 fold.

Similar fatty acid esters have been widely employed to promote intestinal lymphatic transport, the best known being the current commercial oral formulation of testosterone—also an undecanoate ester (Andriol) [14,237]. In the case of testosterone undecanoate, formation of the lipophilic ester promotes drug association with intestinal lymph lipoproteins in the enterocyte and therefore enhances drug transport into the intestinal lymph after oral administration [238,239]. Since the lymph drains directly into the systemic circulation, *via* the major veins in the neck, and does not pass through the liver, stimulating intestinal lymphatic transport can lead to significant increases in bioavailability for drugs like testosterone where first pass metabolism is highly significant [98,240]. Formation of the fatty acid ester of testosterone also allowed ready incorporation into a lipid based soft gelatin capsule formulation. Recent studies have examined a range of lipophilic prodrugs with the principle intent of increasing intestinal lymphatic transport [241,242], but where synthesis of the prodrug increased lipid solubility and ease of incorporation into LBF. These prodrugs were either simple aliphatic esters or lipophilic glyceride esters employed to mimic dietary triglycerides. In all cases the prodrugs were able to promote lymphatic transport, with attendant benefits in increasing drug concentrations in the lymph and reducing first pass hepatic metabolism. However, in the current context, formation of lipophilic prodrugs also significantly elevated lipid solubility, thereby enhancing incorporation in LBF. In the case of lymph directing prodrugs this is critical as coadministered lipid is required to drive intestinal lipoprotein production. For a more detailed review of prodrug strategies in general the interested reader is directed to Stella et al. [233].

8.2. Ionic liquids and LBF

Ionic liquids (ILs) are defined as organic salts with melting temperatures below 100 °C [243]. As such they can exist in a liquid or solid form at room temperature and when in the solid form, ionic liquid

structure may be crystalline or amorphous. The unique physical properties of ionic liquids have been reviewed elsewhere [244].

ILs are a well-established class of materials with existing industrial applications in a number of areas including biomass processing, renewable energy, synthesis and analytical chemistry [243]. In comparison, the potential applications of ionic liquids in drug delivery have only recently been explored, but show great promise. These applications may be grouped into two general areas; (i) the use of ionic liquids as functional excipients or (ii) the transformation of drugs into ionic liquids in order to enhance drug properties.

Interest in the former largely stems from the "designer" solvent properties of ionic liquids since this can lead to higher drug loadings if ionic liquids are used in drug delivery systems [245]. For example, a commercially available ionic liquid, 1-hexyl-3-methylimidazolium hexafluorophosphate, shows a high degree of solvency towards both hydrophobic compounds (*e.g.* danazol and albendazole), and hydrophilic compounds (*e.g.* caffeine and acetaminophen) [246]. This may in part be attributable to favourable interactions between the hydrophobic portions of the drug and *e.g.* the alkyl chains in the IL. Additionally, hydrogen bonding between the drug and the ionic component of the IL may further enhance solvation properties for PWSD. Some ILs are also miscible with lipids and surfactants commonly used in LBF. For example, recent studies have shown that a series of N-alkylnicotinate ester ILs and N-alkyl-3-methylpyridinium alkyl sulphate ILs are able to increase solubility of danazol and itraconazole in LBF by up to 500-fold when compared to a standard LBF [247]. After oral administration to rats, the IL containing LBF resulted in similar exposure to a SEDDS formulation, however drug absorption from the IL containing formulation was sustained over 6–8 h.

Similarly, ILs have been described as having advantageous solvency properties in topical drug formulations. For example, ionic liquid-in-water emulsions have been explored for the topical delivery of etodolac [248], since etodolac solvency in the ionic liquids enabled much higher drug loading (374.3 mg/ml in 1-butyl-3-methylimidazolium hexafluorophosphate compared to <175 mg/ml in other lipids, surfactants and cosolvents).

A further application of ILs in drug delivery is the transformation of the drug itself into an ionic liquid form [249]. These resultant low melting temperature drugs are typically described as drug-ILs or active pharmaceutical ingredient (API)-ILs. The physicochemical properties of API-ILs have led to their use to avoid crystal polymorphism [250], to increase aqueous solubility and dissolution rate for oral [251,252] or parenteral delivery [252] and as a means to deliver stabilised cationic and anionic drug pairs [253].

Of relevance to the subject matter of this review, recent studies have explored the potential utility of generating lipophilic ILs from ionisable drugs in order to enhance solubility in LBF excipients. This approach was based on the hypothesis that reduction/removal of the crystalline drug lattice (manifest in a reduction in melting point), and the use of highly lipid soluble counter ions would enhance lipid solubility and facilitate the formation of LBF for drugs where lipid solubility was otherwise limiting [254]. An additional potential advantage of using an ionic liquid approach to improve lipid solubility is that it may not raise the same issues of receptor promiscuity and potential off-target toxicity that are commonly associated with highly lipophilic analogues since the lipophilicity of the drug remains unchanged, (although the fate and toxicity of many API-IL lipophilic counterions are as yet, unknown). Sahbaz et al. generated a series of ionic liquid drugs using a range of poorly water soluble weak bases and acidic lipophilic counterions [254]. For the PWSD cinnarizine, the IL form of the drug resulted in a 7-fold increase in solubility in LBF, which translated to a higher drug loading in the SEDDS without significant *in vitro* drug precipitation post dispersion and digestion. *In vivo* administration of solubilised IL drug in a SEDDS was achieved at doses significantly in excess of that which could be achieved using cinnarizine free base *i.e.* the equivalent free base formulation was a SEDDS suspension. Comparison of cinnarizine exposure after oral administration of SEDDS containing cinnarizine IL in solution or cinnarizine free base as a suspension, confirmed that exposure was higher after administration of the API-IL formulation. At matched doses to LBF where cinnarizine free base was in solution (which was only possible at low dose, 35 mg/kg) the cinnarizine IL formulation performance was comparable to the SEDDS formulation (Fig. 12A). Similar results were reported for itraconazole which is both water and lipid insoluble, with a docusate ionic liquid form showing a > 50-fold increase in solubility in lipid formulations. A 2–3-fold increase in exposure over the currently marketed amorphous drug formulation (Sporanox®) was observed after oral administration. Generation of lipid soluble API-ILs is also achievable for more hydrophilic drugs. A dextromethorphan decylsulphate IL, for example, was reported to be more soluble in medium chain SEDDS than the free base (93.3 mg/g for the API-IL and 23.5 mg/g for the free base) providing the potential to formulate relatively polar drugs in LBF.

A significant number of drugs currently on the market or in development are ionisable and are formulated as salts. There is therefore wide scope for the application of IL technologies to enhance drug solubilisation in lipids for oral drug delivery. Lipid soluble API-ILs may be synthesised using alkyl sulphates, docusate and fatty acid based anionic counterions, materials that have been widely used in oral drug products (either as counterions, excipients or excipient components), and therefore their toxicity risks are not anticipated to be significant.

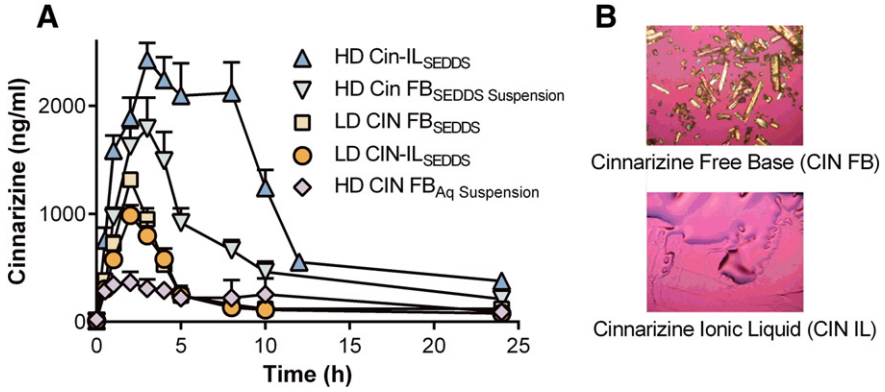

Fig. 12. (A) Pharmacokinetic profiles of cinnarizine free base formulated in suspensions, SEDDS or as an ionic liquid in suspensions and SEDDS. Cin-IL—cinnarizine ionic liquid, Cin FB—cinnarizine free base, HD—High dose (125 mg/g), LD—Low dose (35 mg/g) (B) differences in morphology of cinnarizine and cinnarizine ionic liquid as measured under cross polarised light. Data adapted from Sahbaz et al. [254].

9. Solid LBF development—generating alternative dosage forms for PWSD

Most LBFs are liquid or semisolid at room temperature and require encapsulation or solidification to facilitate ease of dosing. Multiple approaches have therefore been taken to achieve these ends and they are outlined below.

9.1. Encapsulation

Typically, encapsulated LBFs are filled into either soft or hard shell capsules with the choice of shell dependent on formulation compatibility and the mass of formulation to be dosed. Soft and hard shell capsules are typically gelatin based (though other polymeric alternatives to gelatin are also available [255]), and have their own inherent advantages and disadvantages. Soft gelatin capsules, due to their thicker walls, are more compatible with hygroscopic excipients such as PEGs or high HLB surfactants and can be filled almost to maximal capacity [51]. However the presence of plasticizers in soft gelatin capsules may also cause drug migration to the capsule shell affecting drug release [256]. In addition, soft gelatin capsules must be filled at comparatively low temperatures (40 °C) limiting their use for semisolid formulations and capsule filling is usually outsourced to specialised contract manufacturers. On the other hand, hard gelatin capsules are compatible with filling temperatures up to 70 °C, can be filled in-house and the absence of plasticizer reduces the likelihood of drug migration into the capsule shell. Additionally, hard gelatin capsules have lower water content which limits possible water exchange with the capsule fill [257]. The risk of leakage is higher from hard capsules but can be reduced through careful selection of filling parameters, or the use of semi solid fill materials, while capsule banding or sealing can be employed to provide further protection [257]. A common issue with both hard and soft gelatin capsules is moisture sorption either from the fill material or from the environment, which can significantly alter capsule properties [257], although these issues can be attenuated by appropriate secondary packaging. The presence of impurities (particularly peroxides [258]) in formulation excipients can cause crosslinking of gelatin shells, impacting drug release rates over time [51]. The chemical and physical stability of liquid LBFs must therefore be considered prior to encapsulation [258,259].

Despite these complexities, some of which are applicable to both solid and liquid fills, encapsulation is flexible, well tolerated in the clinic, widely employed and remains the primary marketed means of LBF delivery [52].

9.2. Solidification for powder fills or tabletting

Solid lipid formulations have gained interest over recent years, due in part to the desire to harness the benefits of lipids in a variety of different dosage formats, such as sachets, powder for suspension, multiparticulates and tablets. They are particularly attractive where conventional capsules are not suitable *e.g.*, where there are capsule compatibility challenges that cannot be solved using semi-solid formulations, or for clinical reasons, for example in patients with difficulty swallowing or where controlled release is required. Solidification is typically achieved by either combination with carriers or additives to form powders or by the use of high melting lipids to generate semi solid or solid formulations that may be encapsulated or processed to form lipid-based multiparticulates.

Adsorption onto solid carriers, spray drying, melt granulation, melt extrusion, freeze drying and solvent evaporation (Fig. 13) have all been used to convert LBF to solid forms and have been well described in recent reviews by Tan and coworkers [260] and by Jannin and colleagues [258].

Generation of powder or granular forms of LBF is an appealing solidification strategy as these types of solidified formulations are generally compatible with pre-existing tabletting, capsule or sachet filling procedures and equipment. Indeed the generation of solid state emulsions began over 50 years ago with spray drying and lypophilisation strategies [261,262]. In a more recent study by Hansen, Holm and Schulz [263], spray-dried emulsions of MCT were combined with a sugar, gelatine and Neusilin in an attempt to generate directly compressible LBF loaded powders. The study showed that the loading of the MCT solution as well as the particle size of the insoluble silica carrier and sugar directly affected tabletability. Subsequent *in vivo* studies did not show significant differences in oral absorption of the model drug Lu 28–179 when compared to a MCT solution and a HPMC dry emulsion indicating that the solidification process did not significantly impact LBF performance [264].

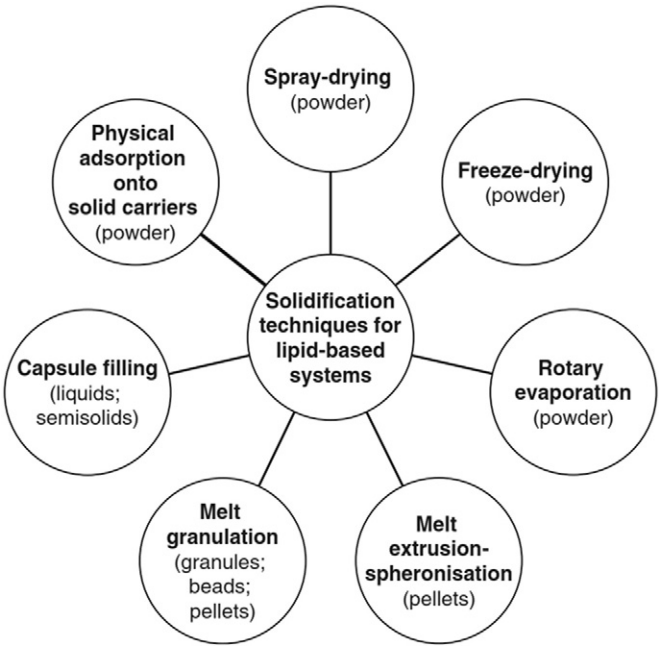

Fig. 13. Overview of solidification techniques commonly used for transforming liquid and semisolid lipid-based formulations into solid dosage forms. All methodologies (excepting rotary evaporation) are viable for scale-up to commercial manufacture. Reproduced from [260] with permission.

Pickering emulsions of LBFs stabilised by silica have been utilised by the Prestidge group to generate solid LBFs capable of controlling lipolysis rates, drug release and reducing food effects [265–267]. For example, a SLH formulation of celecoxib increased lipid hydrolysis when compared to an oil solution, and oral absorption of celecoxib in fasted beagles was significantly greater for the SLH formulation when compared to an oil formulation or when celecoxib was administered to fed dogs [266]. These studies led to a first in man study of SLH for oral PWSD delivery where a two-fold increase in bioavailability was evident relative to a commercial formulation. This was attributed to improved solubilisation of the model drug ibuprofen [268].

Silica finds further application in solidified LBF as a physical adsorbent. Studies utilising mesoporous silica as a carrier for LBFs have been described by multiple groups in recent years [269–271]. Mesoporous adsorbents increase the surface area of contact of a PWSD with solubilising media and may facilitate improved dissolution rates even in the absence of lipids [272,273]. In the context of LBF, these high surface area adsorbents can accommodate up to 100% *w*/w lipid formulation loadings, allowing tabletting of LBF, but this must be balanced with maintenance of adequate powder flowability and compressibility. Furthermore, studies by Van Speybroeck et al. have shown that incomplete desorption of SEDDS from uncompacted silica carriers may alter *in vitro* and *in vivo* performance [271]. In these studies, the model drug, danazol, was not found to strongly adsorb to the carrier and the lack of *in vivo* absorption was attributed to incomplete

desorption of the surfactant in the SEDDS which in turn altered the solvation capacity and increased the particle size of the redispersed formulation. Other studies have also shown that the formation of a gel layer may retard *in vitro* drug release from SEDDS adsorbed onto a Neusilin carrier [270]. Sander and Holm observed reduced bioavailability of adsorbed self-emulsifying formulations of cyclosporine after administration to dogs but found that the addition of a superdisintegrant markedly improved oral bioavailability with similar *in vivo* performance to an encapsulated LBF [274]. These differences in performance may be both drug-specific and formulation dependent and careful screening of adsorption and desorption of formulation excipients and the drugs used is advisable both before and after tabletting.

9.3. Solidification using thermoplastic excipients

Solid LBFs using thermoplastic (or meltable) surfactants and polymers and other solidifying matrices have also been widely explored. For a detailed review of solvent-free melt techniques for solid oral LBF preparation (extrusion, melt coating, granulation etc), the interested reader is directed to a recent publication by Becker et al. which comprehensively outlines the approaches available for generation of solid LBFs from excipient selection to manufacturing technique selection [275]. Shulka et al. have also reviewed the formulation of oral LBFs administered as multiparticulates (from liposomes to self-emulsifying pellets) [276]. However, the impact of these solidification processes on PWSD solubilisation or absorption has been less extensively explored. In a study examining hot melt LBF extrudates for oral delivery of propranolol [277], Mehuys and colleagues showed that oral bioavailability in humans was greater for the extruded system than for a comparable commercial formulation. In preceding studies [278,279], dissolution rates of the formulations were evaluated but the impact of digestion on drug solubilisation or precipitation was not evaluated. Hassan and Mader recently showed that PEG-30-di-(polyhydroxystearate) based semisolid SNEDDS protected the model drug progesterone from digestion-induced precipitation [280] and work from the Serajuddin group has explored the use of PEG and block copolymers to generate solid self-emulsifying formulations [281,282]. The latter formulations form spontaneous emulsions on redispersion, however, the impact of digestion on performance is as yet undescribed. Abdalla and Mader investigated the utility of solid self-emulsifying pellets as drug delivery vehicles for diazepam [283]. They showed that spheronzied solutol HS-15 containing pellets that self-emulsified resulted in faster drug release than formulations without solutol HS-15. However, the *in vitro* evaluation of drug release was limited to dissolution testing. Similarly, spheronised multiparticulate formulations based on gelatin have been reported by Aguirre et al. [284], where extrusion spheronisation was used to solidify an emulsion formulation containing permeation enhancers and salmon calcitonin, a poorly permeable peptide. *In vivo* absorption of sCT was evaluated in jejunal and colonic loop installations and absorption was greater in the presence of the permeation enhancers. Similar formulations for sustained release of cyclosporine are currently in clinical trials [3].

From the studies outlined above it can be seen that a number of different approaches to solidification of LBFs can be taken, although to this point formulation evaluation has been largely based on dissolution data alone. As with liquid LBFs, the performance of these solid LBFs might usefully be evaluated in the presence of hydrolytic enzymes and physiologically relevant concentrations of BS/PL in order to more fully understand their utility and to fine tune performance criteria.

10. Conclusions/perspectives

Drug discovery candidates that fall close to or outside the traditional 'rule-of-5' drug space remain an inconvenient reality. In spite of ever increasing efforts to minimising lipophilicity, molecular size and the numbers of rotatable bonds, h-bond donors and h-bond acceptors, these molecular characteristics appear to correlate with binding affinity for a range of drug targets. Indeed, as indicated by Bergström and colleagues in this issue [186], for some targets, highly lipophilic, and therefore poorly water soluble drugs appear to be a requirement. Fortunately, the challenge of low water solubility is significantly more tractable than, for example, low permeability and a range of formulation approaches have been developed to promote intestinal solubility [6]. Of these, lipid formulations and solid dispersion formulations have had perhaps the most commercial success and continue to be widely applied.

Lipid formulations provide an attractive option for enhancing the oral bioavailability of b-r-o-5 compounds. Unlike many formulation approaches, they have the potential to address solubility, permeability and presystemic metabolic liabilities, although the data base to support the latter two opportunities is both limited and equivocal. That lipid formulation can enhance apparent solubility in the GIT and promote the absorption of PWSD, however, is without question. Realising the limited data set, where LBFs can be employed they almost always appear to out-perform other enabling formulation technologies, including solid dispersion formulations.

Lipid formulations provide further advantage in terms of ease of assembly, flexibility (especially preclinically where varying doses must be orally gavaged to small animals) and scale, and several commercially and clinically successful examples are evident [52]. However, LBF do not provide a universal panacea and challenges remain. These include the need to access liquid filling capabilities, for either sealed hard gelatin or soft gelatin capsules, a situation that often requires outsourcing; the need to consider solution stability and capsule compatibility, particularly for drugs predisposed to oxidation and water sensitivity; and perhaps most importantly, the desire for single phase capsule fill materials, a situation that requires good drug solubility in the fill matrix. Whilst lipid suspension formulations are possible, and often effective, two phase liquid filling is complex and complicates material transfer and content uniformity. As such isotropic, homogeneous, lipid solution formulations are preferred. This typically limits the application of LBF to lipophilic, low melting drugs where solubility in non-aqueous vehicles is high (or the dose is low). To address this limitation, recent advances have identified alternative salt forms (ionic liquids), that reduce melting point and promote lipid solubility and provide new approaches to expand the potential applicability of LBF to drugs that are otherwise insufficiently lipid soluble. Lipophilic prodrugs or analogues are similarly able to increase solubility in non-aqueous vehicles, and in doing so, facilitate administration as a liquid fill capsule. Concerns over the development of highly lipophilic drug molecules due to the potential for increasing metabolic liabilities and receptor promiscuity dictate that ionic liquid or prodrug approaches that provide for 'temporary' increases in lipophilicity may be preferred over the generation of highly lipophilic analogues.

Alongside increasing focus on matched drug design and formulation design strategies, understanding of the mechanisms of drug absorption from LBF has increased, as have the technologies and models employed to assess formulation performance. For many years, the underlying paradigm for drug absorption from LBF was that their advantage stems from avoidance of classical solid–liquid dissolution (since the drug is in solution in the lipid vehicle) and enhancement in drug solubilisation in the GI fluids that are swollen with bile salts and lipid digestion products. This led to widespread use of *in vitro* lipid digestion models to evaluate formulation performance under simulated GI conditions and the overarching assumption that maximising the proportion of the drug that remained solubilised on GI digestion would maximise drug absorption. It is now apparent that whilst this holds true for many drugs, for some drugs, absorption may still be robust, even in light of significant precipitation on *in vitro* formulation digestion. Indeed, supersaturation rather than solubilisation is emerging as a significant driver of absorption and for highly permeable drugs, only transient periods of supersaturation may be sufficient to drive

absorptive drug flux. Nonetheless *in vitro* digestion testing remains a useful and simple challenge test and formulations that retain solubilisation capacity on *in vitro* digestion almost always perform well *in vivo*.

Finally, great strides are being made to develop LBF dosage forms that retain the advantage of traditional liquid fill materials, but that can be solidified to improve stability, manufacturability or ease of administration. The approaches employed range from the use of high surface area microporous materials which allow subsequent granulation or tabletting to the use of thermoplastic excipients for the generation of melt extrudates, pellets or multiparticulates compatible with different modes of administration (capsules, sprinkles, sachets *etc.*).

LBF therefore provide a flexible and highly effective means of enhancing exposure for b-r-o-5 compounds. The largest evidential data base supports the use of LBF to enhance apparent solubility in the GIT, but opportunities in permeability enhancement, controlled release and metabolic/transporter inhibition are increasing. As discovery programmes continue to identify increasing numbers of discovery candidates that are close to, or beyond the r-o-5, LBF provide a vital part of the drug delivery armoury.

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Appendix 2

‘Stealth’ lipid-based formulations: Poly(ethylene glycol)-mediated digestion inhibition improves oral bioavailability of a model poorly water-soluble drug

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'Stealth' lipid-based formulations: Poly(ethylene glycol)-mediated digestion inhibition improves oral bioavailability of a model poorly water soluble drug

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ABSTRACT

For over 20 years, stealth drug delivery has been synonymous with nanoparticulate formulations and intravenous dosing. The putative determinants of stealth in these applications are the molecular weight and packing density of a hydrophilic polymer (commonly poly(ethylene glycol) (PEG)) that forms a steric barrier at the surface of the nanoparticle. The current study examined the potential translation of the concepts learned from stealth technology after intravenous administration to oral drug delivery and specifically, to enhance drug exposure after administration of oral lipid-based formulations (LBFs) containing medium-chain triglycerides (MCT). MCT LBFs are rapidly digested in the gastrointestinal tract, typically resulting in losses in solubilisation capacity, supersaturation and drug precipitation. Here, non-ionic surfactants containing stealth PEG headgroups were incorporated into MCT LBFs in an attempt to attenuate digestion, reduce precipitation risk and enhance drug exposure. Stealth capabilities were assessed by measuring the degree of digestion inhibition that resulted from steric hindrance of enzyme access to the oil–water interface. Drug-loaded LBFs were assessed for maintenance of solubilising capacity during *in vitro* digestion and evaluated *in vivo* in rats. The data suggest that the structural determinants of stealth LBFs mirror those of parenteral formulations, i.e., the key factors are the molecular weight of the PEG in the surfactant headgroup and the packing density of the PEG chains at the interface. Interestingly, the data also show that the presence of labile ester bonds within a PEGylated surfactant also impact on the stealth properties of LBFs, with digestible surfactants requiring a PEG Mw of ~1800 g/mol and non-digestible ether-based surfactants ~800 g/mol to shield the lipidic cargo. *In vitro* evaluation of drug solubilisation during digestion showed stealth LBFs maintained drug solubilisation at or above 80% of drug load and reduced supersaturation in comparison to digestible counterparts. This trend was also reflected *in vivo*, where the relative bioavailability of drug after administration in two stealth LBFs increased to 120% and 182% in comparison to analogous digestible (non-stealth) formulations. The results of the current study indicate that self-assembled "stealth" LBFs have potential as a novel means of improving LBF performance.

1. Introduction

Drug bioavailability from an oral formulation in the gastrointestinal tract (GIT) is heavily reliant on favourable physiochemical characteristics, including adequate solubility and permeability and resistance to metabolism. However, increasing numbers of new chemical entities (NCE) derived from, e.g., combinatorial and high throughput screening processes, do not meet these criteria [1] and as a result, attrition rates in early stage clinical development are rising [2,3]. Lipid-based drug delivery systems are well established as a means to circumvent the low solubility issues associated with hydrophobic drugs [2,4,5]. The past 20 years have seen lipid-based formulations (LBFs) advance from simple one-excipient or binary systems to more complex multi-component self-emulsifying drug delivery systems (SEDDS) [6–11]. Despite this progressive rise in popularity, however, LBFs occupy less than 4% of the oral market and the development of LBFs remains largely empirical [2].

One limitation to the wider use of medium-chain triglyceride (MCT) containing LBFs is the realisation that these formulations often exhibit a rapid loss in drug solubilisation capacity when subjected to digestion by pancreatic enzymes [12–16]. Under digesting conditions, there is a risk that dissolved drug will precipitate into a more slowly dissolving crystalline form, leading to reduced bioavailability. Judicious design of lipidic formulations is therefore required to generate formulations that are able to withstand the solubilisation challenges encountered on digestion.

Lipid digestion in the GIT is catalysed by the lipase superfamily of interfacially active enzymes [17]. Nonspecific adsorption of the inactive lipase/co-lipase enzyme complex to the surface of an emulsified oil

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droplet results in a conformational change in the enzyme to the active form. The interfacial activation of pancreatic lipase renders the lipolysis reaction highly sensitive to changes at the oil–water interface [17,18]. The presence of commonly used polyethoxylated non-ionic surfactants has previously been reported to modify the rate of *in vitro* lipid digestion [19–24]; however, the available data are contradictory. Thus, increases in lipid digestion rates have been observed on surfactant addition to LBFs, and tentatively attributed to enhanced solubilisation of digestion products [19], while reduced digestion has also been reported and attributed to steric hindrance of lipase access to the oil–water interface [21–24].

PEGylated (stealth) drug delivery vehicles comprising a polyethoxylated steric stabilisation layer have been extensively utilised in parenteral drug delivery to enhance plasma circulation times and to promote drug accumulation at sites of hypervascularisation such as tumours or inflamed tissues [25–27]. The stealth effect attributed to these drug delivery systems can be in part attributed to their polymeric PEG coatings that prevent nonspecific protein adsorption (opsonisation) and therefore reduce recognition and clearance from the blood by the mononuclear phagocyte system (MPS). PEGylated drug delivery systems typically exhibit prolonged circulation half-lives and improved accumulation at sites of increased vascular permeability when compared to their unmodified counterparts.

The aim of the current study was to examine the potential to translate the materials and concepts that have been successfully employed to enable parenteral stealth applications, to an advantageous role in oral drug delivery. This has been achieved via the systematic evaluation of a series of LBFs with varying degrees of PEG-mediated steric stabilisation. Parenteral stealth formulations aim to evade recognition by the immune system through PEG-mediated prevention of opsonisation. Here, oral 'stealth' formulations are defined as formulations that use a hydrophilic polymeric interfacial layer to similarly prevent nonspecific protein binding, in this case the adsorption of pancreatic lipase-colipase to the surface of a lipid droplet. In this way, the proposed oral stealth LBF aim to evade lipolysis and in doing so prolong drug solubilisation in the GIT and improve drug absorption.

The development of self-assembled stealth LBFs was informed by the structural principles that govern effective stealth attributes in parenteral formulations, namely, surfactant PEG chain molecular weight (Mw) and PEG chain density [26–31]. Here we hypothesised that if oral stealth LBFs behaved in a similar fashion to their parenteral counterparts, digestion inhibition would correlate with the Mw of the surfactant PEG headgroup and the density of the PEG layer. The degree of PEG-mediated reduction in non-specific protein adsorption (as manifest by changes in lipid digestion) was assessed via *in vitro* lipolysis experiments. Changes in drug solubilisation and supersaturation were measured for selected formulations using danazol as a model drug, and these formulations were subsequently administered orally to male Sprague–Dawley rats. Danazol bioavailability was compared after administration of the stealth formulations and structurally analogous formulations that were readily digested.

2. Methods

2.1. Materials and reagents

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) were from Sigma-Aldrich (St. Louis, MO, USA). Captex® 355 (C_8 and C_{10} MCT) was donated by Abitec Corporation (Janesville, WI, USA) and was used as received. Etocas™ 5, 15, 35, 200 (PEGylated castor oils—CO); Croduret™ 7, 25, 40 (PEGylated hydrogenated castor oils—HCO); Myrj™ S8, 20, 40, 50, 100 (PEGylated stearic acids); Brij™ S2, 10, 20, 100, 200 (PEGylated stearyl alcohols); and Brij™ O2, 3, 5, 10, 20 (PEGylated oleyl alcohols) were kindly donated by Croda International PLC (Yorkshire, England). Jeechem® CAH 16 (PEG 16 hydrogenated castor oil) and Jeechem® CA 25 (PEG 25 castor oil) were donated by Jeen® International Corporation (Fairfield, NJ, USA). Kolliphor® EL (PEG 35 castor oil) and RH40 (PEG 40 hydrogenated castor oil) were donated by BASF Corporation (Washington, NJ, USA). Nikkol® HCO 100 (PEG 100 hydrogenated castor oil) was donated by Nikko Chemicals Co. Ltd. (Chuoku, Tokyo, Japan). Kolliphor® RH60 (PEG 60 hydrogenated castor oil) was purchased from Ingredients Plus (Notting Hill, VIC, Australia). Soybean oil (C_{18} long chain triglycerides—LCT); Triton™ X15, 165, 305, 705 (PEGylated branched octyl phenols); sodium taurodeoxycholate > 95% (NaTDC); porcine pancreatin (8× USP specification activity); and 4-bromophenylboronic acid (4-BPB) were obtained 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%) was obtained from Baxter Healthcare (Old Toongabbie, QLD, Australia). Sodium hydroxide 1.0 M, which was diluted to obtain 0.6 M and 0.2 M NaOH titration solutions, 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. Hypergrade solvents were used for UPLCMS/MS analysis.

2.2. Formulation preparation

2.2.1. Blank formulations

All formulations were prepared as binary mixtures of MCT (Captex® 355) and surfactant (50/50% w/w). The surfactants used are summarised in Table 1. All lipids and surfactants were heated to 37 °C and mixed prior to use to ensure excipient homogeneity. Semisolid excipients (typically surfactants with PEG Mw 800 and above) were heated to 50 °C prior to use. Formulations were vortexed for 30 s after preparation and equilibrated overnight at 37 °C before use.

2.2.2. Drug-loaded formulations

The equilibrium solubility of danazol in each of the drug-loaded LBFs was determined using previously described methodologies [32,33]. Equilibrium solubility was assessed in triplicate and 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. Danazol containing formulations had drug incorporated at a loading of 80% saturated solubility (based on measured values at equilibrium at 37 °C; Fig. S6). Danazol was accurately weighed into a glass screw cap vial with the required mass of formulation, vortexed and equilibrated for 24 h prior to assay. Danazol content was confirmed by HPLC assay prior to formulation use [16].

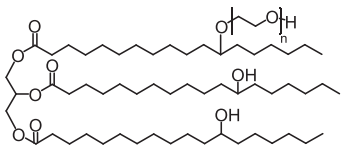
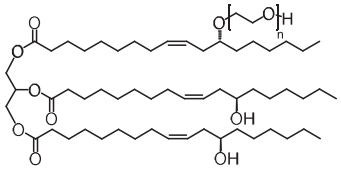
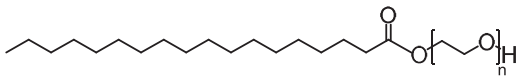
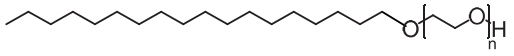
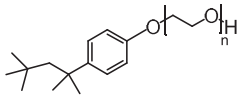
2.3. In vitro dispersion and digestion of formulations

2.3.1. Digestion conditions

For formulation screening experiments, *in vitro* dispersion and digestion were conducted as previously reported by the LFCS Consortium [34,35] (Standard conditions Table 2). For experiments conducted on the formulations that were ultimately progressed into rat bioavailability studies, adjustments to volume, enzyme activity and sample mass were made to better reflect a rat model of *in vitro* lipolysis as previously described by Anby et al. [36] (Rat conditions Table 2).

In vitro dispersion was monitored for 15 min and digestion monitored for 30 min. Lipolysis curves were generated from titration of fatty acids with NaOH. As fatty acids liberated during digestion are likely to be partially ionised, titre values were corrected for the presence of unionised fatty acid by back-titration at pH 9 [37,38]. Titre values were compared to the moles of fatty acid that were expected to be released from the formulation to yield a % digestion value. Details of the total extent of digestion calculations can be found in the supplementary material.

Table 1
Details of the six series of PEGylated surfactant studied during development of stealth LBFs.

PEGylated surfactant series (Trade name(s))	Surfactant general structure	PEG Mw (g mol^{-1})	PEG units [n] (approx.)	HLB*	Digestible***
Hydrogenated castor oils (HCO)** (Croduret™, Kolliphor®, Jeechem®, Nikkol®)		308[b]	7	4.9	
		705[d]	16	8.6	
		1102[b]	25	10.8	
		1763[c]	40	13.0	Yes
		2644[c]	60	14.7	
		4407[e]	100	16.5	
Castor oils (CO)** (Etocas™, Kolliphor®, Jeechem®)		220[b]	5	3.8	
		661[b]	15	8.3	
		1102[d]	25	10.8	
		1542[c]	35	12.5	Yes
		1763[b]	40	13.1	
		8814[b]	200	18.1	
Stearyl esters (SEs) (Myrj™S)		353[b]	8	11.1	
		881[b]	20	15.1	
		1763[b]	40	17.2	Yes
		2201[b]	50	17.7	
		4407[b]	100	18.8	
Stearyl ethers (SEt) (Brij™ S)		88[b]	2	5.0	
		441[b]	10	12.4	
		881[b]	20	15.3	No
		4407[b]	100	18.8	
		8814[b]	200	19.4	
Oleyl ethers (OEt) (Brij™ O)		88[b]	2	4.8	
		132[b]	3	6.4	
		220[b]	5	8.8	No
		441[a]	10	12.2	
		881[a]	20	15.2	
Branched octylphenols (bOP) (Triton™ X)		198[a]	4.5	9.8	
		419[a]	9.5	13.4	
		705[a]	16	15.5	No
		1322[a]	30	17.3	
		2424[a]	55	18.4	

Suppliers.
[a] Sigma Aldrich.
[b] Croda.
[c] BASF.
[d] Jeen.
[e] Nikko.
* HLB calculated from surfactant general structure and PEG Mw according to method proposed by Griffin [39].
** PEGylation of castor oil and hydrogenated castor oils results in a mixture of surfactants with PEG groups at any (or multiple) points on the triglyceride. The PEG unit values for these complex systems represent the number of moles of PEG reacted with 1 mol of triglyceride. The general structure is simplified.
*** Digestible surfactants are those containing an ester bond which may be labile to hydrolysis by gastrointestinal enzymes.

To accurately capture surfactant-mediated changes to both the initial rate of lipolysis and the extent of digestion, the degree of 'digestion inhibition' was expressed as the area under the % digestion curve (% digestion.min). To compare relative changes in digestion in the presence of structurally related surfactants, these AUC values were plotted against the molecular weight of the surfactant PEG headgroup. These digestion inhibition plots were compared to the AUC for MCT digestion in the absence of surfactant to provide an indication of the relative change to lipolysis over the entire period of digestion.

Table 2
Conditions for *in vitro* experiments employing standard and rat models of digestion. Table adapted from Anby et al. [36].

Digestion model (dilution/enzyme activity)	Standard (high / high)	Rat (high / low)
Mass formulation (mg)	1000	125
Digestion medium	Bile salt / phospholipid micelles	Bile salt/phospholipid micelles
Pancreatic enzyme (mL)	4 (high)	0.010 (low)
Total volume (mL)	40	5
Sample volume (mL)	1	0.2
pH Probe	iUnitrode	Biotrode
Vessel capacity (mL)	20–90 mL	5–70 mL
Propeller stirrer	Titrando 802 (25 mm, 3 propeller)	Titrando 802 (20 mm, 4 propeller)
Formulation dilution	40 (high)	40 (high)
Enzyme activity	High	Low
Equivalent to formulation dose (mg)	~1000	~30

For drug solubilisation during digestion experiments, formulations were loaded at 80% of the saturated solubility of drug in the formulation (Fig. S6). Aliquots (sample volumes as described in Table 2) were taken from the dispersion/digestion media throughout the experimental period at t = −10, −5, 0, 5, 15 and 30 min relative to initiation of digestion. Lipase inhibitor (4-BPB, 5 μL/mL of a 1.0-M solution in methanol) was added to prevent further lipolysis. All samples were centrifuged at 37 °C for 10 min at 21,000 g (Heraeus Fresco 21 microcentrifuge, Thermo Scientific, Langenselbold, Germany) in order to pellet drug that precipitated on dispersion and/or digestion. The digestion phases post-centrifugation were recovered according to previously reported protocols [34] and assayed for danazol content by HPLC using a validated HPLC method as previously described [16] (validation results detailed in supplementary material). Blank (drug free) formulations were also subjected to dispersion and digestion to obtain aqueous colloidal phases at the time points outlined above for determination of danazol

equilibrium solubility in the dispersed phase (AP_{DISP}) and digested phase (AP_{DIGEST}). These values were then utilised to calculate the maximum supersaturation ratio as described by Anby et al. [16]. Graphpad Prism for windows (Version 6.0, Graphpad Software Inc., CA, USA) was used to statistically analyse differences in pairs of *in vitro* data using unpaired parametric t-tests. Data were expressed as the mean ($n = 3$) ± standard deviation (SD). A difference was considered statistically significant when $p \leq 0.05$.

2.4. Oral bioavailability studies

2.4.1. Surgical and experimental procedures

All surgical and experimental procedures were approved by the Monash Institute of Pharmaceutical Sciences Animal Ethics Committee and were conducted in accordance with 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 fasted male Sprague–Dawley rats (270–330 g) maintained on a 12-h light/dark cycle. Rats were anaesthetised via inhalation of isoflurane (5% v/v induction, 2.5% v/v maintenance; Abbott Laboratories, NSW, Australia) for the duration of the surgical procedure. A cannula (polyethylene tubing of 0.96 mm o.d. × 0.58 mm i.d.) was inserted into the right carotid artery to allow serial blood collection. Cannulae were exteriorised to the back of the neck and were connected to a swivel tether system. Rats were transferred to individual metabolic cages and were fasted for at least 12 h prior to and 5 h post dose. Drinking water was provided ad libitum. The non-specific CYP inhibitor, 1-aminobenzotriazole (ABT), was pre-dosed 12 h prior to administration of the LBF to avoid complications to data interpretation due to potential differences in first pass metabolism [36,40]; 1.2 mL of 100 mg/g ABT was dosed via oral gavage to lightly anaesthetised rats. Rats were allowed to recover overnight prior to dosing of lipid formulations.

2.4.2. Formulation administration and sample collection

Rats were dosed with 30 mg of each of the oral lipidic formulations dispersed in 470 mg water and administered via oral gavage. Drug doses were 0.5, 1.1, 0.8 and 1.3 mg/kg for the HCO7, HCO40, CO5 and CO35 formulations, respectively. After oral administration of the formulation, a 0.5 ml water flush was administered via a clean oral gavage needle. Blood samples (250 μl) were obtained at pre-dose, 15, 30, 45, 60, 90 min, 2, 3, 4 and 5 h after oral administration. In all cases, cannulae 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 sodium heparin and centrifuged for 5 min at 6700 ×*g* (Eppendorf minispin plus, Eppendorf AG, Hamburg, Germany). Plasma was collected and stored at −20 °C until assayed for danazol content.

2.5. Quantitation of danazol in plasma samples by UPLC-MS/MS

All plasma analyses were performed using a validated method on a Waters Xevo TQS UPLC MS/MS system (Waters, Milford, MA, USA). Run parameters and method validation results are detailed in the supplementary information.

2.6. Pharmacokinetic data analysis

The data were plotted as danazol concentration (ng/mL) vs. time (h) (normalised to a nominal dose of 1 mg/kg danazol). Non-compartmental pharmacokinetic parameters were calculated using Phoenix™ 64 Software (WinNonlin® version 6.3, Pharsight Corporation, CA, USA). Graphpad Prism for windows (Version 6.0, Graphpad Software Inc., CA, USA) was used to statistically analyse differences in pairs of *in vivo* data using unpaired parametric *t*-tests. All data represented are expressed as the mean ± standard error of mean (SEM). A difference was considered statistically significant when $p \leq 0.05$.

3. Results

3.1. In vitro evaluation

3.1.1. Stealth LBF development—effect of surfactant PEG Mw

To evaluate the impact of PEG molecular weight on the ability of non-ionic surfactants to modulate lipid digestion, a series of commercially available hydrogenated castor oil (HCO) surfactants of increasing PEG Mw (Table 1) were formulated with MCT and subjected to digestion experiments under standard lipolysis conditions. Fig. 1 shows *in vitro* lipolysis data for triglyceride alone and the series of HCO surfactants. The extent of digestion of MCT in the absence of surfactant was only 50% after 30 min (Fig. 1A). The addition of an HCO of PEG Mw 308 g/mol (approximately 7 PEG units, HCO 7) increased the initial rate of lipolysis; however, the overall extent of digestion was slightly lower than that of MCT alone (Fig. 1B). Further increasing the PEG Mw of the surfactant to 705 and 1102 g/mol (Fig. 1C, HCO 16 and Fig. 1D, HCO 25) had a marked impact on the rate and extent of *in vitro* lipolysis with the overall extent of digestion reducing to less than 20%. Maximal digestion inhibition (2% digestion) was equivalent to a 15-fold

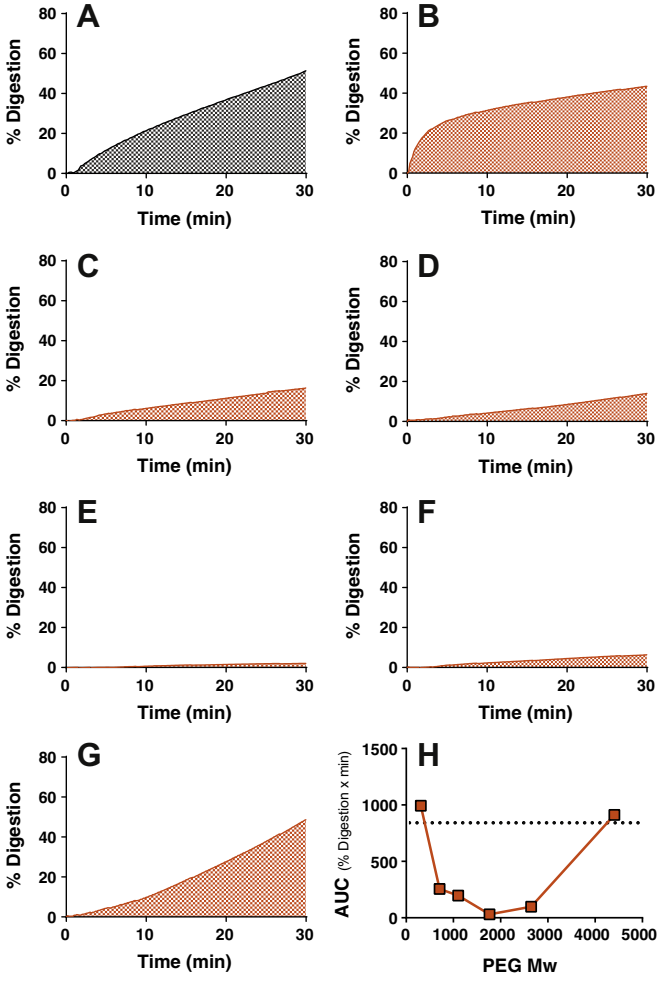


Fig. 1. (A–G) *In vitro* lipolysis profiles for formulations comprising MCT and hydrogenated castor oil surfactants of increasing PEG Mw. Shaded areas represent the AUC calculated using the linear trapezoidal method and this was used to plot the digestion inhibition curve in H. (A) MCT alone, (B) HCO 7 (PEG Mw 308), (C) HCO 16 (PEG Mw 705), (D) HCO 25 (PEG Mw 1102), (E) HCO 40 (PEG Mw 1763), (F) HCO 60 (PEG Mw 2644) and (G) HCO 100 ((PEG Mw 4407). (H) Digestion inhibition curve for the hydrogenated castor oil surfactants plotting AUC (% digestion.min) against PEG Mw.

reduction in lipolysis and was observed for the HCO 40 surfactant (Fig. 1E, PEG Mw 1763 g/mol, Kolliphor® RH40), a surfactant that has been previously used in more complex self-emulsifying formulations *in vitro* and *in vivo* [41,42]. Further increasing the PEG Mw to 2644 g/mol (Fig. 1F, HCO 60, Kolliphor® RH60) resulted in a recovery in digestion to 6%, indicating that this surfactant was marginally less effective at preventing lipid digestion. When the surfactant hydrophilic headgroup was increased to a molecular weight of 4407 g/mol (Fig. 1G, HCO 100), the digestion inhibiting properties of the PEG headgroup were lost.

Plotting these lipolysis profiles as area under the lipolysis curve (% digestion.min) versus surfactant PEG Mw in Fig. 1H reveals a parabolic relationship between the Mw of the PEG groups in the surfactants and the rate and extent of lipolysis. The dotted line shows the degree of digestion of the MCT formulation alone (i.e., in the absence of surfactant). Formulations below the dotted line reduced the extent of lipid digestion. The formulations that occupy the minima of these digestion inhibition curves were considered to be the most effective 'stealth' formulations, preventing adsorption of enzyme and thus lipolysis of the co-formulated triglyceride.

To gain a better understanding of the relationship between surfactant PEG Mw and LBF stealth properties, five further series of formulations comprising structurally diverse surfactants were screened using the same *in vitro* lipolysis assay. Figs. S1 to S5 in the supplementary material show *in vitro* lipolysis and digestion inhibition curves for a range of ethoxylated castor oils (CO, Fig. S1), stearyl esters (SEs, Fig. S2), stearyl ethers (SEt, Fig. S3), oleyl ethers (OEt, Fig. S4) and branched octyl phenols (bOP, Fig. S5). All surfactant series showed a parabolic relationship between PEG Mw and the rate and extent of digestion with the exception of the oleyl ethers and branched octyl phenols. These two data sets were truncated to lower PEG Mw headgroups due to difficulty in sourcing a commercial supply of the high Mw PEG variants for each surfactant. Interestingly, the minimum rate of digestion obtained for the castor oil (CO, Fig. S1E) formulation series corresponds to the Kolliphor® EL surfactant, which has been extensively employed in the development of self-emulsifying drug delivery systems and is well tolerated *in vivo* [16,41,42]. As lipolysis rates are also influenced by surface area [17], the particle size of two series of stealth LBFs was measured by photon correlation spectroscopy to ensure that reductions in digestion rate were not attributable to a reduction in surface area available for enzyme interaction. Figs. S7 and S8 in the supplementary material show the particle size distribution of the formulations containing the hydrogenated castor oil and castor oil series of surfactants, respectively. The particle size data display a similar parabolic relationship between surfactant PEG Mw and particle size, indicating that in general as the PEG Mw increases, the dispersed droplet size decreases (and therefore surface area increases, in contrast to the reduction in digestion). However, at low and very high PEG Mw, the formulations become more polydisperse with multiple populations and the presence of large oil droplets.

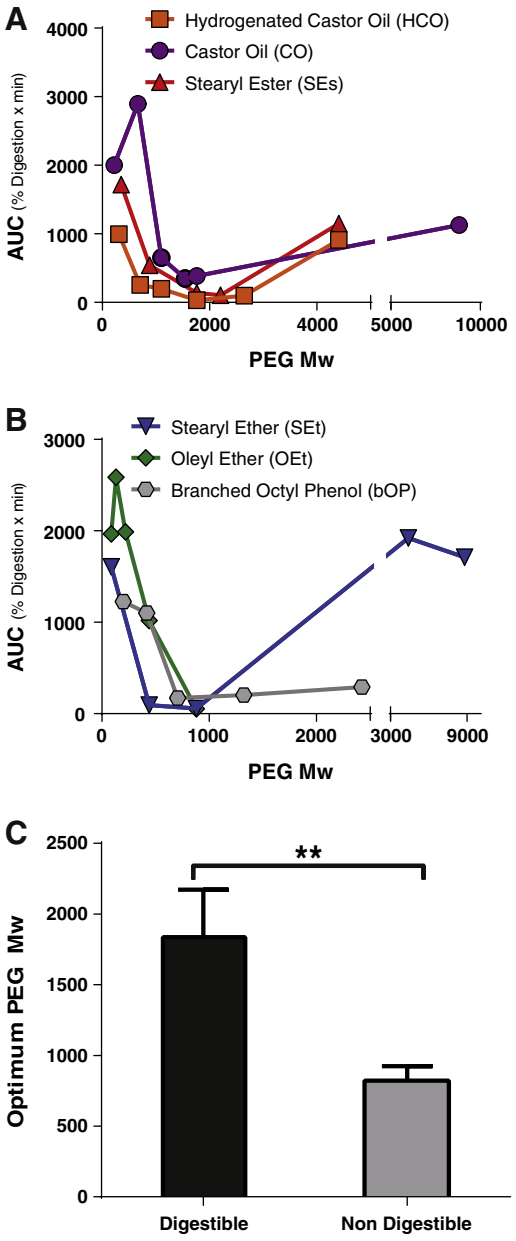


Fig. 2. Overlaid digestion inhibition curves for MCT formulations comprising six series of PEGylated surfactant (A) digestible surfactants, (B) non-digestible surfactants (C) optimal PEG Mw for digestible (black bar) and non-digestible (grey bar) surfactant components of stealth lipid-based formulations. **Statistically significant difference ($p < 0.05$).

3.1.2. Stealth LBF development—effect of surfactant digestibility

To further probe the structural determinants of stealth in regard to oral LBFs, digestion inhibition plots were separated into digestible (esters) and non-digestible (ether) groups. A trend in optimum surfactant PEG Mw and digestion inhibition was apparent. Fig. 2A shows overlaid digestion inhibition curves for the three series of digestible surfactants, namely, ethoxylated hydrogenated castor oils, ethoxylated castor oils and ethoxylated stearyl esters. Maximal digestion inhibition was achieved in a molecular weight range of ~1500–2000 g/mol. Fig. 2B shows overlaid digestion inhibition curves for the three series of non-digestible surfactants; ethoxylated stearyl ethers, ethoxylated oleyl ethers and ethoxylated branched octylphenol ethers. The molecular weight range that resulted in maximal digestion inhibition was ~700–900 g/mol. Fig. 2C provides summary data showing the optimal PEG Mw for digestible and non-digestible surfactant components of stealth LBFs.

3.1.3. Stealth LBF development—effect of PEG chain density

Pairs of structurally analogous surfactants with the same PEG Mw were compared to evaluate the potential for structural differences in the surfactant hydrophobe to influence the digestion of co-formulated MCT. Fig. 3 shows the impact of fatty acid chain saturation/linearity (and indirectly, PEG packing density) on digestion inhibition. The non-digestible ethoxylated stearyl and oleyl ethers (SEt, OEt) were compared at PEG molecular weights of 88, 441 and 881 g/mol. The lower molecular weight PEG surfactant formulations show a clear difference between the straight chain saturated stearyl ether and the unsaturated oleyl ether, with the former showing a greater digestion inhibition effect. When the surfactant PEG Mw was increased to 881 g/mol, there were no discernible differences in digestion inhibition between

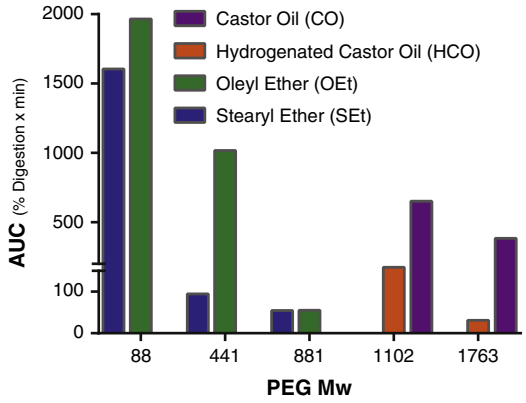


Fig. 3. Comparison of digestion inhibition capability for matched pairs of formulations at equivalent PEG Mw. Formulations differ in fatty acid saturation on the surfactant hydrophobe moiety. Purple bars, PEGylated castor oils; orange bars, PEGylated hydrogenated castor oils; green bars, PEGylated oleyl ethers; blue bars, PEGylated stearyl ethers.

formulations; however, this molecular weight also corresponded with maximal digestion inhibition for both formulations. The effect of differences in the structure of the surfactant hydrophobe was also evaluated using pairs of digestible surfactants. Ethoxylated HCO and CO surfactants differ in fatty acid saturation in the hydrophobe chains, the former comprising an ethoxylated triglyceride based on 12-hydroxy stearic acid while the latter is based on a triglyceride comprising unsaturated ricinoleic acid. At a PEG Mw of 1102 and 1763 g/mol, the saturated fatty acid HCO formulations were again more potent digestion modulators than the equivalent unsaturated CO formulation.

3.1.4. Impact of stealth on drug solubilisation in vitro

Fig. S6 shows the equilibrium solubility of danazol in two series of formulations comprising PEGylated castor oils and hydrogenated castor oils; these surfactant series were selected as HCO 40 and CO 35 are well tolerated *in vivo*. Four formulations, one digestible formulation and one stealth formulation from each group (namely; HCO 7, HCO 40, CO 5 and CO 35) were selected for further *in vitro* analysis.

Drug solubilisation experiments were conducted based on the rat *in vitro* digestion model proposed by Anby et al. (Table 2) [36]. Formulations were loaded with danazol at 80% saturated solubility in the formulation. Fig. 4A shows overlaid drug solubilisation and digestion profiles for the low molecular weight PEG surfactant (HCO 7) formulation. Under rat lipolysis conditions (where lipase activity is lower than the comparable conditions in the dog or human), this formulation maintains drug solubilisation for 15 min. At 30 min, the formulation shows extensive precipitation which correlates to a concomitant increase in digestion. Maximal supersaturation ratios (S^M) for this formulation during dispersion and digestion were 1.2 and 4.6, respectively. Overlaid solubilisation and digestion profiles are also plotted for the analogous stealth formulation HCO 40 (Fig. 4B).

The stealth formulation did not undergo digestion in the rat model of lipolysis (<1%) and this was reflected in the maintenance of drug solubilisation and relatively unchanged maximal supersaturation ratios (S^M) throughout the digestion period. Similar data for the castor oil surfactant-based formulations were observed and are summarised in Fig. 4C and D. The digestible CO 5 formulation (Fig. 4C) shows a loss in drug solubilisation and increase in S^M from 0.7 to 6.6 following digestion of the formulation. The stealth CO formulation (CO 35, Fig. 4D), however, shows little digestion of the formulation (<1%) and results in sustained drug solubilisation and supersaturation.

3.2. In vivo evaluation of stealth LBFs

The mean plasma concentration versus time profiles for danazol following oral administration of stealth and equivalent digestible formulations are depicted in Fig. 5. A summary of the pharmacokinetic

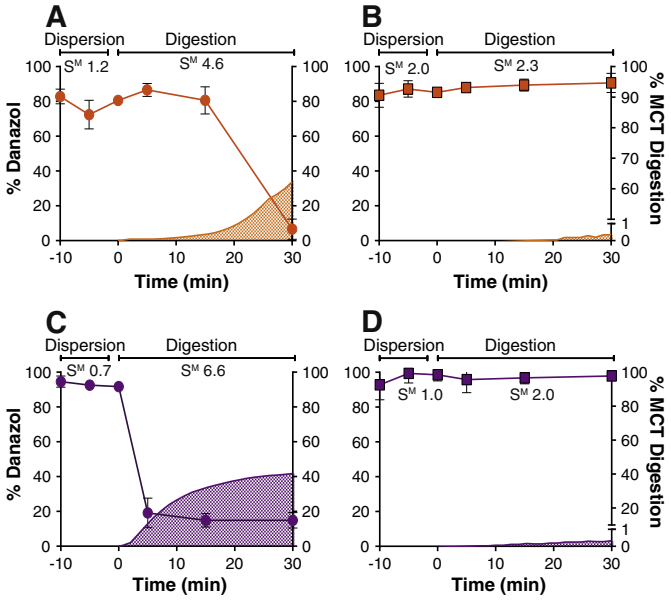


Fig. 4. Danazol solubilisation profiles (filled circles, left *Y* axis) during dispersion and digestion of PEGylated hydrogenated castor oil (orange) and castor oil formulations (purple) (shaded areas represent lipolysis curves, right *Y* axis) [mean ± SD ($n = 3$)] using a rat model of digestion, i.e., high dilution, low enzyme activity. (A) HCO 7, (B) HCO 40, (C) CO 5 and (D) CO 35. Danazol was incorporated into all formulations at 80% saturated solubility.

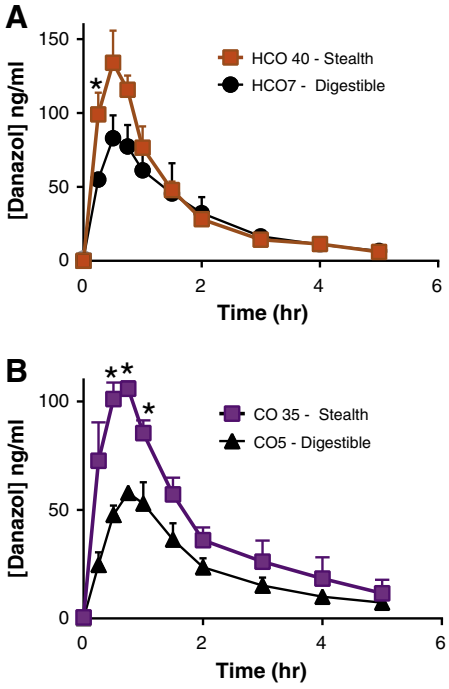


Fig. 5. Mean danazol plasma concentration vs. time profiles for PEGylated hydrogenated castor oil formulations (A) and PEGylated castor oil formulations (B) after oral administration. Digestible formulations were HCO 7 and CO 5 (black circles and diamonds, respectively) and stealth formulations were HCO 40 (orange squares) and CO 35 (purple squares). Data normalized to a 1 mg/kg dose of danazol, [mean ± SEM ($n = 3$)]. *Statistically significant difference ($p < 0.05$).

Table 3
Pharmacokinetic parameters for danazol after oral administration of digestible and stealth formulations with danazol incorporated at 80% saturated solubility [mean ± SEM ($n = 3$)] to fasted male Sprague–Dawley rats.

	Digestible formulations		Stealth formulations	
	HCO 7	CO 5	HCO 40	CO 35
$AUC_{0-\infty}$ (ng.h/mL)[a]	169.0 ± 13.3	134.4 ± 18.2	202.2 ± 27.5	244.4 ± 67.4[b]
C_{max} (ng/mL)	86.5 ± 8.0	61.1 ± 5.8	139.8 ± 16.0	105.8 ± 3.4[b]
T_{max} (h)	0.6 ± 0.1	0.8 ± 0.1	0.6 ± 0.1	0.8 ± 0.0
$t_{1/2}$ (h)	1.4 ± 0.2	1.5 ± 0.2	1.4 ± 0.0	1.8 ± 0.6
Rel bioavailability (%)	N/A	N/A	119.6	181.8

[a] Data normalized to a 1 mg/kg danazol dose to facilitate comparison across differing drug doses.
[b] Statistically significant increase when compared to CO5 formulation, $p < 0.05$.

parameters is provided in Table 3. To permit cross-comparison, pharmacokinetic data were normalised to a 1 mg/kg drug dose. The results of the *in vivo* study show that $AUC_{0-\infty}$ and C_{max} for danazol after administration of the stealth LBFs was greater than those obtained after administration of the corresponding digestible formulations. This trend was replicated for both hydrogenated castor oil and castor oil-based formulations.

The relative bioavailability (defined as the ratio of $AUC_{0-\infty}$ for the stealth LBF and its structurally analogous digestible LBF) was 120% and 182% for the ethoxylated HCO and CO formulations, respectively, illustrating that PEG-mediated digestion inhibition (stealth) improved formulation performance and consequently danazol bioavailability. No significant differences in T_{max} were observed between the stealth formulations and the corresponding digestible formulations.

4. Discussion

Increasing numbers of drug candidates emerging from lead optimisation programs have intrinsically low water solubility and high lipophilicity [3]. This has driven a need to develop enabling formulations that facilitate the oral delivery of poorly water-soluble drugs. Significant recent attention has focused on the development of formulation approaches that enhance GIT solubility and dissolution rate [3]. Of these, lipid-based formulations (LBF) have grown in popularity and have been shown to markedly improve the oral bioavailability of a number of poorly water soluble drugs [2,3]. Despite this, it is becoming increasingly apparent that digestion-mediated changes to drug solubilisation can have a detrimental impact on the *in vivo* performance of LBF, particularly those containing medium-chain triglycerides (MCT). MCT are popular excipients in LBF since they typically allow higher drug doses to be dissolved in the formulation than their long chain counterparts.

Losses in drug solubilisation capacity during formulation digestion can lead to a transiently supersaturated state, which has the potential to promote drug absorption [16]. In instances where supersaturation is significant and/or prolonged, however, it may also promote precipitation. In some cases, the addition of polymeric inhibitors to prevent crystal seeding and reduce drug precipitation can recover these formulations and improve *in vivo* outcomes [11,16,43], but this is not always the case [16]. In contrast, the prevention of digestion-mediated losses in solubilisation capacity, through manipulation of the lipolysis process itself, has, to date, not been explored as an avenue to improved LBF performance *in vivo*. Lipolysis is an inherently surface sensitive reaction, and changes to the oil–water interface might be expected to alter the rate of digestion [17,18]. Tan et al. have utilised the surface sensitivity of the digestion process to increase lipolysis rates, demonstrating that this in turn improves drug absorption from silica lipid nanoparticles [44,45]. Conversely, the potential for non-ionic PEGylated surfactants to modulate or inhibit triglyceride digestion has been documented [17,19–23]; however, the utility of this inhibitory effect has not been explored in detail nor translated into differences in *in vivo* bioavailability.

PEG is a neutral non-immunogenic polymer that is miscible with water and has high motility in aqueous media and a large hydrodynamic volume [46,47]. These properties render the polymer resistant to protein adsorption. PEGylated interfaces find multiple biological applications where nonspecific protein adsorption is unwanted, most notably in nanomedicine where PEG surface coatings are used to prolong the circulation half-life of injectable nanomaterials through avoidance of opsonisation and phagocytosis. This PEG shielding effect was coined 'stealth' by Allen and co-workers in 1991 [48]. The long-circulating performance of a stealth nanoparticle can be directly attributed to a combination of the molecular weight and surface density of the PEG mantle [26–31]. An understanding of these key structural determinants of stealth may provide a template for the development of improved LBFs after oral administration since the adsorption of lipase to the droplet interface (and therefore the possibility of digestion-mediated drug precipitation) is analogous to the opsonisation of a nanoparticle in the systemic circulation. The prevalence of PEGylated non-ionic surfactants in lipid-based drug delivery provides a promising platform from which to develop stealth LBFs.

The main objective of the current study was therefore to develop 'stealth' oral lipid-based formulations from PEGylated surfactants and MCT, to evaluate the impact of these formulations on drug solubilisation and supersaturation *in vitro* and to assess whether this was manifest in improved *in vivo* drug absorption.

4.1. PEGylated surfactants as digestion modulators

4.1.1. Stealth properties are dependent on surfactant PEG Mw

Parenteral stealth formulations require a polymeric steric stabilisation layer of adequate size and surface coverage to prevent protein adsorption and to evade capture by the cells of the MPS. For example, Mori et al. [28] described a relationship between PEG molecular weight and immunoliposome circulatory half-life in Balb/c mice, while Torchilin and co-workers [27,29] demonstrated that long-circulating liposomes required optimised polymer-to-lipid ratios for adequate surface coverage and that this was directly dependent on the Mw of the PEG group. This putative PEG molecular weight/stealth relationship has since been extensively employed to guide the development of stealth nanoparticulate drug delivery vehicles.

More recently, it has been shown that PEGylated surfactants may change the rate and extent of lipolysis of homogenised emulsions in a PEG molecular weight dependant manner [23]. These data from Wulff-Perez and co-workers showed that the digestion of preformed sunflower oil emulsions containing different surfactants, two ethoxylated stearyl esters and two triblock co-polymers, was dependent on PEG Mw. However, the authors failed to find a comprehensive explanation for differences in digestion inhibition for two surfactants of equal PEG Mw.

The current studies applied the principles of stealth drug delivery to lipid-based formulation development by systematically investigating the impact of surfactant PEG Mw and packing density (hydrophobe structure) on the digestion of triglycerides. These data then informed the development of self-assembled stealth lipid-based formulations for oral administration of a poorly water-soluble drug. Fig. 1 shows the direct impact of PEGylated surfactants on the digestion of MCT while Fig. 2 shows a parabolic relationship between PEG Mw and the rate and extent of digestion for six structurally diverse series of PEGylated surfactants. As the PEG Mw of the surfactants increased, the AUC of the digestion profiles (% digestion.min) decreased, suggesting that enzyme adsorption had been reduced, presumably via the generation of a polymeric steric barrier. Figs. S7 and S8 show that the reduction in digestion is not attributable to a reduction in surface area as the particle size of dispersed formulations decreased (and therefore surface area increased) with increasing PEG Mw.

As the PEG headgroup of the surfactant increased further and exceeded a critical maximum, the digestion inhibition effect was

reduced. The latter effect may be attributable to an imbalance between the large motile hydrophilic headgroup and the smaller hydrophobic tail of the surfactant. At increasingly higher molecular weights of the hydrophilic headgroup, the emulsifiers may become unstable at the surface of the lipid droplet and dissociate into the aqueous digestion environment, displaying at best, a transient inhibitory effect. This is consistent with the particle size data in Figs. S7 and S8, which show that surfactants with high Mw PEG headgroups were less efficient emulsifiers. These formulations dispersed to form multiple populations of droplets with very large (>5000 nm) phase separated oil droplets. Similar observations have been made in relation to the use of amphiphilic surface modifiers in liposome development [49].

4.1.2. Digestible surfactants require larger PEG Mw to impart stealth properties

Further examination of the digestion inhibition data in Figs. 3 and 4 reveals a statistically significant correlation ($p < 0.05$) between the digestibility of the surfactant and the optimal surfactant PEG Mw for maximal digestion inhibition or 'stealth' capability. As a rule of thumb, the digestible surfactants required a PEG Mw of ~1800 g/mol to maximally shield the surfactant ester group and the co-formulated triglycerides from digestion. The non-digestible surfactants employed, however, did not possess labile ester bonds and as a result the PEG Mw required to sterically shield the MCT payload was significantly lower (approximately 800 g/mol).

4.1.3. The structure of the surfactant hydrophobe influences the extent of digestion inhibition

Parenteral stealth formulations have been developed based on optimising the quantity of polymer on the surface of the carrier (or the density of the PEG chains) [26,27,29,49]. Accurately controlling this parameter for the development of self-assembled stealth LBFs is limited by the commercial availability of PEGylated surfactants where the hydrophobic moiety has been changed systematically. Despite these limitations, Fig. 5 provides some information as to the impact of the surfactant hydrophobe on PEG packing at the oil–water interface. Comparing pairs of formulations at constant surfactant PEG Mw but with differing degrees of saturation of the hydrophobe moiety provides insight into the influence of surfactant packing at the droplet interface on digestion inhibition. Thus, unsaturated, non-linear (or 'kinked') surfactant hydrophobes (that presumably pack less efficiently at the interface [50]) were less effective at reducing digestion than their straight chain counterparts. This trend was evident for simple non-digestible PEGylated fatty alcohols and more complex digestible PEGylated triglycerides.

4.2. Stealth LBFs improve drug solubilisation in vitro and drug absorption in vivo

Fig. 4 shows the profound impact of stealth LBFs on the solubilisation of danazol during *in vitro* digestion. After a 30-min lipolysis period, the stealth formulations had been digested by less than 1%, and this was reflected in the maintenance of drug solubilisation at >80% of initial load. In contrast, the readily digestible, low Mw PEG equivalent formulations were rapidly digested and lost solubilisation capacity for the formulated drug. These results were also reflected in the maximal supersaturation ratios (S^M) generated for each formulation type, with stealth LBFs maintaining or marginally increasing S^M while digestible LBFs showed significant increases in S^M from dispersion to digestion. As has been previously reported [16,35], S^M values greater than 3 are typically associated with systems that are prone to precipitation and less likely to perform efficiently *in vivo*.

Finally, the plasma profiles in Fig. 5 and the pharmacokinetic parameters presented in Table 3 reveal differences in *in vivo* exposure of danazol after oral administration of stealth and digestible formulations. Two series of surfactants were investigated, polyethoxylated hydrogenated castor oils and polyethoxylated castor oils. The trends between the two data sets were similar; the generation of a self-assembled PEG mantle around the lipid droplet prevented rapid digestion and precipitation of the formulated drug. This in turn resulted in improved drug absorption where the increase in relative bioavailability of the stealth vs non-stealth formulations was 120% and 182% for HCO and CO formulations, respectively.

5. Conclusions

The current studies have explored the potential translation of concepts that have been successfully employed to enable parenteral stealth applications to oral drug delivery. The data suggest that similar to parenteral stealth formulations, the presence of a hydrophilic PEG mantle on the surface of a lipid droplet in the gastrointestinal tract is able to reduce protein binding (in this case, the binding of digestive enzymes). Furthermore, the data suggest that the degree of stealth activity is dependent on the Mw of the PEG surfactant headgroup as well as the packing of the polymer at the surface of the oil droplet. Interestingly, the presence of hydrolysable groups within the surfactant structure affected the performance of the oral stealth LBFs, with digestible surfactants requiring larger Mw PEG headgroups to generate an effective stealth barrier.

Controlling the rate of formulation hydrolysis through the generation of a stealth PEG barrier also altered the propensity of a formulation to supersaturate on digestion, reducing the maximal supersaturation ratio (S^M) and thus reducing the risk of drug precipitation. Finally, *in vivo* administration of stealth and analogous digestible formulations to male SD rats resulted in increased exposure for groups dosed with stealth formulations, confirming that the prevention of rapid supersaturation and precipitation in the GIT is an effective strategy for improving the oral absorption of poorly water soluble drugs.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jconrel.2014.07.037.

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Supplementary Information:

‘Stealth’ Lipid Based Formulations: Poly(ethylene glycol) Mediated Digestion Modulation Improves Oral Bioavailability of a Model Poorly Water Soluble Drug.

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Keywords: Lipid based formulation, drug absorption, stealth; PEGylation; *In vitro* digestion; Lipid based drug delivery; lipolysis; pharmacokinetics

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Calculation of total extent of digestion

To determine the total extent of digestion, back titrations were performed at the end of the digestion experiments (30 min). At the conclusion of the digestion period, 1 M sodium hydroxide was rapidly added to the reaction vessel to increase the pH to 9.0. The quantity of sodium hydroxide added was used to calculate the number of moles of unionized FA present. This was then used to calculate an ionisation ratio of ionised to total fatty acid; equation 1.

$$\text{Ionisation Ratio} = \frac{\text{mmol FA at 30min}}{\text{total mmol FA (ionised+unionised)}} \quad (1)$$

Lipolysis profiles were then adjusted for ionisation in order to more accurately estimate and plot the extent of digestion (% digestion) over time for each formulation. Lipolysis is a dynamic process and ionisation of fatty acids is anticipated to vary over time as the quantity of fatty acids increase and the apparent pKa of the fatty acids changes with the changing digestion environment. Utilisation of ionisation ratios acquired at the end of the lipolysis experiment provides a maximum value of ionisation ratio, this method, while oversimplifying the lipolysis and fatty acid ionisation processes, allows for more accurate estimation of the extent of digestion than in systems where unionised fatty acids are not quantified.

$$\%\ \text{Digestion} = \frac{\text{mmol FA}_{\text{(at time t)}} \text{ x } 100}{\text{ionisation ratio x Theoretical Max mmol FA}} \quad (2)$$

All digestion experiments were corrected for fatty acids released from the digestion media in the absence of formulation. Calculation of theoretical molar maxima of fatty acids was based on the assumption that 1 mole triglyceride will be hydrolysed to yield one mole of monoglyceride and two moles of fatty acid in the presence of pancreatic lipase. Triglyceride based surfactants were assumed to be subject to similar patterns of hydrolysis. Fatty acid based surfactants were expected to yield one mole of fatty acid per mole surfactant. The fatty acid contribution of both lipid and surfactant (where applicable) was factored into the calculation of extent of digestion (% digestion).

HPLC-UV method parameters and validation

Digested samples were analysed on a Waters Alliance 2695 separation module and Waters 486 tunable absorbance detector (Waters Instruments, Milford, MA). Chromatographic separation was carried out on a Waters Symmetry C18 column (150 × 15 mm, 5 µm) coupled to a C18 security guard cartridge (4 × 2.0 mm, Phenomenex), the column was maintained at ambient temperature. The injection volume was 50 µL and UV absorbance was monitored at 286 nm. The mobile phase consisted of methanol and water in a 75:25 v/v ratio at a flow rate of 1 mL/min. Assay validation was conducted by multi-day matrix analysis of validation samples (n = 6 per group) of 0.5, 5, and 50 µg/mL danazol in blank aqueous phases. System precision, % RSD (n = 5) 2.4%; interassay precision, % RSD (n=18) 8.2%; intermediate precision, % RSD (n = 12) 7.6%; accuracy, (n = 24) 89.5 – 107.4%; recovery from spiked blank digesta, (n = 6) oil 93%, pellet 95.2%, aqueous phase 98.4%; linearity, R^2 0.999 – 1; specificity, no interfering peaks at RT of Danazol.

HPLC-MS/MS method parameters and validation

Plasma samples were analysed on a Waters Xevo TQS UPLC MS/MS system (Waters, Milford, MA, USA), autosampler temperature was 4 °C and column temperature was 25 °C. Chromatographic separation was conducted on a Kinetex phenyl hexyl column (50 × 2 mm, 2.7 µm) (Phenomenex, Torrence, CA), coupled to a Gemini C6-phenyl security guard cartridge (4 × 2.0 mm) (Phenomenex). The injection volume was 5 µL. The mobile phase consisted of 0.1% formic acid in Mili Q water (A) and 0.1% formic acid in acetonitrile (B). Gradient elution at a constant flow rate of 0.5 mL/min was performed as follows: 41% B linearly increasing to 55% B in 3.75 min.

Under these conditions the retention times of danazol and internal standard (IS) progesterone were 2.1 and 1.9 min, respectively. Elution was immediately succeeded by a 1.25 min wash step. Total run time was 5 min including the wash step.

The MS/MS conditions were optimised as follows: source temperature, 150 ºC; desolvation temperature, 200 ºC; cone gas flow, 150 L/h, desolvation gas flow, 900 L/h; collision gas flow, 0.16 mL/min; cone voltage, 31 kV; capillary voltage, 3.68 kV; collision energy, 24 kV. Data acquisition and peak integration were performed using MassLynx software, version 4.1 (Waters).
Unknown plasma concentrations were determined by interpolation from a weighted (1/X) calibration curve of danazol:IS peak response plotted as a function of danazol concentration. Assay validation was conducted by multi-day matrix analysis of validation samples (n = 6 per group) of 0.5, 5, and 125 ng/mL danazol in blank plasma with IS. System precision, % RSD (n = 5) 3.4%; interassay precision, % RSD (n=18) 11.5%; intermediate precision, % RSD (n = 18) 14.6%; accuracy, (n = 24) 81.3 – 126.4%; recovery, (n = 6) 79%; linearity, R^2 0.995 – 0.999; specificity, no interfering peaks in blank plasma extracts. The limit of quantitation was arbitrarily set at 0.5 ng/mL; the lowest concentration of the validated concentration range.

Particle Size Determination for Stealth LBF dispersions

Photon correlation spectroscopy (Malvern Nano-ZS zetasizer, Malvern Instruments, Worcestershire, U.K.) was used to determine the average particle size (average particle diameter in nm based on light scattering by volume), the % of each size population in the sample and the polydispersity index (PDI) of the self-emulsified LBFs. Formulations were dispersed to match the concentrations obtained in lipolysis experiments, thus 0.1g formulation was dispersed in a total of 3.6 ml digestion media. Samples were stirred at 37’C for 15 min and maintained at 37’C throughout particle size analysis. Size data was plotted as the mean for three separate sample measurements (mean ± SD (n = 3).

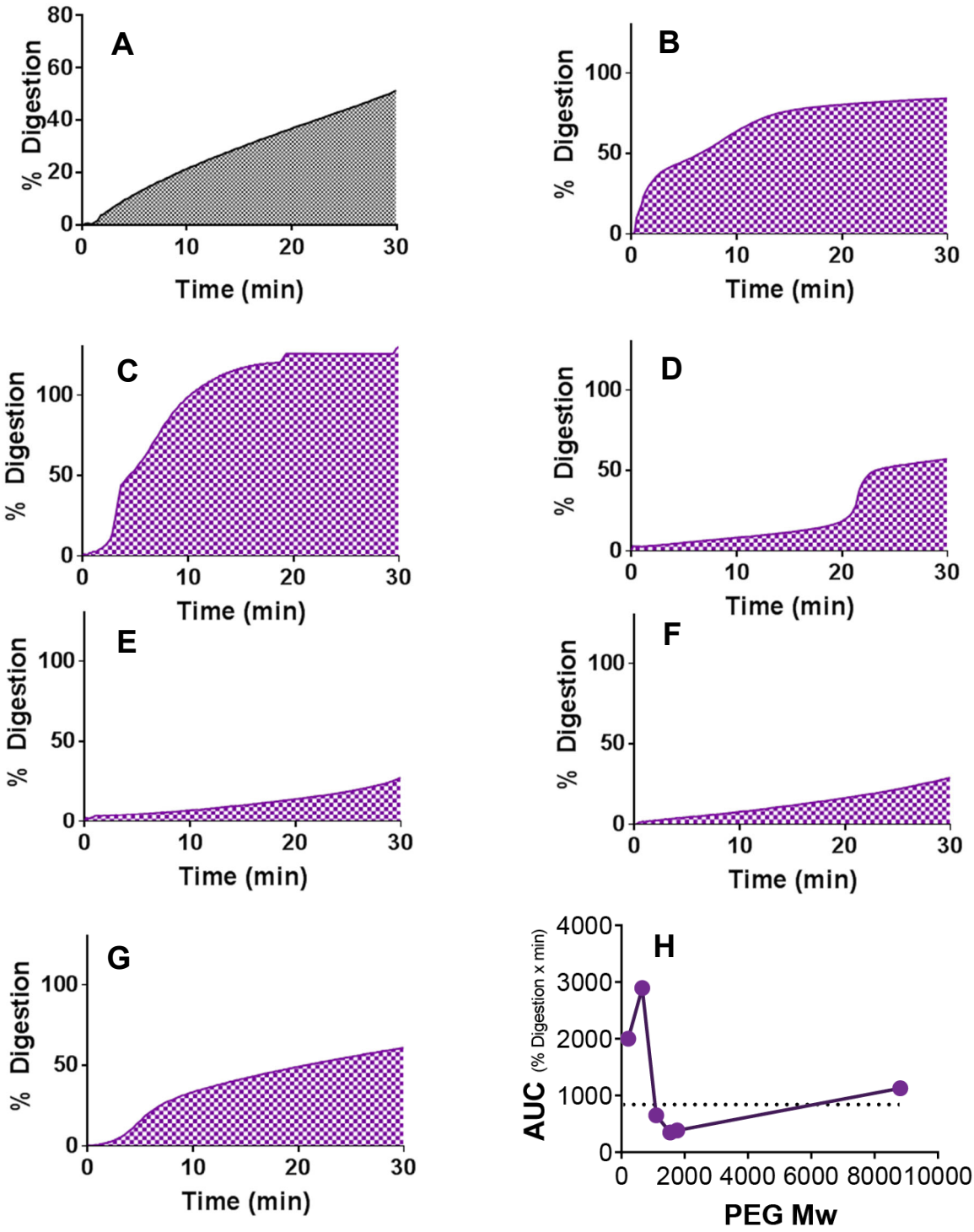

Figure S1. (A – G) *In vitro* lipolysis profiles for formulations comprising MCT and castor oil surfactants of increasing PEG Mw. Shaded areas represent the AUC calculated using the linear trapezoidal method and were used to plot the digestion modulation curve in H. (A) MCT alone, (B) CO 5 (PEG Mw 220), (C) CO 15 (PEG Mw 661), (D) CO 25 (PEG Mw 1102), (E) CO 35 (PEG Mw 1542), (F) CO 40 (PEG Mw 1763), and (G) CO 200 ((PEG Mw 8814). (H) Digestion modulation curve for castor oil surfactants plotting AUC (% digestion.min) against PEG Mw. The dashed line represents the AUC for MCT in the absence of surfactant.

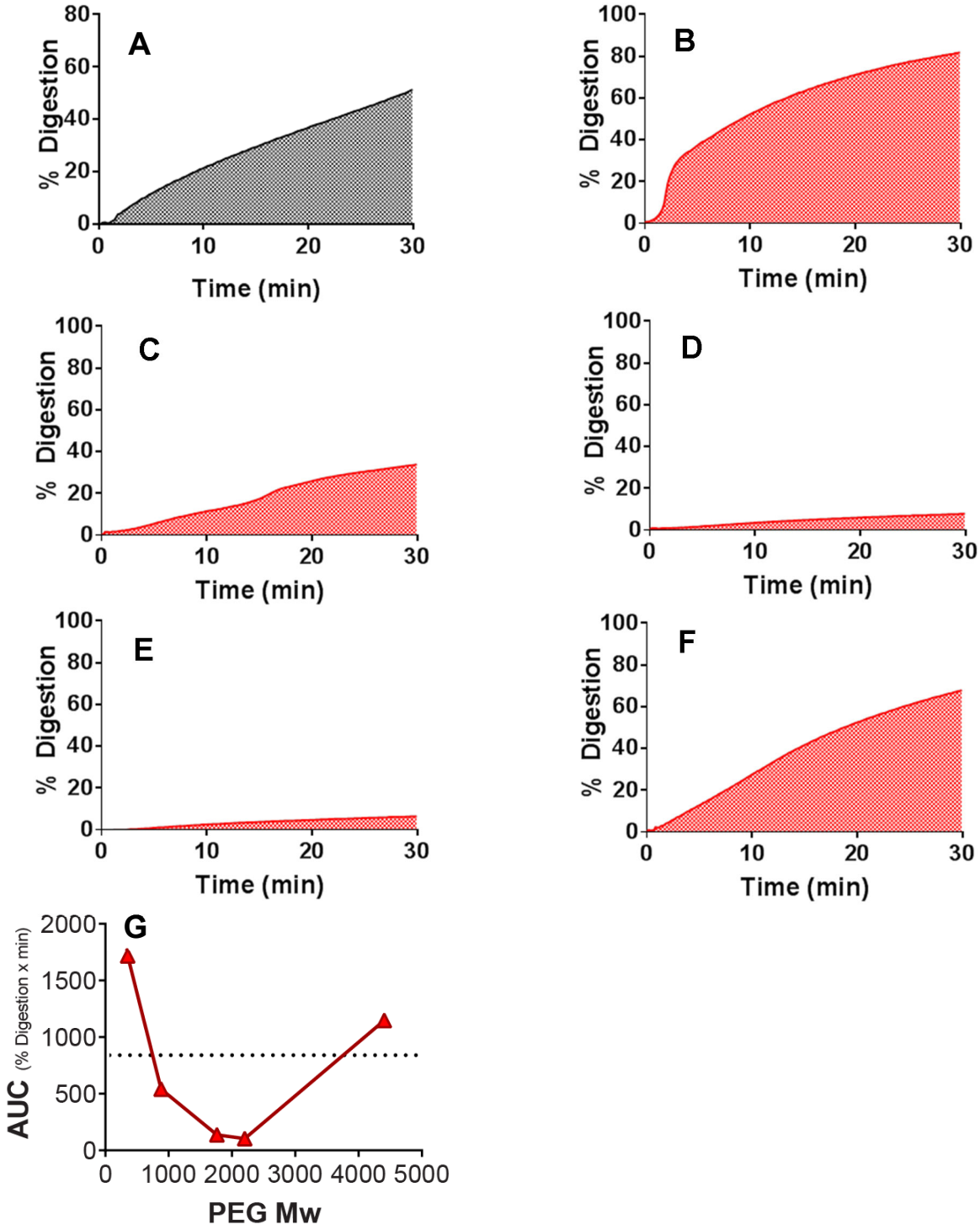

Figure S2. (A – F) *In vitro* lipolysis profiles for formulations comprising MCT and stearyl ester surfactants of increasing PEG Mw. Shaded areas represent the AUC calculated using the linear trapezoidal method and were used to plot the digestion modulation curve in G. (A) MCT alone, (B) SEs 8 (PEG Mw 353), (C) SEs 20 (PEG Mw 881), (D) SEs 40 (PEG Mw 1763), (E) SEs 50 (PEG Mw 2201) and (F) SEs 100 (PEG Mw 4407). (G) Digestion modulation curve for stearyl ester surfactants plotting AUC (% digestion.min) against PEG Mw. The dashed line represents the AUC for MCT in the absence of surfactant.

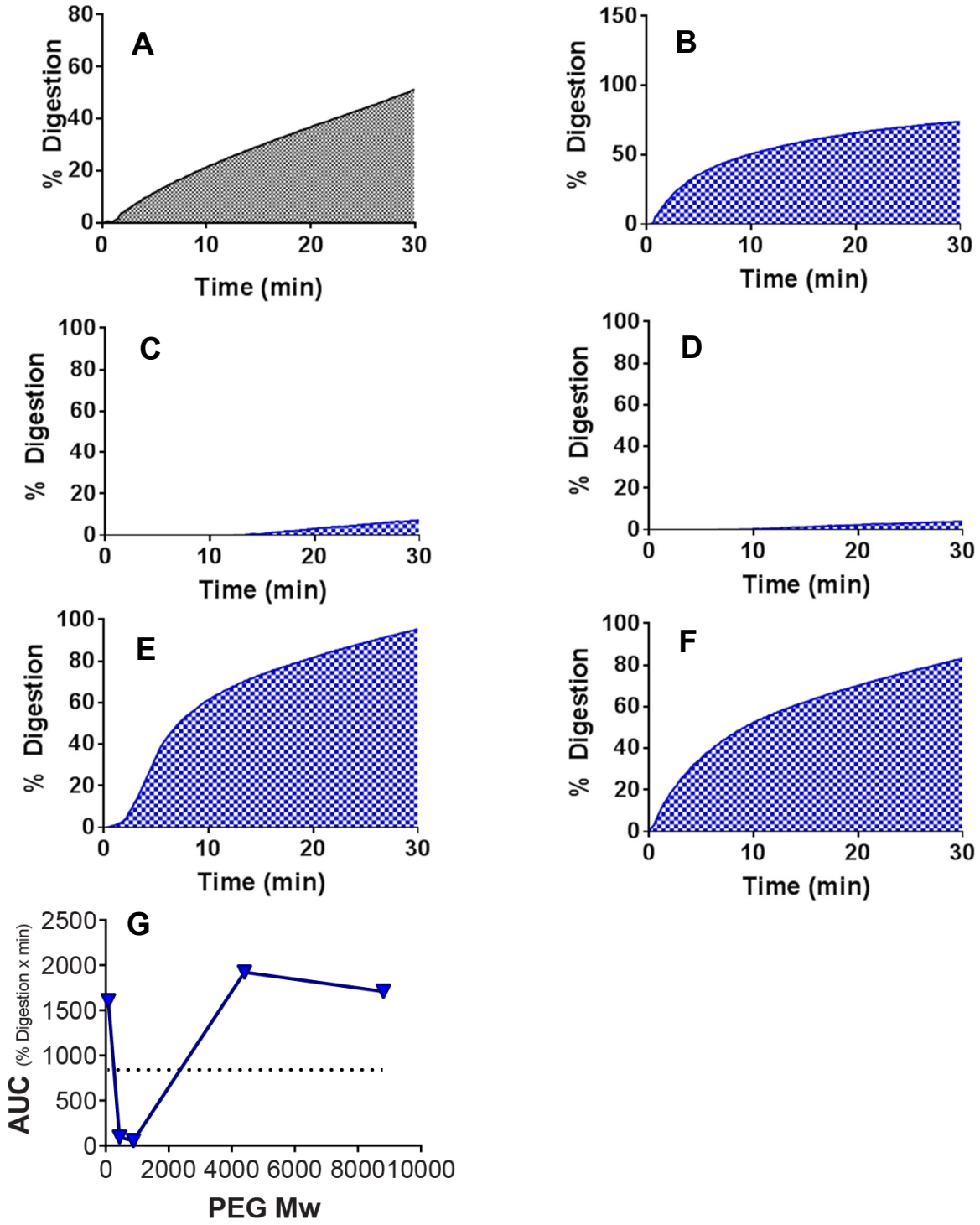

Figure S3. (A – F) *In vitro* lipolysis profiles for formulations comprising MCT and stearyl ether surfactants of increasing PEG Mw. Shaded areas represent the AUC calculated using the linear trapezoidal method and were used to plot the digestion modulation curve in G. (A) MCT alone, (B) SEt 2 (PEG Mw 88), (C) SEt 10 (PEG Mw 441), (D) SEt 20 (PEG Mw 881), (E) SEt 100 (PEG Mw 4407) and (F) SEt 200 (PEG Mw 8814). (G) Digestion modulation curve for stearyl ether surfactants plotting AUC (% digestion.min) against PEG Mw. The dashed line represents the AUC for MCT in the absence of surfactant.

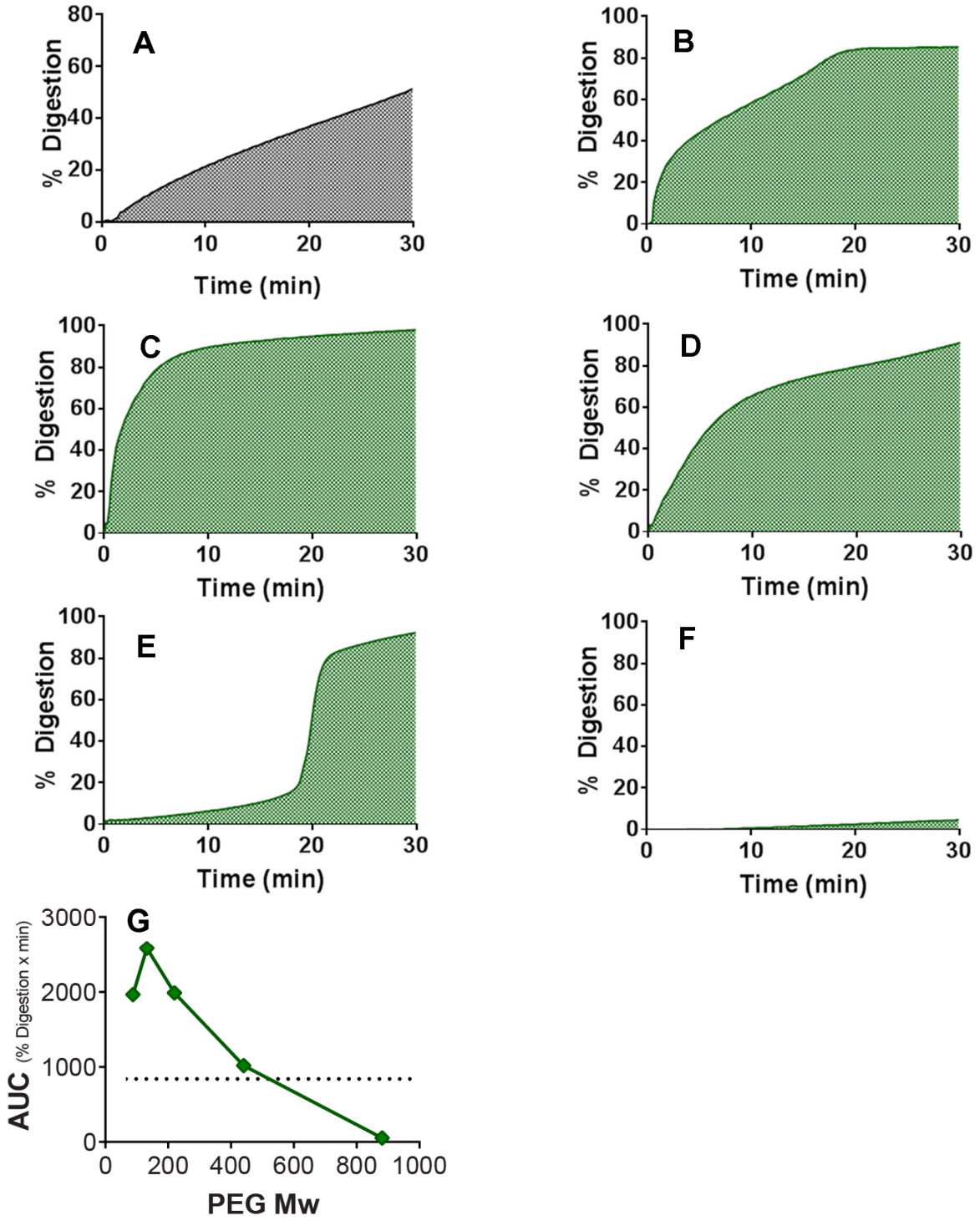

Figure S4. (A – F) *In vitro* lipolysis profiles for formulations comprising MCT and oleyl ether surfactants of increasing PEG Mw. Shaded areas represent the AUC calculated using the linear trapezoidal method and were used to plot the digestion modulation curve in G. (A) MCT alone, (B) OEt 2 (PEG Mw 88), (C) OEt 3 (PEG Mw 132), (D) OEt 5 (PEG Mw 220), (E) OEt 10 (PEG Mw 441) and (F) OEt 20 (PEG Mw 881). (G) Digestion modulation curve for oleyl ether surfactants plotting AUC (% digestion.min) against PEG Mw. The dashed line represents the AUC for MCT in the absence of surfactant.

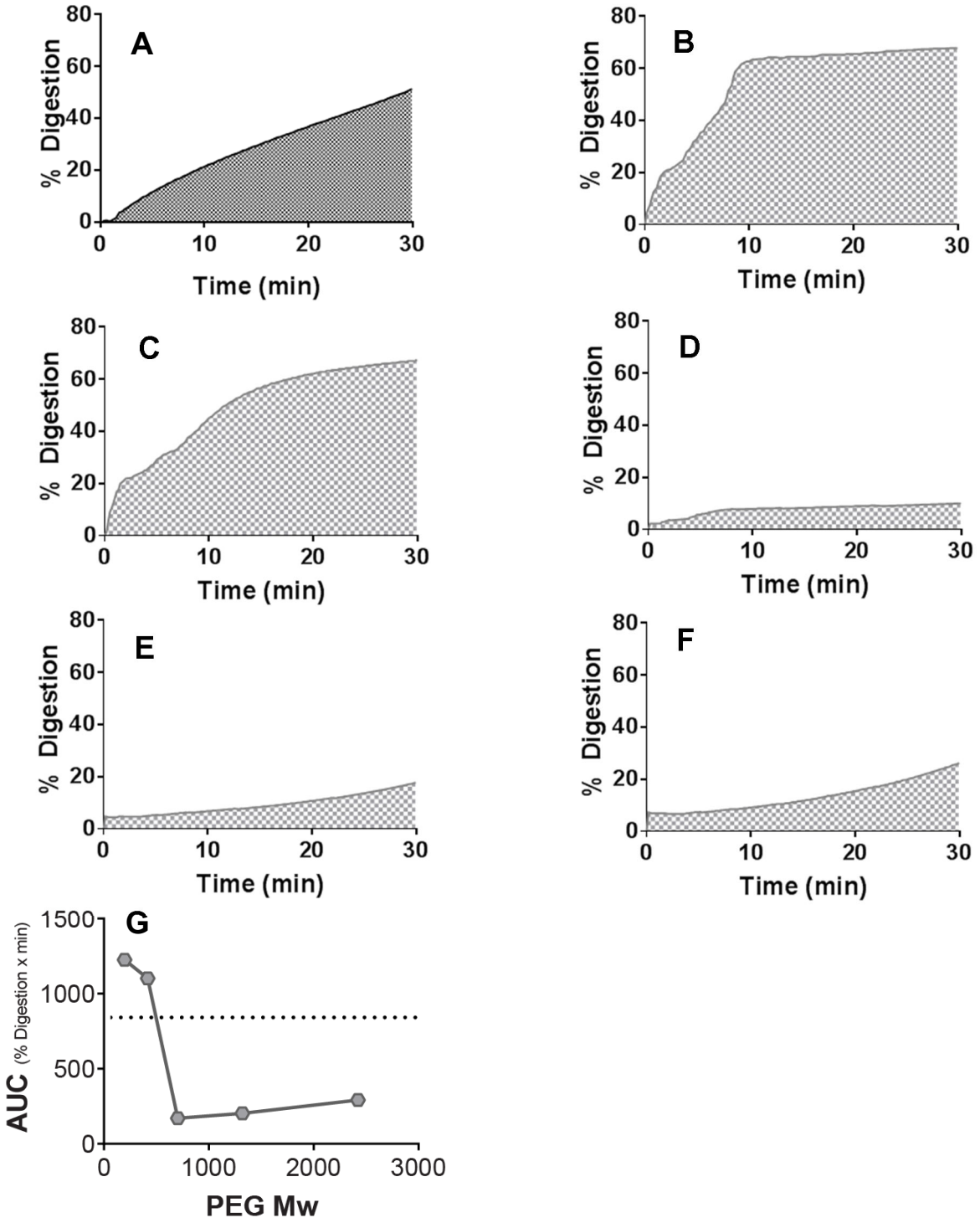

Figure S5. (A – F) *In vitro* lipolysis profiles for formulations comprising MCT and branched octyl phenol surfactants of increasing PEG Mw. Shaded areas represent the AUC calculated using the linear trapezoidal method and were used to plot the digestion modulation curve in G. (A) MCT alone, (B) bOP 4.5 (PEG Mw 198), (C) bOP 9.5 (PEG Mw 419), (D) bOP 16 (PEG Mw 705), (E) bOP 30 (PEG Mw 1322) and (F) bOP 55 (PEG Mw 2424). (G) Digestion modulation curve for branched octyl phenol surfactants plotting AUC (% digestion.min) against PEG Mw. The dashed line represents the AUC for MCT in the absence of surfactant.

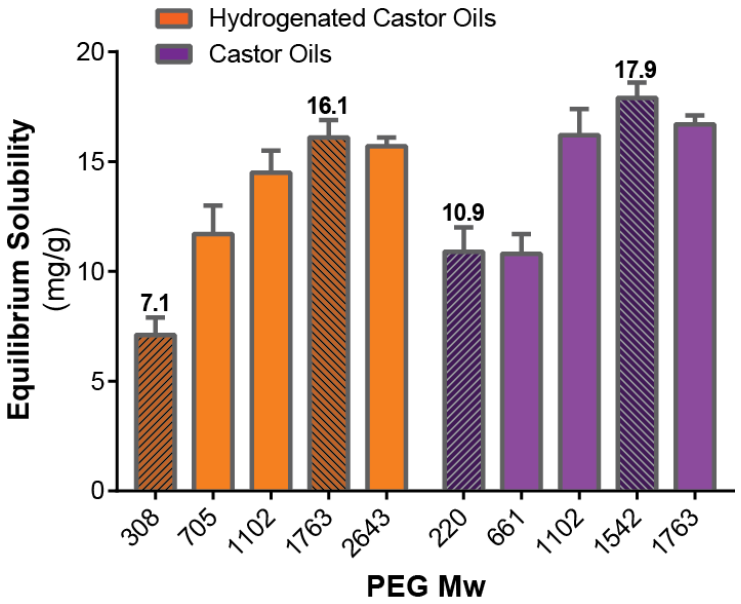

Figure S6. Danazol equilibrium solubility in LBFs formulated from the series of PEGylated hydrogenated castor oil surfactants (orange bars) and PEGylated castor oil surfactants (purple bars) [mean ± SD (n = 3)]. Darker shaded bars in both groups represent the formulations progressed for *in vitro* characterisation and *in vivo* administration. Equilibrium solubility values are annotated above the formulations.

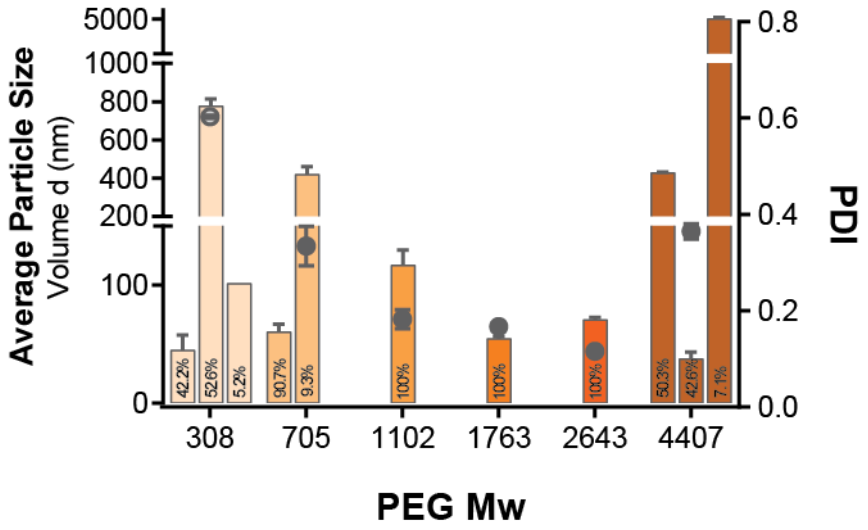

Figure S7. Average particle size (bars, left axis) and polydispersity index (dots right axis) for LBFs formulated from the series of PEGylated hydrogenated castor oil surfactants. Each size population is plotted as a separate bar with % volume annotated inside the bar. [Mean ± SD (n = 3)].

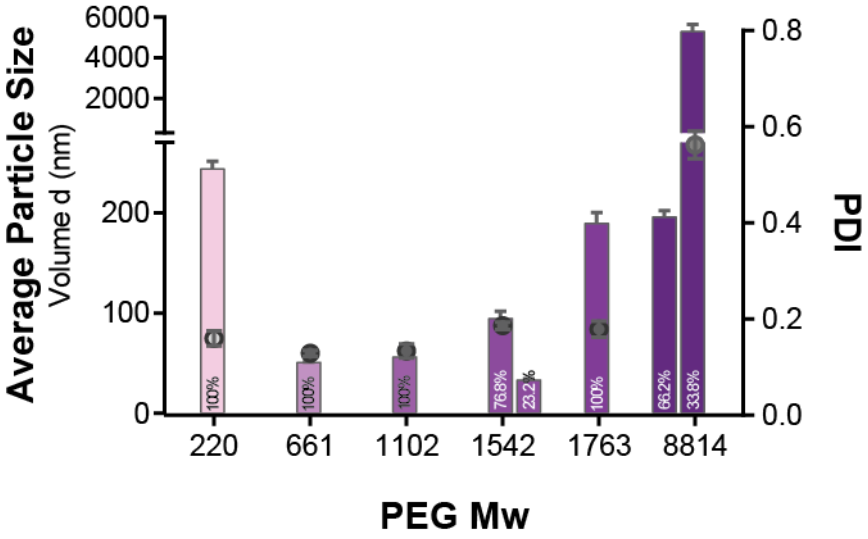

Figure S8. Average particle size (bars, left axis) and polydispersity index (dots right axis) for LBFs formulated from the series of PEGylated castor oil surfactants. Each size population is plotted as a separate bar with % volume annotated inside the bar. [Mean ± SD (n = 3)].

Appendix 3

An *in vitro* Digestion Test That Reflects Rat Intestinal Conditions To Probe the Importance of Formulation Digestion vs First Pass Metabolism in Danazol Bioavailability from Lipid-based Formulations

Mette U. Anby, Tri-Hung Nguyen, Yan Yan Yeap, Orlagh M. Feeney, Hywel D. Williams, Hassan Benameur, Colin W. Pouton and Christopher J. H. Porter

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An in Vitro Digestion Test That Reflects Rat Intestinal Conditions To Probe the Importance of Formulation Digestion vs First Pass Metabolism in Danazol Bioavailability from Lipid Based Formulations

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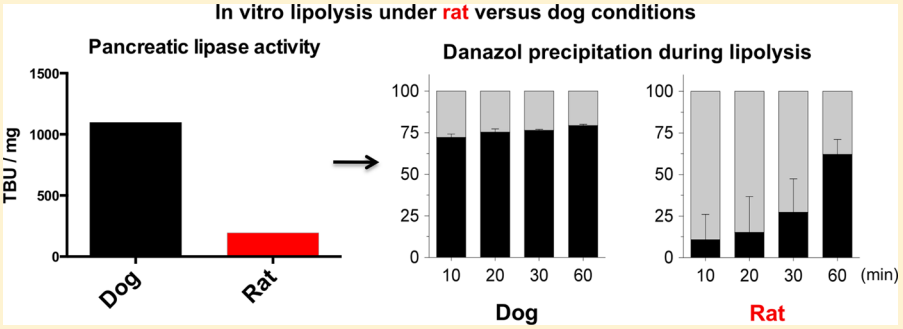
Supporting Information



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

KEYWORDS: *absorption, lipid-based drug delivery systems, in vitro digestion, supersaturation, danazol, first pass metabolism, bioavailability*

INTRODUCTION

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

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Table 1. Composition (% w/w) of Self-Emulsifying Lipid-Based Formulations Containing Danazol[d]

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

[a]Lipid Formulation Classification System (LFCS) as described by Pouton et al.[26,27] [b]Equivalent to 40% of the saturated solubility in the formulation at 37 °C. [c]Equivalent to 80% of the saturated solubility in the formulation at 37 °C. [d]Captex 300 (medium-chain triglycerides); Capmul MCM (medium-chain mono-, di-, and triglycerides); Cremophor EL (surfactant); ethanol (cosolvent).

In response to dilution, and interaction of the formulation with pancreatic and biliary fluids, the solubilization 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 solubilization capacity and the generation of transient supersaturation.[9−11] Supersaturation ultimately provides a driver for precipitation, and where this is significant, drug absorption from SEDDS is typically compromised. Realization that drug precipitation on digestion may provide an indication of formulation performance has led to increasingly common utilization of in vitro models of lipid digestion to assist in the design and evaluation of candidate formulations.[12−15]

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

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

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

In light of this, the objectives of the current studies were 2-fold. First, we sought to further explore the role of drug dose on the bioavailability of danazol from SEDDS and in particular to evaluate the potential limitations of first pass metabolism. Studies were conducted in rats to allow more facile comparison of the impact of first pass metabolism versus gastric and intestinal processing on danazol bioavailability. The results of these studies, however, raised a question regarding the ability of previous in vitro lipid digestion models to accurately describe events in the rat. As such, a second objective of the current studies was to identify modifications to the in vitro lipolysis model that allow better alignment with events in the rat GI tract. The data suggest that, in the rat, lipid digestion may be less efficient than it is in the dog (or potentially in humans), that danazol absorption from SEDDS formulations is relatively high (>50%), and that the principal limitation to danazol bioavailability is first pass metabolism.

MATERIALS AND METHODS

Materials. Danazol (pregna-2,4-dien-20-yno[2,3-*d*]isoxazol-17-ol) was supplied by Coral Drugs PVT (New Delhi, India), and progesterone and 1-aminobenzotriazole (ABT) were from Sigma-Aldrich (St. Louis, MO, USA). Captex 300 and Capmul MCM EP were kindly donated by Abitec Corporation (Janesville, WI, USA). Soybean oil, Cremophor EL (polyoxyl 35 castor oil), sodium taurodeoxycholate >95% (NaTDC), porcine pancreatin (8 × USP specification activity), glyceryl tributyrate, 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). Anesthetics: Parnell ketamine (100 mg/mL) from Parnell Laboratories, NSW, Australia, Ilium xylazine-100 (100 mg/mL) from Troy Laboratories, NSW, Australia), and ACP 10 (13.5 mg of acepromazine maleate equivalent to 10 mg of acepromazine) from Ceva Delvet, NSW, Australia were used as received. Sodium hydroxide 1 M, which was diluted to obtain 0.6 M NaOH titration solution, was from Merck (Darmstadt, Germany), and water was purified with a Milli-Q (Millipore, Bedford, MA, USA) system. All other chemicals and solvents were of analytical purity or high performance liquid chromatography (HPLC) grade.

Formulation Preparation. *SEDDS Formulations for Oral/Intraduodenal Administration.* All formulations were prepared as previously described,[22] and danazol, molecular structure illustrated in Table 1 (aqueous solubility 0.59 μg/mL,[23] log *P* of 4.53[24]), was incorporated at low (L) and high (H) drug loading (40% or 80% of saturated solubility in the formulation based on measured values at equilibrium at 37 °C).

Drug solubility in each formulation was assessed in triplicate as previously described.[9,25] The formulations are summarized in Table 1. After mixing, the formulations were vortexed for 30 s and equilibrated overnight at 37 °C.

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

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

BIOAVAILABILITY STUDIES IN RATS

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

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

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

Formulation Administration and Sample Collection. Rats were fasted for 12 h prior to dosing and remained fasted until the conclusion of the study. Drinking water was provided ad libitum. In groups pretreated with ABT (100 mg/kg), a single bolus dose (1.2 mL) of ABT in saline (25 mg/mL) was administered via oral gavage postsurgery, approximately 14 h prior to IV, oral, or ID dosing.[30,31] A similar dosing protocol has been shown previously to provide almost complete inhibition (93%) of CYP-mediated antipyrine clearance.[30]

The intravenous formulation (2.3 mg/kg) was administered over 0.5 min by infusion pump (1 mL/min) via the indwelling jugular vein cannula. The cannula was subsequently flushed with 0.5 mL of saline. Blood samples (300 μL) were taken predose 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 predispersed in water (100 mg of formulation + 400 mg of Milli-Q water) immediately prior to administration. For ID dosing, the predispersed formulations were administered into the duodenum via the ID cannula over 30 min at a constant infusion rate (1 mL/h) followed by infusion of 0.5 mL of Milli-Q water to flush the cannula. Blood samples (300 μL) were taken predose and at 15, 25, 35, 45, 60, and 90 min and 2, 3, and 5 h after ID dosing. For oral administration, rats were lightly anesthetized via inhalation of isoflurane (2.5% v/v), and the predispersed formulations were dosed via oral gavage followed by 0.5 mL of Milli-Q water to rinse the gavage tube. Blood samples were taken predose and at 15, 30, 45, 60, and 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 heparinized saline (2 IU/mL) between samples to ensure patency of the cannula. Samples were centrifuged for 5 min at 6700g (Eppendorf minispin plus, Eppendorf AG, Hamburg, Germany) to separate plasma. Collected plasma samples were stored in eppendorfs at −80 °C until analysis.

Quantification of Danazol in Plasma Samples by LC–MS. Plasma samples and calibration standards for danazol were prepared and quantified by LC–MS as previously described.[34]

Pharmacokinetic Data Analysis. The peak plasma concentrations (C_{max}) and the time for their occurrence (T_{max}) were noted directly from the individual plasma concentration vs time profiles. The areas under the plasma concentration vs time profiles to the last measured time point (AUC_{0-tz}) were calculated using the linear trapezoidal method. In the absence of ABT treatment, the AUC was in general very low and the terminal phase poorly defined. The mean terminal elimination rate constant from the IV study was therefore used to extrapolate the AUC to infinity ($AUC_{0-\infty}$). In the presence of ABT, the AUCs were higher and extrapolated AUC was based on elimination rate constants obtained from individual plasma profiles. Clearance (Cl), volume of distribution (Vd_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.

IN VITRO EXPERIMENTS

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

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

For experiments using ex vivo gastric fluids, male Sprague–Dawley (SD) rats (300–400 g) were fasted for 12 h prior to surgery with free access to drinking water. Animals were anesthetized with isoflurane (5% v/v) and a ligature tied around the esophageal and duodenal apertures of the stomach. The stomach was excised and rinsed with 900 μL of Milli-Q (the

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

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

[a]Source of porcine pancreatic extract as previously published[37] [b]Ex vivo rat pancreatic/biliary fluid contains bile. Experiments were therefore carried out in digestion buffer without added bile salt. In vitro digestions using porcine pancreatic extract were performed in digestion medium containing bile salt micelles (5 mM NaTDC and 1.25 mM phosphatidycholine (PC)). [c]The total volume of pancreatic enzyme added was kept constant (1 mL). The 1 mL volume was made up of 17 μL enzyme extract and 983 μL digestion medium. The quantity of enzyme extract is listed here to emphasize comparison with other groups. The additional volume of digestion medium is included in the total volume of media employed.

volume of fluid dosed with the formulations in the oral bioavailability experiments). The rinsing fluid ("ex vivo stomach fluid") was collected and utilized in low volume dispersion experiments as described above.

In Vitro Digestion of SEDDS Formulations. The impact of digestion on the solubilization 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 summarized in Table 2 and described below.

Dog Digestion Model: High Dilution/High Enzyme Activity. In Vitro Digestion Model (High Dilution) Using 100% Porcine Pancreatin Extract (1000 TBU/mL). This in vitro model was used in previous in vitro studies[9] and was designed to estimate the impact of digestion on SEDDS formulations in larger species such as dogs or humans.[12] In these studies, 1 g of formulation was dispersed in 36 mL of digestion medium in a thermostatically controlled (37 °C) vessel and digestion initiated by addition of porcine pancreatin extract (4 mL).

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

In Vitro Rat Digestion Model (Low Dilution) Using 1.7% Porcine Pancreatin Extract. In vitro digests were also performed using the "rat" digestion protocol described above (high dilution/low enzyme) but using a quantity (17 μL) of porcine enzyme that provided a similar enzyme activity to that of 1 mL ex vivo rat pancreatic fluid. The activity of the ex vivo rat pancreatic/biliary fluid and the quantity of porcine pancreatic fluid required to mimic this activity were evaluated using a tributyrin assay as described below.

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

In Vitro Rat Digestion Model (Low Dilution) Using 1.7% Porcine Pancreatin Extract. In vitro digestion experiments were also conducted as described above, but with ex vivo rat bile/pancreatic fluid replaced with 17 μL of porcine pancreatic extract.

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

titration unit (Radiometer, Copenhagen, Denmark). Digestion was followed for 30 min and lipase activity in TBU (μmol of titratable butyric acid per minute) calculated from the initial rate of digestion (i.e., via the slope of the titration profile (i.e., fatty acids (mmol) liberated per min) multiplied by the molar strength of titrant (0.5 M)).

To determine the relative lipase activity of ex vivo rat pancreatic fluid and the porcine pancreatin extract used previously, the lipase activity of 1 mL of porcine pancreatin enzyme extract was also examined under the same conditions (although in this case the digestion buffer contained 5 mM NaTDC and 1.25 mM phosphatidycholine (PC) since the enzyme preparation did not contain bile). Porcine pancreatic lipase extract was prepared by dispersing 1000 mg of porcine pancreatin (8 × USP specification activity) in 5 mL of digestion buffer. The mixture was stirred for 15 min and centrifuged for 10 min (2880*g* at 5 °C, Eppendorf 5804 R centrifuge, Eppendorf AG, Hamburg, Germany). The supernatant was separated and used for digestion studies on the day of preparation.

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

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

Sample Workup for in Vitro Dispersion/Digestion Experiments. The lipase inhibitor 4-bromophenylboronic acid[42] (4-BPB; 9 μL of a 0.5 M solution in methanol per mL of digestion medium) was added to all in vitro digestion samples to prevent further digestion of the lipid components during phase separation. Dispersion samples were centrifuged for 10 min and digestion samples for 30 min at 21100*g* (Heraeus Fresco 21 microcentrifuge, Thermo Scientific, Germany) to form a pellet phase and a solubilized aqueous phase (AP). Samples obtained from each phase were diluted (aqueous phase, 50 to 950 μL in ACN; precipitate (pellet) and oil phase, initially in 50 μL of chloroform/methanol (2:1 v/v) and this further diluted with 950 μL of MeOH) and then analyzed by HPLC as below.

Quantification of Danazol in in Vitro Experiments. Danazol concentrations in samples taken from in vitro dispersion and digestion experiments were measured by HPLC as described previously.[9] The results are presented as the % drug distribution in the aqueous phase (AP), i.e., the proportion of the drug initially dissolved in the formulation that was recovered in the aqueous phase postcentrifugation of the dispersion or digestion media.

RESULTS

In Vivo Evaluation. *Intravenous Pharmacokinetics of Danazol.* The mean plasma concentration versus time profile for danazol following intravenous administration of a 15% HP-β-CD solution containing danazol at 1.2 mg/mL to rats is shown in Figure 1 with corresponding mean pharmacokinetic

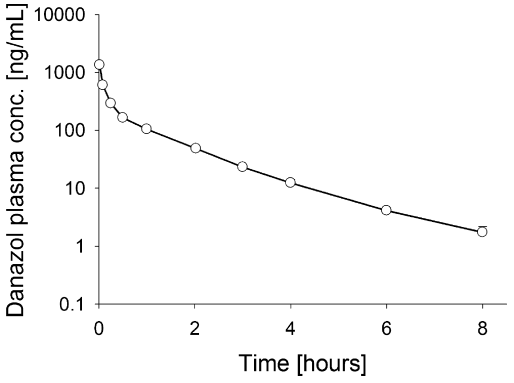

Figure 1. Danazol plasma profile after IV administration of 15% HP-β-CD solution containing danazol at 1.2 mg/mL (total dose 2.3 mg kg^{-1}) to rats [mean ± SEM (n = 5)].

Table 3. Pharmacokinetic Parameters after Intravenous Administration of a 15% HP-β-CD Solution Containing 1.2 mg/mL Danazol [Mean ± SEM (n = 5)]

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

parameters summarized in Table 3. Plasma concentrations declined biexponentially. 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 was relatively short (1.2 h).

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

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

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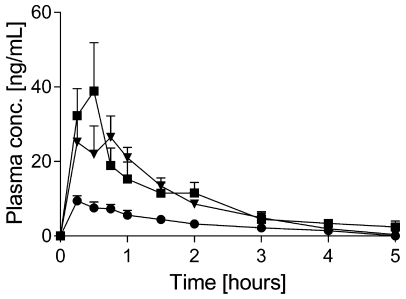

Figure 2. Danazol plasma concentration profiles following oral administration to rats of SEDDS-III at low ($SEDDS_L$-III, circle), and high ($SEDDS_H$-III, square) dose and SEDDS-IV (triangle). All plasma concentration profiles are presented as mean ± SEM (n = 4) and the administered doses are tabulated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

compared to SEDDS-III. However, even when accounting for the difference in dose, the absolute bioavailability of danazol after administration of 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 nonlinear with dose and a ∼2-fold increase in absolute danazol bioavailability was evident at the higher dose. In all cases, absolute bioavailability was low (<5%) (Table 4).

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 Figures 3A, 3B, and 3C, respectively. The corresponding mean pharmacokinetic parameters following ID administration are summarized in Table 5, and dose normalized plasma profiles are presented in S5 in the Supporting Information.

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

Impact of First Pass Metabolism on Danazol Bioavailability from SEDDS. The impact of first pass metabolism was explored by administration of a nonspecific cytochrome P450 inhibitor, 1-aminobenzotriazole (ABT), prior to danazol administration. The influence of ABT on danazol elimination was evaluated following IV administration of a 15% HP-β-CD solution containing danazol at 1.2 mg/mL in ABT-pretreated rats (a comparison of the mean plasma concentration versus time profiles for danazol in the presence and absence of ABT (S1) and the corresponding tabulated mean pharmacokinetic parameters (S2) can be found in the Supporting Information).

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

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

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

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

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

IN VITRO EVALUATION

Impact of Gastric Dispersion and ex Vivo Gastric Fluid on Drug Precipitation from SEDDS. The impact of formulation processing under gastric conditions on the in vitro performance of the investigated formulations (SEDDS-IV/$SEDDS_L$-III) was evaluated in a series of dispersion studies (Figure 5).

Dispersion of SEDDS-IV under high dilution conditions at pH 1.2 led to rapid drug precipitation, and only 40% of the initial drug load was retained in a solubilized state after 30 min. In comparison, no drug precipitation was observed on dispersion of $SEDDS_L$-III under similar conditions (Figure 5A). Experiments were also conducted under lower dilution

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

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

[a] *F:* % Absolute bioavailability relative to IV data (refer Table 3). [b] Statistically significant different compared to $SEDDS_L$-III (P < 0.05)

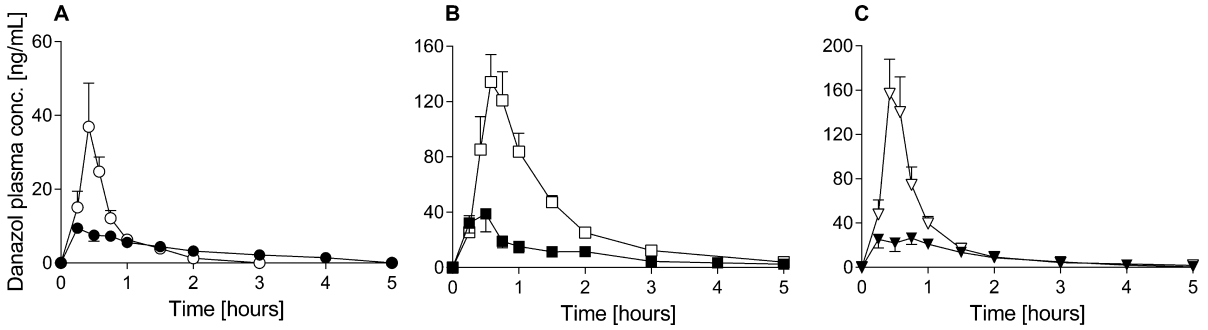

Figure 3. Danazol plasma concentration profiles comparing oral (filled) versus intraduodenal (ID) (open) administration of (A) $SEDDS_L$-III (circle), (B) $SEDDS_H$-III (square), and (C) SEDDS-IV (triangle). All plasma concentration profiles are illustrated as mean ± SEM (n = 4), and the administered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

Table 5. Pharmacokinetic Parameters for Danazol after ID Administration to Rats [Mean ± SEM (n = 4)]

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

[a]F: % absolute bioavailability relative to IV data (refer to Table 3). [b]Statistically significantly different compared to $SEDDS_L$-III (P < 0.05). [c]Statistically significantly different compared to oral administration of equivalent formulation (P < 0.050) (see Figure 2 and Table 4).

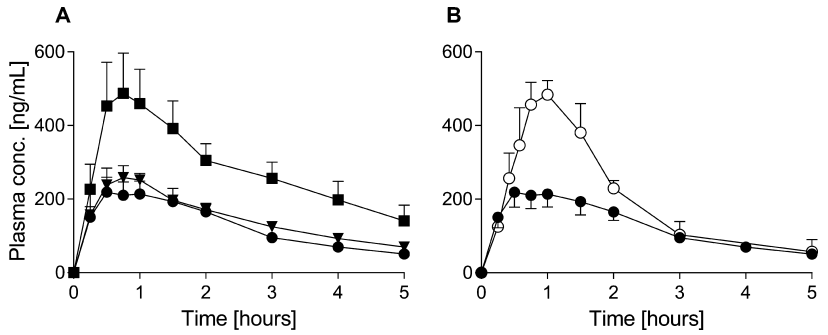

Figure 4. Danazol plasma concentration profile from ABT-pretreated rats following (A) oral administration of $SEDDS_L$-III (filled circle), $SEDDS_H$-III (filled square), and SEDDS-IV (filled triangle) and (B) intraduodenal (open) versus oral (filled) administration of $SEDDS_L$-III. All plasma concentration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

Table 6. Pharmacokinetic Parameters for Danazol after Oral and Intraduodenal Administration to ABT Pretreated Rats [Mean ± SEM (n = 4)]

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

[a]F: % absolute bioavailability calculated relative to IV data in ABT-pretreated rats (data in Supporting Information Figure S2). [b]Oral administration (O). [c]Intraduodenal administration (ID). [d]Statistically significantly different compared to oral administration of $SEDDS_L$-III (P < 0.050).

conditions (1 in 10) and at pH 5.5 (Figure 5B). Decreasing the volume of dispersion medium did not affect drug solubilization patterns when compared to high volume conditions for either $SEDDS_L$-III or SEDDS-IV. Increasing the drug load in SEDDS-III, however, resulted in ∼20% drug precipitation for $SEDDS_H$-III (Figure 5B).

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

Impact of Intestinal Digestion on in Vitro Performance of Danazol SEDDS. *Development of a Rat in Vitro Digestion Model. Rat Digestion Model: High Dilution/Low Enzyme Activity.* To evaluate potential differences in formulation processing between species, in vitro digestions were initially conducted using ex vivo rat pancreatic enzymes and biliary fluids (Table 2: rat digestion model (high/low)) rather than the porcine pancreatin extract previously employed.[9] Figures 6A and 6B show the precipitation profiles

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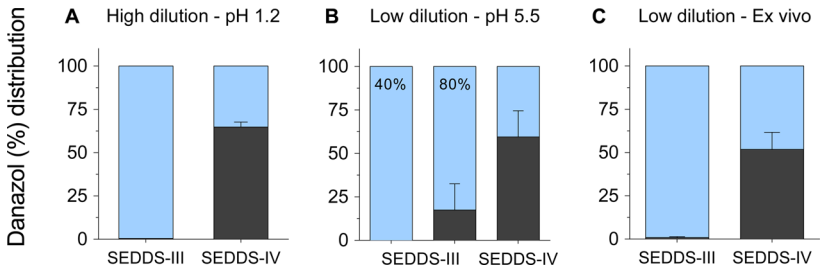

Figure 5. Extent of danazol precipitation after 30 min dispersion of SEDDS-IV and $SEDDS_L$-III (drug loaded at 40% saturated solubility) under gastric conditions [mean ± SD (n = 3)] in (A) 40 mL of 0.1 N HCl pH 1.2; (B) 0.9 mL of buffer, pH 5.5; (C) 0.9 mL of ex vivo rat gastric fluid pH 4.8. In addition,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

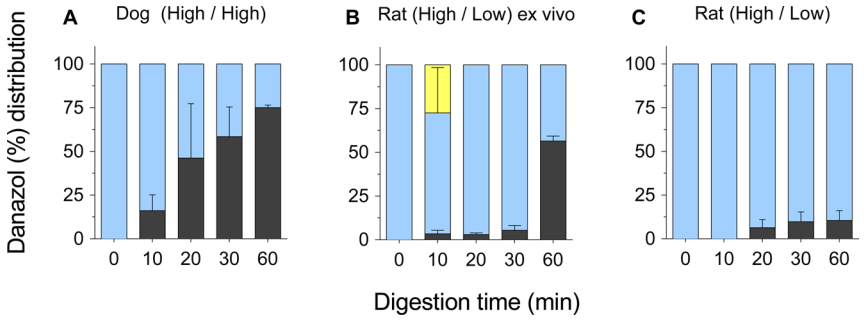

Figure 6. Danazol solubilization during digestion of $SEDDS_L$-III under three conditions: (A) in vitro digestion utilizing the previous dog digestion model (high dilution/high enzyme activity) using 4 mL of porcine pancreatin extract (data from Anby et al.[9]), (B) a rat digestion model (high dilution/low enzyme activity) using 1 mL of rat pancreatic/biliary fluid, and (C) a rat digestion model (high dilution/low enzyme activity) using 17 μL of porcine pancreatin extract to match the activity of ex vivo rat pancreatic/biliary fluid. Bars represent danazol in aqueous colloidal (light blue), oil phase below colloidal phase (light yellow), and precipitate (dark gray) [mean ± SD (n = 3)].

for danazol over 60 min during in vitro digestion of $SEDDS_L$-III using the previous in vitro lipolysis model (dog digestion model (high dilution/high enzyme activity)) and the initial rat digestion model (Table 2: rat digestion model (high dilution/low activity)). Compared with the previous in vitro digestion model, the use of ex vivo rat pancreatic fluid to stimulate digestion resulted in much lower drug precipitation during the initial 30 min of digestion. Drug precipitation became more apparent after 60 min, suggesting that the extent of precipitation may not vary significantly, but that there is a significant delay when using ex vivo rat pancreatic fluid rather than porcine pancreatic extract.

The much lower effect of ex vivo rat pancreatic enzymes on danazol solubilization 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.

Evaluation of ex Vivo Lipase Activity. The activity of ex vivo rat pancreatic fluids was quantified using a tributyrin assay and compared to a "standard curve" of the activity of known volumes of porcine pancreatin extract (data presented in Figure 6 and tabulated in S3 in the Supporting Information). In the standardized tributyrin lipolysis test, 1 mL of porcine pancreatin resulted in an effective lipase activity of 1097 TBU. Decreasing the volume of porcine pancreatin added resulted in a nonlinear 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 nonlinear relationship between enzyme activity and mass of porcine pancreatic extract, 17 μL (1.7%) of porcine pancreatic extract gave the same activity (194 TBU) as that provided by 1 mL of ex vivo rat pancreatic fluid (Figure 7).

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

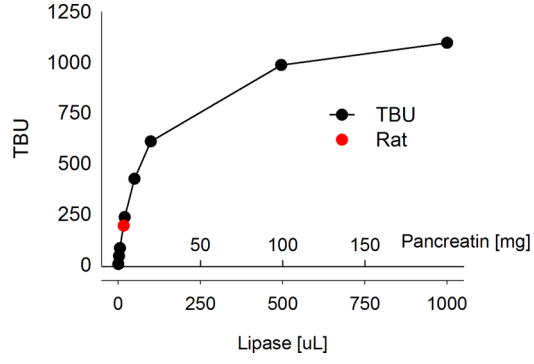

Figure 7. Volume of porcine pancreatic extract (and the corresponding quantity of pancreatin powder) added to 10 mL digestions versus the correlating enzyme activity expressed in TBUs. The lipase activity in ex vivo rat pancreatic/biliary fluid is illustrated by the red circle (194 ± 34 TBU). Similar activity is expected from 17 μL of porcine pancreatic extract (~1.7% of the volume utilized previously).

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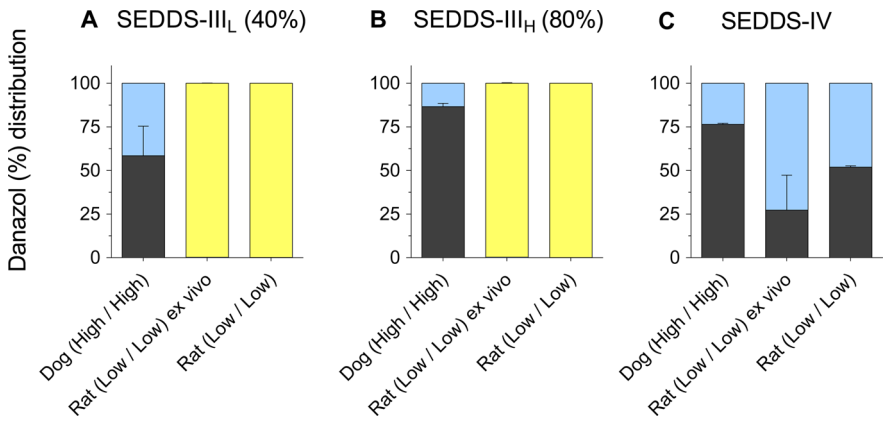

Figure 8. Danazol distribution following 30 min digestion of (A) SEDDS$_L$-III, (B) SEDDS$_H$-III, and (C) SEDDS-IV under three conditions: (1) in vitro dog digestion model (high/high) using 1 mL of porcine pancreatin extract (data from Anby et al.[9]), (2) in vitro rat digestion model (low/low) using 1 mL of ex vivo rat pancreatic/biliary fluid, and (3) in vitro rat digestion model (low/low) using 1 mL of 1.7% porcine lipase to reflect the activity in ex vivo rat pancreatic/biliary fluid. Bars represent danazol in aqueous colloidal (light blue), oil phase below colloidal phase (light yellow), and precipitate (dark gray) [mean ± SD (n = 3)].

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

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

The impact of drug load was also evaluated using the low volume digestion model (Figure 8B). For the SEDDS$_H$-III formulation, addition of ex vivo lipase led to a similar high-density oil phase that contained the majority of the drug. Limited drug precipitation was observed after 30 min as shown in Figure 8B; however, following 60 min digestion some drug precipitation was observed, albeit at a relatively low level (<20%), presumably reflecting the higher drug load. The use of porcine pancreatic extract resulted in a similar profile (Figure 8B), but in this case precipitation did not occur at later time points.

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

DISCUSSION

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

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

Danazol bioavailability after administration of SEDDS-IV, however, was significantly higher (~2-fold) than that observed after administration of SEDDS$_L$-III, in contrast to previous

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studies in beagle dogs where little difference in oral bioavailability was seen across the two formulations.[9] The very low absolute bioavailability of danazol in the rat prompted a more detailed evaluation of the potential causes of the limited exposure. In the first instance, intraduodenal (ID) administration was explored as a means of circumventing potential gastric processing of lipidic formulations, on the basis that drug precipitation in the stomach may reduce drug absorption. The plasma profiles in Figure 3 and the pharmacokinetic parameters presented in Table 5 are consistent with this suggestion and ID administration of both the high cosolvent/surfactant formulation (SEDDS-IV), and the more lipid-rich formulations ($SEDDS_L$-III and $SEDDS_H$-III) led to increased danazol exposure (1.8−3.5-fold) when compared to oral administration (although the increase was not statistically significant for $SEDDS_L$-III). Interestingly, the nonlinearity 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% versus 3.6% for $SEDDS_L$-III (a 3.6-fold increase) whereas after oral administration danazol bioavailability from the same formulations was 3.6% versus 2% (a 1.8-fold increase).

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

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

Comparison of oral and IV plasma AUCs obtained after ABT pretreatment 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-pretreated rats resulted in a sharp increase in apparent bioavailability to 59.7% compared to true bioavailability of 2% in non-ABT-pretreated animals (Figure 4 and Figure 2, respectively). First pass metabolism is therefore a very significant limitation to danazol oral bioavailability in rats under these conditions and at these doses.

Furthermore, in contrast to the data in non-ABT-pretreated rats, increasing the drug load in the $SEDDS_H$-III formulation resulted in similar danazol exposure (51.6%) to that obtained after administration of $SEDDS_L$-III. The lack of difference in danazol bioavailability after administration of the two drug doses in the presence of ABT suggests that the nonlinear 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 preadministration of ABT, danazol bioavailability was slightly higher after administration of $SEDDS_L$-III when compared to the cosolvent/surfactant-based formulation (SEDDS-IV); however, these differences were not significant (Figure 4). This is in contrast to the data obtained in the absence of ABT where danazol bioavailability from SEDDS-IV was higher than that from SEDDS-III. The enhanced bioavailability of danazol after administration of the SEDDS-IV formulation in the absence of ABT is consistent with higher danazol solubility in SEDDS-IV (17.9 mg/g vs 12.1 mg/g in $SEDDS_L$-III) (Table 1) and therefore administration of a slightly higher dose at 40% of the saturation solubility. Realizing the role of first pass metabolism in dictating danazol bioavailability in the rat, it is likely that the higher dose may have led to greater saturation of first pass metabolism in the absence of ABT. Previous studies have also suggested that Cremophor EL (present at high concentrations in SEDDS-IV) is able to inhibit CYP3A in human and rat liver microsomes.[46,47] The results obtained here in the absence of ABT may therefore indicate differences in the ability of the two formulations to inhibit first pass metabolism. More specifically, the higher quantity of Cremophor in SEDDS-IV [65% (w/w)] compared to SEDDS-III [(30% (w/w)] may help to reduce first pass metabolism and promote bioavailability. In contrast, in the presence of ABT, metabolic limitations are circumvented and solubilization events dominate. Under these circumstances, differences between danazol bioavailability from SEDDS-III and SEDDS-IV were not significant (consistent with the previous data in dogs), although exposure was slightly higher from SEDDS-III.

Bearing in mind the increase in exposure in the absence of ABT after ID administration (compared to oral administration), $SEDDS_L$-III was also administered intraduodenally to ABT-pretreated rats to see whether the same trends were apparent. In this case, bioavailability increased to 111.0% after ID administration when compared to 59.7% after oral administration. Absorption of danazol was therefore essentially complete after intraduodenal administration of $SEDDS_L$-III. The increase in bioavailability observed after ID rather than oral administration of $SEDDS_L$-III to ABT-pretreated animals (1.9-fold) was also consistent with the increases seen in non-ABT-pretreated animals (1.8-fold), suggesting that the drivers of enhanced absorption after ID rather than oral administration, in the presence of ABT, were similar to the drivers of enhanced bioavailability in the absence of ABT. Alignment between increases in bioavailability in the presence and absence of ABT suggests that in this case the differences between ID and oral administration may have been mediated by changes to solubilization 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-pretreated 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-pretreated animals was as high as ~52% (and therefore the fraction absorbed must have been at least 52%), the 3.5-fold increase in bioavailability seen after intraduodenal administration in the absence of ABT could not have stemmed (completely) from increases in absorption. The current data therefore indicate that at the higher drug load (i.e., after administration of $SEDDS_H$-III) intraduodenal administration was able to more effectively saturate first pass metabolism than was the case at lower drug doses. This trend was also replicated for SEDDS-IV where increases in ID versus oral bioavailability were slightly higher (2.6-fold) than were evident for $SEDDS\text{-}III_L$ at the lower danazol dose. Intraduodenal delivery therefore seems more able to take advantage of direct delivery to the absorptive site and to subsequently inhibit first pass metabolism when combined with formulations containing higher doses of danazol. Formulation strategies that deliver a high concentration of danazol rapidly to the upper small intestine therefore seem most likely to benefit from increases in bioavailability, at least in the rat.

In summary, the in vivo data suggest that the primary limitation to danazol bioavailability in the rat is first pass metabolism, that increasing drug dose leads to increases in bioavailability via saturation of first pass metabolism, that intraduodenal administration results in increases in bioavailability probably as a result of both increases in absorption and reductions in first pass metabolism, and that, based on the data obtained in the presence of ABT, danazol absorption from the SEDDS formulations examined here is generally good (in contrast to bioavailability). Indeed after ID administration danazol absorption from $SEDDS_L$-III was almost complete. This is surprising based on previous in vitro dispersion/digestion data[9] that show significant drug precipitation after initiation of digestion for both SEDDS-IV and SEDDS-III. Interspecies differences in GI tract conditions may, however, influence formulation processing, and the efficiency of digestion (and subsequent drug precipitation) may be different in the rat when compared to larger species, such as the dog. The solubilization behavior of the SEDDS formulations was therefore also evaluated under in vitro conditions more reflective of the GI tract in the rat, when compared to GI conditions in the dog.

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

The interaction of lingual lipase with medium-chain triglycerides results in the liberation of fatty acid,[48] and lingual lipase activity is reportedly[49] high in rodents. Subsequent experiments were therefore conducted to explore the potential additional impact of ex vivo gastric fluids (containing any available lingual lipase) on danazol precipitation from SEDDS formulation. These data suggest limited effects of lingual lipase on danazol solubilization in SEDDS in the rat stomach (Figure 5C). However, preprocessing of lipids in the stomach may affect subsequent events in the duodenum (i.e., secretion of digestive enzymes and the rate and extent of lipid digestion),[50−52] and as such, gastric digestion by lingual lipase may indirectly affect drug absorption in the small intestine.

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

Recently, Tønsberg et al.[53] examined lipase activity in luminal intestinal samples from rats and reported lower activity (153 U/mL), consistent with the levels utilized 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, while 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 utilizing different formulation dilutions, enzyme activities, and enzyme sources. As expected, the conduct of studies using the high dilution/low enzyme activity model and employing 1 mL of ex vivo rat pancreatic/biliary fluid resulted in much lower lipid digestion and correspondingly lower levels of drug precipitation when compared to the previously employed dog in vitro conditions (Figure 6). Based on in vitro analysis of the activity of the ex vivo pancreatic/biliary fluids, subsequent studies were conducted using a quantity of porcine pancreatic enzyme that was equally active in the TBU test to 1 mL of ex vivo enzyme fluid (Figure 7). Similarly reduced levels of digestion and precipitation were evident, suggesting that substitution of low levels of porcine pancreatic enzyme may be sufficient to broadly mimic the lipolytic activity of ex vivo rat pancreatic fluids. For different digestible substrates, however, different pancreatic enzymes may be required, and a more detailed series of studies would be required to fully characterize the similarity of rat pancreatic/biliary fluids to porcine pancreatic extract.

A digestion model was also evaluated using much lower dilution conditions (the rat low dilution/low activity model). This was designed to better mimic the lower fluid volumes expected in the rat GI tract where the volume of fluid

administered with the formulation was ~1 mL and the flow of fluid from the bile duct is ~1.5 mL/h. Under these conditions, the formulations behaved quite differently, and SEDDS-III formed a dense lipid phase that phase separated below the aqueous phase. This lipid-rich phase contained 80−99% of the incorporated drug. In contrast, no oil phase was generated on digestion of SEDDS-IV, the lipid-free formulation, suggesting that the high density oil phase generated by digestion of SEDDS-III consisted of fatty acids and mono/diglycerides generated via digestion of the lipids present in SEDDS-III.

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

The presence of an oil phase that floats on centrifugation is common during lipid digestion and typically represents poorly digested tri- and diglycerides, and less readily solubilized 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 solubilized aqueous phase and sedimented when left unstirred, consistent with previous observations.[55,56] The current data suggest that in the rat, under conditions of lower dilution in vitro (and potentially in vivo), less readily dispersed lipid phases are formed that are less dense than water. This may also be exacerbated by the lower pH, resulting in greater quantities of less polar un-ionized fatty acid. Where isotropic, partially digested lipid phases are formed under conditions of low enzyme activity and low dilution, the likelihood of drug precipitation appears to be diminished. Whether drug absorption is possible from these phases directly or whether further dispersion into, e.g., bile salt micelles is required is unknown at this point. Continued dilution is likely to occur, and the possibility of transition through different phases, which may not be captured with the low dilution conditions employed here, is likely. This is supported by the difference between oral and ID administration in the presence and absence of ABT, suggesting that differences in phase generation and how the drug is presented at the absorption site are important, and that drug precipitation from SEDDS-III may occur in spite of the low dilution/low enzyme in vitro model.

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

Impact of Animal Model on Danazol Bioavailability from SEDDS. In the current studies the primary limitation to danazol oral bioavailability in the rat was first pass metabolism, and this was reduced (and bioavailability enhanced) by administration of higher doses or by direct infusion of the dose into the duodenum. These data are broadly consistent with previous danazol dose-escalation studies in older beagle dogs where administration of higher doses also resulted in increased drug exposure.[19] However, in beagle dogs the absolute oral bioavailability of danazol was higher (10−26%)[19] than that seen here in rats, and much higher oral bioavailability of danazol in beagle dogs has previously been reported (64%, 82%, and 107%).[21,59] Collectively, the data suggest that where solubilization is ensured, danazol bioavailability in the dog may be more than an order of magnitude higher than that observed here in the rat (<5% after oral administration), and therefore that first pass limitations to bioavailability are likely to be lower. While the rat data presented here indicate a very large first pass effect; under conditions where first pass metabolism was inhibited, absolute bioavailability was high, suggesting that the fraction absorbed was also high. This was not expected based on previous in vitro digestion data showing considerable drug precipitation under simulated dog GI environments,[9,22] which was seemingly reflected in previous bioavailability data in the dog[22,60] showing significant formulation effects on bioavailability. However, much improved absorption in the rat is consistent with the lower extent of precipitation obtained in the in vitro tests conducted in the current studies under the lower dilution conditions and lower digestive enzyme levels expected in the rat. In contrast, under conditions of lower metabolic effects, higher intestinal dilution, and higher digestive enzyme load in the dog, bioavailability appears to be higher and more dependent on continued solubilization. One significant lack of congruence between the current rat studies and previous beagle studies is the differential behavior 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.[9] 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 solubilization. Interestingly, in the presence of ABT (i.e., in the absence of first pass effects) danazol absorption from SEDDS-III and SEDDS-IV was similar in the rat and more consistent with the previous dog data.

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

The current data also raise the question as to whether formulation or dose effects on first pass metabolism may have obscured data interpretation in previous dog studies. In particular, in studies by Cuine et al.,[22] danazol bioavailability

was previously shown to correlate well with differences in solubilization during in vitro digestion experiments and in particular to decrease with the inclusion of increasing proportions of surfactant (Cremophor EL) and cosolvent in SEDDS formulations. In these studies, however, the formulations with the highest quantities of Cremophor EL (and therefore those that might be expected to more significantly inhibit first pass metabolism) resulted in the lowest in vivo danazol exposure. Similarly, drugs were dosed at a fixed proportion of drug solubility in the formulation, and since danazol is more soluble in surfactant and cosolvent than in lipids, the absolute dose in the surfactant and cosolvent rich formulations was also higher. This in turn might be expected to lead to increased saturation of first pass metabolism. In contrast, the reverse was true and the formulations containing the highest absolute danazol doses led to the lowest exposure. These previous data are therefore consistent with the suggestion that while first pass metabolic limitation may dominate danazol bioavailability in the rat, this may not be the case in the dog where correlations with in vitro solubilization profiles appear to provide good rank order indicators of in vivo exposure.

CONCLUSION

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

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

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

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

ASSOCIATED CONTENT

Supporting Information

S1 illustrating the danazol plasma profile after IV administration of 15% HP-β-CD solution containing danazol at 1.2 mg/mL to control and pretreated ABT rats. S2 showing the tabulated pharmacokinetic parameter after intravenous administration of a 15% HP-β-CD solution containing 1.2 mg/mL danazol to control and ABT-pretreated rats. S3 showing the tabulated activity of ex vivo rat bile and lipase fluids in comparison to the activity of porcine pancreatin extract. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS USED

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

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Supplementary Information

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

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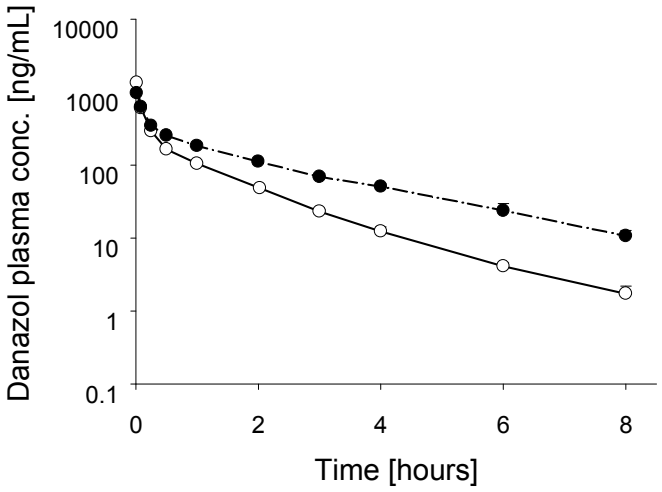
Supplementary Information



S1 Danazol plasma profile after IV administration of 15% HP-β-CD solution containing danazol at 1.2 mg/mL to control (open) [mean ± SD (n = 5)] and pre-treated (filled) ABT rats [mean ± SD (n = 4)].

S2 Pharmacokinetic parameters 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)].

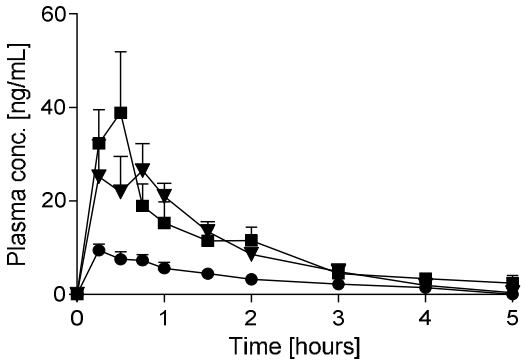
Treatment	Dose	$AUC_{0-\infty}$	Cl	Vd_{β}	$t_{1/2}$
	[mg kg^{-1}]	[ng h mL^{-1}]	[mL min^{-1} kg^{-1}]	[L kg^{-1}]	[h]
Control	2.3	434 ± 18	87 ± 3.3	8.9 ± 0.5	1.2 ± 0.1
ABT pre-treated	2.3	761 ± 22 [a]	50 ± 2.6[a]	7.6 ± 0.4	1.8 ± 0.2[a]

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

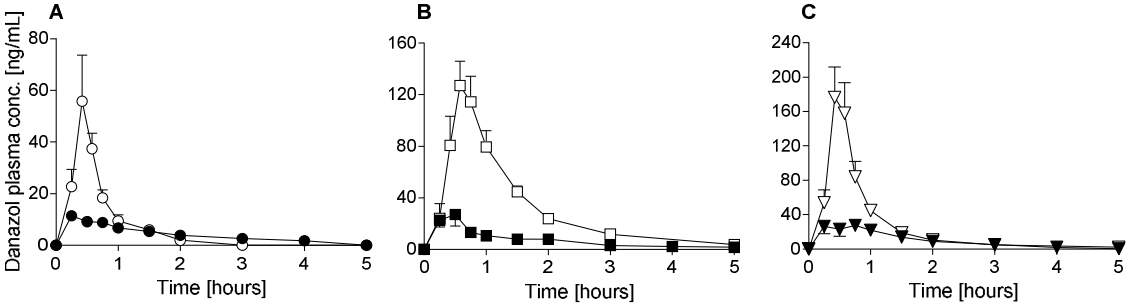
S3 Activity of ex vivo rat bile and lipase fluids in comparison to the activity of porcine pancreatin extract.

	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]

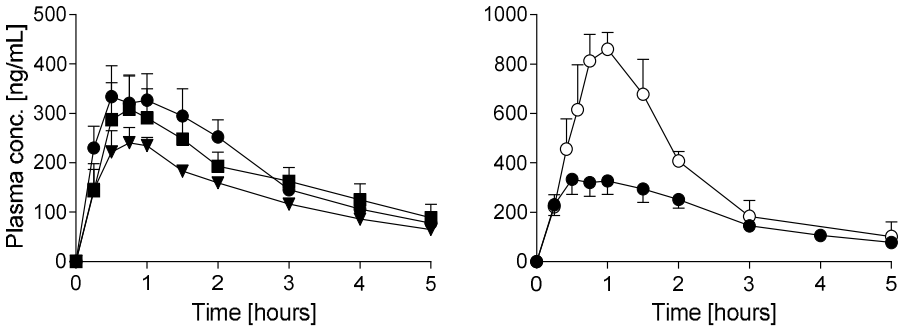
[a] Porcine pancreatic extract was generated by reconstitution of 1 g pancreatin powder in 5 mL buffer followed by centrifugation to separate the aqueous extract.
[b] Quantity of dry porcine pancreatin powder equivalent to the volume of the extract based on the extraction procedure above (i.e. 5 ml extract = 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)]



S4 Dose normalized (6 mg/kg) danazol plasma concentration profiles following oral administration to rats of SEDDS-III at low ($SEDDS_L$-III, circle), and high ($SEDDS_H$-III,square) dose and SEDDS-IV (triangle) [mean ± SEM, n = 4].



S5 Dose normalized (6 mg/kg) 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) [mean ± SEM, n = 4].



S6 Dose normalized (6 mg/kg) danazol plasma concentration profile from ABT pre-treated rats following A: oral administration of $SEDDS_L$-III (filled circle), $SEDDS_H$-III (filled square), SEDDS-IV (filled triangle); B: Intraduodenal (open) versus oral (filled) administration of $SEDDS_L$-III [mean ± SEM, n = 4].

Appendix 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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RESEARCH PAPER

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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ABSTRACT

Purpose To explore the possibility that age-related changes in physiology may result in differences in drug bioavailability after oral administration of lipid based formulations of danazol.

Methods Danazol absorption from lipid formulations with increasing drug load was examined in younger (9 months) and older (8 years) beagles. Age related changes to hepatic function were assessed via changes to systemic clearance and serum bile acid concentrations. Changes to lipolytic enzyme activity and intestinal bile salt concentration were evaluated using *in vitro* lipolysis.

Results Drug exposure increased linearly with dose in younger animals. In older animals, bioavailability increased with increasing dose to a tipping point, beyond which bioavailability reduced (consistent with initiation of precipitation). No differences in hepatic function were apparent across cohorts. Changes to enzyme concentrations in lipolysis studies had little impact on drug precipitation/solubilisation. In contrast, higher bile salt concentrations better supported supersaturation at higher drug loads.

Conclusions Differences in animal cohort can have a significant impact on drug absorption from lipid based formulation. For danazol, bioavailability was enhanced under some circumstances in older animals. *In vitro* experiments suggest that this was unlikely to reflect changes to metabolism or lipolysis, but might be explained by increases in luminal bile salt/phospholipid concentrations in older animals.

KEY WORDS absorption · bile salt · bioavailability · danazol · lipid-based drug delivery systems · non-linear bioavailability · solubility · supersaturation

ABBREVIATIONS

4-BPB	Bromophenyl boronic acid
AP_{DISP}	Colloidal aqueous phase formed on dispersion of a SEDDS formulation
AP_{DIGEST}	Colloidal aqueous phase formed on digestion of a SEDDS formulation

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AUC	Area under the curve
BA	Bioavailability
BS	Bile salt
C_{max}	Peak plasma concentration
CrEL	Cremophor EL
CYP	Cytochrome P450
F	Absolute bioavailability
GI	Gastrointestinal
HPLC	High performance liquid chromatography
HPMC	Hydroxypropyl methylcellulose
IV	Intravenous
LBDDS	Lipid-based drug delivery system
LCMS	Liquid chromatography mass spectrometry
MC	Medium-chain
NaTDC	Sodium taurodeoxycholate
PL	Phospholipid
PPI	Polymeric precipitation inhibitors
S	Supersaturation ratio
SBA	Serum bile acid
SEDDS	Self-emulsifying drug delivery system
$t_{1/2}$	Half life
TBU	Tributyrin units
T_{max}	Time of occurrence of peak plasma concentration
Vd_{β}	Volume of distribution

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 (1). Several formulation approaches have been explored as a means to overcome these limitations (2), 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 (3–6).

In most cases, LBDDS present drug to the GI tract in a molecularly dispersed, solubilised state thereby circumventing traditional dissolution. 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 lipids (bile salts, phospholipids) (7–10). The majority of LBDDS formulations are digested after oral administration, and digestion of the lipids and surfactants present in the formulation commonly results in a reduction in solubilisation capacity (8,10–13). 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 (14). 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 (14,15).

In a previous study (14), 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 observed that increasing the dose of drug in the formulation resulted in an increase in bioavailability on oral administration. Interestingly, this occurred in contrast to traditional drug absorption paradigms that suggest that increases in dose for poorly water-soluble drug are expected to reduce absorption (since the mass of drug that must be dissolved increases) (14).

The current study was therefore initiated to explore in more detail the mechanism(s) by which increasing drug load resulted in increased danazol bioavailability following oral administration to beagle dogs. The working hypothesis that underpinned these investigations was that the increase in bioavailability with dose was a result of either an increase in thermodynamic activity in the colloidal species formed by formulation digestion, or a decrease in first pass metabolism. As part of this investigation, it became evident that the non-linearity in danazol bioavailability with dose (14), 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 possibility that age-related changes in physiology may have resulted in differences in bioavailability across the two dog cohorts was therefore explored. In particular, the potential for differences in hepatic function, lipolytic enzyme activity and intestinal bile salt concentration was addressed in an attempt to explain the *in vivo* data obtained.

MATERIALS AND METHODS

Materials

Danazol (pregna-2,4-dien-20-yno[2,3-d]isoxazol-17-ol) was supplied by Sterling Pharmaceuticals (Sydney, Australia) and progesterone was from Sigma-Aldrich (St Louis, MO, USA). Captex 300, a medium-chain (MC) triglyceride, and Capmul MCM, a blend of medium-chain mono-, di-, and triglycerides, were donated by Abitec Corporation (Janesville, WI, USA). Cremophor EL (polyoxyl 35 castor oil), sodium

taurodeoxycholate 97% (NaTDC) and porcine pancreatin (8×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.

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 (16) 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 μg/ml (17) and a log *P* of 4.53 (18). The drug was incorporated into SEDDS-III at various drug loadings (mg/g) (tabulated in Table I), representing different proportions of saturated solubility in the formulation (based on the measured equilibrium solubility determined at 37°C) as previously described (14).

Oral Bioavailability Studies in Beagle Dogs

All surgical and experimental procedures were approved by the Melbourne University animal ethics committee and were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Table I 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*. (14)

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

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

Study Design

A previously published study (14) (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 SEDDS formulation comprising 30% (w/w) Captex 300, 30% Capmul MCM, 30% Cremophor EL and 10% ethanol (SEDDS-III) and increasing quantities of danazol. In this pre-study, danazol was administered in two capsules each containing 800 mg of the SEDDS-III formulation and with danazol dissolved at either 40% or 80% of saturated solubility in the formulation (equivalent to 12 mg/g and 24 mg/g, respectively) (14). Bearing in mind the non-linearity in exposure in the pre-study, but realising that data was only obtained at two dose levels, the current study sought initially to expand the pre-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 SEDDS-III formulation that was used in the pre-study, but with danazol incorporated (nominally) at 5 mg/g, 9 mg/g, 18 mg/g and 27 mg/g (equivalent to 15%, 30%, 60% and 80% of the saturated solubility in the formulation, respectively). Surprisingly, 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 (average age 9 months) than that employed in the pre-study (14), a second study (study II) was conducted in the original (older) beagle cohort (approximate age 8 years) 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 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).

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-day washout period). Treatments were hand filled into gelatine capsules 2 h prior to dosing as previously described (19). Each treatment was administered in two capsules (2×800 mg formulation) with approximately 50 mL water. Treatments were based on SEDDS-III as above and danazol was incorporated at the drug loads tabulated in Tables II and III. Dogs were fasted for at least 20 h prior to dosing and remained fasted until 10 h post-dose after which they were fed on a daily basis. Water was available *ad libitum*.

Blood samples (3 ml) were collected pre-dose and at 0, 15, 30, 45, 60, and 90 min, then at 2, 3, 4, 6, 8 and 10 h post-dose. Samples were collected via an indwelling catheter inserted in the cephalic vein and additional samples were obtained by individual venepuncture 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×*g* (Eppendorf 5702 R/A-4-38 centrifuge, Eppendorf AG, Hamburg, Germany) and stored at −80°C until sample analysis. Danazol concentrations in plasma were quantified by LC-MS as described previously (14).

Systemic Clearance and Absolute Bioavailability Determination

To evaluate the possibility of differences in systemic clearance across the older and younger dog cohorts and to provide an indication of absolute bioavailability, a subsequent study sought to examine danazol intravenous pharmacokinetics in both dog cohorts. An intravenous formulation of danazol (1.3 mg/mL) was prepared using 20% (w/v) sulphobutyl ether β-cyclodextrin (Captisol®). Danazol and Captisol® were dissolved in 0.9% saline using a magnetic stirrer (Teflon coated stirrer bar, 10×6 mm) at ambient temperature and filtered through a 0.22 μm filter (Millix®-GV) before use.

Study conditions were similar to that described for the oral bioavailability studies, and the intravenous formulation (10 mL to provide an administered dose of approximately 0.85 mg/kg) was administered to fasted dogs by infusion pump (2 mL/min over 5 mins) into a cephalic catheter. Blood samples (3 mL) were taken pre-dose, at −2 min (2 min after start of infusion), 0 (at the conclusion of the infusion), 2, 5, 15, 30, 45, 60, 90, min, then at 2, 3, 4, 6, 8, 10 and 24 h into vaccutainer tubes and samples treated and analyzed as above.

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 ($AUC_{0\text{-}10}$) was calculated using the linear trapezoidal method. Because the danazol plasma concentrations were typically below the limit of quantification at 24, 32 and 48 h post-dose, accurate determination of the terminal elimination rate constant and ($AUC_{0\text{-}\infty}$) was not possible. However, the danazol plasma concentrations at 10 h were low and the extrapolated AUC ($AUC_{10\text{-}\infty}$) was therefore expected to contribute only a minor proportion of the total AUC ($AUC_{0\text{-}\infty}$). Relative bioavailability comparisons were therefore performed using ($AUC_{0\text{-}10}$). Clearance (Cl) and volume of distribution (Vd_β) were calculated from the IV data ($AUC_{0\text{-}\infty}$) using standard 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.

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 and first pass metabolism), 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 (20,21). The test is based on the realisation 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 (20–22). Where liver function is normal, serum bile salt levels remain relatively constant post-prandially (regardless of differences in bile salt secretion) 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 (23–25).

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).

GI and Gall Bladder Bile Salt Concentrations in Young and Old Beagle Dogs

To provide an indication of potential differences in bile salt concentrations in the GI tract of the two beagle cohorts, samples of bile were obtained directly from the gall bladder in one young beagle dog and two older beagles pre- and post-mortem. Data was obtained in a limited subset of animals since euthanasia of the majority of the younger cohort was not justified (the animal that was used developed behavioural problems requiring euthanasia), and one of the older dogs had to be euthanized (tumour growth) prior to the conduct of the bile collection study.

The dogs were fasted for at least 12 h and pre-medicated with a combination of acepromazine (0.03 mg/kg) and methadone (1 mg/kg) and anaesthetised with propofol (4 mg/kg). Animals were subsequently maintained on isoflurane in oxygen for the period of the procedure and did not recover from the anaesthetic. An incision was made to the abdominal wall revealing the gastrointestinal tract and a 23G syringe was used to collect bile directly from the gall bladder. An overdose of sodium pentobarbitone was subsequently administered to

euthanise the animal. Post-mortem, tissue samples of the duodenum, gastric duodenal junction, stomach, liver, pancreas and other tissues were collected for pathological and histopathological analysis. Attempts were made to collect GI fluids prior to sampling gall bladder bile, but were unreliable, presumably due to slowed gastric emptying and reduced gall bladder contraction in anaesthetised animals.

Bile salt concentrations were analysed via a kinetic enzyme cycling reaction in a 96-well plate (Total bile acid kit from Diazyme Laboratories, Poway, USA). Samples were measured at 410 nm via a microplate reader (Fluostar Optima, BMG Labtech GmbH, Ortenberg, Germany) following significant (2000 fold) dilution in milliQ water. The method was validated in the range 5.25–100.5 μM by analysis of 5 replicate standards made up at three different concentrations (5.25, 25.125 and 100.5 μM sodium glycodeoxycholate). Intra-assay variability was accurate to 114.7, 92.1 and 98.7% and precise to ±2.7, 5.1 and 3.1% of 5.25, 25.125 and 100.5 μM. Inter-assay variability was assessed over 2 separate days and was accurate to 111.6, 90.6 and 97.9% and precise to ±1.6, 4.1 and 3.8% of 5.25, 25.125 and 100.5 μM.

In Vitro Evaluation

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 (14). Formulations contained danazol at differing levels of saturated solubility in the formulation as described in Table I, and the *in vitro* solubilisation/precipitation behaviour was examined as described previously (14). 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 (9,26). Samples were centrifuged and phase separated as previously described (14) and each phase was assayed for danazol content by HPLC. Phase separation led to the generation of an 'aqueous phase' (AP) containing dispersed colloidal structures (a combination of bile salt/phospholipid micelles and surfactant micelles swollen with lipid digestion products) and a pellet phase (containing insoluble calcium soaps and any precipitated drug).

Solubility in the Aqueous Phase Pre-Digestion (Dispersion Phase) (AP_{DISP}) and During Digestion (AP_{DIGEST})

The solubility of danazol in dispersed emulsified blank (i.e. drug-free) formulation was evaluated as described previously (14). 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 (14). 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 (14).

Data Analysis for *In Vitro* Experiments

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 (14). 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 solubilised drug concentration in the aqueous phase (AP) obtained after centrifugation of samples collected during dispersion (AP_{DISP}) and

digestion (AP_{DIGEST}) divided by the solubility of danazol in AP_{DISP} and AP_{DIGEST}.

$$S = \frac{\text{Solubilised drug conc. in } AP_{DISP \text{ or } DIGEST}}{\text{Drug solubility in } AP_{DISP \text{ or } DIGEST}} \quad (1)$$

Note that in a previous publication (14), 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*. (14) 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 experiment 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.

RESULTS

In Vivo Evaluation

Effect of Increasing Dose on Danazol Exposure In Vivo

The mean plasma concentration *versus* time profiles for danazol following oral administration of the 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 Fig. 1a. The corresponding mean pharmacokinetic parameters are summarized in Table II and show a dose-proportional increase in maximum danazol plasma concentration (C_{max}) and area under the plasma level time curve (AUC). 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 Fig. 2. The non-linearity with dose observed previously (14) was not evident in this younger cohort (Study I). The study was therefore repeated in the original cohort used in the pre-study (14). This group of animals was considerably older (mean age 8± 1.0 years.) The mean plasma concentration *versus* time profiles for danazol following oral administration to this older cohort (Study II) is shown in Fig. 1b (along with the data obtained in the previously published study). The corresponding mean pharmacokinetic parameters are summarized in Table III showing non-linear increases in C_{max} and AUC, a mean T_{max} of 1.6±0.3 h and a danazol half-life of 3.8±0.5 h across the different doses.

Table II 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 Fig. 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_{0-10h} (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 × 800 mg formulation)

[b] Relative BA was the relative bioavailability (%) expressed in comparison to the danazol AUC_{0-10} 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_{0-10}

A comparison of the dose–response relationships is shown in Fig. 2a where the areas under the plasma concentration *versus* time curves in Fig. 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 (27) 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 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 consistent across individual animals in 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 Supplementary Material, S1).

Table III 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 Fig. 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_{0-10h} (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*. (14)

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

[c] Relative BA was the relative bioavailability (%) expressed in comparison to the danazol AUC_{0-10} 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_{0-10}.

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).

Intravenous Pharmacokinetics and Absolute Bioavailability of Danazol in Both Animal Cohorts

To provide an indication of possible differences in systemic pharmacokinetic behaviour in both animal cohorts, systemic clearance and volume of distribution were evaluated via the conduct of intravenous pharmacokinetic studies. Plasma profiles in both cohorts are shown in S2 in the Supplementary Material and pharmacokinetic parameters are tabulated below in Table IV. No significant differences in either pharmacokinetic parameter were evident in the two groups. The generation of intravenous pharmacokinetic data also allowed for estimation of danazol absolute bioavailability in the previous oral studies. These data are given in S3 and S4 in the Supplementary Material. Absolute bioavailability ranged

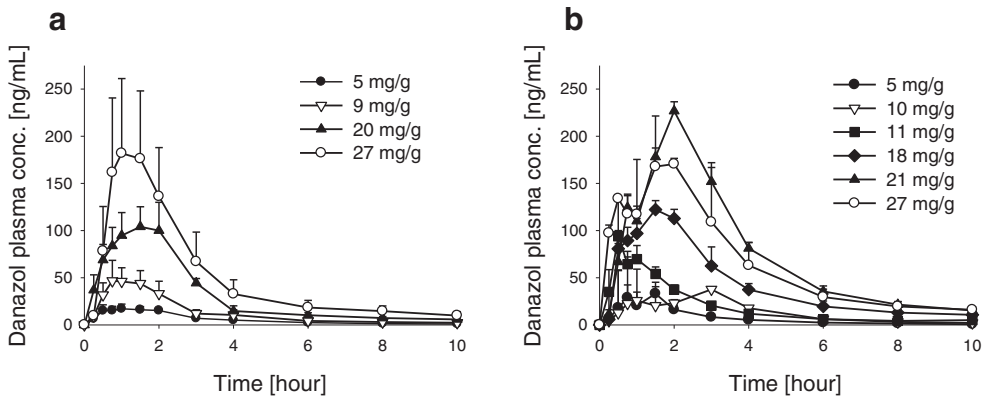

Fig. 1 Mean plasma conc. *vs* time profiles for danazol after oral administration of a SEDDS-III containing [60/30/10% w/w] of [lipid/CrEL/ethanol]. SEDDS-III administered to (**a**) a young beagle cohort (study I) with danazol loading of 5 mg/g (*black circle*), 9 mg/g (*inverted white triangle*), 20 mg/g (*black triangle*) and 27 mg/g (*white circle*) (doses are tabulated in Table II) [mean ± SEM ($n = 4$)], and (**b**) to an older cohort (pre-study and study II) with danazol doses of 5 mg/g (*black circle*), 10 mg/g (*inverted white triangle*), 11 mg/g (*black square*)[a], 18 mg/g (*black diamond*), 21 mg/g (*black triangle*)[a] and 27 mg/g (*white circle*), respectively (doses and equivalent saturation levels are tabulated in Table III [mean ± SEM ($n = 3$)]. [a]Data reproduced from Anby *et al*. (14).

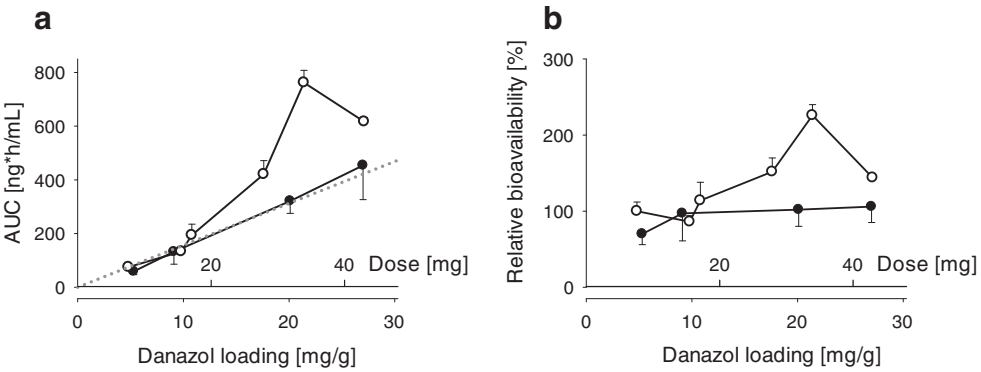

Fig. 2 Dose linearity of (**a**) danazol exposure (AUC) and (**b**) relative bioavailability after oral administration of increasing danazol doses (expressed both as absolute dose (mg) and drug concentration in the formulation (mg/g) in a type III SEDDS formulation to both a younger beagle cohort (*black circle*) [mean ± SEM ($n = 4$)] and an older beagle cohort (*white circle*)[mean ± SEM ($n = 3$)]).

from 8 to 13% in the younger animals and 10 to 26% in the older animals.

Evaluation of Liver Function in Young *Versus* Old Beagle Cohorts

To provide an additional 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 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 (20,25,28). No significant differences in liver function were evident between the two cohorts, although SBA levels were, in general, slightly higher in the older dogs and showed higher variability than the equivalent data for the younger animals.

Table 4 Pharmacokinetic Parameters After Intravenous Administration of A 20% Captisol® Solution Containing Danazol (1.3 mg/mL) to the Younger Beagle Cohort [mean ± SEM ($n = 4$)] and Individual Data for Two Older Beagle Dogs

Treatment	$AUC_{0-\infty}$ [ng h mL^{-1}]	Cl [mL min^{-1} kg^{-1}]	Vd [L kg^{-1}]	$t_{1/2}$ [h]
Younger cohort	1046 ± 70	823.4 ± 54	7.9 ± 0.4	6.6 ± 0.3
Older cohort	1173; 1033	725.5; 823.1	6.1; 7.0	5.9; 5.9
Average[a]	1075 ± 70	799 ± 51	7.2 ± 0.4	6.3 ± 0.2

Data are dose normalized to 0.85 mg/kg

[a] Data from both cohorts was used to calculate absolute bioavailability in S3 and S4 [mean ± SEM ($n = 6$)]

Gall Bladder Bile Salt Concentrations in Young *Versus* Old Beagle Cohorts

Unfortunately, the advanced age of the older animals dictated that ethics approval to conduct further detailed studies could not be obtained. However, approval was obtained to collect bile immediately prior to euthanasia in two of the older animals. Similar data was also obtained in one of the younger animal cohort who was euthanized due to a deteriorating behavioural condition. The data are given in S5 in the Supplementary Material and reveal gall bladder bile salt concentrations in the young animal of 162 mM pre-mortem and 187 mM post-mortem and in the older two dogs of 194 mM and 208 mM pre-mortem and 208 mM and 240 mM post-mortem. The data are broadly consistent with previous data (253 ± 6 mM, $n = 15$ (29), 247 ± 40 mM, $n = 15$ (30) and with the SBA data (Fig. 3) that suggest slightly (but insignificantly) higher BS levels in the older animals.

Since further *in vivo* studies could not be undertaken in the older cohort, experiments were conducted *in vitro* to explore possible differences in drug solubilisation/precipitation (and accompanying differences in supersaturation and thermodynamic

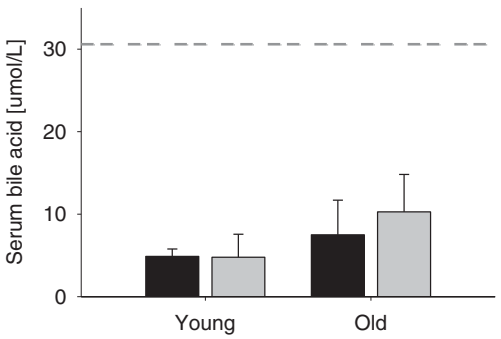

Fig. 3 Serum bile acid (SBA) concentrations measured pre-prandially (*black bars*) and post-prandially (2 h) (*grey bars*) in two beagle dog cohorts. Mean SBA levels are shown for the young [mean ± SD ($n = 4$)] and old cohort [mean ± SD ($n = 3$)]. The *upper dashed line* represents the reference value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

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).

In Vitro Evaluation

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 (14). 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 media 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 also measured. The data are tabulated in Table V 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 by >60% when compared to the solubility in the 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) led to only 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.

Table V 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[d,f]
Solubility in AP_{DIGEST} (60 min) [ug/mL][c]	56 ± 0.9[d,e]	109 ± 7.9[d,f]

[a] Data reproduced from Anby *et al*. (14)

[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] ($P < 0.050$)

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 Fig. 4a with the dotted line illustrating the solubility in the AP over time.

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 Fig. 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 V). 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 Fig. 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 V) 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 supersaturation was not maintained on initiation of digestion.

Increasing the [BS/PL] in the digestion media (Fig. 4b), resulted in moderate (but significant) differences in danazol solubility in the aqueous colloidal phase on dispersion and digestion (dotted line in Fig. 4, Table V). 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

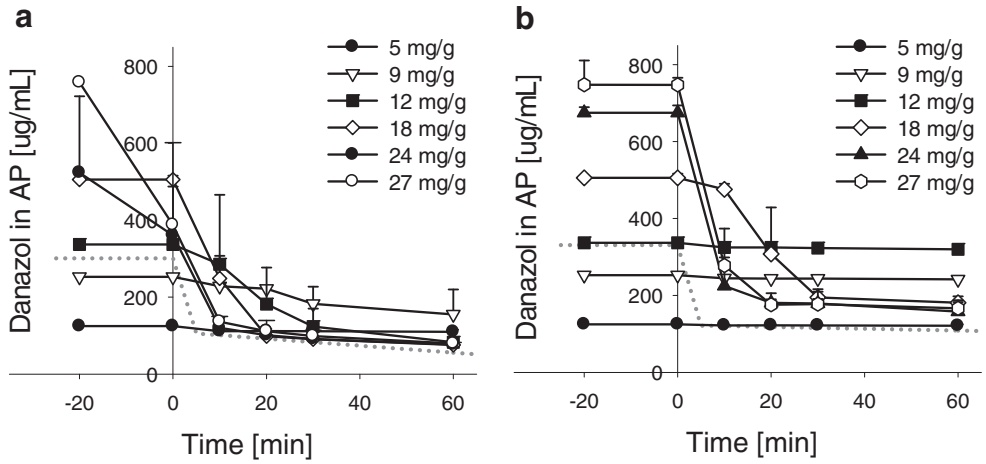

Fig. 4 Drug solubilisation profiles during dispersion (−30 to 0 min) and digestion (0 to 60 min) of SEDDS formulations with danazol loading of; 5 mg/g (*black circle*), 9 mg/g (*inverted white triangle*), 12 mg/g (*black square*)[a], 18 mg/g (*white diamond*), 24 mg/g (*black triangle*)[a] and 27 mg/g (*white circle*) 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*. (14).

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 V), reduced the extent of supersaturation and resulted in the maintenance of higher danazol concentrations in AP_{DIGEST} when compared to low [BS/PL].

Impact of Pancreatic Lipase Activity on Drug Solubilisation During In Vitro Digestion

To simulate the potential for differences in intestinal lipid digestion in the two dog cohorts (as a result of differences in pancreatic lipase secretion) 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 Fig. 5.

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

Figure 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.

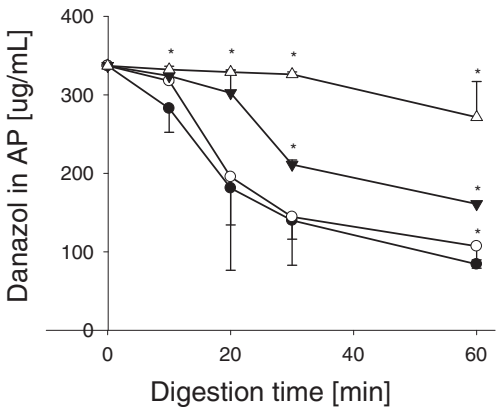

Fig. 5 Drug solubilisation during 60 min *in vitro* digestion of a SEDDS-III formulation as a function of pancreatic enzyme concentration [mean ± SD ($n = 3$)]. Danazol loading was 12 mg/g (equivalent to 40% of the equilibrium solubility in SEDDS-III). The quantities of enzyme employed were 4000 μL (*black circle*)[a], 1000 μL (*white circle*), 400 μL (*inverted black triangle*) and 100 μL (*white triangle*) of pancreatic lipase extract, where 4 mL is the volume added under normal conditions. [a] Data reproduced from Anby *et al*. (14). *Statistically significant different compared to data obtained using 4000 μL lipase extract ($P < 0.050$).

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 (11,31–33). Depending on the drug load in the formulation, this may result in the generation of transiently supersaturated conditions in the GI tract (14). 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 (14). 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.

Effect of Drug Dose on *In Vivo* Exposure in Older *vs*. Younger Beagles

The plasma profiles in Fig. 1 and the pharmacokinetic parameters presented in Tables II and III 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 (Fig. 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 (14). This prompted an extended evaluation of the dose–response in the first (older) cohort (Fig. 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) (Fig. 2a). Relative bioavailability increased with dose until a 'tipping point' was reached at doses equivalent to concentrations of 21 mg/g danazol in the SEDDS-III formulation (71% of drug solubility in the formulation). Above this, relative exposure dropped significantly and approached that obtained in the younger cohort (Fig. 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 (34) and gastric pH is often higher in dogs when compared to humans (35)), gastric emptying (36) and intestinal motility (37,38). Changes in permeability of the intestinal tissue (39) and a reduction in renal and/or hepatic drug elimination have also been described with increases in age (40,41). Several studies have addressed permeability related issues from solubilising formulations, and have shown that permeability is critically dependent on colloid properties (42–45). Age-related changes might therefore be expected to influence permeability directly, via physiological changes to the absorptive membrane, and indirectly via changes to GI luminal conditions, thereby altering colloidal structure.

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 (46). *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 (47). 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.

Differences in Serum Bile Acid Levels and Systemic Clearance 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 and by monitoring for changes in systemic clearance after intravenous administration. Many aspects of 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 (22,48,49). 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 (20,25,28). 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 (50). Differences in GI bacterial growth have also been reported in aged dogs (51), 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 (Fig. 2). Note that double peaks were seen in the early time periods of the mean plasma profiles in the older animals (Fig. 2b), however, this reflected differences in T_{max} in individual dogs rather than the occurrence of double peaks in individual animals, and thus was likely to be 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. The data obtained following intravenous administration of danazol (Table IV and S2 in Supplementary Material) further supports this suggestion and failed to show differences in systemic clearance across the two cohorts. 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.

Age-Related Changes to Drug Absorption from the Gastrointestinal Tract

Several studies have addressed the potential for age related physiological changes to alter drug disposition. These are well reviewed in Cusack (52), Fahey *et al*. (53) and McLean & Le Couteur (54). 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 (52,54). 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. The IV study (Table IV and S2 in Supplementary Material) further supports the lack of significant differences in volume of distribution (Vd) between the two cohorts.

Intestinal permeability has also been reported to increase for some drugs in older animals (rats), due to age-related changes to membrane permeability in the enterocyte (39). 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 (55,56). Increases in gastric pH, delays in gastric emptying and decreases in gastrointestinal motility are also commonly associated with increased age (40). These changes might all be expected to alter absorption and potentially increase absorption (although pH is unlikely to alter the absorption of an non-ionizable drug such as danazol), but are rarely clinically significant (52) 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 (39)). The authors reported a ~2-fold increase in danazol solubility in human intestinal fluid with age, and concluded that this may have an impact on drug absorption profiles and bioavailability in older subjects. 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.

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) (57). 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 Fig. 5.

Under normal conditions, the specific activity of pancreatic lipase in the dog is high (58) and similar to that in humans (59). 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 higher in older dogs when compared to weanlings (60).

Impact of Bile Salt Concentration on Drug Solubilisation in the GI Tract

A subsequent study evaluated the bile salt concentration recovered in the gall bladder from one young and two older beagles from the two cohorts. Acknowledging the limited number of animals, the data supports the suggestion that BS levels in the older dogs may be slightly higher when compared to the younger dogs. These trends were evident both pre- and post-mortem (data are presented in S5 in Supplementary Material) and are consistent with previous studies in humans (39) where a tendency towards elevated bile concentrations was noted with age (7 ± 4 mM *vs* 5 ± 3 mM), although the differences in this case were not statistically significant. It is difficult to predict with accuracy how the differences in gallbladder bile concentrations might be manifest in changes to luminal BS concentrations, since the degree of dilution is unknown. It is also apparent that local concentrations in areas of the GI tract may vary significantly during the dilution process. Nonetheless, the available data support the possibility that in some areas of the GI tract BS levels may be higher in older subjects.

The influence of [BS/PL] on drug solubilisation and the kinetics of drug precipitation during formulation dispersion and digestion were evaluated *in vitro* and the data are shown in Fig. 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) might 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 Fig. 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 (Fig. 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

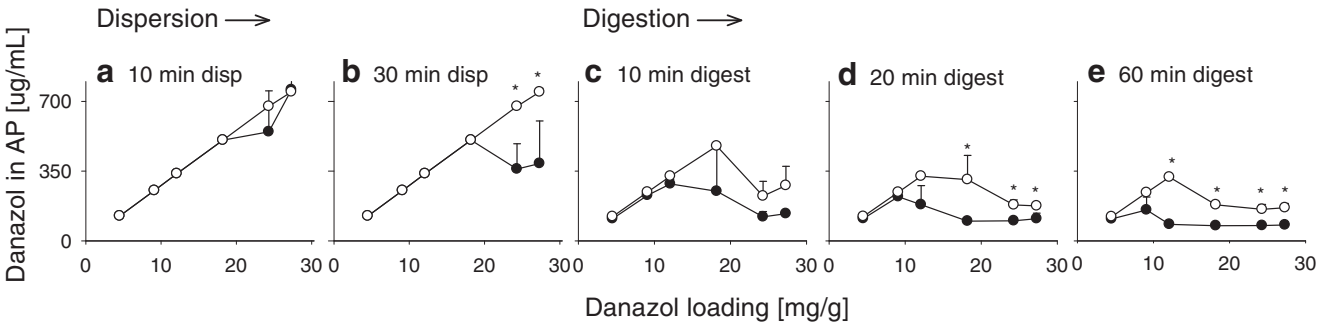

Fig. 6 Drug solubilisation *versus* initial danazol saturation level in the formulation during *in vitro* digestion at low [BS/PL] (*black circle*) and high [BS/PL] (*white circle*) [mean ± SD ($n = 3$)]. Individual figures represent time points in Fig. 4; (**a**) early dispersion (−20 min, ie after 10 min dispersion), (**b**) the end of the dispersion period (time zero, ie after 30 min dispersion); (**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] ($P < 0.050$).

evident at low [BS/PL] and higher drug loads (Fig. 6b). Initiation of digestion resulted in a rapid drop in the solubilisation capacity of mixed colloidal species present in the digest (Fig. 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 V). 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.

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 (Fig. 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 (Fig. 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 (Fig. 6c–e, filled symbols). In contrast, at the higher [BS/PL] (Fig. 6, open symbols), solubilisation increases with dose were more apparent and total quantities of solubilised drug were significantly greater, especially at later time points (Fig. 6c–e, open symbols).

The data in Fig. 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 (39). 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 Fig. 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 Fig. 4, Table V) and yet drug precipitation, was either avoided or delayed in many cases. This results in supersaturation (14), 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 Fig. 7 where the data have been

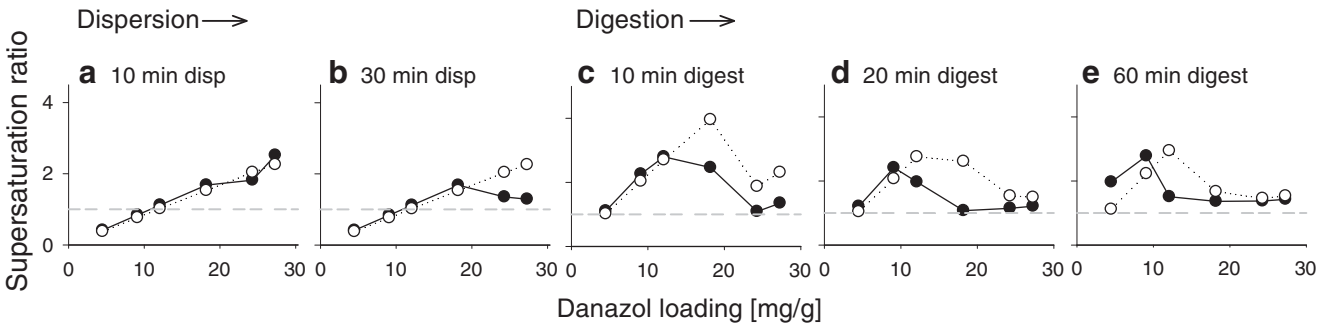

Fig. 7 Supersaturation ratios *versus* danazol load in the formulation during *in vitro* digestion at 5 mM (*black circle*) and 20 mM (*white circle*) bile salt concentration [mean ± SD ($n = 3$)]. Individual figures represent time points in Fig. 4; (**a**) early dispersion (−20 min, ie after 10 min dispersion), (**b**) the end of the dispersion period (time zero, ie after 30 min dispersion); (**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 a supersaturation ratio of 1 with ratios above this line indicating supersaturated conditions.

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).

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 (Fig. 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.

CONCLUSION

In the current study, danazol absorption from SEDDS formulations containing drug at increasing drug loads was examined in two cohorts of beagle dogs, one younger (9 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 70% 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 (47) 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 Fig. 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 (Fig. 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.

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 and clearance data suggest limited differences in hepatic function between the two animal cohorts (at least in steroid

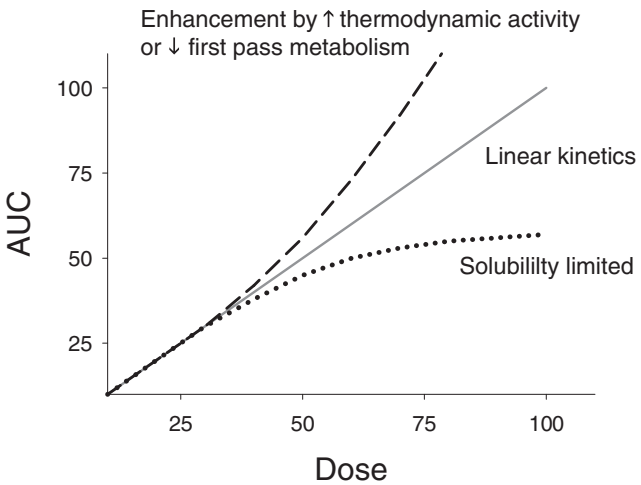

Fig. 8 Theoretical relationship between exposure and dose following oral administration in a solubilized formulation of a poorly water-soluble drug that undergoes first pass metabolism. The plot illustrates the exposure following linear kinetics (*solid line*) and the potential beneficial effects of saturation of first pass metabolism or promotion of supersaturation and thermodynamic activity (*dashed line*) and the unfavourable effect of solubility-limited absorption (*dotted line*)

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 (52,54,61,62). 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 (albeit non-significant increases) in danazol luminal solubility in the elderly (39) and supported by subsequent analysis of gallbladder BS levels in the two cohorts here, although the data set (and the magnitude of the differences) is limited. 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 plausible 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.

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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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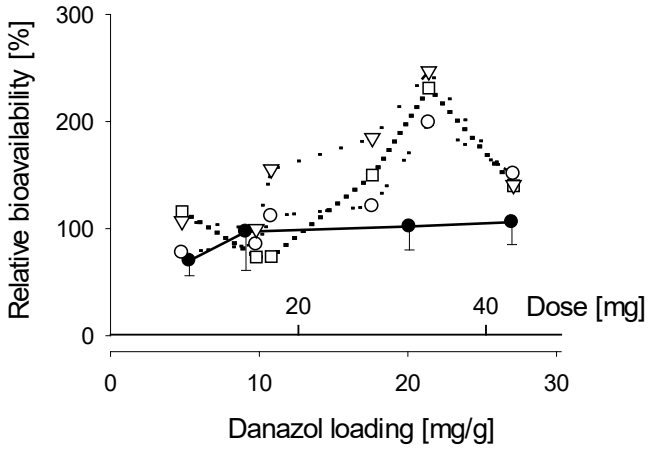
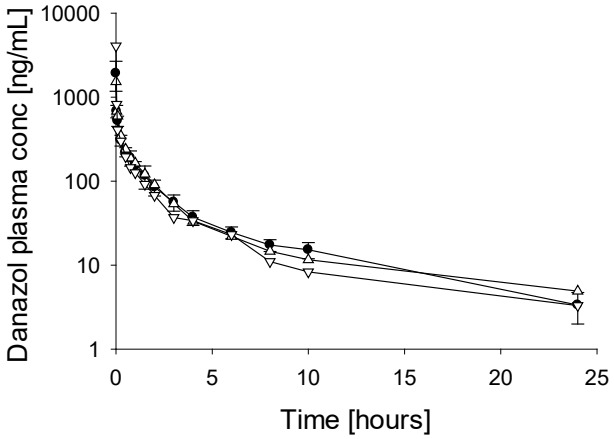
SUPPORTING INFORMATION



S1 Dose linearity of relative bioavailability in individual animals in the older beagle cohort (open symbols) compared to the younger beagle cohort (●) [mean ± SEM (n = 4)] following oral administration of increasing danazol doses (expressed both as absolute dose (mg) and drug concentration in the formulation (mg/g)



S2 Danazol plasma profile after IV administration of a 20% Captisol® solution containing 1.3 mg/mL danazol to the younger beagle cohort (closed symbols) [mean ± SEM (n = 4)] and two animals from the older beagle cohort as individual dogs (open symbols). Pharmacokinetic parameters in main text.

S3 Absolute bioavailability (*F*) of danazol following oral administration of the SEDDS-III formulation containing increasing drug loadings to the young beagle cohort (study I) [mean ± SEM (n = 4)] (Corresponding PK data and plasma profiles are presented in Table 2 and Figure 1A).

	SEDDS-III formulation treatments in younger beagle dogs			
Danazol loading [mg/g]	5	9	20	27
Treatment dose [mg]	8.5	14.5	32.1	43.2
F (%)	8 ± 2	11 ± 4	12 ± 3	13 ± 4

S4 Absolute bioavailability (*F*) of danazol following oral 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 PK data and plasma profiles are presented in Table 3 and Figure 1B).

	SEDDS-III formulation treatments in older beagle dogs					
Danazol loading [mg/g]	5	10	11	18	21	27
Treatment dose [mg]	7.6	15.6	17.3	28.1	34.2	43.4
F (%)	12 ± 1	10 ± 1	13 ± 3	18 ± 2	26 ± 2	17 ± 1

S5 Bile salt concentrations recovered in the gall bladder of a young beagle dog and two older beagles [mean ± SD (n = 3)]

	Pre-mortem	Post mortem
Young dog [mM]	162 ± 27	187 ± 9
Older dog (i) [mM]	194 ± 4	208 ± 9
Older dog (ii) [mM]	206 ± 18	240 ± 2

Appendix 5

Correlating *in vitro* Solubilization and Supersaturation Profiles with *in vivo* Exposure for Lipid-based Formulations of the CETP Inhibitor CP-532,623

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Correlating in Vitro Solubilization and Supersaturation Profiles with in Vivo Exposure for Lipid Based Formulations of the CETP Inhibitor CP-532,623

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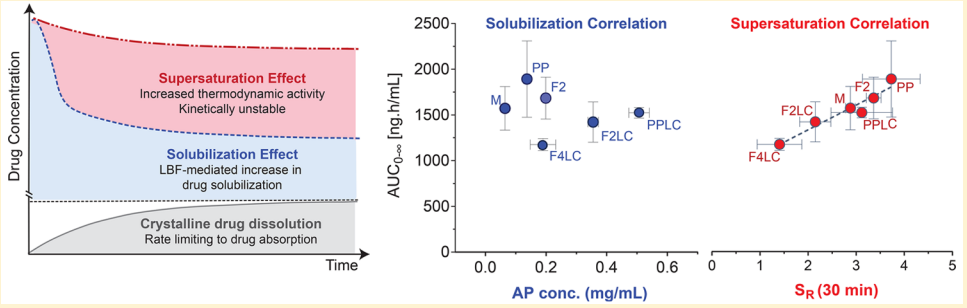

ABSTRACT: Lipid based formulations (LBFs) are a promising formulation strategy for many poorly water-soluble drugs and have been shown previously to enhance the oral exposure of CP-532,623, an oral cholesteryl ester transfer protein inhibitor. In the current study, an in vitro lipid digestion model was used to probe the relationship between drug solubilization and supersaturation on in vitro dispersion and digestion of LBF containing long chain (LC) lipids and drug absorption in vivo. After in vitro digestion of LBF based on LC lipids, the proportion of CP-532,623 maintained in the solubilized state in the aqueous phase of the digest was highest in formulations containing Kolliphor RH 40, and in most cases outperformed equivalent formulations based on MC lipids. Subsequent administration of the LC-LBFs to beagle dogs resulted in reasonable correlation between concentrations of CP-532,623 measured in the aqueous phase of the in vitro digest after 30 min digestion and in vivo exposure (AUC); however, the LC-LBFs required greater in vitro drug solubilization to elicit similar in vivo exposure when compared to previous studies with MC-LBF. Although post digestion solubilization was enhanced in LC-LBF compared to MC-LBF, equilibrium solubility studies of CP-532,623 in the aqueous phase isolated from blank lipid digestion experiments revealed that equilibrium solubility was also higher, and therefore supersaturation lower. A revised correlation based on supersaturation in the digest aqueous phase and drug absorption was therefore generated. A single, linear correlation was evident for both LC- and MC-LBF containing Kolliphor RH 40, but this did not extend to formulations based on other surfactants. The data suggest that solubilization and supersaturation are significant drivers of drug absorption in vivo, and that across formulations with similar formulation composition good correlation is evident between in vitro and in vivo measures. However, across dissimilar formulations, solubilization and supersaturation alone are not sufficient to explain drug exposure and other factors also likely play a role.

KEYWORDS: *lipid based drug delivery, in vitro digestion, bioavailability, poorly water-soluble drug, supersaturation*

INTRODUCTION

The oral bioavailability of many poorly water-soluble drugs (PWSD) is hindered by slow dissolution and low solubility in the aqueous environment of the gastrointestinal tract. Lipid based formulations (LBFs) provide one means to enhance the absorption of PWSD by delivering the drug in solution in the formulation (thus bypassing traditional drug dissolution) and maintaining drug in a solubilized state on formulation dispersion and digestion.[1−3]

LBFs differ from most traditional oral drug delivery systems in that the excipients in the formulation are markedly altered by the gastrointestinal (GI) environment, and in particular by enzymatic digestion. Lipids, whether from dietary or formulation sources, are hydrolyzed by gastric and pancreatic

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lipases to release more amphiphilic lipid digestion products that are more readily solubilized by biliary lipids secreted in bile. Micellar association subsequently promotes lipid solubilization and supports lipid absorption.[4,5] PWSD that are coadministered with lipids may also be solubilized by the bile salt−lipid (mixed micellar) complexes that are formed on lipid digestion. This enhances apparent drug solubility in the GI tract, and commonly leads to increases in drug absorption and bioavailability, a process that has been exploited commercially.[6]

Hydrophilic surfactants and cosolvents are often added to LBFs to increase drug loading in the formulation and to improve formulation dispersion (i.e., self-emulsifying properties).[5,7] However, aqueous dispersion of LBFs containing high quantities of water miscible excipients typically results in drug precipitation as solubilizing power is lost on dilution.[8] Digestion of the lipids in LBFs is also commonly a stimulus for a reduction in solubilizing power as the polarity of lipid digestion products is increased and their ability to swell the mixed colloidal species formed in the GI tract is reduced.[9] Collectively therefore LBF dispersion and digestion typically leads to varying degrees of drug precipitation, and in many cases this has been shown to correlate with reductions in drug absorption.[10] The relationship between drug precipitation on digestion and drug exposure in vivo has led to the increasingly frequent use of in vitro lipid digestion models to differentiate formulation performance.[11]

More recently, however, it has become apparent that drug precipitation on in vitro dispersion and digestion may not necessarily be detrimental to drug absorption in vivo.[12−14] Instead, the emerging paradigm suggests that formulation dispersion and digestion results in a loss of solubilization capacity and the generation of a transiently supersaturated state, and that there is a dynamic interplay between the likelihood that supersaturation drives either absorption or precipitation.[15−17] These processes are dependent on formulation composition and may also be impacted by the state of precipitated drug, i.e., crystalline or unstructured amorphous drug, the latter being generally more amenable to redissolution.[18,19]

Attempts at in vitro−in vivo correlation between aqueous phase (AP) solubilization after in vitro dispersion or digestion and in vivo AUC after oral administration therefore vary in the strength of correlation.[2,20] In previous studies with CP-532,623, a cholesteryl ester transfer protein inhibitor (Figure 1), a series of medium chain lipid based LBFs improved the bioavailability of CP-532,623 when compared to a crystalline powder formulation, however, the difference in in vivo absorption between the LBFs was only moderate when compared to differences in in vitro solubilization, resulting in a nonlinear correlation.[21]

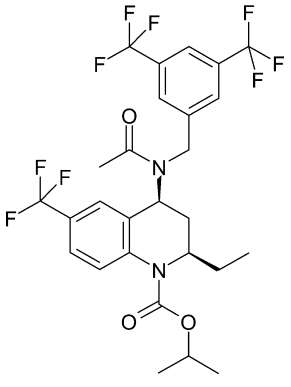

Figure 1. Chemical structure of CP-532,623.

In the current studies, in an attempt to better define the relationship between solubilization, supersaturation, and absorption, the in vitro solubilization range of CP-532,623 has been widened by the inclusion of a series of LBFs similar to those previously examined, but where long chain (LC) lipids were substituted for medium chain (MC) lipids. Digestion of LBFs containing long chain lipids typically results in more robust in vitro solubilization properties when compared to the medium chain equivalent.[22,23] In this way we hoped to provide further evidence (or otherwise) of the putative relationship between drug exposure and in vitro solubilization on lipid digestion. To verify the absolute extent of drug exposure, the absolute bioavailability was measured via the inclusion of an intravenous administration leg to the study. Finally, to explore the likely upper limits of drug exposure, CP-532,623 was administered with higher quantities of formulation to fasted beagles and also to fed animals. The data suggest that drug solubilization and supersaturation are indicators of drug absorption, especially for formulation series comprising similar excipients.

MATERIALS

CP-532,623 and CP-524,515 (internal standard) were supplied by Pfizer Inc. Pharmaceutical Sciences (Groton, CT). CP-532,623 (Catalog No. PZ0225) and CP-524,515 (Catalog No. PZ0226) are now commercially available as reference standards from Sigma-Aldrich. Formulation excipients were obtained from the following suppliers: soybean oil (Sigma-Aldrich Co., St Louis, MO); Maisine 35-1 (Gattefossé, Saint Priest, France); Miglyol 812 N (SASOL North America, Houston, TX); Capmul MCM (Abitec Corporation, Janesville, WI); Labrafil M 2125 CS (Gattefossé, Saint Priest, France); TPGS; α-tocopheryl-polyethylene glycol 1000 succinate (Eastman Chemical Company, Kingsport, TN); polysorbate 80 (Spectrum Laboratory Products, Gardena, CA); Kolliphor RH 40 (previously available under the trade name Cremophor RH 40, a gift from BASF, Ludwigshafen, Germany); triacetin (Sigma Chemical Co., St Louis, MO); propylene carbonate (Huntsman Corp., The Woodlands, TX); 10% Intralipid (Fresenius Kabi AB, Sweden); *N*,*N*-dimethylacetamide (Sigma-Aldrich Co., St Louis, MO). Soft gelatin capsules (a gift from Cardinal Health, Dublin, OH) were used as supplied.

Lipolysis media contained the following: Lipoid E PC S (phosphatidylcholine from egg lecithin) (Lipoid GmbH, Ludwigshafen, Germany); sodium taurodeoxycholate (NaTDC) ≥ 95% (Sigma-Aldrich Co., St. Louis, MO); Trizma maleate (Sigma-Aldrich Co., St. Louis, MO); calcium chloride dihydride (BDH, Victoria, Australia); sodium hydroxide (Merck, Darmstadt, Germany); sodium chloride (Chem Supply, South Australia, Australia); porcine pancreatin 8× USP specifications (Sigma-Aldrich Co., St. Louis, MO). Lipolysis was inhibited by 4-bromophenylboronic acid (Sigma-Aldrich Co., St. Louis, MO).

Water was obtained from a Milli-Q (Millipore, Bedford, MA) water purification system. All solvents used were of HPLC analytical grade and degassed by filtration immediately before use.

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Table 1. Composition (% w/w) of Formulations[a]

formulation	lipid (soybean oil)	cosolvent	surfactant	
			high HLB	low HLB
F1LC	20	15 (triacetin)	50 (polysorbate 80)	15 (Maisine)
F2LC[b]	20	10 (triacetin)	50 (RH40)	20 (Maisine)
F3LC	20	20 (prop carb)	20 (TPGS)	40 (Labrafil)
F4LC[b]		28 (triacetin)	30 (RH40)	42 (Maisine)
F5LC	20	20 (prop carb)	20 (polysorbate 80)	40 (Maisine)
F6LC	20	20 (prop carb)	20 (TPGS)	40 (Maisine)
F7LC[c]	20	30 (triacetin)	35 (RH40)	15 (Maisine)
F8LC	20	30 (triacetin)	20 (polysorbate 80)	30 (Maisine)
F10LC	10	40 (triacetin)	20 (TPGS)	30 (Labrafil)
ILC[c]	20	30 (prop carb)	20 (TPGS)	30 (Maisine)
JLC[c]	20	40 (prop carb)	40 (TPGS)	
MLC	20	30 (triacetin)	20 (RH40)	30 (Maisine)
PPLC[b]	15	15 (triacetin)	40 (RH40)	30 (Maisine)

[a]Prop carb: propylene carbonate. RH40: Kolliphor RH 40. TPGS: α-tocopheryl-polyethylene glycol 1000 succinate. Maisine: Maisine 35-1. Labrafil: Labrafil M 2125 CS. [b]Formulations were assessed in vivo. [c]Formulations were immiscible and excluded from further study.

METHODS

In Vitro Evaluation. *Preparation of Drug Loaded Lipid Based Formulations.* The series of formulations employed in this study were adapted from previous studies with CP-532,623 and torcetrapib,[21,24] but in this case the medium chain lipids (Miglyol 812 and Capmul MCM) previously included have been replaced with equivalent long chain lipids (soybean oil and Maisine 35-1 respectively). Each formulation (Table 1) was loaded at 50 mg/g CP-532,623. Poor miscibility of some of the modified formulations precluded further use in this study. Formulations comprised a combination of long chain triglyceride (soybean oil), surfactant (Kolliphor RH 40, polysorbate 80, or TPGS), cosurfactant (Maisine 35-1 or Labrafil M 2125), and cosolvent (triacetin or propylene carbonate). Semisolid excipients (Maisine 35-1, TPGS, Kolliphor RH 40) were heated to 60 °C and mixed thoroughly prior to formulation preparation. All excipients and drug were weighed directly into glass vials, heated to 37 °C for 1 h to promote wetting of the drug prior to being vortexed regularly (initially every 2−3 h). The formulations were incubated at 37 °C over 48 h to allow equilibration to be attained. Any undissolved drug was removed by centrifugation (20 min, 37 °C, 2205*g*, Eppendorf 5804 R refrigerated centrifuge equipped with an A-4-44 rotor, Eppendorf AG, Hamburg, Germany) and the supernatant transferred to a new vial before use. The concentration of drug in the formulation was assessed by HPLC before use.

In Vitro Dispersion and Digestion Studies. In vitro evaluation of the LBFs described in Table 1 was performed using an in vitro lipid digestion model as described previously.[21] Drug maintained in a solubilized state after dispersion and digestion was measured in the aqueous phase (AP) obtained after separation of residual oil phase (OP) and precipitated drug and insoluble material (pellet, P) by centrifugation.

Briefly, drug loaded formulations (120 mg LBFs containing 6 mg of CP-532,623) were dispersed in 9 g of digestion buffer (50 mM tris maleate, 5 mM $CaCl_2{\cdot}2H_2O$, 150 mM NaCl, and 50 mM NaOH) containing 1.25 mM phospholipid (egg lecithin containing phosphatidylcholine derived from egg yolk) and 5 mM bile salt (sodium taurodeoxycholate) representative of fasted state intestinal conditions. The system was adjusted to pH 6.5 and maintained at 37 °C.

The dispersion and digestion procedures were performed separately to enable measurement of drug precipitation triggered by each event. Dispersion and digestion procedures were initiated by addition of 1 mL of Milli-Q and pancreatin extract solution, respectively. Pancreatin extract contained ∼10,000 TBU of pancreatic lipase (equivalent to ∼1,000 TBU/mL digest) and was prepared by the addition of 1 g of pancreatin powder to 4 mL of Milli-Q water followed by stirring for 10 min prior to centrifugation (20 °C, 15 min, 2205*g*) to isolate the extract (supernatant). Both dispersion and digestion procedures continued for 30 min, during which time stoichiometric titration of the liberated fatty acids (0.5 M NaOH) maintained the mixture at pH 6.5.

At the end of the dispersion or digestion period a 4.2 mL sample was taken and lipolysis inhibited by addition of 42 μL of 3 mM 4-bromophenylboronic acid (in methanol). Ultracentrifugation (336238*g*, 30 min, 37 °C, Optima XL-100 K ultracentrifuge, SW60 rotor, Beckman, Palo Alto, CA) was subsequently used to separate the sample into undigested oil, aqueous, and pellet phases. To quantify the proportion of drug in each phase, the oil phase (OP) was first aspirated from the top of the (polyallomer) centrifuge tube and added to a 5 mL volumetric flask containing 50 μL of 1 M HCl and made to volume with chloroform/methanol (2:1 v/v). Second, the aqueous phase (AP) was collected by piercing the side of the tube with a needle and withdrawing the aqueous phase to avoid contamination by residual oil phase or the pellet phase. The pellet (P) was finally transferred to a 5 mL volumetric flask and treated in the same way as the oil phase sample. All phases were diluted at least 1:10 (v/v) with acetonitrile and assayed for CP-532,623 as described below.

Solubility of CP-532,623 in Isolated Aqueous Phase. The solubility of CP-532,623 in isolated AP was measured for both the medium chain formulations examined previously and the long chain formulations examined in detail here. The in vitro digestion model was used to digest each drug free formulation and the AP collected as described above. Excess drug was added to the blank collected aqueous phase and the mixture vortexed regularly and maintained at 37 °C. The drug/AP mixture was centrifuged at regular intervals and a sample of the supernatant collected, diluted in acetonitrile, and assayed

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for drug content by HPLC. Samples were collected at short intervals (0.5, 1, 2, 4, and 6 h) initially to reflect the relevant time period of drug absorption. Later time points (24, 48, 72, and 168 h) were also collected to examine the change in solubility over time.

Calculation of Supersaturation Ratio. The supersaturation ratio (S_R) provides a measure of the degree of supersaturation of drug in the AP during formulation digestion. S_R was determined as the AP concentration measured after in vitro digestion of a drug loaded LBF divided by the equilibrium solubility of drug in the AP (eq 1). S_{max} is the theoretical maximum supersaturation ratio and is calculated using the initial concentration of drug in the dispersion/digestion system (i.e., the total amount of drug loaded in the formulation divided by the volume of the dispersion media) divided by the equilibrium solubility of drug in the AP. The equilibrium AP concentration was obtained after 24 h dissolution of excess drug in AP isolated after 30 min of in vitro digestion of drug free formulation.

$$S_R = \frac{\text{AP concentration after in vitro digestion}}{\text{equilibrium AP solubility}} \quad (1)$$

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Polarized Light Microscopy (PLM). Digestion pellets containing precipitated CP-532,623 were analyzed using a Zeiss Axiolab microscope (Carl Zeiss, Oberkochen, Germany) equipped with crossed polarizing filters. After in vitro digestion a portion of the pellet was carefully transferred to a microscope slide immediately after ultracentrifugation. Images were recorded using a Canon PowerShot A70 digital camera (Canon, Tokyo, Japan). A control sample was prepared by in vitro digestion of a drug free formulation, which was then spiked with crystalline CP-532,623 (equivalent to the amount that precipitated in the drug loaded formulation) prior to ultracentrifugation.

X-ray Powder Diffraction (XRPD). A representative formulation (PPMC) was selected to verify the absence of crystalline CP-532,623 in the pellets obtained after in vitro digestion and to verify the observations by PLM. Digestion pellets were allowed to dry at 37 °C to reduce background signal due to moisture prior to loading onto a poly(methyl methacrylate) back loading sample holder. The solid state of CP-532,623 was assessed by comparison with a control sample of crystalline CP-532,623. XRPD was performed using a Bruker D8 Advance powder diffractometer (Bruker, Sydney, Australia) equipped with a copper (Cu) tube anode and Kα radiation source (λ = 1.542 Å). The samples were scanned between 5° and 40° (2θ), with a step size of 0.02° and a scanning speed of 2 s/step. The applied voltage and current were 45 kV and 40 mA respectively. The data were analyzed using DIFFRACPLUS software (Bruker).

Quantification of CP-532,623 in in Vitro Experiments by HPLC. Quantification of CP-532,623 in each of the separated phases (AP/OP/P) after formulation dispersion or digestion was performed using a previously developed and validated assay.[21,25]

Preclinical Bioavailability Studies in Beagle Dogs. All animal studies were approved and proceeded in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans http://www.wma.net/e/policy/b3.htm and EC Directive 86/609/EEC for animal experiments http://europa.eu.int/scadplus/leg/en/s23000.htm and were approved by the local Institutional Animal Experimentation Ethics Committee. Two separate crossover studies were conducted in four fasted male beagle dogs (12−22 kg). In the first study, each animal received three different LC-LBFs, whereas the second study included a total of four treatments (one intravenous and three oral treatments) as detailed below (composition of formulations marked with "*b*" in Table 1) with a minimum washout period of 7 days between doses.

In Vivo Evaluation of CP-532,623 after Oral Administration of LC-LBFs. Based on the in vitro digestion studies, three lipid based formulations were selected and assessed in vivo to examine the relationship between aqueous phase drug solubilization after in vitro digestion and in vivo exposure and to identify the formulation characteristics/components that support drug solubilization and absorption.

The LBFs employed were selected on the basis of high aqueous phase solubilization after in vitro digestion to maximize in vivo drug solubilization and the potential for enhanced bioavailability. Formulations F4LC, PPLC, and F2LC were chosen and comprised varying proportions of soybean oil, Maisine, Kolliphor RH 40 and triacetin, the major differences being increasing Kolliphor RH 40 concentrations (30, 40, and 50% w/w Kolliphor RH 40 respectively in F4LC, PPLC, and F2LC). Each treatment consisted of 30 mg of CP-532,623 (50 mg/g) dissolved in 600 mg of LBF and filled into a single soft gelatin capsule. The capsule was administered orally with 50 mL of water.

For the fasted studies, dogs were fasted for at least 12 h prior to dosing and remained fasted until 10 h after dosing. Water was available ad libitum. In fed studies, dogs were fed 400 g of "My Dog" canned dog food up to 1 h before commencement of studies with water provided ad libitum. Nutritional composition: crude protein 7.0%, crude fat 5.0%. The primary source of lipids in the food administered were LCT from animal fat and vegetable oil.

The concentration of drug in the plasma was monitored over time. Blood samples (3 mL) were collected prior to dosing and at 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, and 10 h post dose via an indwelling cannula positioned in the cephalic vein and at 24 h by individual venipuncture. Blood samples were taken into tubes containing 20 U/mL heparin. Plasma was separated within 2 h of sample collection by centrifugation (1328*g*, 10 min) in an Eppendorf 5702 R/A-4-38 centrifuge (Eppendorf AG, Hamburg, Germany) and stored at −80 °C until sample analysis. Plasma samples were prepared for analysis by liquid−liquid extraction, and CP-532,623 was quantified using a validated LCMS method.[21]

In Vivo Evaluation of Absolute Bioavailability and Maximum Drug Exposure. To verify the extent of drug exposure and to explore the upper limits of CP-532,623 bioavailability, a second in vivo study was conducted with the addition of an intravenously administered formulation to enable calculation of the absolute bioavailability of the oral formulations.

The oral treatments included a repeat administration of PPLC (30 mg of CP-532,623 in 600 mg of formulation) to fasted dogs (this also allowed a cross study control to evaluate reproducibility), administration of the same treatment to fed dogs, and a third treatment comprising the same dose but in a higher quantity of the PPLC formulation (30 mg of CP-532,623 in 2000 mg of formulation).

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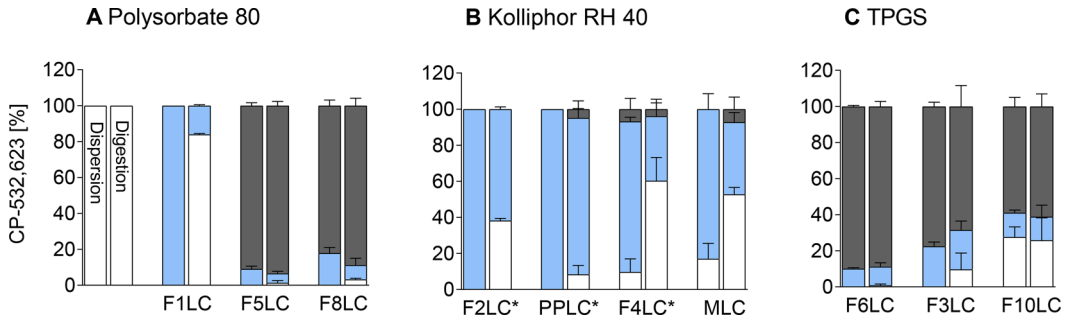

Figure 2. Drug distribution profiles after in vitro dispersion (left column for each formulation) and digestion (right column) of lipid based formulations. Individual bars—white, blue, and dark gray—represent the proportion of drug distributed to the pellet, aqueous, and oil phases respectively after in vitro dispersion and digestion [mean ± SD (n = 3)]. Each formulation contained 50 mg/g CP-532,623. (*) Formulations were subsequently assessed in vivo. Formulations are grouped by the surfactant present: (A) polysorbate 80; (B) Kolliphor RH 40 (in order of decreasing Kolliphor content); (C) TPGS.

The iv formulation was administered by infusion (1 mL/min over 5 min) via an indwelling cannula placed in the cephalic vein, in the opposite leg used for blood sampling. A total dose of 3 mg of CP-532,623 was administered in 5 mL of Intralipid. Due to the rapid clearance of intravenously administered treatments, plasma sample time points were more frequent in the first 2 h after dosing and concluded at 8 h post dosing. Blood samples (3 mL) were collected as follows: prior to dosing, immediately after infusion of the iv formulation, and every 10 min for the first hour, then every 15 min for the next hour, then at 2, 2.5, 3, 3.5, 4, 6, and 8 h.

Oral Capsule Preparation. Air filled soft gelatin capsules (a kind gift from Catalent Pharma Solutions, Braeside, Australia) were filled with formulation using a syringe and needle (21G) no more than 24 h prior to dosing. The physical stability of filled capsules was tested by comparing the time taken for the capsule to rupture in simulated gastric medium immediately after filling and again after 24 h. Capsule rupture was assessed using a USP paddle dissolution apparatus containing 200 mL of 0.1 M HCl at 37 °C and 60 rpm. The time taken to rupture was approximately 7−10 min and was not affected by storage for 24 h.

Intravenous Formulation Preparation. The poor aqueous solubility of CP-532,623 dictated the use of an Intralipid based intravenous formulation to facilitate CP-532,623 solubilization. This approach was attractive as it had been used successfully in a previous study.[25] The iv formulation was prepared by incremental addition (5 μL increments to a total of 160 μL) of a 37.5 mg/mL solution of CP-532,623 in *N*,*N*-dimethylacetamide:triacetin (3:5 v/v) to 10 mL of 10% Intralipid. The formulation was ultrasonicated between additions (Misonix XL 2020 ultrasonic processor (Misonix, Farmingdale, NY) equipped with a 3.2 mm microprobe which was pulsed (2 min on/20 s off) at an amplitude of 240 μm and a frequency of 20 kHz). The Intralipid emulsion was cooled in an ice bath during preparation. A final dose of 3 mg of CP-532,623 was administered in 5 mL of Intralipid. Iv formulations were sterile filtered through a 0.2 μm syringe filter immediately prior to infusion (1 mL/min over 5 min). An aliquot of the filtered formulation was collected to verify dose by HPLC.

Analytical Methods. Plasma concentrations of CP-532,623 after oral administration of the LBF and the intravenous formulation were determined using previously described analytical methods.[21]

Pharmacokinetic Data Analysis. Peak plasma concentrations (C_{max}) and the time of occurrence (T_{max}) were noted directly from the plasma concentration vs time profiles. First order terminal elimination rate constants (k) were determined from the gradient of the terminal log−linear phase of individual plasma drug concentration vs time profiles and the elimination half-life ($t_{1/2}$) subsequently calculated as 0.693/k. The area under the plasma concentration vs time profiles from time zero to the last measured concentration (AUC_{0-24h}) was calculated using the linear trapezoidal method. The area under the plasma concentration vs time profiles from time zero to infinity ($AUC_{0-\infty}$) was calculated by adding the area obtained by extrapolation from the last plasma sample (C_{last}) to infinite time (C_{last}/k) to AUC_{0-24h}. Absolute bioavailability (F) of CP-532,623 was calculated by comparison of dose-normalized AUC values following oral and intravenous administration (eq 2), where D_{po} and D_{iv} are the oral and intravenous doses administered, and AUC_{po} and AUC_{iv} represent the $AUC_{0-\infty}$ in plasma following oral and intravenous dosing, respectively.

$$F_{total} = (AUC_{po}/D_{po})/(AUC_{iv}/D_{iv}) \times 100\% \quad (2)$$

.

Relative bioavailability was assessed for statistically significant differences by ANOVA followed by a post hoc Tukey test for multiple comparisons at a significance level of α = 0.05. All statistical analysis was performed using IBM SPSS Statistics for windows version 20.0.0 (SPSS Inc., Chicago IL).

RESULTS

In Vitro Evaluation. *Solubilization of CP-532,623 after in Vitro Dispersion and Digestion of LC-LBFs.* The formulations under investigation were type III formulations according to the lipid formulation classification system[7] and comprised a combination of long chain lipid (soybean oil), high HLB (hydrophobic lipophilic balance) surfactant (polysorbate 80, Kolliphor RH 40, or TPGS), low HLB surfactant (Maisine 35-1 or Labrafil M 2125), and cosolvent (triacetin or propylene carbonate). The composition of the LBFs was based on the MC-LBFs investigated previously[21,24] and was modified by replacement of medium chain lipids (Miglyol N 812, Capmul MCM) with long chain lipids (soybean oil, Maisine 35-1) in an attempt to enhance solubilization of CP-532,623 in the more lipophilic solubilized micellar environment created by digestion of LC lipids. The formulations examined were restricted to those mixtures that were isotropic at 37 °C. Long chain lipid versions of formulations F7, I, and J were not isotropic and were excluded from further study.

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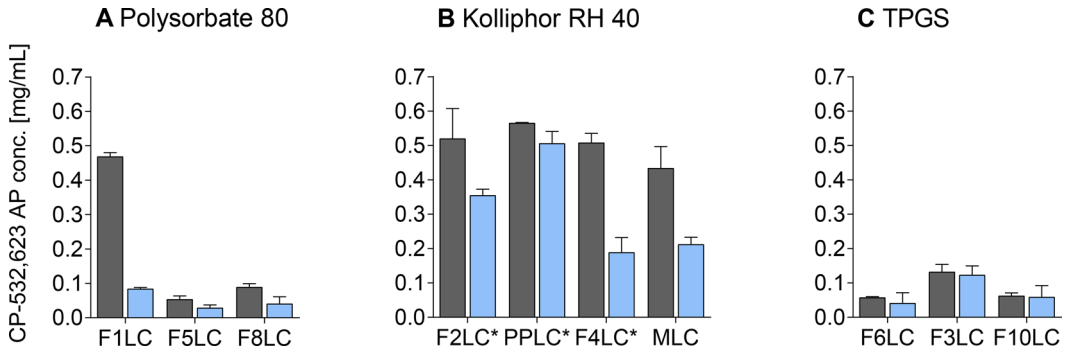

Figure 3. Aqueous phase drug solubilization profiles after in vitro dispersion (gray bars, left) and digestion (blue bars, right) of lipid based formulations. (*) Formulations were subsequently assessed in vivo. Formulations are grouped by surfactant present: (A) polysorbate 80; (B) Kolliphor RH 40 (in order of decreasing Kolliphor content); (C) TPGS. Each formulation (120 mg) contained 50 mg/g CP-532,623 and was digested at pH 6.5 for 30 min [mean ± SD (n = 3)].

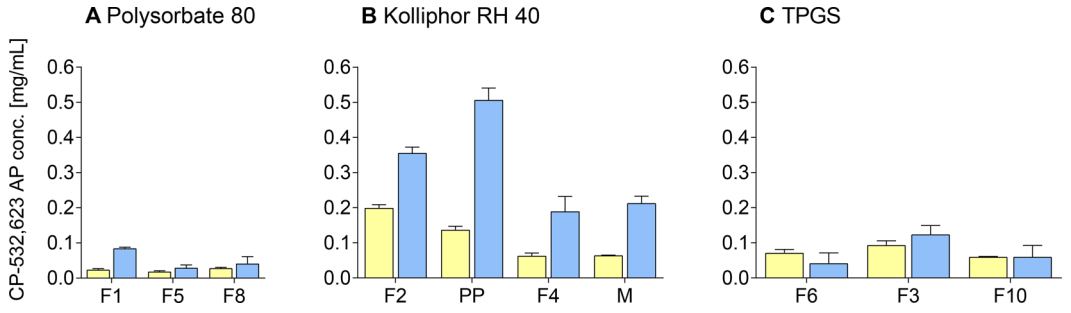

Figure 4. Comparison of aqueous phase drug solubilization after in vitro digestion of formulations based on medium (yellow bars, left) and long chain lipids (blue bars, right). MC formulations are analogous to the LC formulations in Table 1, but where the medium chain lipids Miglyol and Capmul MCM are present in place of the long chain lipids soybean oil and Maisine 35-1. Formulations are grouped by surfactant present: (A) polysorbate 80; (B) Kolliphor RH 40 (in order of decreasing Kolliphor content); (C) TPGS. Each formulation (120 mg) contained 50 mg/g CP-532,623 and was digested at pH 6.5 for 30 min [mean ± SD (n = 3)]. MC-LBF digestion data reprinted from previous study.[21]

The solubilization of CP-532,623 after in vitro dispersion and digestion of the different LC-LBFs is summarized in Figure 2 (as % drug distribution across the aqueous (AP), oil (OP), and pellet (P) phases obtained postcentrifugation of the dispersion/digestion media) and Figure 3 (as AP concentration). The AP concentrations after in vitro digestion of MC and the equivalent LC formulations are compared in Figure 4. To aid interpretation, the profiles after in vitro dispersion and digestion have been grouped by the high HLB surfactant present since this excipient group has the greatest influence on formulation dispersion and drug solubilization.[21] Formulations containing polysorbate 80, Kolliphor RH 40, and TPGS are shown in panels A, B, and C respectively.

The in vitro dispersion characteristics of the LC-LBFs were largely dictated by the type and quantity of surfactant present. Formulations containing 20% w/w polysorbate 80 (F5LC, F8LC) were poorly dispersed with 90 and 82% drug sequestered in a phase separated OP. Increasing the surfactant content to 50% in formulation F1LC improved dispersion, and increasing quantities of drug were recovered in the AP. Formulations containing Kolliphor RH 40 were well dispersed with 100% drug solubilized in the AP after dispersion and ultracentrifugation of formulations F2LC and PPLC (50 and 40% Kolliphor RH 40 respectively). Increasing the proportion of cosolvent at the expense of surfactant in formulations F4LC and MLC (30 and 20% Kolliphor RH 40 respectively), however, resulted in drug precipitation (10 and 17% respectively) on dispersion.

TPGS formulations F6LC, F3LC, and F10LC were poorly dispersed with 59 to 90% drug sequestered in the oil phase post dispersion. For formulations F3 and F10 this may have reflected the presence of Labrafil[21] in the formulations as these two formulations were the only two to contain Labrafil in place of Maisine (cosurfactant). In general, TPGS/lipid mixtures required high cosolvent levels to promote dispersion (i.e., to reduce the OP), however, miscibility was compromised in these formulations and therefore they were not investigated further.

Stimulation of in vitro digestion typically resulted in a decrease in solubilized drug concentrations (Figure 3), however, LBFs containing long chain lipids and Kolliphor RH 40 retained solubilization capacity reasonably effectively, resulting in the most robust AP drug concentrations (Figure 3, panel B). In almost all cases, drug concentrations in the AP were higher after digestion of the LC formulations when compared to the medium chain equivalent (Figure 4). In contrast, F1LC (containing 50% polysorbate 80) was particularly susceptible to drug precipitation (84% CP-532,623 precipitated) despite being fully solubilized during dispersion, suggesting that polysorbate 80 was more susceptible to digestion mediated drug precipitation when compared to Kolliphor RH 40 containing formulations.

The most poorly dispersed formulations [e.g., those containing TPGS (F6LC, F3LC, F10LC) or polysorbate 80 (F5LC, F8LC)], where drug was isolated in the oil phase after ultracentrifugation, remained resistant to drug precipitation during digestion. However, the affinity of CP-532,623 for the

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poorly dispersed oily phase prevented significant improvements in AP solubilization.

In summary, replacing MC lipids with LC lipids enhanced post digestion drug solubilization, and formulations containing Kolliphor RH 40 were more effective at maintaining drug in a solubilized state in the aqueous phase compared to those containing polysorbate 80 or TPGS.

Characterization of Precipitated CP-532,623 Post Digestion. The pellet phases collected after in vitro digestion of all formulations were observed under polarized light microscopy to check for the presence of crystalline drug. In all cases crystalline drug was not evident by microscopy. PPMC was selected for further study (Figure 5) since it had the largest amount of precipitated drug in vitro and yet was relatively well absorbed in vivo.[21] The pellet collected after in vitro digestion of drug loaded formulation was compared to pellet spiked with an equivalent mass of crystalline CP-532,623 and the pellet from a blank digest of the same formulation. CP-532,623 was evident as large needle shaped crystals in the spiked pellet (panel A), and there was no comparable structure observed in the pellet collected from digested drug free formulation (panel C). Upon digestion of drug loaded formulation (panel B) no crystalline material was observed by polarized light microscopy, despite the recovery of 17.5 mg of CP-532,623 in the pellet after digestion of 480 mg of formulation in 40 mL of digestion medium, as determined by HPLC. The absence of crystalline drug in the precipitated material obtained post digestion was further examined by X-ray powder diffraction (Figure 6). In this instance, there was evidence of some crystalline material (second diffractogram in Figure 6), however, the crystalline peaks in the XRPD signal obtained from analysis of the pellet from the digestion experiment were significantly lower than those of a spiked pellet of matched drug mass, indicating that the material was present in both amorphous and crystalline forms, but that the amorphous form was dominant. The apparent disparity between the polarized light microscopy (no evidence of crystalline drug) and XRPD data (some evidence of crystalline drug) may be explained by the lag time for XRPD analysis which may have resulted in drug crystallization during dry down and storage prior to XRPD. In contrast polarized light microscopy was performed immediately at the end of the digestion experiment.

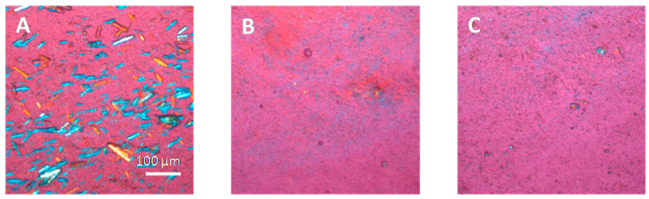

Figure 5. Polarized light microscope images of pellets collected after in vitro digestion of a representative formulation (PPMC). Panel A shows the pellet after digestion of a drug free formulation spiked with crystalline CP-532,623 prior to ultracentrifugation. Panel B shows the pellet after digestion of a typical drug loaded formulation (50 mg/g CP-532,623). Panel C is the pellet obtained after digestion of drug free formulation. Panels A and B contain an equivalent amount of CP-532,623 as determined by HPLC.

Equilibrium Solubility of CP-532,623 in Digested Aqueous Phase. The solubilization capacity of dispersed LBFs that contains glyceride lipids (i.e., type I, II, IIIa, and IIIb formulations) is typically reduced during digestion, commonly leading to drug precipitation.[26−31] The concentration of drug maintained in a solubilized state, however, may be maintained at a level above the equilibrium AP solubility for a finite period (i.e., supersaturated). Supersaturation is the inevitable precursor to precipitation, but may also drive increases in drug absorption in vivo. To evaluate the degree of supersaturation of drug in the AP obtained post digestion of the LBFs examined in this study, and to compare to the previously examined MC lipid containing formulations, drug solubility in the colloidal phases obtained post digestion of both MC- and LC-LBFs was also measured.

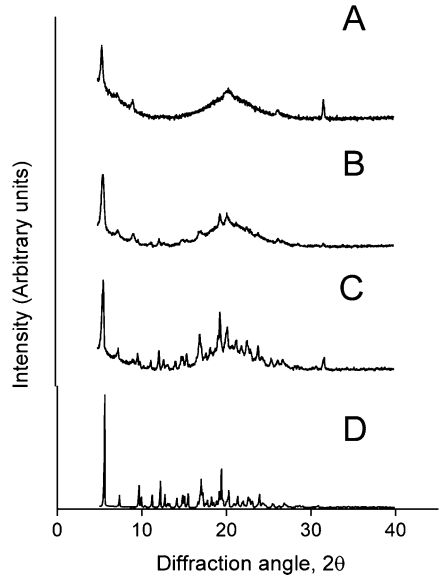

Figure 6. X-ray powder diffractograms of pellets obtained after in vitro digestion of PPMC formulation. Top to bottom: (A) drug free pellet; (B) digest of drug loaded formulation (50 mg/g CP-532,623); (C) pellet spiked with equivalent quantity of crystalline CP-532,623 to that in the pellet in panel B; (D) crystalline CP-532,623.

Drug solubility over time was measured in predigested AP (obtained after 30 min digestion of the MC and LC formulations included in the vivo studies). The solubility vs time profiles are presented in Figure 7. Note that the time axis here shows the variation in measured drug concentrations as a function of time during the solubility experiment and is not related to the time scale of the digestion experiment. Drug solubility was measured in the AP obtained at a single time point after digestion (30 min). In all cases (Figure 7), an equilibrium solubility was difficult to define due to the dynamic nature of the colloidal AP obtained post digestion. Thus, the "equilibrium" solubility study dictated sampling to longer time points in order to evaluate if the solubilization capacity of the colloids fluctuated less with time. However, the relative instability of the colloidal species formed, especially on digestion of long chain lipids, suggests that at longer time periods the nature of the colloids in the AP was likely very different from that present during the original digestion study. Coupled to this, the absorptive events in the GI lumen are unlikely to extend to the 168 h sampling time in these solubility studies. Data obtained at intermediate time points most likely provides the most representative indication of drug solubility in the colloids produced 30 min post lipid digestion. Solubility data were only generated at a single digestion time point (30 min) since previous studies have shown that drug solubility in

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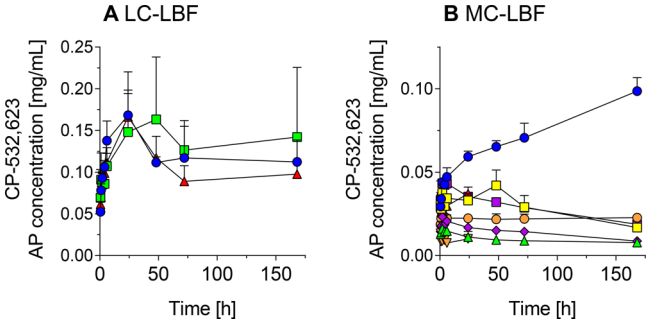

Figure 7. Solubility of CP-532,623 in aqueous phase samples taken 30 min after digestion of blank formulations. Panel A shows solubility as a function of dissolution time in digested LC formulations: F2LC (blue circle), F4LC (green square), and PPLC (red triangle). Panel B shows the solubility in digested MC formulations: F2 (blue circle), F3 (yellow square), F6 (green triangle), F8 (orange inverted triangle), F10 (purple diamond), M (orange circle), PP (red triangle), and Miglyol (purple square). AP was collected after in vitro digestion of 120 mg of drug free formulations at pH 6.5 for 30 min [mean ± SD (n = 3)].

type III formulations drops usually within the first 30 min post digestion initiation.[15]

In the current studies, the solubility profile of CP-532,623 in the AP obtained from digested LC formulations was variable, however, the kinetic changes observed were common to the three LC formulations examined. Thus, solubilization was initially quite rapid, reaching peak solubility in the first 24−48 h. Beyond this point, however, the drug concentration declined, most likely due to instability of the more hydrophobic LC lipids in the AP colloids.[9] In contrast, the dissolution profile for CP-532,623 in the AP obtained after digestion of the medium chain formulations was lower and more regular and remained stable beyond 24 h. The notable exception to this was the data for formulation F2MC, where drug solubility continued to increase over time. An explanation for this anomalous behavior is not clear at this time. In all cases, the apparent solubility of CP-532,623 in digested LC formulations was greater than in digested MC formulations.

Supersaturation of CP-532,623 in the Aqueous Phase Obtained after in Vitro Digestion of MC- and LC-LBFs. Supersaturation of the intestinal milieu during formulation digestion can provide a driving force for either drug absorption or precipitation.[15,32] The supersaturation ratio (S_R) describes the kinetic or transient solubility in the aqueous phase during digestion compared to the intrinsic (equilibrium) aqueous phase solubility. A solution is supersaturated at S_R values >1 and has higher thermodynamic activity and may therefore promote drug absorption. Alternatively, however, high S_R values may also increase the tendency to precipitate.

Digestion of lipid based formulations containing CP-532,623 resulted in supersaturation in all cases, although the degree of supersaturation was very low for F4LC (Table 2). Consistent with previous studies, where the initial or maximum supersaturation (S_{max}) ratio was high, precipitation was more likely.[11,17] After 30 min of digestion, however, the degree of supersaturation was lower and differed less across the formulations examined.

In vivo evaluation. *Relative Bioavailability of CP-532,623 after Oral Administration of LC-LBFs.* LC-LBFs that displayed enhanced AP solubilization after in vitro digestion were selected for in vivo assessment to expand the range of data previously collected for analogous MC formulations. PPLC, F2LC, and F4LC (Table 1) were selected due to high AP solubilization and the absence of a residual OP post digestion.

The study was conducted as a four way crossover in beagle dogs. Summary pharmacokinetic parameters are provided in Table 3, and the mean plasma profiles are presented in Figure 8. The plasma concentration vs time profiles are normalized to a nominal dose of 1.5 mg/kg CP-532,623.

Table 2. Supersaturation Ratio (S_R) Values Obtained after in Vitro Digestion of LC- and MC-LBFs Included in the in Vivo Bioavailability Studies[a]

	AP concn				
formulation	theor max	after in vitro digestion	equilib AP solubility	S_{max}	$S_{R(30min)}$
F2LC	0.6	0.36	0.17	3.6	2.1
F4LC	0.6	0.19	0.15	4.0	1.3
PPLC	0.6	0.51	0.17	3.6	3.0
F2MC	0.6	0.20	0.06	10.1	3.4
F3MC	0.6	0.09	0.03	18.1	2.8
F6MC	0.6	0.07	0.01	54.3	6.4
F8MC	0.6	0.03	0.01	60.0	2.8
F10MC	0.6	0.06	0.02	35.7	3.6
MMC	0.6	0.06	0.02	26.8	2.9
PPMC	0.6	0.14	0.04	16.2	3.7
Miglyol	0.6	0.09	0.04	17.0	2.5

[a]Aqueous phase concentrations were measured after 30 min in vitro digestion of LBFs and compared to equilibrium drug solubility to provide $S_{R(30min)}$. S_{max} was calculated from the ratio of the theoretical maximum AP concentration and the equilibrium AP solubility. The equilibrium solubility was measured after 24 h dissolution of excess CP-532,623 in the AP obtained after in vitro digestion of drug free LBFs.

Table 3. Summary Pharmacokinetic Parameters of CP-532,623 after Oral Administration in LC-LBFs to Fasted Beagle Dogs (Data Normalized to 1.5 mg Dose per kg Dog Weight) [Mean ± SD (n = 4)][a]

	F2LC	F4LC	PPLC
AUC_{0-t} (ng·h·mL^{-1})	1328 ± 420	1103 ± 131	1435 ± 103
$AUC_{0-\infty}$ (ng·h·mL^{-1})	1422 ± 440	1176 ± 133	1524 ± 116
C_{max} (ng/mL)	414 ± 117	438 ± 55	518 ± 72
T_{max} (h)	2.2 ± 1.2	1.4 ± 0.2	1.8 ± 0.3
elimination $t_{1/2}$ (h)	11.2 ± 5.1	12.9 ± 5.6	11.0 ± 3.2
BA	86 ± 27	71 ± 8	92 ± 7

[a]Bioavailability was calculated using the data in the iv leg of the study reported in Table 4. PPLC was administered in both studies providing a means of cross study control. The AUCs for PPLC were not statistically different across the two studies (1311 ± 538 versus 1435 ± 103 ng·h·mL^{-1}).

Despite the increase in AP solubilization observed after in vitro digestion of PPLC, F2LC, and F4LC, the plasma exposure of CP-532,623 was within the range of AUCs obtained after administration of the equivalent MC formulations. The presence of LC lipids in the modified formulations had no significant impact on the rate of CP-532,623 absorption or elimination, with T_{max} (1.8 ± 0.8) and the average terminal half-life ($t_{1/2}$) (11.7 ± 4.4 h) being consistent with previous studies.[21,25] F4LC showed a trend toward slightly lower exposure, lower C_{max}, and a slightly flatter plasma level time

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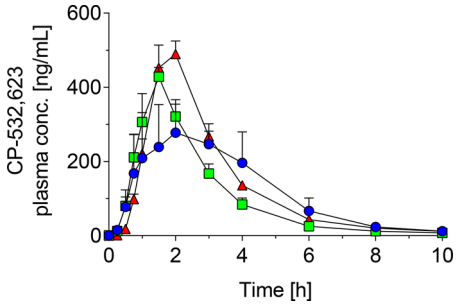

Figure 8. Mean plasma concentration vs time profiles for CP-532,623 after oral administration of LC-LBFs to fasted beagle dogs [mean ± SEM (n = 4)]. Formulations administered are F2LC (blue circle), F4LC (green square), and PPLC (red triangle). Profiles of oral lipid based formulations are truncated to 10 h for clarity.

profile, however, these differences were not statistically significant.

Absolute Bioavailability of CP-532,623 Administered in PPLC LBFs and the Impact of the Quantity of Lipid/Formulation Administered. A second study was conducted to assess the absolute bioavailability of the best performing LC formulation (PPLC) and also to probe the effect of the quantity of coadministered formulation/lipid on drug exposure. Figure 9 shows the plasma versus time profiles for three oral formulations as well as the plasma versus time profile for an iv formulation. The summary pharmacokinetic parameters are provided in Table 4. Administration of the PPLC formulation to fasted beagle dogs resulted in an AUC of 1340 ± 542 ng·h/mL. This was not statistically significantly different from that obtained in the first study, i.e., Table 3 (1435 ± 103 ng.h/mL). Administration of the same formulation to fed dogs increased the exposure of CP-532,623 by ∼40% to an AUC of 2002 ± 407 ng·h/mL. A third treatment was also administered and included the 30 mg dose of CP-532,623 but in a larger quantity (2000 mg vs 600 mg) of the PPLC formulation. Increasing the mass of formulation 3-fold had no statistically significant effect on in vivo drug exposure.

Comparison of the AUCs of CP-532,623 obtained after oral administration with those obtained after intravenous administration suggests that absolute bioavailability was essentially complete after fed administration and slightly lower and similar for both the 600 mg and 2000 mg quantities of formulation containing 30 mg of CP-532,623 in the fasted state (81 and 86% respectively, relative to the iv data obtained in the first study). The comparable in vivo exposure obtained for CP-532,623 after administration of the PPLC formulation in study one and study two provided some confidence in cross study comparison, i.e., applying the data obtained after iv administration in this study to the oral exposure data obtained in the first study. The data suggests that the bioavailability of CP-532,623 was 86, 71, and 92% after administration of F2LC, F4LC, and PPLC respectively.

Correlations. A comparison between in vitro AP solubilization and in vivo exposure is presented in Figure 10. Contrary to the marked increase in AP solubilization of CP-532,623 after in vitro digestion of LC formulations compared to MC formulations, the $AUC_{0-\infty}$ values obtained after administration of the LC formulations (1176 ± 133 to 1596 ± 323 ng.h/mL) fell within the range of the MC formulations (1092 ± 663 to 1892 ± 835 ng·h/mL). Interestingly, however, within a group both LC-LBFs and MC-LBFs seemed to show reasonable correlation between exposure and in vitro solubilization, although the LC systems required higher in vitro solubilization to drive similar extents of in vivo exposure.

AP solubilization alone may not be the most appropriate indicator of in vivo formulation performance since drug absorption is thought to occur from the free drug concentration in equilibrium with the solubilized reservoir. Under these circumstances the most appropriate correlant is arguably the supersaturation ratio since this provides a relative indication of thermodynamic activity (and therefore free concentration). This data is presented in Figure 11. The symbol for each formulation in Figure 11 identifies the surfactant present in the formulation. The formulations containing Kolliphor RH 40 show a linear response (panel B) where AUC increases linearly with S_R. With the exception of F3, all the MC formulations containing the other surfactants appeared to show a similar correlation, but were shifted such that higher degrees of supersaturation were required to drive exposure.

DISCUSSION

LBFs have been shown to enhance the oral bioavailability of the CETP inhibitor CP-532,623 as well as the related structural analogues CP-529,414 (Torcetrapib) and CP-524,515.[21,24,25] Previous studies have shown that, for a series of MC-LBFs, some correlation is evident between CP-532,623 solubilization in the AP obtained after in vitro digestion and in vivo exposure

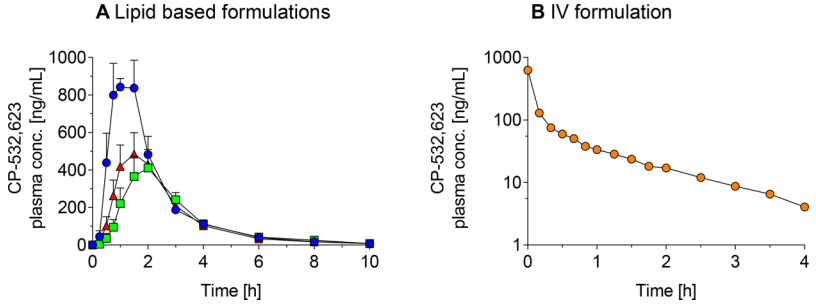

Figure 9. Mean plasma concentration vs time profiles for CP-532,623 after administration of lipid based formulations to either fed or fasted beagle dogs [mean ± SEM (n = 4)]. Formulation PPLC (600 mg of PPLC containing 50 mg/g CP-532,623) was administered to fed (blue circle) and fasted (green square) beagle dogs. Formulation PPLC with the higher lipid dose (2000 mg of PPLC containing 15 mg/g CP-532,623) was administered to fasted beagle dogs (red triangle). The dose of CP-532,623 was equal (30 mg of CP-532,623) in all formulations, however, the volume of formulation was increased for the second PPLC treatment. Profiles are truncated to 10 h for clarity. The iv formulation contained 3 mg of CP-532,623 solubilized in 5 mL of Intralipid and was administered over 5 min into the cephalic vein.

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Table 4. Summary Pharmacokinetic Parameters after Oral Administration of CP-532,623 in Lipid Based Formulations and Intravenous Administration of an Intralipid Solution, to Fed and Fasted Beagle Dogs (Data Normalized to 0.15 and 1.5 mg Dose per kg Dog Weight for Iv and Oral Doses Respectively) [Mean ± SD (n = 4)]

	5 mL of Intralipid, 3 mg of CP-532,623 (fasted)	600 mg of PPLC, 30 mg of CP-532,623 (fed)	600 mg of PPLC, 30 mg of CP-532,623 (fasted)	2000 mg of PPLC, 30 mg of CP-532,623 (fasted)
AUC_{0-t} (ng·h·mL^{-1})	160 ± 20	1967 ± 401	1311 ± 538	1364 ± 429
$AUC_{0-\infty}$ (ng·h·mL^{-1})	166 ± 19	2002 ± 407	1340 ± 542	1412 ± 427
C_{max} (ng/mL)	624 ± 197	1021 ± 74	470 ± 163	572 ± 138
T_{max} (h)	0.0 ± 0.0	1.2 ± 0.4	1.6 ± 0.5	1.5 ± 0.4
elimination $t_{1/2}$ (h)	1.0 ± 0.1	6.8 ± 3.7	6.0 ± 3.6	10.1 ± 4.0
Cl (mL·kg^{-1}·h^{-1})	934 ± 211			
V_D (mL/kg)	1390 ± 338			
BA		122 ± 25	81 ± 33	86 ± 26

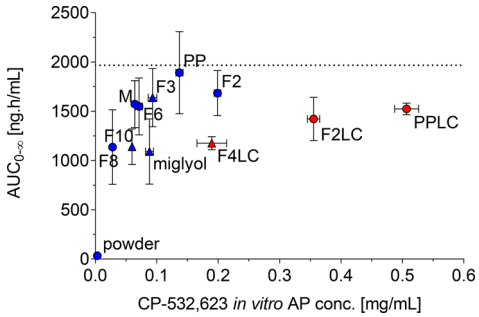

Figure 10. In vitro−in vivo correlation plotted as the area under the plasma concentration−time curve (AUC) [mean ± SEM (n = 4)] after oral administration to beagle dogs (30 mg of CP-532,623) vs the concentration of drug solubilized in the aqueous phase after in vitro digestion [mean ± SEM (n = 3)]. Formulations based on medium chain and long chain lipids are shown as blue and red data points, respectively. Aqueous phase concentrations for formulations with a residual oil phase after in vitro digestion are shown as triangles. Aqueous phase concentrations for formulations where no residual oil phase was apparent after in vitro digestion are shown as circles. Exposure obtained after fed administration of PPLC is shown as the dotted horizontal line.

after oral administration.[21] However, the impact of formulations containing LC lipids on the solubilization and subsequent in vivo exposure of CP-532,623 was not evaluated. In the current studies, the relationship between in vitro solubilization and oral bioavailability of CP-532,623 has therefore been explored further by generating a series of formulations based on LC lipids (rather than MC lipids) since previous studies have shown that LC lipid containing formulations typically generate more robust drug solubilization on lipid digestion.[22,33] Further studies have extended these observations to evaluate the impact of coadministration of larger quantities of lipids either by administering larger quantities of formulation or by coadministering with food.

Impact of Lipid Chain Length on CP-532,623 Solubilization after in Vitro Dispersion and Digestion of LBFs. Drug loading and self-emulsification properties are typically more favorable for MC lipid containing formulations than the equivalent LC lipid systems. LC lipid based formulations, however, commonly lead to enhanced drug solubilization post digestion since LC fatty acid digestion products more effectively intercalate into endogenous bile salt micelles, thereby swelling the mixed micellar core.[9] The benefit of micellar swelling, however, is drug specific. For example, formulation of danazol,[23] halofantrine,[33] cinnarizine,[34] cyclosporine,[35] and vitamin D_3[36] with long chain lipids has been shown to be advantageous, whereas incorporation of long chain lipids into formulations of griseofulvin[37] and penclomedine[38] was unfavorable and, in the case of seocalcitol[39] or dexamethasone,[37] made no significant difference.

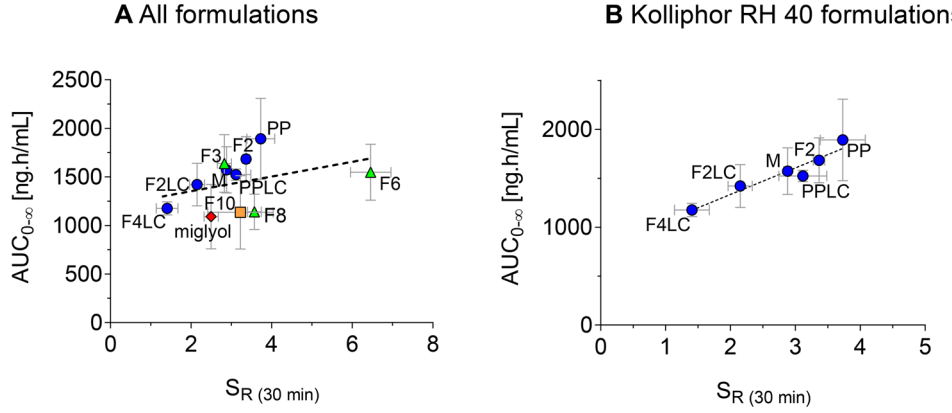

Figure 11. In vitro−in vivo correlation plotted as the area under the plasma concentration−time curve (AUC) [mean ± SEM (n = 4)] after oral administration to beagle dogs (30 mg of CP-532,623) vs the supersaturation ratio (S_R) of CP-532,623 in aqueous phase after in vitro digestion [mean ± SEM (n = 3)]. The surfactant present in each formulation is identified by the data symbol: Kolliphor RH 40 (blue circle), polysorbate 80 (orange square), TPGS (green triangle), or no surfactant (red diamond). (A) Poor correlation was evident when all formulations dosed were compared (R^2 0.13). (B) Analysis of Kolliphor RH 40 formulations only showed a clear correlation between degree of supersaturation and in vivo exposure for formulations comprising similar excipients (R^2 0.92).

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After in vitro digestion, the original series of MC based formulations developed for CP-532,623 achieved AP solubilization in the range ~0.02 to 0.2 mg/mL (F5MC and F2MC respectively). These values are well above the aqueous phase solubility of crystalline CP-532,623 (0.003 mg/mL), however, a large proportion of the drug initially dissolved in the formulation precipitated,[21] suggesting that further formulation optimization was possible. Substitution of the MC lipids in the formulations to generate LC formulations was undertaken to further improve post digestion AP solubilization, however, this came at the expense of formulation miscibility. LC versions of formulations I and J were not isotropic, and the miscibility of formulations F3LC and MLC became unstable below 37 °C.

Nonetheless, the LC formulations that were possible were effective, and they prevented drug precipitation during dispersion for all formulations except F10MC (Figure 2, left bars). In vitro digestion of the LC formulations also achieved higher (F1LC, F2LC, F4LC, MLC, PPLC) or similar (F5LC, F8LC, F3LC, F6LC, F10LC) AP solubilization when compared to the MC equivalents (Figure 4). Notably, formulations containing Kolliphor RH 40 maintained superior AP solubilization for CP-532,623 in all cases. This trend is consistent with that seen previously for the MC formulations.[21]

The AP concentrations achieved at the end of the in vitro digestion experiment were compared to the equilibrium solubility of drug in the AP obtained post digestion to provide an indication of the extent of supersaturation. Interestingly, the marked improvements in drug solubilization achieved with the LC formulations were associated with relatively low levels of supersaturation (S_R 1.3−3.0). In contrast, the MC formulations reached significantly higher $S_{R(30min)}$ values of 2.5−6.4. The higher supersaturation ratios obtained with the MC formulations occurred in parallel with increases in precipitation, consistent with previous studies that suggest increased likelihood of precipitation when S_R values increase above 3.[17,31] However, while precipitation was evident in vitro, increases in supersaturation and thermodynamic activity may also drive increases in absorption, and it remains possible that, for highly permeable drugs, increased thermodynamic activity in vivo may promote absorption, reducing supersaturation and reducing the stimulus for precipitation.[15,40] In this case in vitro studies may significantly overestimate the likelihood of in vivo precipitation. It was also apparent that CP-532,623 precipitated from the MC-LBFs in the amorphous form. Previous studies have suggested that precipitation in the amorphous form may result in increased redissolution and therefore higher bioavailability than might be expected based on the significant precipitation in vitro.[19]

In Vivo Bioavailability of CP-532,623 LC-LBFs. In spite of significantly greater in vitro drug solubilization in the aqueous phase after dispersion and digestion of the LC-LBF formulation, in vivo exposure of CP-532,623 remained within the range seen previously for MC formulations.[21] The lack of increase in in vivo exposure promoted further exploration of the absorption of CP-532,623, via the coadministration of increasing quantities of LBFs, and coadministration with food, in an attempt to maximize possible lipid-mediated increases in absorption. An evaluation of absolute bioavailability was also conducted to estimate whether the relatively small range of in vivo exposures obtained were similar and high, or similar and low. The data obtained showed that coadministration with higher quantities of lipid had little impact on oral bioavailability, but that coadministration with food was able to increase absorption by ~40%. The increase in exposure in the fed state is likely to reflect prolonged drug solubilization conditions, but may also reflect increases in lymphatic transport of CP-532,623 (as seen previously)[25] resulting in a reduction in first pass metabolism. Absolute bioavailability was, in general, high, ranging from 71% for the least effective formulation up to being essentially complete after coadministration with food. Interestingly, after coadministration with food, absolute bioavailability was greater than 100%. Similar trends have been observed previously for halofantrine[41] and were shown to reflect differences in systemic pharmacokinetics in the fed and fasted state. Thus, in the fed state where systemic lipid levels are high, the association of highly lipophilic drugs with plasma lipoproteins is increased, reducing clearance and volume of distribution. This may be particularly evident for drugs such as halofantrine and CP-532,623 where intestinal lymphatic transport plays a role in drug transport to the systemic circulation[25,41,42] and where drug association with lipoproteins is inherently high. In summary, in all cases, LBFs were able to significantly enhance the oral bioavailability of CP-532,623, and the relatively small differences in exposure obtained across the series likely reflect, at least in part, the efficiency of absorption.

Relationship between in Vitro Solubilization and Oral Bioavailability. Traditionally, in vitro digestion models have been employed to predict the rank order performance of LBFs, on the premise that the ability of different formulations to maintain drug in a solubilized reservoir after the challenges of formulation dispersion and digestion provides an indication of the relative ability to promote drug absorption.[10,14] However, the interplay of lipid digestion, drug solubilization, and drug absorption is difficult to replicate in a simple model,[40,43] and in some cases, correlations between solubilization after in vitro digestion and drug absorption have been less easy to identify.[2,20,44] Nonetheless, digestion remains a critical aspect of formulation processing in vivo, and the current studies aimed to shed further light on the complex relationship between drug solubilization, supersaturation, and absorption. Interestingly, in the current studies good correlation was seen between solubilization of CP-532,623 during in vitro lipid digestion of the LC-LBFs and in vivo absorption (Figure 10), however, this correlation did not overlap with previous data obtained for similar medium chain lipid containing formulations. Instead, the correlation for the LC-LBFs was shifted to higher in vitro solubilization levels, suggesting that higher in vitro solubilization was required for the LC-LBFs to generate the same degree of exposure as the MC formulations. Nevertheless, within a class (i.e., LC-LBFs or MC-LBFs), reasonable correlation between in vitro and in vivo end points was evident.

To better understand the drivers of drug absorption in vivo, the in vitro solubilization data was also analyzed to give an indication of supersaturation. The rationale behind this analysis was that the relative extent of supersaturation provides a surrogate indication of thermodynamic activity (and therefore drug concentration in free solution) and that for solubilized drug this might provide a better indication of absorption potential than simple solubilization.

Figure 11B shows a remarkable correlation (R^2 0.92) between the degree of drug supersaturation after digestion and in vivo exposure for all the LBFs where Kolliphor RH 40 was used as the surfactant, regardless of whether the formulations contained MC or LC lipids. Thus, the LC lipid formulations resulted in greater post digestion solubilization (Figure 10), however, they also increased equilibrium solubility

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in the colloids produced during digestion. As such the degree of supersaturation was lower (Figure 11). Correction of the correlation for supersaturation therefore brings these values into line with the medium chain formulations. Interestingly the formulations that were not based on Kolliphor also showed some evidence of a similar trend, although in this case the correlation was displaced in Figure 11, suggesting that for the non-Kolliphor containing formulations higher supersaturation was required to promote similar exposure.

The data therefore suggest that correlations between drug solubilization, supersaturation, and absorption are evident, but that these correlations are not a simple "one-size-fits-all" relationship where the degree of solubilization or supersaturation is able to entirely explain differences in absorption. Instead, good correlation is evident with formulation series containing varying proportions of the same or structurally similar components.[2] Thus, formulations containing medium chain lipids appear to provide a different solubilization relationship from those containing long chain lipids, and those containing Kolliphor as surfactant appear to behave differently from those containing polysorbate or TPGS. In all cases, however, within a series, formulations that resulted in higher in vitro solubilization and supersaturation appeared to perform better in vivo.

An explanation for the apparent "shifts" in formulation performance for different classes of formulation is not clear at this time, but at the same degree of solubilization or supersaturation different colloidal species are seemingly more/less able to promote absorption. This argues against the suggestion that drug absorption is simply a function of the free concentration in equilibrium with a solubilized reservoir. Instead, these data indicate that the nature of the solubilized reservoir, with respect to both kinetic changes to the colloids and chemical composition, may also influence the free drug concentration.

Previous studies have shown that for CP-532,623 drug absorption occurs, at least in part, via the intestinal lymphatics.[25,45] Lymphatic drug transport is more effectively stimulated by long chain rather than medium chain lipids[45−47] and is typically maximally enhanced by food.[25,47] The increase in exposure of CP-532,623 on coadministration with food might, therefore, reflect increased lymphatic transport. However, it remains unclear whether increases in lymphatic transport per se are able to increase drug absorption, since drug association with intestinal lipoproteins (the key step in redirecting drug into the lymph) occurs after drug absorption across the apical membrane of enterocytes. Thus, lymphatic transport is only likely to promote absorptive flux if the transport sink provided by the lymph is able to increase the absorptive concentration gradient across the apical membrane. It is clear, however, that intestinal lymphatic transport is able to circumvent hepatic first pass metabolism and may reduce enterocyte based metabolism. As such increases in lymphatic transport may enhance the bioavailability of CP-532,623 by reducing first pass metabolism, in particular after food. Arguing against a significant role for lymphatic transport, however, and especially in altering drug exposure after fasted administration of the different formulations, is the fact that LC-LBFs were, in general, no more effective at promoting drug exposure than MC-LBFs. Indeed the data in Figure 10 suggest that LC lipids were less effective at promoting bioavailability, at the same levels of solubilization, when compared to MC lipids, at least for CP-532,623. In contrast, LC lipids are typically more effective at promoting lymphatic transport.

Long chain triglycerides are digested more slowly and have slower absorption rates than MCT.[48] Consequently, LC lipid formulations may supersaturate in vivo at a slower rate than MCT or drug absorption may occur more rapidly than lipid absorption, desaturating the dispersed LBFs and reducing precipitation risk but also reducing thermodynamic drive for absorption.[40]

Alternatively, the physical nature of the colloids formed post digestion may play a role in drug absorption and this may vary with different formulation components. For example, differences in the particle size of the colloids assembled from the products of digestion of LC lipid formulations versus MC lipid formulations might result in different rates of diffusion across the unstirred water layer and therefore differences in patterns of drug absorption. Unfortunately, accurate measurement of the particle size of the species present in the in vitro digest here was not possible since the systems were heterogeneous, and this suggestion therefore remains speculative. Subsequent studies, however, might usefully attempt to better characterize the particle size of post digestion colloidal species and incorporate this information into the interpretation of in vitro solubilization/absorption relationships. Similarly, intestinal P-glycoprotein and intestinal first pass metabolism, separately or in concert, may reduce the transfer of intact drug from the intestinal lumen through to the portal blood[49−55] It is possible therefore that the differences seen here reflect differential inhibition of efflux or first pass metabolism due to the presence of different formulation components. However, the role of P-gp or enterocyte based metabolism on the absorption and bioavailability of CP-532,623 is not known at this time.

CONCLUSION

Lipid based formulations are a promising formulation strategy to enhance the oral bioavailability of poorly water-soluble drugs and have been shown previously to promote the absorption of the CETP inhibitor CP-532,623 after administration to beagle dogs. In the current studies, an in vitro digestion model has been used to further probe the relationship between simple in vitro indicators of utility (drug solubilization and estimates of supersaturation) and bioavailability for a series of long chain LBFs of CP-532,623. The data have also been compared to previous studies of analogous formulations containing MC lipids. The data confirm the utility of LBFs for enhancing CP-532,623 bioavailability and provide further evidence that, within related formulation types, good correlation between in vitro indicators of solubilization and, in particular, supersaturation and drug exposure in vivo are possible. The data also suggest, however, that these are not the only drivers of drug absorption from LBFs (at least for CP-532,623) and that across dissimilar formulation groups other factors may shift the relationship between absorption and physicochemical indicators of in vitro performance such as solubilization or supersaturation.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

LBF, lipid based formulation; LC, long chain lipid; MC, medium chain lipid; PWSD, poorly water-soluble drug; GI, gastrointestinal; AP/OP/P, aqueous/oil/pellet phases (obtained after in vitro digestion); S_R, supersaturation ratio; S_{max}, maximum supersaturation ratio; PLM, polarized light microscopy; XRPD, X-ray powder diffraction; HLB, hydrophilic−lipophilic balance

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